UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS
DEPARTAMENTO DE FARMACOLOGIA
CAMPUS DE BOTUCATU

EFEITOS DA DOXICICLINA RELACIONADOS À INIBIÇÃO DAS METALOPROTEINASES EM MODELO DE PRÉ-ECLÂMPSIA INDUZIDA POR L-NAME EM RATAS

REGINA APARECIDA DO NASCIMENTO

Tese apresentada ao Instituto de Biociências da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Botucatu, como requisito para obtenção do título de Doutora em Farmacologia e Biotecnologia

Orientador: Prof. Dr. Carlos Alan Candido Dias Junior

Botucatu-SP
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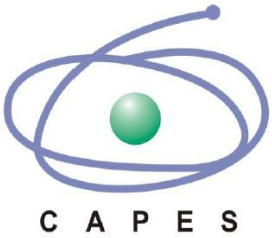

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Dedicatória

Ao único Deus, cujo nome é Senhor somente tu, és Altíssimo sobre toda a terra.
Salmo 83:18.

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***Ao meu Deus**, por estar sempre ao meu lado. "Portanto, ao **Rei eterno, imortal, invisível, Deus único**, seja honra e glória pelos séculos dos séculos. Amém! Invocarei ao **Senhor** enquanto viver, cantarei ao **SENHOR**; entoarei louvores ao meu **Deus,** enquanto eu existir". 1 Timóteo 1:17 e Salmo 116:2; 104: 33.*

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***À minha família,** que sempre esteve me apoiando e incentivando a seguir em frente!*

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Prefácio

A tese intitulada: “Efeitos da doxiciclina relacionados à inibição das metaloproteinases em modelo de pré-eclâmpsia induzida por L-NAME em ratas

Primeiramente a tese consta de uma introdução a respeito da gestação e das desordens hipertensivas gestacionais. São resumidamente abordados alguns fatores de predisposição que levam ao processo de má formação placentária e posterior disfunção endotelial características da hipertensão gestacional e posteriormente, descreve os principais efeitos da doxiciclina como inibidor de metaloproteinases associada à desordem hipertensiva gestacional.

O segundo capítulo traz o artigo entitulado “*Hypertension, augmented activity of matrix metalloproteinases-2 and -9 and angiogenic imbalance in hypertensive pregnancy are attenuated by doxycycline*” publicado na revista *European Journal of Pharmacology* onde nós descrevemos os efeitos do tratamento com um inibidor de metaloproteinases, num modelo experimental de hipertensão gestacional induzida pelo inibidor da enzima óxido nítrico sintase (NOS) Nω-nitro-L-arginina-metil éster (L-NAME).

O capítulo três traz o manuscrito *“Nitric oxide downregulates activity of matrix metalloproteinases-2 and -9 during pregnancy, but reduced-nitric oxide-induced hypertensive pregnancy increases activity of these gelatinases”* submetido para publicação à revista *Nitric Oxide*. Neste manuscrito, avaliamos a atividade das MMP-2 e -9 em diferentes períodos da gestação e mudanças nas atividades de MMPs com diminuição da biodisponibilidade do NO ao longo da prenhe em ratas com hipertensão gestacional induzida por (L-NAME).

A quarta parte consta de uma breve discussão dos achados nos dois artigos que foram anteriormente apresentados e a última parte da tese apresenta algumas considerações finais e conclusões a respeito do estudo descrito na tese.

Também, no decorrer deste curso de doutoramento, outras atividades foram desenvolvidas com o objetivo de enriquecer a formação acadêmica e estas são apresentadas a seguir:

Disciplinas cursadas

Disciplinas	Ano	Período Letivo	Créditos	Carga Horária	Freq	Conceito
Tópicos Avançados em Farmacologia e Biotecnologia	2015	1º semestre	3	45	92	B
Tópicos Avançados em Farmacologia e Biotecnologia	2015	2º semestre	3	45	100	A
Modelos experimentais em farmacologia da reprodução: enfase no papel dos reguladores endócrinos e na diferenciação sexual hipotalâmica	2016	1º semestre	2	30	100	A
Prática de ensino de Farmacologia	2016	2º semestre	4	60	83	B
Aspéctos Farmacológicos e Bioquímicos da Síndrome Metabólica	2017	1º semestre	4	60	100	A
Metabolismo energético	2017	1º semestre	4	60	100	A
Créditos aproveitados do mestrado			15	225		
Total geral créditos em disciplinas			35	525		
Total de Créditos em atividades complementares			70	1050		
Total geral créditos			120	1800		

Artigos publicados

1. **Nascimento RA,** Possomato-Vieira JS, Gonçalves-Rizzi VH, Bonacio GF, Rizzi E, Dias-Junior CA. Hypertension, augmented activity of matrix metalloproteinases-2 and -9 and angiogenic imbalance in hypertensive pregnancy are attenuated by doxycycline. Submetido à revista *Eur J Pharmacol*. doi: 10.1016/j.ejphar.2018.10.017.

2. Possomato-Vieira JS, Gonçalves-Rizzi VH, **Nascimento RA**, Wandekin RR, Caldeira-Dias M, Chimini JS, Santos da Silva ML, Dias-Junior CA. Clinical and experimental evidences of hydrogen sulfide involvement in lead-induced hypertension. *Bio Med Res Int.2018 Mar 28*.

3. Gonçalves-Rizzi VH, Possomato-Vieira JS, **Nascimento RA**, Caldeira-Dias M, Dias-Junior CA. Maternal hypertension and feto-placental growth restriction is reversed by sildenafil: Evidence of independent effects of circulating nitric oxide levels. *Eur J Pharmacol*. 2018 Jan 17.

4. Zhu ML, Zhao JP, Cui N, Gonçalves-Rizzi VH, Possomato-Vieira JS, **Nascimento RA**, Dias-Junior CA. Cardiac myeloperoxidase activity is elevated in hypertensive pregnant rats. *J HuazhongUnivSciTechnolog Med Sci*. 2017 Dec;37(6):904-909.

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Artigo submetido para publicação

1. **Nascimento RA,** Possomato-Vieira JS, Bonacio GF, Rizzi E, Dias-Junior CA. Nitric oxide downregulates activity of matrix metalloproteinases-2 and -9 during pregnancy, but reduced-nitric oxide-induced hypertensive pregnancy increases activity of these gelatinases. Submetido à revista *Nitric oxide* Manuscript: NOX_2018_244

Participação em eventos científicos

1. XXII Simpósio Brasileiro de Fisiologia Cardiovascular. Faculdade de Medicina da Universidade de São Paulo – Ribeirão Preto, Brasil (2018).

2. 49th Brazilian Congress of Pharmacology and Experimental Therapeutics – Ribeirão Preto, Brasil (2017).

3. IV Simpósio em Biologia Vascular – Faculdade de Ciências Farmacêuticas de Ribeirão Preto da Universidade de São Paulo – Ribeirão Preto, Brasil (2017).

4. Simpósio de Farmacologia e Biotecnologia – SIMFARTEC -UNESP/Botucatu, (Brasil) 2017.

5. 7th International Symposium of Graduate and Reserarch. Faculdade de Medicina da Universidade de São Paulo – Ribeirão Preto, Brasil (2016).

6. 19º Encontro Nacional de Biomedicina, realizado na cidade de Botucatu-SP, (Brasil) 2016.

7. V Simpósio de Farmacologia da UNESP - SIMFAR (UNESP/Botucatu), (Brasil) 2015.

Resumos em eventos científicos

1. **Nascimento, R.A**; Possomato-Vieira, J.S. ; Gonçalves-Rizzi, VH ; Dias-Junior, C.A. Doxycycline reduces blood pressure and reestablishes the antioxidant capacity without changes in feto-placental restriction in hypertensive pregnant rats. XXII Simpósio Brasileiro de Fisiologia Cardiovascular. Faculdade de Medicina da Universidade de São Paulo – Ribeirão Preto, Brasil (2018.

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3. Possomato-Vieira, J.S.; Gonçalves-Rizzi, VH ; **Nascimento, R.A** ; Silva, K.P ; Caldeira-Dias, M. ; Sandrim, V. C. ; Dias-Junior, C.A. Hydrogen Sulfide (H2S) Donor Reduces Systolic Blood Pressure and Stimulates Nitric Oxide Production in rats with L-NAME-Induced Hypertension in Pregnancy. 48th Brazilian Congress of Pharmacology and Experimental Therapeutics, 2016, Foz do Iguaçu.

4. Gonçalves-Rizzi, VH ; Possomato-Vieira, J.S. ; **Nascimento, R.A** ; Silva, K.P ; Caldeira-Dias, M. ; Sandrim, V. C. ; Dias-Junior, C.A. Placental-fetal interface is affected positively by sodium nitrite and sildenafil and concomitantly shows reductions in hypertension-in-pregnancy. 48th Brazilian Congress of Pharmacology and Experimental Therapeutics and 21th Latin American Congress of Pharmacology, 2016, Foz do Iguaçu.

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6. Gonçalves-Rizzi, VH. ; Possomato-Vieira, J.S. ; Mendes, G ; **Nascimento, R.A** ; Silva, K.P ; Pupo, A.S ; Dias-Junior, CA. Sodium Nitrite Reduces Systolic Blood Pressure in Preeclampsia. Experimental Biology, 2015, Boston. The FASEB Journal. v. 29. p. 4-4.

Lista de abreviaturas e Siglas

ABTS - Ácido 2,2-azino-bis (3-etilbenzotiazolino-6-sulfónico)

$CaCl_2$ – cloreto de cálcio

EDTA - ácido etilenodiamino tetra-acético

ELISA – ensaio de imunoabsorção enzimática

eNOS – óxido nítrico sintase endotelial

Early- Período inicial da prenhez, entre os dias 4 e 8 da gestação

Early Preg – ratas prenhas no período inicial da prenhez entre os dias 4 e 8 da gestação

Early-Preg+L-NAME – ratas prenhas hipertensas no período inicial da prenhez, entre os dias 4 e 8 da gestação

cGMP – guanosina monofosfato cíclico

HTN-Preg – prenhas hipertensas

HTN-Preg+Doxy- prenhas hipertensas tratadas com doxiciclina

iNOS – óxido nítrico sintase induzível

KDa - Kilodauto

L-NAME - Nω-nitro-L-arginina-metil éster

Late - período final da prenhez entre os dias 14 e 20 da gestação

Late-Preg- ratas prenhas no período final da prenhez entre os dias 14 e 20 da gestação

MDA – malondialdeído

Mid - período intermediário da prenhez entre os dias 9 e 15 da gestação

Mid Preg- ratas prenhas no período intermediário da prenhez entre os dias 9 e 15 da gestação

MMPs- metaloproteinases

MMP-2 – Metaloproteinases de matrix2

MMP-9 – Metaloproteinases de matrix 9

mmHg – milímetro de mercúrio

nNOS – óxido nítrico sintase neuronal

NO – óxido nítrico

Norm-Preg – prenhe normal

NOS – óxido nítrico sintase

$O_2^{\bullet -}$ - ânion superóxido

$OONO^-$ - peroxinitrito

PE – pré-eclâmpsia

PLGF – fator de crescimento placentário

Preg+Doxy- prenhas tratadas com doxiciclina

ROS – espécie reativa de oxigênio

RUPP – redução da pressão de perfusão uterina

SBP – pressão arterial sistólica

SDS – duodecil sulfato de sódio

sFlt-1 – fms-like tirosina quinase-1 solúvel

TBA – ácido tiobarbitúrico

TBARS – espécies reativas ao ácido tiobarbitúrico

TEAC – capacidade antioxidante equivalente do Trolox

VEGF – fator de crescimento endotelial vascular

Zn^{2+} - Zinco

Sumário

Sumário

Resumo

Resumo

O óxido nítrico (NO) é um gás produzido principalmente por células endoteliais, e durante a época gestacional, contribui para evitar aumento exacerbado da resistência vascular sistêmica (RVS), uma vez que, durante esse período, aumenta-se o volume sanguíneo e a frequência cardíaca. Quando, há disfunções endoteliais, reduz-se a biodisponibilidade de NO, provocando vasoconstrição e esta, por sua vez, consequentemente aumenta atividade das MMPs. Essa queda do NO e aumento das MMPs culminam em desordens hipertensivas gestacionais. Segundo estudos anteriores realizados em modelos de hipertensão em machos a doxiciclina, um antibiótico derivado das tetraciclinas, diminuiu a pressão arterial sistólica devido a sua capacidade de inibir a atividade das MMPs. No entanto, há escassez de trabalhos que desenvolveram estudos sobre os efeitos da doxicicilina em modelos animais de hipertensão gestacional. Por esse motivo, investigamos os efeitos da doxiciclina na inibição das MMPs na hipertensão gestacional, induzida pelo Nω-nitro-L-arginina-metil éster (L-NAME) em ratas. Para tanto, realizamos zimografia para avaliar a atividade da MMP-2 e -9 na placenta, no útero e na aorta torácica. Ainda avaliamos a pressão arterial sistólica, o desenvolvimento feto-placentário e os metabólitos do NO. Também foi avaliada a capacidade antioxidante plasmática, os níveis plasmáticos de *soluble fms-like tyrosine kinase-1* (sFlt-1) e o fator de crescimento placentário (PLGF). Nossos dados mostram que o tratamento com L-NAME aumentou a pressão arterial e diminuiu a prole destes animais. Por outro lado, a doxiciclina preveniu a hipertensão gestacional induzida por L-NAME sem afetar a prole. Observou-se também uma diminuição da biodisponibilidade de NO em ratas hipertensas. O tratamento com doxiciclina atenuou o aumento da atividade da MMP-2 e -9, provocados pela hipertensão induzida por L-NAME. Concomitantemente, a doxiciclina restaurou o equilíbrio angiogênico por meio do aumento dos níveis de PLGF e redução dos níveis de sFlt-1. A doxiciclina também aumentou a capacidade antioxidante do plasma nas ratas hipertensas. Por

causa disso, compreendemos que o aumento da atividade da MMP-2 e -9 deveu-se a uma redução do NO, causada pelo tratamento com L-NAME. Cabe ressaltar que o aumento da ativação de MMPs e o desequilíbrio angiogênico causados pelo modelo de hipertensão gestacional induzido por L-NAME estão associados ao aumento da pressão arterial sistólica, enquanto a doxiciclina restabeleceu todos esses parâmetros. Visto a importância do NO durante a gestação saudável, quanto durante a gestação hipertensiva devido a sua diminuição, realizamos um segundo estudo, em que avaliamos a atividade de MMP-2 e -9 em três diferentes períodos gestacionais: inicial (Early), intermediário (Mid) e tardio (Late). Nesse segundo estudo, observamos que a pressão arterial sistólica apresentou-se elevada nos três períodos gestacionais propostos, evidenciando-se principalmente nos dois últimos períodos (intermediário e tardio) em ratas hipertensas. Paralelamente, nesse mesmo grupo de animais, os níveis de NO plasmático apresentaram-se insuficientes, principalmente nos períodos gestacionais intermediário e tardio, em que há uma maior demanda de NO. Além disso, os parâmetros feto-placentários foram comprometidos em ratas hipertensas. Os níveis plasmáticos de malondialdeído e a atividade da MMP-2 e -9 apresentaram aumentos ao longo dos períodos gestacionais previamente adotados em ratas hipertensas. Em suma, os dados apresentados tanto no primeiro quanto no segundo estudo sugerem que o processo gestacional por si requer uma maior biodisponibilidade de NO e atuação do NO na modulação da atividade das MMPs ao longo da gestação, sendo essa demanda maior nos períodos finais da gestação.

Palavras chaves: Hipertensão gestacional; doxiciclina; metaloproteinases; óxido nítrico; ratas

Abstract

Abstract

Nitric oxide (NO) is a gas produced mainly by endothelial cells, and during the gestational period contributes to avoid an exacerbated increase in systemic vascular resistance (SVR), since during this period, there is a rise in blood volume and heart rate. When there are endothelial dysfunctions, the bioavailability of NO is reduced, causing vasoconstriction and this, in turn, consequently increases the activity of MMPs. This decrease in NO and increase in MMPs culminates in gestational hypertensive disorders. According to previous studies in non-gestational hypertension models, doxycycline, an antibiotic derived from tetracyclines, lowered systolic blood pressure due to its ability to inhibit MMP activity. However, there is a shortage of works that have developed studies on the effects of doxycycline in animal models of gestational hypertension. For this reason, we investigated the effects of doxycycline on the inhibition of MMPs in gestational hypertension, induced by Nω-nitro-L-arginine-methyl ester (L-NAME) in rats. We performed a zymography to evaluate the activity of MMP-2 and -9 in the placenta, uterus and thoracic aorta. We also evaluated systolic blood pressure, fetal-placental development and NO metabolites. Plasma antioxidant capacity, plasma levels of soluble fms-like tyrosine kinase-1 (sFlt-1) and placental growth factor (PLGF) were also evaluated. Our data shows that the treatment with L-NAME increased blood pressure and decreased the offspring of these animals. On the other hand, doxycycline prevented L-NAME-induced gestational hypertension and the reduction of offspring. A decrease in NO bioavailability in hypertensive rats was also observed. Treatment with doxycycline attenuated the increased activity of MMP-2 and -9, caused by L-NAME-induced hypertension. Concomitantly, doxycycline restored the angiogenic balance by increasing PLGF levels and reducing levels of sFlt-1. Doxycycline also increased plasma antioxidant capacity in hypertensive rats. For this reason, we understand that the increased activity of MMP-2 and -9 was due to a reduction of NO caused by the treatment with L-NAME. It should be noted that

the increase in MMP activation and angiogenic imbalance caused by the L-NAME-induced gestational hypertension model are associated with increased systolic blood pressure, while doxycycline reestablished all of these parameters. Considering the importance of NO during a healthy gestation and during a hypertensive pregnancy due to its decrease, we performed a second study, in which we evaluated the activity of MMP-2 and -9 in three different gestational periods: initial, intermediate and late. In this second study, we observed that systolic blood pressure was elevated in the three proposed gestational periods, evidencing mainly in the last two periods (intermediate and late) in hypertensive rats. At the same time, in this same group of animals, plasma NO levels were insufficient, especially in the intermediate and late gestational periods, where there is a greater demand for NO. In addition, the fetal-placental parameters were compromised in hypertensive rats. Plasma levels of malondialdehyde and MMP-2 and -9 activity increased gradually over the gestational periods previously adopted in hypertensive rats. In summary, the data presented in both the first and second studies suggests that the gestational process itself requires a greater bioavailability of NO, and NO action in the modulation of the MMP activity during pregnancy, being this demand is greater in the final periods of gestation.

Keywords: Gestational hypertension; doxycycline; metalloproteinases; nitric oxide; rats

Introdução

1 Introdução

1.1 A gestação e as mudanças hemodinâmicas

Devido à ocorrência da gestação, há inúmeras mudanças ocorrendo no corpo da mãe, ocasionadas, sobretudo por alterações hormonais. Relaciona-se com esse processo o remodelamento vascular e mudanças hemodinâmicas na circulação materna para o desenvolvimento fetal (Majed e Khalil, 2012), incluindo o aumento do volume sanguíneo, do débito cardíaco (Lopes Van Balen *et al.*, 2013) e do volume plasmático (Rodger *et al.*, 2015).

No primeiro trimestre, durante a sexta semana gestacional, o volume sanguíneo começa a aumentar, isso é notável até o inicio do segundo trimestre (Pisani *et al.*, 2017). O aumento de volume sanguíneo chega a progredir 50% entre 34 semanas gestacionais e o final do terceiro trimestre e o volume plasmático aumenta mais que 50–60% (Soma-Pillay *et al.*, 2016). O volume sanguíneo atinge em média 73-96 mL/kg a mais do que os valores normais de uma mulher não gestante (Ouzounian e Elkayam, 2012). Além disso, as mulheres no terceiro trimestre podem apresentar aumento do volume intersticial e plasmático, com maior volume de líquido extracelular no espaço intravascular em gestantes comparadas às não gestantes (Bird, Zhang e Magness, 2003).

O débito cardíaco atinge um aumento de 20% ainda na oitava semana gestacional e atinge 40% de aumento entre a vigésima e a vigésima oitava semana (Soma-Pillay *et al.*, 2016), podendo alcançar os 100%, no final do período gestacional, quando comparado a uma mulher não gestante (Lopes Van Balen *et al.*, 2013).

Esses parâmetros aumentados consequentemente poderiam promover o aumento da pressão arterial, no entanto, concomitantemente a essas várias mudanças, ocorrem também no sistema cardiovascular, durante toda a gestação, modificações para dilatar os vasos e diminuir a resistência (Dang *et al.*, 2013).

A redução da resistência vascular tem sido atribuída principalmente ao remodelamento das artérias espiraladas (James, Saghian et al. 2018), realizado por meio da migração dos trofoblastos, que substituem as células endoteliais da artéria espiralada uterina (Whitley and Cartwright 2010). Essa diferenciação dos citotrofoblastos deve-se ao papel das metaloproteinases (Zhang, Qi et al. 2010). Essas alterações dilatam os vasos de alta resistência, transformando-os em grandes vasos, com um fluxo sanguíneo aumentado e uma pressão bem reduzida.

A redução da resistência vascular sistêmica deve-se ao aumento de agentes vasodilatadores como óxido nítrico e prostaciclinas (Gongora and Wenger 2015). A resistência vascular reduzida é uma importante adaptação para manter a pressão arterial sanguínea em níveis normais (Burton *et al.*, 2009; Ouzounian e Elkayam, 2012).

Dentre os mediadores envolvidos no tônus vascular, o principal é o óxido nítrico (NO) (Furchgott e Zawadzki, 1980). O NO é um mediador gasoso que está envolvido em diversos processos fisiológicos, ele é produzido enzimaticamente no endotélio, principalmente pela isoforma endotelial da NO sintase (eNOS), enzima ativada pelo cálcio intracelular. Essa ativação converte o aminoácido L-arginina em NO e citrulina, com isso, o NO produzido na célula endotelial vascular difunde-se para a célula muscular lisa, ativando a enzima guanilato ciclase solúvel a qual converte guanosina trifosfato em guanosina monofosfato cíclico (GMF). A GMF em seguida ativará a Proteína Kinase dependente do guanosina monofosfato cíclico, promovendo a redução de cálcio intracelular de três formas: aumento do recrutamento de cálcio para o retículo endoplasmático, ativação do efluxo de cálcio intracelular e inibição dos canais para entrada de cálcio, resultando em vasodilatação (Takimoto, 2012). No entanto, estudos sugerem que havendo comprometimento dessa via do NO, poderá haver a ocorrência de hipertensão, durante a gestação.

1.2 Complicações Hipertensivas Gestacionais

O período gestacional pode ser o tempo mais propenso a complicações hemodinâmicas (Santos e Couto, 2018), tendo sido associado a vários distúrbios materno-fetais, como parto prematuro, complicações vasculares e o desenvolvimento de doença cardiovascular a longo prazo (Rich-Edwards *et al.*, 2014; Wu *et al.*, 2017). Desordens hipertensivas gestacionais afetam até 10% das mães em todo o mundo (Sutton, Harper e Tita, 2018). Elas são classificadas como:

- ✓ Hipertensão crônica/pré-existente: hipertensão (sistólica ≥ 140 ou diastólica ≥ 90 mmHg), diagnosticada antes da gestação ou antes da 20ª semana gestacional ou durante a gestação e não regride após o parto (Maloney, Heller e Baergen, 2012).
- ✓ Hipertensão gestacional: hipertensão diagnosticada após a 20ª semana gestacional, sem presença de proteinúria ou complicações sistêmicas. Há a regressão do quadro hipertensivo em aproximadamente 12 semanas após o parto (Sibai, 2003).
- ✓ Pré-eclâmpsia: hipertensão (sistólica ≥ 140 ou diastólica ≥ 90 mmHg) diagnosticada após a 20ª semana de gestação com presença de proteinúria (≥0,3g/24h) (Roberts *et al.*, 2003).
- ✓ Hipertensão crônica/pré-existente sobreposta por pré-eclâmpsia: mulheres com hipertensão crônica que desenvolvem proteinúria ou outra alteração clínica ou laboratorial característica da pré-eclâmpsia (Tuuli *et al.*, 2011)
- ✓ Eclâmpsia: é definida como a ocorrência de conseqüências neurológicas causadas como convulsões em associação com a pré-eclâmpsia (Mahran *et al.*, 2017).
- ✓ Síndrome HELLP: apresenta características severas de pré-eclâmpsia com hemólise, níveis elevados de enzimas hepáticas, e baixos níveis de plaquetas (Kongwattanakul *et al.*, 2018).

1.3 Fisiopatologia da PE

A PE está entre as complicações médicas mais comuns da gravidez (Lavie *et al.*, 2018). É uma síndrome exclusiva da gestação humana e sua fisiopatologia ainda não está completamente elucidada (Kallela *et al.*, 2016). A PE continua a ser uma das principais causas de morbidade e mortalidade materna e perinatal (Spracklen *et al.*, 2016), sendo a principal causa de parto prematuro, a fim de proteger a mãe e o feto (Hashemi *et al.*, 2017). Atualmente não existe tratamento eficiente para PE, a não ser a retirada da placenta (Bortolotto, Francisco e Zugaib, 2018). Os médicos realizam esse procedimento porque, apesar da PE não ter etiologia totalmente esclarecida, há o alívio dos sintomas após o parto. Isso nos sugere que a placenta tem um papel importante associado a esta desordem.

A placenta é o órgão materno-fetal que começa a se desenvolver nos períodos iniciais da gestação e tem a função de realizar as trocas de nutrientes e gases entre a mãe e o feto. Estudos sugerem que diversos fatores de risco podem estar relacionados à ocorrência de uma falha no processo de placentação e ao desenvolvimento de desordens hipertensivas da gestação. Entre esses fatores, estudos mostram que a genética tem se mostrado importante no desenvolvimento da PE (Founds *et al.*, 2009), com uma maior incidência em mulheres afro-americanas do que em mulheres asiáticas (Rosenberg *et al.*, 2005).

De acordo com Lamminpaa., et al (2012), a idade também parece ser um fator de risco, uma vez que mulheres gestantes mais velhas têm apresentando maior prevalência de PE do que mulheres jovens. Além disso, o aumento de resposta imune pode causar a apoptose de células trofoblásticas e prejudicar a invasão trofoblástica, com inadequado remodelamento das artérias espiraladas e consequente má formação placentária (Redman e Sargent, 2010; Negishi *et al.*, 2018).

O desenvolvimento anormal da placenta e consequente isquemia/hipóxia placentária se relacionam com o primeiro estágio do desenvolvimento da PE (Rodriguez *et al.*, 2018). A

isquemia/hipóxia placentária propicia o segundo estágio do desenvolvimento da PE por promover a liberação de diversos fatores bioativos circulantes que atuam sobre o endotélio e desencadeiam a disfunção endotelial sistêmica, causando a vasoconstrição generalizada, aumento da resistência vascular periférica e hipertensão como ocorre na PE (Ali e Khalil, 2015).

Dentre os diferentes fatores ativos que podem contribuir para o dano endotelial durante a hipertensão gestacional, foi identificado o fms-like tirosina quinase-1 solúvel (sFlt-1), o qual é um receptor solúvel para o Fator de Crescimento Endotelial Vascular (VEGF). O sFlt-1 se liga às moléculas de VEGF e do Fator de Crescimento Placentário (PLGF) circulantes, impedindo que esses fatores angiogênicos se acoplem aos seus receptores usuais na membrana celular (Di-Marco et al., 2009). Por esse motivo, estudos sugerem que o sF1t-1 possui papel importante no dano às células endoteliais e que o excesso de sFlt-1 poderia ter papel na fisiopatologia da PE por abolir as respostas vasodilatadoras mediadas por VEGF e pelo PLGF (Maynard *et al.*, 2003).

Estudos experimentais mostraram que os níveis de sFlt-1 encontram-se elevados em modelos animais de hipertensão gestacional (Agunanne *et al.*, 2010; Baijnath *et al.*, 2017) e também em mulheres com PE (Bian, Shixia e Duan, 2015). Ainda, devido a um aumento de espécies reativas de oxigênio (ROS), há a rápida reação do O^{2-} (ânion superóxido) com NO para formar peroxinitrito ($OONO^-$) e diminuição da biodisponibilidade de NO (Di Marco *et al.*, 2009).

Como a disfunção endotelial é caracterizada por uma diminuição da liberação de fatores de relaxamento e um aumento da liberação de fatores de contração derivados do endotélio, a diminuição do NO, um importante fator de relaxamento do endotélio vascular, pode estar relacionada, conforme estudos experimentais em ratas do modelo de hipertensão

gestacional (Baijnath, Soobryan et al. 2014) e em mulheres com PE (Ehsanipoor, Fortson et al. 2013), com essa diminuição do relaxamento vascular.

É claramente notável o importante o papel do NO e de suas interações com diversas substâncias de interesse na hipertensão gestacional. Uma maneira de modular farmacologicamente a síntese endógena do NO é utilizando ω-Nitro-L-arginine methylester (L-NAME), um inibidor não seletivo da síntese de NO. O mecanismo de ação do L-NAME consiste em um falso substrato para sintase NO, ou seja, o L-NAME compete com a L-Arginina pela Nos. Dessa maneira, não há a síntese de NO e citrulina.

Tem-se utilizado o L-NAME como agente farmacológico indutor de hipertensão arterial em ratas prenhes por causá-la e por restringir o crescimento fetal, mimetizando exatamente o que ocorre na PE.

1.4 Metaloproteinases

Assim como o NO, as metaloproteinases também se mostram alteradas durante a hipertensão gestacional. Elas são enzimas envolvidas na degradação de componentes da matrix extracelular, exercendo remodelamento e reparação tecidual nos processos fisiológicos, como a angiogênese e a morfogênese (Stamenkovic, 2003; Theocharis *et al.*, 2016).

Atualmente são identificados 28 tipos de metaloproteinases, que são classificadas em seis grupos: **(1)** colagenases (MMP-1, MMP-8, MMP-13 e MMP-18), **(2)** gelatinases (MMP-2 ou gelatinase A e MMP-9 ou gelatinase B), **(3)** estromelisinas (MMP-3 ou estromelisina 1 e MMP-10 ou estromelisina 2), **(4)** matrilisinas (MMP-7 ou matrilisina 1 e MMP-26 ou matrilisina 2), **(5)** MMPs de membranas (MT1-MMP(MMP-14), MT2- MMP (MMP-15), MT3-MMP (MMP-16), MT4-MMP, [MT5-MMP e MT6-MMP (MMP- 25)] e **(6)** outras

MMPs (MMP-12, MMP-20, MMP-21, MMP-23, MMP-27 e MMP-28) (Visse e Nagase, 2003).

As MMPs são secretadas como zimogênios inativos e requerem uma ativação proteolítica a qual ocorre após a remoção do pró-peptídeo que disponibiliza o sitio ativo para catálise (Lu *et al.*, 2011; Khokha, Murthy e Weiss, 2013). As MMPs podem ser ativadas por vários fatores, dentre eles, hormônios, estresse oxidativo e estresse de cisalhamento (Rizzi *et al.*, 2009).

1.5 O papel das MMP-2 e MMP-9 em doenças cardiovasculares:

As metaloproteinases estão envolvidas em muitos processos fisiológicos, entretanto essas mesmas enzimas participam de processos patológicos (Barbosa, Gerlach e Tanus-Santos, 2006), e de diversos eventos cardiovasculares (Castro e Tanus-Santos, 2013), especificamente MMP-2 e MMP-9, que se destacam nas doenças cardiovasculares (Wang e Khalil, 2018).

As gelatinases são capazes de clivar diferentes tipos de colágeno, incluindo o tipo IV, V e XI (Xu *et al.*, 2001). O colágeno tipo IV é o componente mais importante da membrana basal vascular (Hudson, Reeders e Tryggvason, 1993). Em modelo experimental com ratos espontaneamente hipertensivos, houve mudanças morfológicas na parede aórtica, com significativo aumento da atividade e expressão da MMP-2 (Antonio *et al.*, 2014). Neste contexto, estudos têm mostrado que a atividade excessiva ou desequilibrada de MMP-2 e MMP-9 estão associadas à patogênese de hipertensão e remodelamento vascular em modelos animais (Pereira *et al.*, 2018) e em humanos (Kostov *et al.*, 2016).

Curiosamente, estudos experimentais mostraram MMP-2 aumentada em tecidos de útero e aorta de ratas prenhas (Dang *et al.*, 2013) e em todas as regiões da placenta de mulheres gestantes hipertensas (Sahay *et al.*, 2018b). Além disso, um estudo clínico observou

mulheres em diferentes períodos de gestação e mostrou uma aumentada concentração de MMP-2 no plasma sanguíneo de gestantes a partir da vigésima segunda semana gestacional (Myers *et al.*, 2005). Foi demonstrado que a MMP-2 também se encontra em concentrações elevadas na urina de gestantes prestes a desenvolver a PE (Martinez-Fierro *et al.*, 2018). Ademais, aumentada atividade de MMP-9 tem sido correlacionada de forma positiva com os sintomas de hipertensão gestacional (Xiao *et al.*, 2018). Alguns estudos também mostraram aumentados níveis circulantes de ambas gelatinases MMP-2 e MMP-9 em mulheres com pré-eclâmpsia, quando comparado a gestantes normais (Eleuterio *et al.*, 2015).

Pelo exposto, fica claro a escassez de estudos e o importante papel desempenhado pelas MMPs durante desordens hipertensivas da gestação e que a inibição dessas enzimas pode ser uma estratégia terapêutica interessante para essa patologia. Estudos utilizando modelos animais de hipertensão mostraram que a doxiciclina atua inibindo as MMPs e por consequência impede alterações anormais na estrutura vascular associadas à hipertensão arterial (Castro, Tanus-Santos e Gerlach, 2011).

1.6 Doxiciclina, uma estratégia para inibir as metaloproteinases aumentadas durante a hipertensão gestacional

A doxiciclina é um antibiótico semi-sintético derivado das tetraciclinas, capaz de inibir a atividade das MMPs, entretanto o mecanismo de ação dessa inibição não está totalmente esclarecido. Sabe-se que as tetraciclinas são quelantes de Zn^{2+}, sugere-se assim que a doxiciclina possa se ligar próximo ao Zn^{2+}, bloqueando o sítio catalítico e inibindo a atividade de MMPs (Garcia *et al.*, 2005). Especula-se ainda que o mecanismo de ação da inibição de MMPs ocorra por meio da indução da atividade de inibidores endógenos teciduais (Yao *et al.*, 2007).

Em modelos experimentais com ratos, a doxiciclina inibiu a atividade da MMP-9 na dosagem de 5mg/dia e sua expressão foi coibida na dose de 10 mg/dia (Lee *et al.*, 2006). Em humanos, a atividade da MMP-2 foi reduzida pela doxiciclina na dose de 20mg/dia (Schulze *et al.*, 2013).

A doxiciclina é considerada o inibidor mais potente e não seletivo de MMPs (Castro *et al.*, 2012). Além disso, em estudos experimentais, não foi encontrada teratogenicidade em ratos com dose superior a 100 vezes a dose humana de doxiciclina (Nahum,et al., 2006).

A doxiciclina é de baixa toxicidade oral, em um estudo experimental de toxicidade crônica com diversas doses de doxiciclina em ratos, hamsters, porcos, cães e macacos, notou-se um efeito NOEL (nenhum nível de efeito observado) na dose 25mg/kg (figura 1), durante um mês de tratamento. Por esse motivo, concluiu-se que não há evidências de toxicidade reprodutiva ou de desenvolvimento como também nenhuma evidência de potencial genotóxico, (EMA/MRL, 1997).

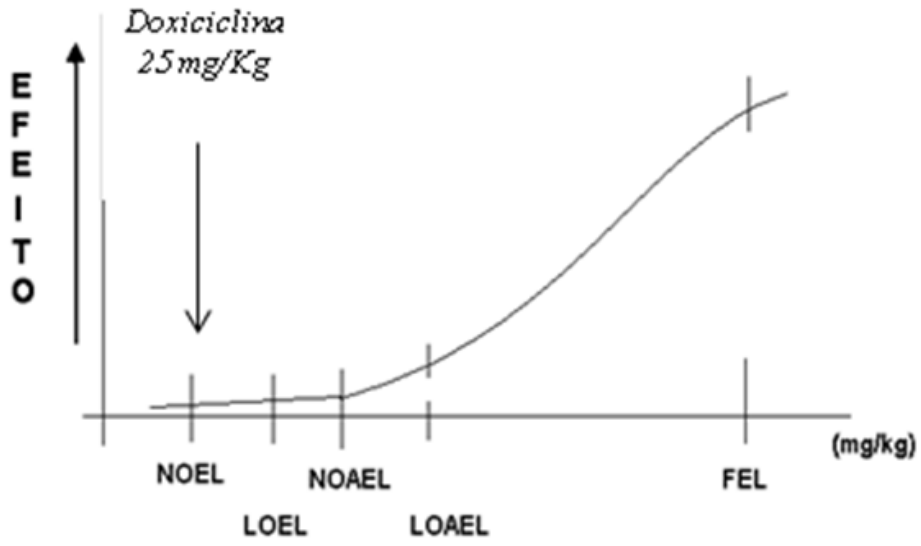

Figura 1: Gráfico do Efeito versus dosagem recebida. Sendo NOEL nenhum nível de efeito observado; LOEL baixo nível de efeito observado; NOAEL nenhum nível de efeito adverso observado; LOAEL baixo nível de efeito adverso observado e FEL Franco nível de efeito.

Em suma, a hipótese deste trabalho seria a de que a atividade das MMPs estaria aumentada devido à diminuição dos níveis de NO durante a hipertensão gestacional induzida por L-NAME. Para confirmá-la, investigamos se a doxiciclina reduz a atividade da MMP-2 e da -9 e atenua a hipertensão gestacional no período final da gestação. Além disso, com a finalidade de entendermos um pouco mais a relação dessas metaloproteinases com a formação endógena do NO durante as mudanças uteroplacentárias e o remodelamento vascular em diferentes períodos de tempo da gestação saudável e hipertensiva, realizamos um segundo estudo, em que avaliamos a pressão arterial sistólica, a biodisponibilidade do NO, os parâmetros feto-placentário e a atividade de MMP-2 e -9 em três diferentes períodos gestacionais: inicial (Early), intermediário (Mid) e tardio (Late) em ratas tratadas com salina ou L-NAME.

Capítulo I

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Hypertension, augmented activity of matrix metalloproteinases-2 and -9 and angiogenic imbalance in hypertensive pregnancy are attenuated by doxycycline

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ABSTRACT

Preeclampsia is manifested as maternal hypertension and fetal growth restriction. Matrix metalloproteinases (MMPs) are involved in hypertension and doxycycline reduces blood pressure by inhibition of MMPs. Moreover, excessive levels of MMPs and reduced nitric oxide (NO) bioavailability have been related to preeclampsia. We investigated the involvement of MMPs in hypertension in pregnancy induced by Nω-Nitro-L-arginine methyl ester (L-NAME) in rats. To this end, zimography was performed to evaluate the activity of MMPs -2 and -9 in placenta, uterus and thoracic aorta, and systolic blood pressure, feto-placental development and metabolites of NO were evaluated. Also, plasma antioxidant capacity, plasma levels of soluble fms-like tyrosine kinase-1 (sFlt-1) and placental growth factor (PLGF) were examined. Doxycycline prevented hypertensive pregnancy and significant reductions in number of pups induced by L-NAME. Low NO bioavailability was found in hypertensive pregnant rats treated (or not) with doxycycline. Increased activity of placental MMP-2 and MMP-9 and uterine MMP-2 were attenuated by doxycycline. MMP-2 activity of thoracic aorta showed no change after hypertension. Increases in PLGF with concomitant decreases in sFlt-1 levels were found with doxycycline treatment. Also, plasma antioxidant capacity was improved with doxycycline. Also, elevations of plasma antioxidant capacity were observed in hypertensive rats treated with doxycycline. Therefore, we suggest that L-NAME reduced NO and this triggered the increases in MMP-2 and -9 activities during hypertensive pregnancy. Importantly, increases in MMPs activation and angiogenic imbalance were attenuated by doxycycline and these effects were associated with decreases in systolic blood pressure.

1. Introduction

Normal pregnancy is marked by physiological adaptations (Pisani et al., 2017), including increased blood volume (Osol and Bernstein, 2014) and cardiac output (Osol et al., 2017) with concomitant decreases in systemic vascular resistance (Mazzuca et al., 2014; Tong et al., 2017), thus preventing increases in blood pressure. Vascular tone during gestation is modulated by nitric oxide (NO), that contributes to the decreased vascular resistance (Cavalli et al., 2012) in pregnant women (Hodzic et al., 2017) and rats (Valdes et al., 2009).

Since normal pregnancy has been associated with increased NO production (Hodzic et al., 2017; Valdes et al., 2009), non-selective NO synthases (NOS) inhibitor, L-NAME, which reduces NO formation by competing with the precursor L-arginine for NOS, has been used to promote hypertension-in-pregnancy in rats (Altoama et al., 2016; Goncalves-Rizzi et al., 2018).

Preeclampsia is a hypertensive disorder of gestation (Say et al., 2014) characterized by hypertension after the 20th gestational week (ACOG, 2013; Banoth and Chaudhary, 2018) and is a leading cause of intrauterine growth restriction (von Dadelszen and Magee, 2014), threatening the life of both mother and fetus. Moreover, women with preeclampsia (Motta-Mejia et al., 2017) and animal models of hypertensive pregnancy (Goncalves-Rizzi et al., 2018) have shown decreases in NO levels.

MMPs are zinc-dependant enzymes that degrade extracellular matrix and are involved in several physiological and pathological

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conditions (Wang and Khalil, 2018). Particularly, the isoenzymes-2 and -9 are gelatinolytic MMPs which activities are up-regulated in the uterus and aorta during pregnancy of different animals, such as rats (Dang et al., 2013; Li et al., 2014; Yin et al., 2012), bitches (Schafer-Somi et al., 2005) and bovines (Ulbrich et al., 2011). Although increased activity of MMPs during normal pregnancy may be required to a proper vascular adaptation for placentation (Cohen et al., 2010), an excessive MMPs activity may contribute to pathophysiological processes (Grasso and Bonnet, 2014) as hypertension (Pereira et al., 2018) and hypertension-in-pregnancy (Martinez-Fierro et al., 2018; Myers et al., 2005). Importantly, NO is capable of modulating MMPs (Demacq et al., 2008; O'Sullivan et al., 2014) and inhibition of NO synthesis due to L-NAME promoted increases in MMP-2 and -9 activities (Del Mauro et al., 2017; Souza-Costa et al., 2005), linking reduced NO formation and increased MMPs activity (Metzger et al., 2012). Thus, reduction of excessive MMPs activity in hypertensive pregnancy may be interesting to understand the relationship between NO and MMPs. In this context, experimental studies have shown that doxycycline inhibits MMPs and attenuates hypertension (Castro and Tanus-Santos, 2014; Mata et al., 2015; Nascimento et al., 2015). However, this is uncertain in hypertensive pregnancy. Therefore, we hypothesized that activity of MMPs is enhanced due to decreases in NO levels in hypertensive pregnancy induced by L-NAME. Also, we investigated whether doxycycline may reduce MMP-2 and -9 activities and attenuate hypertension in pregnancy.

2. Materials and methods

2.1. Animals and experimental protocol

Wistar rats (200–250 g) were used. Rats were maintained in controlled conditions (22 ± 2 °C, 12-hr light/dark cycle) and given free access to standard rat chow and tap water. Each female rat was separately mated overnight and day one of pregnancy was defined as the day when spermatozoa were found in a vaginal smear.

On pregnancy day 13, female were randomized and assigned into four groups: pregnant rats receiving daily 0.9% saline solution injections (intraperitoneal - i.p.) and 0.9% saline solution (gavage) (Norm-Preg, n = 10); pregnant rats receiving injections (i.p.) of (L-NAME) 60 mg/Kg/daily and 0.9% saline solution (gavage) (HTN-Preg, n = 10); pregnant rats receiving daily 0.9% saline solution injections (i.p.) and doxycycline 20 mg/kg (gavage - Preg + Doxy, n = 10); pregnant rats receiving injections (i.p.) of L-NAME 60 mg/Kg daily and doxycycline in a dose of 20 mg/kg (gavage - HTN-Preg + Doxy, n = 10). Doxycycline dosage was chosen based on previous studies showing antihypertensive effect and reduced activity of MMP in rats (Antonio et al., 2014; Bouvet et al., 2005; Guimaraes et al., 2011).

On pregnancy day 21, female were killed under overdose of isoflurane followed by exsanguination. Blood samples were collected in lyophilized ethylenediaminetetraacetic acid (EDTA) and lyophilized heparin containing tubes (Vacuntainer Becton-Dickinson, BD, Oxford, UK) and immediately centrifuged. Plasma was separated and stored at − 80 °C until use for biochemical analysis.

Fetal and placental weights, litter size, number of viable fetuses and number of resorptions were recorded. Viable fetuses were determined as those which showed no macroscopical sign of malformation and could apparently have a normal outcome with the progression of the pregnancy, as previously reported (Ma et al., 2010).

All procedures for animal experimentation were approved by the Ethics Committee, Biosciences Institute of Botucatu, São Paulo State University (Protocol #741/2015), which is complied with international guidelines of the European Community for the use of experimental animals.

2.2. Blood pressure measurements

Systolic blood pressure was measured on gestational days 13, 14, 16, 18 and 20 by tail cuff plethysmography (Insight, Ribeirao Preto, Sao Paulo, Brazil, # EFF-306) (Goncalves-Rizzi et al., 2018; Nascimento et al., 2015). Briefly, rats were restrained and pre-warmed in a warm-box (Insight, Ribeirao Preto, Sao Paulo, Brazil, # EFF-307) at 40 °C for 10 min. Then, systolic blood pressure was determined as the average of the cuff inflation-deflation (3–6 cycles) by a trained operator. Measurements of systolic blood pressure on gestational day 13 were performed before randomization of the animals into different groups (before gavage or i.p. injections) and represent baseline values. Measurements on gestational days 14, 16, 18 and 20 were performed 6 h after drugs or saline administrations.

2.3. Determination of plasma levels of nitrite/nitrate (total NOx)

Plasma total NOx concentrations were determined using Griess reagents followed by reduction of nitrous species with vanadium chloride III (Miranda et al., 2001). Briefly, before addition of Griess reagents, samples were incubated with 100 µl of saturated solution of vanadium chloride III for 3 h at 37 °C with agitation. After incubation, 50 µl of 1% sulfanilamide solution in 5% phosphoric acid was added and plate incubated for 10 min protected from light. Then, 50 µl of 0.1% N-(1-Naphthyl)- ethylenediamine dihydrochloride solution were added followed by 10-min incubation in dark. Absorbance at 535 nm was read in spectrophotometer (Synergy 4, BIOTEK, Winooski, VT) and NOx concentration was calculated using a standard curve of sodium nitrite (1.56–100 µM). The metabolites levels of NO (NOx) in plasma were expressed in µmol/L.

2.4. Determination of fms-like tyrosine kinase-1 (sFlt-1) and placental growth factor (PLGF)

Commercial enzyme immunoassay (ELISA) kits for sFlt-1 (RayBiotech Inc., Norcross, GA, #ELM-VEGFR1) and PLGF (Elabscience Inc., Houston, TX, #E-EL-R0742) were used to determine plasma levels. Assays were performed according to the manufacturer's instructions. Plasma levels of sFlt-1 and PLGF were expressed in pg/ml.

2.5. Determination of plasma antioxidant capacity

The trolox equivalent antioxidant capacity (TEAC) was performed as previously described (Erel, 2004). In summary, 100 µg of Trolox (6-hidroxy-2,5,7,8 - tetramethylchroman- 2-carboxylic-acid, Sigma, St. Louis, MO, USA, catalogue #238813) was mixed in 1 ml of sodium acetate buffer (0.4 M, $C_2H_3NaO_2{\cdot}3H_2O$) + glacial acetic acid (0.4 M) to establish a standard curve. Initially, 20 µl of plasma samples were added to 200 µl of sodium acetate buffer + glacial acetic acid and the absorbance at 660 nm was read with the spectrophotometer (Synergy 4, BIOTEK, Winooski, VT, USA). In a second moment, 20 µl of sodium acetate buffer (0.03 M) and glacial acetic acid (0.03 M) + H_2O_2 + ABTS (2,20-azino- bis(3-ethylbenzthiazolin- 6 sulfonic acid), Sigma, St. Louis, MO, USA, catalogue #1888) was added to the samples and incubated for 5 min. A second spectrophotometer read was performed at 660 nm and the second reading values were subtracted from the values found in the first reading. The plasma antioxidant activity was showed as mmol of Trolox equivalent/L.

2.6. Determination of lipid peroxidation

Lipid peroxidation was assessed through measurements of thiobarbituric acid reactive substances (TBARS). Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA), which is the end product of lipid peroxidation to form a magenta color that is read by spectrophotometer at wavelength of 532 nm (Perico et al., 2015). In test tubes, a reaction

mixture containing 100 µl of distilled water, 50 µl of 8.1% sodium dodecyl sulfate (SDS), 100 µl of plasma samples, 375 µl of acetic acid 20% and 375 µl of TBA 0.8% were incubated in water-bath at 95 °C for 1 h and thereafter, mixture was centrifuged at 1792 g for 10 min. Standard curve was made in a similar form, replacing samples with 25 µl of known concentrations of MDA. Plasma levels of TBARS were calculated compared to a standard curve of MDA (20–320 nmol). MDA concentrations were expressed in nmol/ml.

2.7. Zymography for MMP-2 and -9 activities in placenta, uterus and aorta

Gelatin zymography was used to determine MMP-2 and − 9 activities in placenta, uterus and aorta as previously described (Li et al., 2014; Rizzi et al., 2010, 2013). Briefly, frozen samples were homogenized in ice-cold RIPA buffer (1 mM 1,10 -ortho-phenanthroline, 1 mM phenylmethanesulfonyl fluoride, and 1 mM N- ethylmaleimide) and protease inhibitor (4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), E-64, bestatin, leupeptin, aprotinin, and EDTA) in a proportion of 100 µl RIPA + protease inhibitor for each 10 mg of tissue sample. The samples were placed in ice for 2 h, with gentle stirring and then centrifuged at 11200 g for 10 min. The protein concentrations were measured using the Bradford assay (Sigma Aldrich). 10 µg of uterus and aorta proteins and 5 µg of placenta proteins were diluted 1:1 with sample buffer (final concentration): 2% SDS, 125 mM Tris–HCl, pH 6.8, 10% glycerol, and 0.001% bromophenol blue and subjected to electrophoresis on 12% SDS–polyacrylamide gel electrophoresis copolymerized with gelatin (0.05%) as the substrate.

After electrophoresis was completed, gel was washed and incubated at 37 °C for 18 h in Tris–HCl buffer, pH 7.4, containing 10 mmol/L $CaCl_2$. Gels were stained with 0.05% Coomassie Brilliant Blue G-250 and then destained with 30% methanol and 10% acetic acid. Gelatinolytic activity was detected as an unstained band against the background of Coomassie blue-stained gelatin. Enzyme activity was assayed using ImageJ software by optical densitometry and the integrated protease activity density was measured as pixel intensity × mm^2, which was normalized to fetal bovine serum intensity, used as standard, to correct for loading and inter-gel variation, and the results were expressed as arbitrary units. The MMP-2 forms were identified as bands at 75, 72 and 64 kDa and MMP-9 as band at 92 kDa. Total MMP-2 was taken as a sum of the different isoforms.

2.8. Statistical analysis

Using commercially available statistical software (Graph Pad Prism® 6.0 for Windows, San Diego, CA, USA), a Shapiro-Wilk test was applied to verify normality of data distribution. Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons among groups. Differences were statistically significant when $P < 0.05$. All values are expressed as mean ± S.E.M.

3. Results

3.1. Doxycycline attenuates hypertension in pregnancy induced by reduced NO formation

There were no significant differences in basal systolic blood pressure among 40 rats on the 13th gestational day ($P > 0.05$, Fig. 1A), as well as on the 14th gestational day ($P > 0.05$, Fig. 1A). However, on gestational days 16, 18 and 20, systolic blood pressure was increased in animals from HTN-Preg compared to Norm-Preg (*$P < 0.05$, Fig. 1A) and Preg + Doxy ($^{+}P < 0.05$, Fig. 1A) groups. Moreover, increases in systolic blood pressure were blunted in HTN-Preg + Doxy group on gestational days 16, 18 and 20 compared to HTN-Preg group ($^{\#}P < 0.05$, Fig. 1A).

The NO formation was measured by Griess reaction (which is expressed as plasma concentrations of nitrite and nitrate – total NOx). We found significantly decreased total NOx in HTN-Preg when compared to Norm-Preg group (*$P < 0.05$, Fig. 1B). Doxycycline treatment in HTN-Preg + Doxy group did not change the decreases in NOx levels ($P > 0.05$, Fig. 1B). Moreover, plasma NOx in Preg + Doxy did not differ from Norm-Preg group ($P > 0.05$, Fig. 1B).

3.2. Doxycycline prevents decreases in total number of pups and in number of viable fetuses, without affecting fetal and placental weights in hypertensive pregnancy

Litter size was significantly reduced in HTN-Preg compared to Norm-Preg (*$P < 0.05$, Fig. 2A) and Preg + Doxy ($^{+}P < 0.05$, Fig. 2A) groups. Also, number of viable fetuses were significantly reduced in HTN-Preg *versus* Norm-Preg (*$P < 0.05$, Fig. 2B) and Preg + Doxy ($^{+}P < 0.05$, Fig. 2B) groups. However, HTN-Preg + Doxy group showed no significant changes in litter size and number of viable fetuses compared to Norm-Preg group ($P > 0.05$, Fig. 2A and B, respectively).

The number of reabsorbed fetuses showed no significant differences among four groups ($P > 0.05$, Fig. 2C). While fetal weights were significantly decreased in HTN-Preg and HTN-Preg + Doxy compared to Preg + Doxy group ($^{+}P < 0.05$, Fig. 2D), no significant reductions were found when compared to Norm Preg group ($P > 0.05$, Fig. 2D). Placental weights presented no significant changes among four groups ($P > 0.05$, Fig. 2E).

3.3. Anti-angiogenic/pro-angiogenic imbalance is reversed by doxycycline treatment in hypertensive pregnancy

Significant increases were found in plasma levels of sFlt-1 in HTN-Preg compared to Norm-Preg group (*$P < 0.05$, Fig. 3A). However, plasma levels of sFlt-1 were significantly lower in HTN-Preg + Doxy compared to HTN-Preg group ($^{\#}P < 0.05$, Fig. 3A). Plasma levels of sFlt-1 were not statistically different among Norm-Preg, Preg + Doxy and HTN-Preg + Doxy groups ($P > 0.05$, Fig. 3A). Also, while plasma levels of PLGF were decreased in HTN-Preg when compared to Norm-Preg group (*$P < 0.05$, Fig. 3B), HTN-Preg + Doxy group presented higher plasma levels of PLGF than HTN-Preg group ($^{\#}P < 0.05$, Fig. 3B) and that were similar to levels found in Norm-Preg and Preg + Doxy groups ($P > 0.05$, Fig. 3B).

3.4. Doxycycline treatment elevates plasma antioxidant capacity in hypertensive pregnancy

We examined plasma antioxidant capacity among the four experimental groups. No significant decreases in plasma antioxidant capacity were observed in HTN-Preg when compared to Norm-Preg ($P > 0.05$, Fig. 4A). However, plasma antioxidant capacity was reduced in HTN-Preg compared to Preg + Doxy group ($^{+}P < 0.05$, Fig. 4A). Importantly, doxycycline treatment in HTN-Preg + Doxy group increased plasma antioxidant capacity when compared to Norm-Preg (*$P < 0.05$, Fig. 4A) and HTN-Preg ($^{\#}P < 0.05$, Fig. 4A) groups. In addition, TBARS assay was used to assess plasma levels of MDA, which indicates oxidative stress. However, there were no significant differences among the four groups ($P > 0.05$, Fig. 4B).

3.5. Increased activity of placental MMP-2 and MMP-9 and uterine MMP-2 found in hypertensive pregnancy were attenuated by doxycycline

A representative zymogram of placental tissue homogenate is shown in Fig. 5A, which shows four bands corresponding to 92 kDa pro-MMP-9 and three isoforms of MMP-2: 75, 72, and 64 kDa. HTN-Preg rats presented an increase in 92 kDa pro-MMP-9 activity compared to Norm-Preg ($^{*}P < 0.05$) and Preg + Doxy ($^{+}P < 0.05$) groups (Fig. 5B). Moreover, doxycycline treatment in HTN-Preg + Doxy group decreased 92 kDa pro-MMP-9 activity compared to HTN-Preg group ($^{\#}P < 0.05$, Fig. 5B). Also, MMP-2 increased in HTN-Preg group to 75, 72 and 64

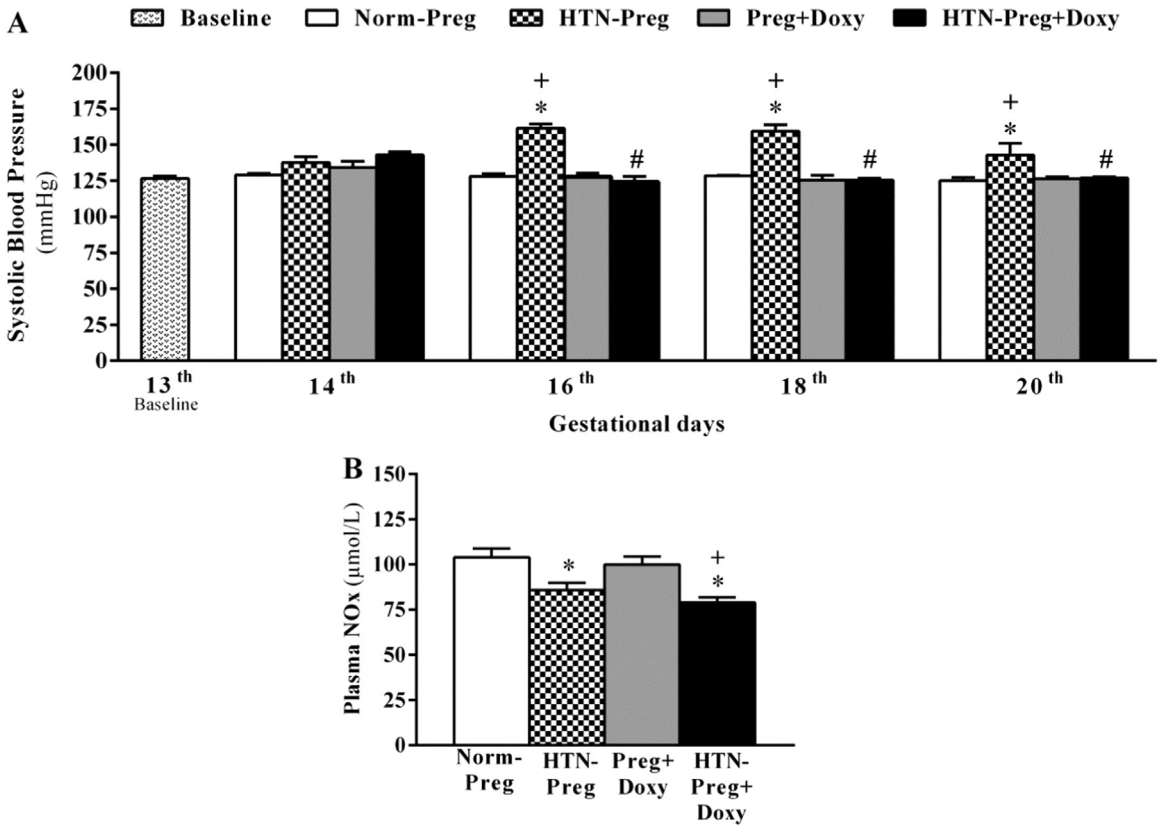

Fig. 1. Systolic blood pressure measurements **(A)** and plasma levels of nitrate + nitrite **(B)**. Systolic blood pressure measured by tail-cuff plethysmography on days 13, 14, 16, 18 and 20 of gestation. Baseline blood pressure represents a measure of systolic blood pressure in all animals prior to randomization into four groups: Normal pregnancy (Norm-Preg), pregnant rats treated with L-NAME (HTN-Preg), pregnancy + doxycycline (Preg + Doxy), and pregnant rats treated with L-NAME and doxycycline (HTN-Preg + Doxy). Values represent mean ± S.E.M. *P < 0.05 *versus* Norm-Preg; $^{+}$P < 0.05 *versus* Preg + Doxy; $^{\#}$P < 0.05 *versus* HTN-Preg.

isoforms and total KDa MMP-2 compared to Norm-Preg group (*P < 0.05, Fig. 5C-F). However, treatment with doxycycline in HTN-Preg + Doxy animals only reversed the increases observed in HTN-Preg group in 75 kDa MMP-2 ($^{\#}$P < 0.05, Fig. 5C). Moreover, 92 kDa pro-MMP-9, 75, 72 and 64 kDa isoforms and total MMP-2 in Preg + Doxy did not differ from the Norm-Preg group (P > 0.05, Fig. 5B-F).

A representative zymogram of uterus homogenate is shown in Fig. 5G. No corresponding band to 92 kDa pro-MMP-9 appeared in zymography gel, thus, zymogram shows three isoforms of MMP-2: 75, 72, and 64 kDa (Fig. 5G). MMP-2 activity increased in HTN-Preg group in 75, 72 and 64 isoforms and total KDa MMP-2 compared to Norm-Preg group (*P < 0.05, Fig. 5I-L). HTN-Preg + Doxy presented decreased activity of 75 and 64 kDa isoforms and total KDa MMP-2 compared to HTN-Preg group ($^{\#}$P < 0.05, Figs. 5I, 5K and 5L). However, HTN-Preg + Doxy animals did not show decreases in activity of pro-active 72 kDa MMP-2 (P > 0.05, Fig. 5J). Moreover, in Preg + Doxy group 75, 72 and 64 kDa isoforms and total MMP-2 did not differ from the Norm-Preg group (P > 0.05, Fig. 5I-L).

A representative zymography of aorta tissue homogenate is shown in Fig. 6A, which shows three bands corresponding to three isoforms of MMP-2: 75, 72, and 64 kDa and total MMP-2. However, there was no significant difference among the four groups in none of MMP-2 isoforms and total MMP-2 (P > 0.05, Fig. 6B-E).

4. Discussion

The main findings of the present study are that doxycycline attenuated L-NAME-induced hypertension in pregnant rats and reduced activity of placental MMP-2 and MMP-9 and uterine MMP-2. In addition, restored angiogenic balance could also be related with beneficial effects on systolic blood pressure and may therefore represent an important potential therapeutic target for doxycycline in hypertensive disorders of pregnancy.

In our hands, following doxycycline treatment, systolic blood pressure was attenuated in hypertensive pregnant rats (HTN-Preg + Doxy group), which is in agreement with previous studies. Doxycycline blunted the increase in systolic blood pressure in spontaneously hypertensive (Antonio et al., 2014), renovascular hypertension (Castro et al., 2012) and lead-induced hypertension (Nascimento et al., 2015) in rats.

We observed a decrease in NO levels in HTN-Preg group. This is in accordance with previous findings from our and other groups showing that inhibition of NOS with L-NAME promoted a decrease in circulating NO of pregnant rats (Baijnath et al., 2014; Goncalves-Rizzi et al., 2018; Possomato-Vieira et al., 2016). Also, we found that doxycycline treatment did not change the plasma levels of NO, thus, reductions in systolic blood pressure observed in HTN-Preg + Doxy group suggest that decreased activity of placental MMP-2 and -9, and uterine MMP-2, underlies antihypertensive effects.

Doxycycline treatment prevented the significant decreases in litter size and in the number of viable fetuses, but did not affect the low fetal weight caused by L-NAME. Importantly, low birth weight is a hallmark of hypertensive pregnant disorders (Chisholm et al., 2018) and improvements in fetal weight would be beneficial during treatment of hypertension in pregnancy.

Anti-angiogenic/pro-angiogenic imbalance may play a key role in the pathogenesis of hypertensive pregnancy (Osol et al., 2017). On the one hand, sFlt-1 is an important anti-angiogenic factor that has been shown to be elevated during hypertensive pregnancy (Goncalves-Rizzi et al., 2016; Maynard et al., 2003); on the other hand, PLGF is a pro-angiogenic factor that has been shown to be decreased in women with preeclampsia (Bian et al., 2015; Molvarec et al., 2015) and in animal

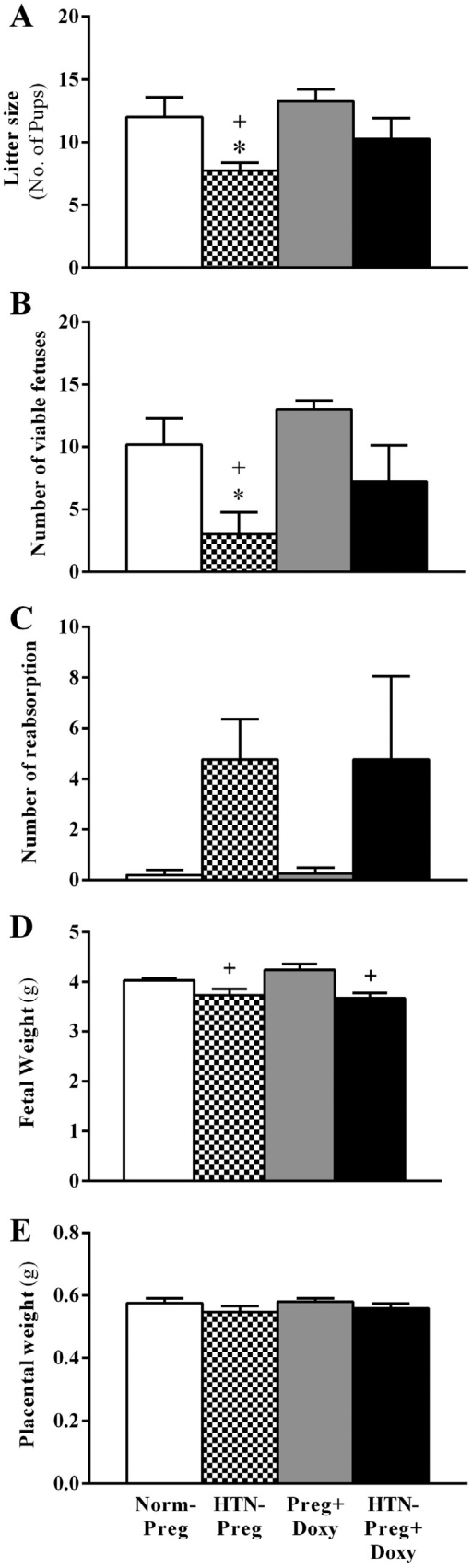

Fig. 2. Fetal and placental parameters: **A)** Litter size, **B)** Number of viable fetuses, **C)** Number of reabsorption, **D)** Fetal weight and **E)** Placental weight were evaluated in 21th day gestational, among the four groups: normal pregnancy (Norm Preg), pregnant rats treated with L-NAME (HTN-Preg), pregnancy + Doxycycline (Preg + Doxy), and pregnant rats treated with L-NAME and Doxycycline (Preg + Doxycycline). Values represent mean ± S.E.M. *P < 0.05 *versus* Norm-Preg. $^{+}$P < 0.05 *versus* Preg + Doxycycline and $^{\#}$P < 0.05 *versus* HTN-Preg.

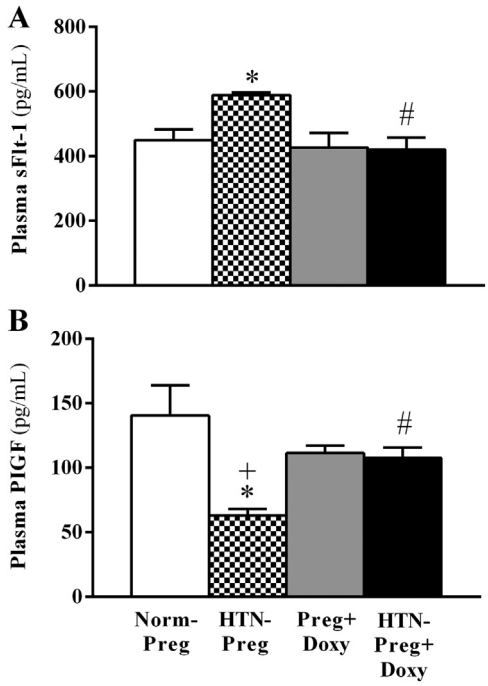

Fig. 3. Plasma levels of antiangiogenic (**A**, sFlt-1) and pro-angiogenic (**B**, PLGF) factors were evaluated among the four groups: normal pregnancy (Norm-Preg), pregnant rats treated with L-NAME (HTN-Preg), pregnancy + doxycycline (Preg + Doxy), and pregnant rats treated with L-NAME and doxycycline (HTN-Preg + Doxy). Values represent mean ± S.E.M. *P < 0.05 *versus* Norm-Preg, $^{+}$P < 0.05 *versus* Preg + Doxy and $^{\#}$P < 0.05 *versus* HTN-Preg.

models of hypertensive pregnancy (Agunanne et al., 2010; Gilbert et al., 2007). Therefore, we sought to investigate doxycycline treatment effects on anti-angiogenic/pro-angiogenic balance. Then, we observed an increase in plasma levels of sFlt-1 in HTN-Preg rats. This is in accordance with previous results from our and other groups showing increases in sFlt-1 levels following L-NAME-induced hypertension in pregnancy (Baijnath et al., 2017; Goncalves-Rizzi et al., 2016; Possomato-Vieira et al., 2016). Moreover, plasma levels of PLGF were decreased in HTN-Preg group, which corroborate previous findings (Agunanne et al., 2010; Baijnath et al., 2017; De Vivo et al., 2008). Importantly, doxycycline treatment reduced sFlt-1 and increased PLGF levels in HTN-Preg + Doxy group. Therefore, the reverse in anti-angiogenic/pro-angiogenic imbalance promoted by doxycycline suggests that angiogenic balance could improve blood flow into uteroplacental circulation. In fact, during pregnancy, reduction in systemic vascular resistance is an important vascular adaptation to assure the maintenance of blood pressure (Ouzounian and Elkayam, 2012); thus increases in blood flow of uteroplacental circulation due to augmented angiogenesis could further contribute to the reduction in systolic blood pressure followed doxycycline treatment.

There is evidence that doxycycline may exert antioxidant effects and counterbalance oxidative stress (Antonio et al., 2014; Tilakaratne and Soory, 2014). Moreover, increases in oxidative stress may play an important role in the pathophysiology of hypertensive disorders of pregnancy (Cornelius and Lamarca, 2014; Ferguson et al., 2017; Hansson

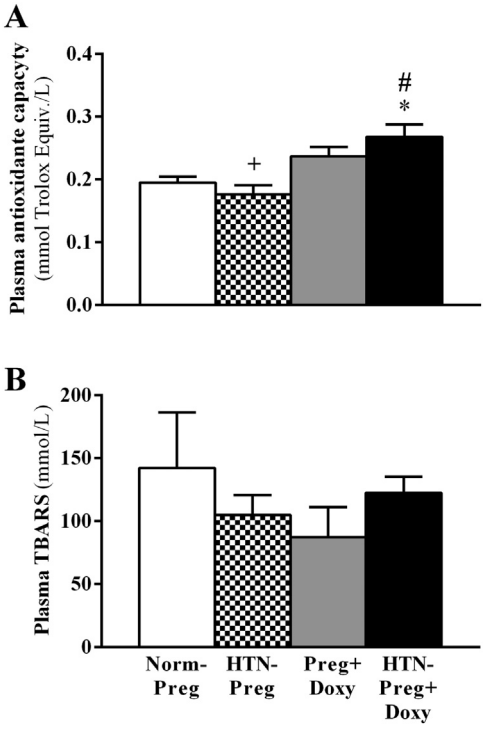

Fig. 4. Plasma antioxidant capacity **(A)** and MDA levels **(B)** were evaluated among the four groups: normal pregnancy (Norm-Preg), pregnant rats treated with L-NAME (HTN-Preg), pregnancy + doxycycline (Preg + Doxy), and pregnant rats treated with L-NAME and doxycycline (HTN-Preg + Doxy). Values represent mean ± S.E.M. $^{*}P < 0.05$ *versus* Norm-Preg, $^{+}P < 0.05$ *versus* Preg +Doxy and $^{\#}P < 0.05$ *versus* HTN-Preg.

et al., 2014). Therefore, we evaluated the plasma antioxidant capacity and plasma levels of MDA, which may indicate oxidative stress (Atiba et al., 2016; Bakacak et al., 2015). We found that doxycycline treatment promoted increases in plasma antioxidant capacity in animals from HTN-Preg + Doxy and Preg + Doxy groups when compared to HTN-Preg group. According to Matsubara et al. (2015) decreases in antioxidant capacity results in elevated oxidative stress, which leads to pathological conditions, such as hypertension. However, we observed no significant changes in MDA levels among four experimental groups. Nevertheless, doxycycline reestablished the antioxidant capacity, corroborating with previous findings (Lai et al., 2010; Zeydanli et al., 2011). Moreover, increases in plasma antioxidant capacity may protect cells against reactions of peroxidation and preserve cellular membrane integrity and thus saving vasculature from harm due to reactive oxygen species and maintain the vascular function (Matsubara et al., 2015; Raijmakers et al., 2004).

During healthy pregnancies, an increase in levels/activity of MMPs may be required to a proper vascular adaptation and placentation (Cohen et al., 2010), however, an excessive increase in MMPs activity may contribute to the pathophysiology of hypertension in pregnancy (Martinez-Fierro et al., 2018; Myers et al., 2005). Hence, decreased MMP activity by doxycycline may be related with the beneficial effects observed in our study. MMPs are enzymes involved in the degradation of many components of the extracellular matrix (Li et al., 2014). MMP-2 and -9 are found in several tissues and organs (Mayer et al., 2018), such as uterus (Lombardi et al., 2018), placenta (Sahay et al., 2018), myometrial cells (Cardozo et al., 2018) and lungs (Souza-Costa et al., 2005).

We found that increases in systolic blood pressure in HTN-Preg group were associated with significant increases in placental MMP-2 and -9 and uterine MMP-2 activities, while doxycycline treatment attenuated both arterial hypertension and increased activity of MMPs. It has been shown that excessive hemodynamic forces, such as shear stress and hydrostatic forces, may lead to increases in mechanical stretch of vascular smooth muscle and therefore, increase MMPs activity (Seo et al., 2013, 2015). We speculate that decreased levels of NO may induce a greater vasoconstriction and consequently increase these hemodynamic forces in vessels of utero-placental interface. Such mechanism would contribute, at least in parts, for the increases in MMPs activities in our study.

MMP-2 activity is increased in uterus and aorta during late pregnancy in rats (Dang et al., 2013) and in plasma of women with preeclampsia (Myers et al., 2005). Moreover, MMP-2 has been shown to be increased in all regions of the preterm preeclamptic placentae (Sahay et al., 2018). Also, elevated MMP-2 activity may indicate intensified endothelial activation and harm the vascular function (Montagnana et al., 2009). Also, overexpression of MMP-9 was positively correlated with the symptoms of preeclampsia (Xiao et al., 2018) and increased MMP-2 and -9 activities were found during inflammation and renal injury in preeclampsia (Wang et al., 2015). Faced with these previous evidences, we hypothesized that generalized increases in MMP-2 and 9, in placenta, uterus and aorta could occur, however, no significant changes in MMP-2 were found in aorta tissue. Therefore, our results suggest that hypertensive pregnancy-associated increases in MMPs are localized in the uteroplacental, but not in aorta tissues.

Of note, some limitations should be considered. Firstly, we used only one dose of doxycycline. Although we chose this dose based on previous evidences showing that similar doses significantly reduced systolic blood pressure with further decrease in the activity of MMPs in hypertensive rats (Guimaraes et al., 2011), future studies should investigate different doses of doxycycline for MMP inhibition in hypertensive pregnancy. Secondly, no signs of teratogenicity were observed in our study and in doses of doxycycline more than 100 times the human dose (Cross et al., 2016; Nahum et al., 2006). However, future studies are needed to evaluate doxycycline fetotoxicity, since doxycycline may cause brown deposits in milk teeth (Vennila et al., 2014). Lastly, doxycycline has been shown to increase antioxidants enzymes (Lai et al., 2010) and in our hands, doxycycline increased antioxidant capacity of plasma, which was followed by unchanged levels of oxidative stress, thus, antioxidants effects of doxycycline in the modulation of MMPs activity during hypertensive pregnancy deserve further investigation.

Thus, the present findings are in accordance to the hypothesis that reductions of NO formation may be correlated to increased MMP-2 and -9 activities, suggesting that there may be a crosstalk between reduced NO synthesis and increases in MMPs activity in hypertension in pregnancy. However, upon NOS inhibition, doxycycline reduces systolic blood pressure and attenuates the increases in MMP-2 and -9 activities.

5. Conclusions

Author contribution statement. Nascimento RA: conceptualization, data curation, formal analysis, investigation, methodology, writing original draft, review and editing final version. Possomato-Vieira JS: data curation, formal analysis, writing original draft. Gonçalves-Rizzi VH: data curation, formal analysis and writing original draft. Bonacio GF: data curation, formal analysis, investigation and writing original draft. Rizzi E: data curation, formal analysis, investigation, methodology, writing original draft, review and editing final version. Dias-Junior CA: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, visualization, writing original draft, review & editing final version.

Taken together, our results suggest that doxycycline blunts increases in systolic blood pressure induced by L-NAME in hypertensive pregnant rats. Importantly, increased activity of MMPs promoted by reduction of NO formation was attenuated by doxycycline treatment, and this could contribute to the antihypertensive effects observed in our study. Authors contribution statement. Conceptualization, data

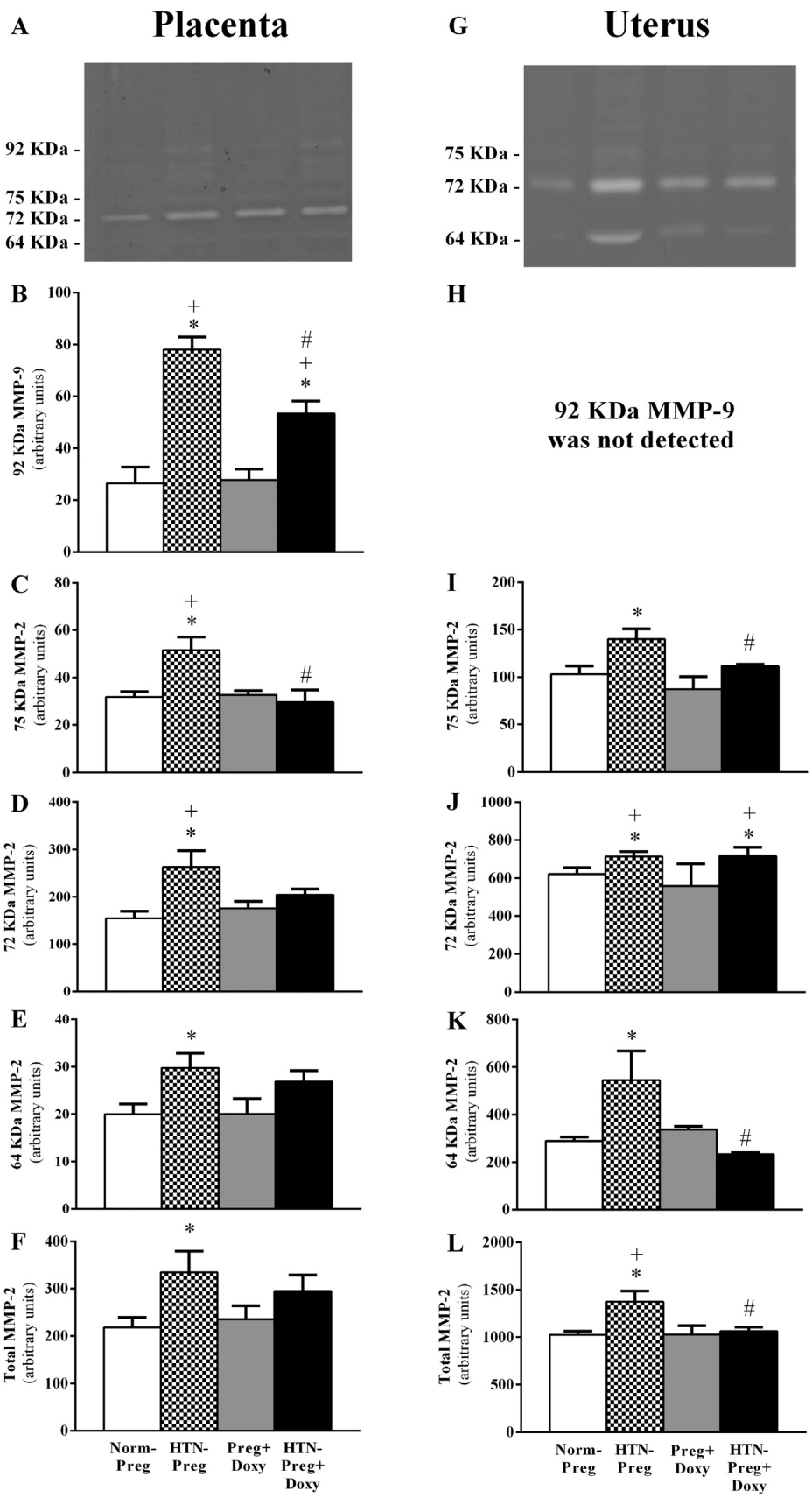

Fig. 5. Representative zymography gel of tissue homogenate from the placenta **(A)** and uterus **(G)**. Gelatinase activity of placental (**B–F**) and uterine (**H–L**) MMP-2 and -9 isoforms were assessed among the four groups: normal pregnancy (Norm-Preg), pregnant rats treated with L-NAME (HTN-Preg), pregnancy + doxycycline (Preg + Doxy), and pregnant rats treated with L-NAME and doxycycline (HTN-Preg + Doxy). Values represent mean ± S.E.M. *P < 0.05 *versus* Norm-Preg, $^{+}$P < 0.05 *versus* Preg +Doxy and $^{\#}$P < 0.05 *versus* HTN-Preg.

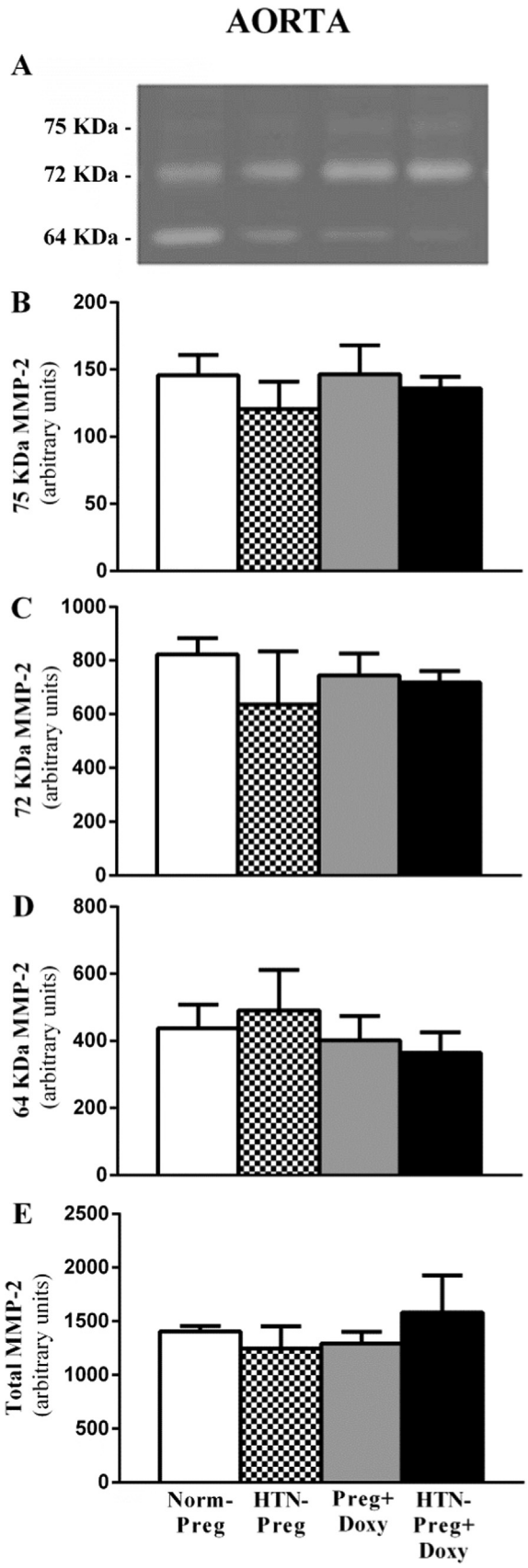

Fig. 6. Representative zymography gel of tissue homogenated from the aorta (**A**), Gelatinase activity of aorta (**B–E**) MMP-2 isoforms were assessed among the four groups: normal pregnancy (Norm-Preg), pregnant rats treated with L-NAME (HTN-Preg), pregnancy + doxycycline (Preg + Doxy), and pregnant rats treated with L-NAME and doxycycline (HTN-Preg + Doxy). Values represent mean ± S.E.M. *P < 0.05 *versus* Norm-Preg, $^{+}$P < 0.05 *versus* Preg + Doxy and $^{\#}$P < 0.05 *versus* HTN-Preg.

curation, formal analysis, investigation, methodology, writing original draft: Nascimento RA, Possomato-Vieira JS, Gonçalves-Rizzi VH, Dias-Junior CA; Data curation and formal analysis: Bonacio GF, Rizzi E; Funding acquisition, project administration, resources, supervision, review & editing final version: Dias-Junior CA.

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Conflict of interest

There are no known conflicts of interest.

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Capítulo II

Nitric oxide downregulates activity of matrix metalloproteinases-2 and -9 during pregnancy, but reduced-nitric oxide-induced hypertensive pregnancy increases activity of these gelatinases

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Abstract

Hypertensive disorders of pregnancy have been associated with reduced nitric oxide (NO) bioavailability and increased activity of matrix metalloproteinases (MMPs) -2 and -9. However, it is unclear if MMPs activation is regulated by NO during pregnancy. To this end, we examined activity of MMP-2 and MMP-9 in plasma, placenta, uterus and aorta, and NO bioavailability, oxidative stress, systolic blood pressure (SBP) and feto-placental development at the different periods of gestation (early, mid and late) in normal pregnancy and Nω-Nitro-L-arginine methyl ester (L-NAME)-induced hypertensive pregnancy. We found that, in normal pregnancy, there are increases in NO bioavailability in mid- and late-pregnancy, with concomitant reductions in MMP-9 in plasma and placenta of late-pregnant and reductions in MMP-2 in uterus and aorta in mid- and late-pregnancy. Also, while L-NAME increased SBP in virgin and all periods of pregnancy, decreases in NO in virgin, mid- and late-, but not in early-pregnancy were observed. L-NAME reduced fetal growth and increased MMP-9 in plasma and placenta only in late-pregnancy. Increased uterine MMP-2 of early-, mid- and late-pregnancy, increased placental MMP-2 of mid- and late-pregnancy and increased MMP-2 in aorta of early-, mid- and late-pregnancy were observed in L-NAME-treated rats. Also, L-NAME increased oxidative stress in mid- and late-, but not in early-pregnant compared to virgin rats. Elevated oxidative stress was found at the late period of normal and hypertensive pregnancy. Our findings support the hypothesis that NO bioavailability may regulate MMPs activation during normal and hypertensive pregnancy.

Keywords: hypertensive pregnancy; metalloproteinases; nitric oxide; rats

1 Introduction

During pregnancy, the uterus undergoes significant and expansive hypertrophy and distension to provide the adequate space for the growing fetus, while placental remodeling and cytotrophoblast invasion of spiral arteries occur to supply blood flow required for the fetal developing [1; 2]. Also, hemodynamic changes as increases in maternal blood volume and cardiac output counterbalanced by systemic vasodilation following decreases in vascular resistance occur during gestational period [3; 4]. These utero-placental and vascular changes may be modulated by nitric oxide (NO)[5; 6] and matrix metalloproteinases (MMPs)[7].Particularly, the gelatinases MMP-2 and MMP-9 have been implicated in these pregnancy-associated changes [8; 9]. However, no previous study has examined if the activity of MMP-2 and MMP-9 is regulated by NO during pregnancy.

In normal pregnancy, there are increases in endogenous NO synthesis [5; 6; 10; 11], while hypertensive disorders of pregnancy are associated with reductions of NO bioavailability in humans [12; 13; 14; 15; 16]and in animals [17; 18; 19].Because normal pregnancy involves significant utero-placental and vascular remodeling[7; 20] and MMPs play important roles in tissue remodeling[21], changes in activity of MMPs have also been found during normal and hypertensive pregnancy [7; 20].

In normal pregnancy, previous studies have found that early-pregnant animals present increases in activity of MMP-2 in uterus [22] and increases in placental MMP-9[23]. Also, other authors reported increases in activity of MMP-2 and MMP-9 in the uterus and aorta during mid- and late-pregnancy, but decreases in placental activity of these two gelatinases were observed in late- compared to mid-pregnancy in animals [24; 25]. Furthermore, in hypertensive disorders of pregnancy, excessive gelatinolytic

activity of MMP-2 and MMP-9 in plasma [26; 27] and increases in MMP-2 in urine [28]have been found in women.

Of note, earlier studies have focused at the end of pregnancy, however, considering the potential role of NO to dow nregulate the MMP-2 and MMP-9 [29; 30; 31; 32; 33; 34], we hypothesized that reductions in NO bioavailability caused by Nω-Nitro-L-arginine methyl ester (L-NAME) could increase the activity of MMPs throughout periods of pregnancy[35; 36]. The L-NAME-induced hypertensive pregnancy results infeo-placental growth restriction because of decreases in utero-placental perfusion caused by aberrant vascular remodeling in the utero-placental circulation [37; 38].

Therefore, in search for the mechanisms involved in the changes in utero-placental and vascular remodeling during hypertension in pregnancy, we examined systolic blood pressure, NO bioavailability, feto-placental development, gelatinolytic activity of MMP-2 and MMP-9 in early-, mid- and late-pregnancy in rats treated (or not) with L-NAME.

2 Materials and methods

2.1 Animals and experimental protocol

Female Wistar rats (200-250 g) were maintained in controlled conditions (22 ± 2 ºC, 12-hr light/dark cycle) and given free access to standard rat chow and tap water. Experiments on virgin rats were conducted during estrous cycle; vaginal secretion of female rats was collected to analyze the presence of cornified squamous cells[39].The experiments with pregnant rats were conducted as following: each female rat was separately mated overnight, and the first pregnancy day was defined when spermatozoa and estrous cells were found in a vaginal smear.

Normotensive virgin and pregnant rats received intraperiotoneal (i.p.) injections of saline throughout experimental protocol (n = 8 per group). Virgin rats were evaluated for 6 days (**Virgin group**). Pregnant rats were studied according to different periods of gestation as following: in early pregnancy from gestational day 3-8 (**Early Preg group**); in middle pregnancy from gestational day 9-15 (**Mid Preg group**); and in late pregnancy from gestational day 13-20 (**Late Preg group**).

Hypertensive virgin and pregnant rats received i.p. injections of L-NAME (60 mg/Kg/daily, n = 8 per group) as following: in virgin rats from days 1-6 (**Virgin+L-NAME**); in early pregnancy from gestational day 3-8 (**Early Preg+L-NAME group**); in middle pregnancy from gestational day 9-15 (**Mid Preg+L-NAME group**); and in late pregnancy from gestational day 13-20 (**Late Preg+L-NAME group**). Age-matched virgin rats were used in Virgin and Virgin+LNAME groups.

On the last day of each period, dams were killed under overdose of isoflurane followed by exsanguination. Blood samples were collected in lyophilized ethylenediamine tetra acetic acid (EDTA) (Vacuntainer Becton-Dickinson, BD, Oxford, UK) and lyophilized heparin containing tubes (Vacuntainer Becton-Dickinson, BD, Oxford, UK) and immediately centrifuged. Plasma was separated and stored at -80ºCuntil use for biochemical analysis.

Litter size (number of pups) was observed in all periods, while number of viable fetuses, number of resorptions, fetal and placental weights were observed only for the periods middle and late pregnancy. Viable fetuses were determined as those which showed no macroscopic signal of malformation and could apparently have a normal outcome with the advancement of the pregnancy, as previously reported[40].

The animals used in the present study were handled according to the guiding principles published in the National Institutes of Health Guide for the Care and Use of

Laboratory Animals and all procedures for animal experimentation were approved by Committee for Ethics in Animal Experimentation of the Biosciences Institute, Sao Paulo State University, Botucatu, Sao Paulo, Brazil (Protocol nº. 741-CEUA). All animals were obtained from the colony at Sao Paulo State University.

2.2 Blood pressure measurements

Systolic blood pressure was recorded in virgin rats from days 1-6 and in pregnant rats on gestational days 3, 4, 6 and 8 (early pregnancy period), 9, 11, 13 and 15(middle pregnancy period) and 13, 14, 16, 18 and 20(late pregnancy period). Systolic blood pressure was measured by tail cuff plethysmography (Insight, Ribeirao Preto, Sao Paulo, Brazil, # EFF-306) [41; 42].

The first measure of systolic blood pressure was performed in all animals prior to randomization into saline-treated or L-NAME-treated groups, before i.p. injections and represent baseline values (BL) on day 1 (Virgin groups), on day 3 (Early groups), on day 9 (Mid groups) and on day 13 (Late groups). The other measurements were taken 6 hours after i.p. injections. Briefly, rats were restrained and pre-warmed in a warm-box (Insight, Ribeirao Preto, Sao Paulo, Brazil, # EFF-307) at 40ºC for 10 min. Then, systolic blood pressure was determined as the average of the cuff inflation-deflation (3–6 cycles) by a trained operator.

2.3 Determination of plasma levels ofnitrite/nitrate (total NOx)

Plasma total NOx concentrations were determined using Griess reagents followed by reduction of nitrous species with vanadium chloride III [43]. Briefly, before addition of Griess reagents, samples were incubated with 100 µL of saturated solution of vanadium chloride III for three hours at 37ºC with agitation. After incubation, 50 µL

of 1% sulfanilamide solution in 5% phosphoric acid was added and plate incubated for 10 minutes protected from light. Then, 50 µL of 0.1% N-(1-Naphthyl)-ethylenediamine dihydrochloride solution were added followed by 10-minute incubation in dark. Absorbance at 535 nm was read in spectrophotometer (Synergy 4, BIOTEK, Winooski, VT) and NOx concentration was calculated using a standard curve of sodium nitrite (1.56-100 µM). The metabolites levels of NO (NOx) in plasma were expressed in µmol/L.

2.4 Determination of lipid peroxidation

Lipid peroxidation was assessed through measurements of thiobarbituric acid reactive substances (TBARS)[44]. Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA), which is the end product of lipid peroxidation to form a magenta color that is read by spectrophotometer at wavelength of 532 nm. In test tubes, a reaction mixture containing 100 µL of distilled water, 50 µL of 8.1% sodium dodecyl sulfate (SDS), 100 µL of plasma samples, 375 µL of acetic acid 20% and 375 µL of TBA 0.8% were incubated in water-bath at 95ºC for one hour and thereafter, mixture was centrifuged at 4000 rpm for 10 minutes. Standard curve was made in a similar form, replacing samples with 25 µL of known concentrations of MDA. Plasma levels of TBARS were calculated compared to a standard curve of MDA (20-320 nmol). MDA concentrations were expressed in nmol/mL.

2.5 Zymography for MMP-2 and MMP-9 activity in placenta, uterus and aorta.

Gelatin zymography was used to determine MMP-2 and MMP-9 activity in plasma,placenta, uterus and aorta as previously described [45; 46; 47]. Briefly, frozen samples were homogenized in ice-cold RIPA buffer (1 mM 1,10 -ortho-phenanthroline,

1mM phenylmethanesulfonyl fluoride, and 1mM N- ethylmaleimide) and protease inhibitor (4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), E-64, bestatin, leupeptin, aprotinin, and EDTA) in a proportion of 100µLRIPA+protease inhibitor for each 10mg of tissue sample. The samples were placed in ice for 2 hours with gentle stirring and then centrifuged at 12.000 rpm for 10 min. The protein concentrations were measured using the Bradford assay (Sigma Aldrich). 10µg of uterus and aorta proteins and 5µg of placenta proteins were diluted 1:1 with sample buffer (final concentration): 2% SDS, 125 mMTris–HCl, pH 6.8, 10% glycerol, and 0.001% bromophenol blue, and were subjected to electrophoresis on 12% SDS–polyacrylamide gel electrophoresis copolymerized with gelatin (0.05%) as the substrate. After electrophoresis was completed, gel was washed and incubated at 37 ºC for 18 hours in Tris–HCl buffer, pH 7.4, containing 10 mmol/L $CaCl_2$.Gels were stained with 0.05% Coomassie Brilliant Blue G-250 and then destained with 30% methanol and 10% acetic acid. Gelatinolytic activity was detected as an unstained band against the background of Coomassie blue-stained gelatin. Enzyme activity was assayed using ImageJ software by optical densitometry and the integrated protease activity density was measured as pixel intensity × mm^2, which was normalized to fetal bovine serum intensity, used as standard, to correct for loading and inter-gel variation, and the results were expressed as arbitrary units. The MMP-2 forms were identified as bands at 75, 72 and 64 kDa and MMP-9 as band at 92 KDa. Total MMP-2 was taken as a sum of the different isoforms.

2.6 Statistical analysis

Using commercially available statistical software (Graph Pad Prism® 6.0 forWindows, San Diego, CA, USA), a Shapiro-Wilk test was applied to verify normality ofdata distribution. Data were first analyzed using one-way analysis of

variance (ANOVA).When a statistical difference was observed, data were further analyzed using Tukey's correction for multiple comparisons. Student's *t-test* was used for comparison of two means. A probability value $P < 0.05$ was considered statistically significant. All values are expressed as mean ± S.E.M.

3 Results

In virgin rats, increases in systolic blood pressure occurred after one day of L-NAME injections when compared to day 1 of virgin groups (*P<0.05), and systolic blood pressure remained higher in Virgin+L-NAME compared to respective days in Virgin group ($^{*,\#}$P<0.05, Fig. 1A).

In early-pregnancy, increases in systolic blood pressure were observed only after five days of L-NAME injections (gestational day 8) when compared to gestational day 3 (*P<0.05) or respective day in Early-Preg group ($^{\#}$P<0.05, Fig. 1A).

In mid-pregnancy, systolic blood pressure rose after four days of L-NAME injections (gestational day 13) and remained elevated (gestational day 15) when compared to gestational day 9 (*P<0.05) or respective days in Mid-Preg group ($^{*,\#}$P<0.05, Fig. 1A).

In late-pregnancy, increases in systolic blood pressure occurred after one day of L-NAME injection (gestational day 14) compared to gestational day 13 (*P<0.05) and remained higher in Late+L-NAME compared to respective days in Late-Preg group ($^{*,\#}$P<0.05, Fig. 1A).

The NO bioavailability showed significant increases in Late-Preg compared to Virgin, Mid-Preg and Ealry-Preg groups ($^{*,\ +,\ ++}$P<0.05) and in Mid-Preg compared to Early-Preg group ($^{+}$P<0.05). However, reduced NO levels were found in Virgin+L-

NAME, Mid-Preg+L-NAME, Late-Preg+L-NAME, but not in Early-Preg+L-NAME compared to saline-treated respective group (*,#P<0.05, Fig. 1B).

Feto-placental parameters were affected negatively by L-NAME in late-pregnancy, presenting significant reductions in litter size (#P<0.05, Fig. 2A) and number of viable fetuses (#P<0.05, Fig. 2B) with concomitant increases in number of resorptions (#P<0.05, Fig. 2C). Also, fetal weight (#P<0.05, Fig. 2D), but not placental weight (P>0.05, Fig. 2E) presented reductions in Mid-Preg+L-NAME and Mid-Preg+L-NAME compared to Mid-Preg and Late-Preg group, respectively (#P<0.05, Fig. 2D).

In normal pregnant rats, while oxidative stress has reduced in Early-Preg and Mid-Preg, increases in Late-Preg were found, when compared to Virgin group (*P<0.05, Fig. 3). Moreover, oxidative stress in Late-Preg+L-NAME was increased when compared to Early-Preg+L-NAME and Mid-Preg+L-NAME groups (+, ++P<0.05, Fig. 3). Also, Virgin+L-NAME, Early-Preg+L-NAME and Mid-Preg+L-NAME presented reduced oxidative stress compared to Virgin group (*P<0.05, Fig. 3). Furthermore, Mid-Preg+L-NAME presented increases in oxidative stress compared to Mid-Preg group (#P<0.05, Fig. 3).

A representative zymogram of gelatinolytic activity of plasma samples is shown in Fig. 4A.Corresponding bands to 92 KDapro-MMP-9 and two isoforms of MMP-2: 75KDa and 72KDa. No corresponding band to 64 KDa MMP-2 appeared in zymography gel. Saline-treated pregnant rats presented reductions in MMP-9 activity only in Late-Preg compared to Virgin group (*P<0.05, Fig. 4B). However, increase in MMP-9 activity was found only in Late-Preg+L-NAME compared to Late-Preg group (#P<0.05, Fig. 4B). No significant differences were observed at the early and mid-pregnancy (P>0.05, Fig. 4B-F).

A representative zymogram of gelatinolytic activity of uterine tissue homogenate is shown in Fig. 5A. No corresponding band to 92 KDa pro-MMP-9 appeared in zymography gel, thus zymogram shows three isoforms of MMP-2: 75, 72, and 64 kDa. Saline-treated pregnant rats presented reductions activity of all MMP-2 isoforms and total MMP-2 in Mid-Preg and Late-Preg (*P<0.05), but not in Early-Preg compared to Virgin group (Fig. 5C-F). However, increased activity of 75 and 72 KDa MMP-2 were observed in all L-NAME-pregnant rats (early-, mid- and late-pregnancy) compared to respective saline-treated pregnant group($^{\#}$P<0.05, Fig. 5C-D). Also, increased activity of 64 KDa and total MMP-2 were observedin Mid-Preg+L-NAME and Late-Preg+L-NAME, but not in Early-Preg+L-NAME compared to respective saline-treated pregnant group ($^{\#}$P<0.05, Fig. 5E-F).

A representative zymogram of gelatinolytic activity of placental tissue homogenate is shown in Fig. 6A. Corresponding band to 92 KDa pro-MMP-9 and three isoforms of MMP-2: 75 KDa, 72 KDa and 64 KDa. Saline-treated pregnant groups showed decreased activity of MMP-9 (Fig. 6B), 64 KDa (Fig. 6E) and total MMP-2 (Fig. 6F) in Late-Preg compared to respective Mid-Preg group ($^{++}$P<0.05). Also, decreased activity of MMP-9 (Fig. 6B) and of all isoforms and total MMP-2 (Fig. 6C-F) were found in Late-Preg+L-NAMEwhen compared to Mid-Preg-L-NMAE group ($^{++}$P<0.05). However, all isoforms of MMPs were increased in Late-Preg+L-NAME compared to Late-Preg group ($^{\#}$P<0.05, Fig. 6B-F).

A representative zymogram of gelatinolytic activity of aorta homogenate is shown in Fig. 7A. No corresponding band to 92 KDa pro-MMP-9 appeared in zymography gel, thus zymogram shows three isoforms of MMP-2: 75, 72, and 64 kDa. Saline-treated pregnant rats presented reductions activity of 75 KDa MMP-2 in Early-, Mid- and Late-pregnant compared to Virgin group (*P<0.05, Fig. 7C). Also, reduced

activity of 72 KDa MMP-2 were found in Mid-Preg and Late-Preg, but not in Early-Preg compared to Virgin group (*P<0.05, Fig. 7D). However, increased activity of 75 and 64 KDa were found in Early-Preg+L-NAME, Mid-Preg+L-NAME and Late-Preg+L-NAME compared to respective Early, Mid- and Late-Preg group ($^{\#}$P<0.05, Fig. 7C and E). Also, increased activity of 72 KDa and total MMP-2 were observed in Mid-Preg+L-NAME and Late-Preg+L-NAME, but not in Early-Preg+L-NAME compared to respective Early-, Mid- and Late-Preg group ($^{\#}$P<0.05, Fig. 7D and F).

4. Discussion

The main findings of this study are:**(1)** during normal pregnancy, there are increases in NO bioavailability in mid- and late- compared to early-pregnant and virgin rats, with concomitant reductions in MMP-9 activity in plasma and placenta at the late-pregnancy. and reductions in MMP-2 activity in uterus and aorta at the mid- and late-pregnancy; **(2)** while L-NAME increased systolic blood pressure in virgin and in early-, mid- and late-pregnant rats with concomitant decreases in NO bioavailability in virgin, mid- and late-pregnant, but not in early-pregnant rats; **(3)** L-NAME affected negatively fetal growth only in late-pregnant rats; **(4)**L-NAME increased only activity of MMP-9 in plasma of late-pregnant rats;**(5)**increases in activity of MMP-2 in uterus of early-, mid- and late-pregnant rats, as well as increases in activity of MMP-9 in placenta of late-pregnant rats, increases in activity of MMP-2 in placenta of mid- and late-pregnant rats and increases in activity of MMP-2 in aorta of early-, mid- and late-pregnant rats were observed in L-NAME-treated animals. The present findings support the hypothesis that NO bioavailability may regulate MMPs during normal and hypertensive pregnancy.

The rises in blood pressure promoted by L-NAME throughout early-, mid- and late-pregnancy periods found in the preset study are in accordance with previous reports

[35; 36; 48; 49]. Although we did not observe decreases in NO bioavailability in virgin and early-pregnant rats treated with L-NAME, our results regarding to the reduced NO followed by increases in systolic blood pressure during mid- and late-pregnancy indicate that the demand for NO must be increased at the later stages of pregnancy.

Our results in normal pregnant rats are supported by previous studies showing that urinary excretion of NO metabolites increased throughout gestational periods in rats, reaching peak levels on late stages of pregnancy[10]and also that blood pressure increased in pregnant endothelial NO synthase (eNOS) knockout mice, whereas blood pressure remains unaltered in eNOS knockout virgin mice[50].Importantly, the rise in blood pressure occurred in a more "abrupt" manner in late-pregnant rats when compared to the latency observed in virgin, early and mid-pregnant rats treated with L-NAME, further indicating that the demand for NO must be increased in later stages of pregnancy. Also, we found that fetal development was negatively affected only during later stages of pregnancy, whereas the fetal and placental growths did not change significantly in early and mid-pregnant rats treated with L-NAME, which confirms a greater involvement of NO at the end of pregnancy.

To examine if NO bioavailability modulates the activity of MMPs during normal and hypertensive pregnancy, gelatinolytic activity of MMPs was determined in target tissues as the uterus, which undergoes remodeling to accommodate the fetal growing; the placenta, which provides nutrients supply to the fetal developing; and the plasma and the aorta, which reflect the vascular changes into maternal circulation. In this regard, we sought to investigate the alterations of the activity of MMP-2 and MMP-9 in specific stages of normal pregnancy and L-NAME-induced hypertensive pregnancy.

In general, we observed that during healthy pregnancy, there is an increase in NO bioavailability followed by decreases in the activity of MMP-2 in uterus, placenta

and aorta and decreases in MMP-9 in plasma and placenta of late-pregnant rats. Although some isoforms presented no changes in their activities in pregnant compared to virgin rats, our data suggest that activity of MMPs is down regulated by endogenous NO and this may control, at least, part of the physiological process involved in the utero-placental and vascular remodeling during normal pregnancy [51].

Conversely, L-NAME reduced NO bioavailability and this effect was associated with increases in activity of MMP-2 in uterus, placenta and aorta, and MMP-9 in plasma and placenta of hypertensive compared to normal-pregnant rats. Importantly, an excessive increase in MMPs activity[26; 27; 28] may be associated with reduced NO bioavailability [12; 13; 14; 15; 16; 17; 18; 19], thus contributing to the pathophysiology of hypertension in pregnancy. These suggestions are aligned with our previous findings showing that activation of MMPs induced by L-NAME was attenuated by doxycycline, a non-selective MMP inhibitor, in late-hypertensive pregnant rats [52].

Our results suggest that NOdownregulatesMMP-2 and MMP-9 during normal pregnancy, because increases in activity of MMPs were found under reduced NO bioavailability caused by L-NAME. Thus, this may represent an important mechanism enrolled in hypertensive disorders of pregnancy, since reductions in NO levels have been found in gestational disorders[12; 53]. Taken together, our results indicate that there may be a demand for increased NO with the advancement of healthy pregnancy, while reductions in NO bioavailability induced by L-NAME during mid- and late-pregnancy periods may result in greater activity of MMPs, and further contributing to hypertension in pregnancy.

We also observed that L-NAME increased oxidative stress in mid- and late-pregnant, but not in early-pregnant, compared to virgin rats. It has been proposed that oxidative stress may trigger the activation of MMPs [54]and therefore we suggest that

increases in oxidative stress may also contribute to MMPs activation observed in hypertensive pregnant rats. Of note, although high oxidative stress was similar in normal and hypertensive pregnant groups at the late-pregnancy period, increases in oxidative stress counterbalanced by abundant antioxidant defenses may occur even during healthy pregnancies, due to higher metabolic demands required for fetal development at the end of pregnancy[55; 56]. Thus, we suggest that impaired antioxidant system associated with increase in oxidative stress caused by L-NAME[42]could also contribute to trigger the activation of MMPs during hypertension in pregnancy [57].

5. Conclusion

In summary, our data indicate that NO demand may be greater during later periods of gestation, and that NO may negatively modulate the MMPs during healthy pregnancy. Also, reductions in NO bioavailability were associated with increases in activity of MMPs during hypertension in pregnancy. Therefore, our present results are in accordance with the hypothesis that NO modulates the activity of MMPs in pregnancy.

6. Author contributions

R.A.N., J.S.P.V., G.F.B. and E.R performed experimental procedures, statistical analyses and contributed to discussion and revision of manuscript. C.A.D.J. contributed to statistical analyses and reviewed the discussion, edited the manuscript and gave final approval of the version to be published.

7. Conflict of interest

There are no known conflicts of interest.

8. Acknowledgements

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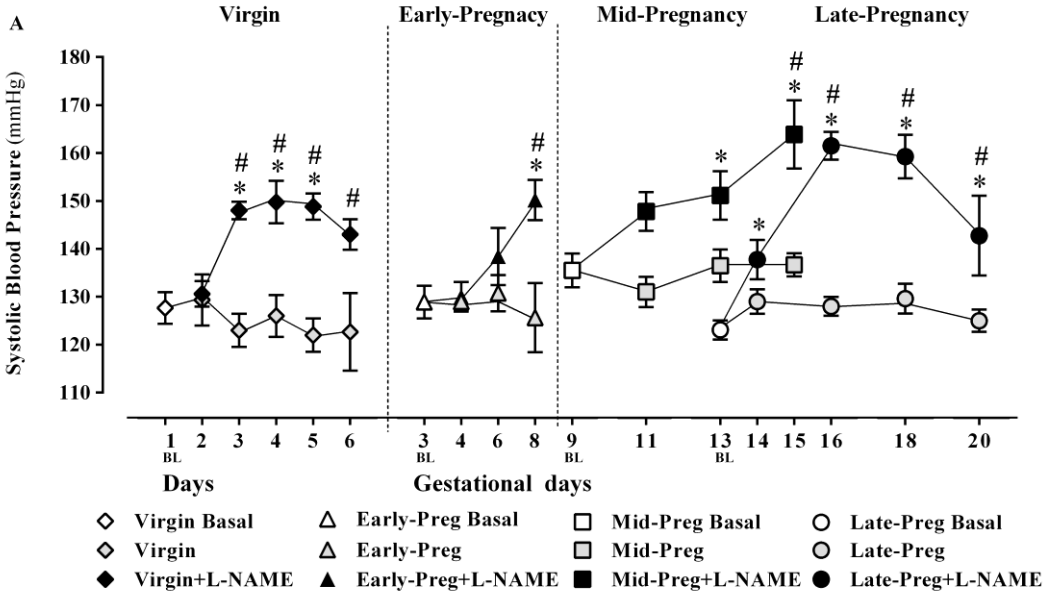
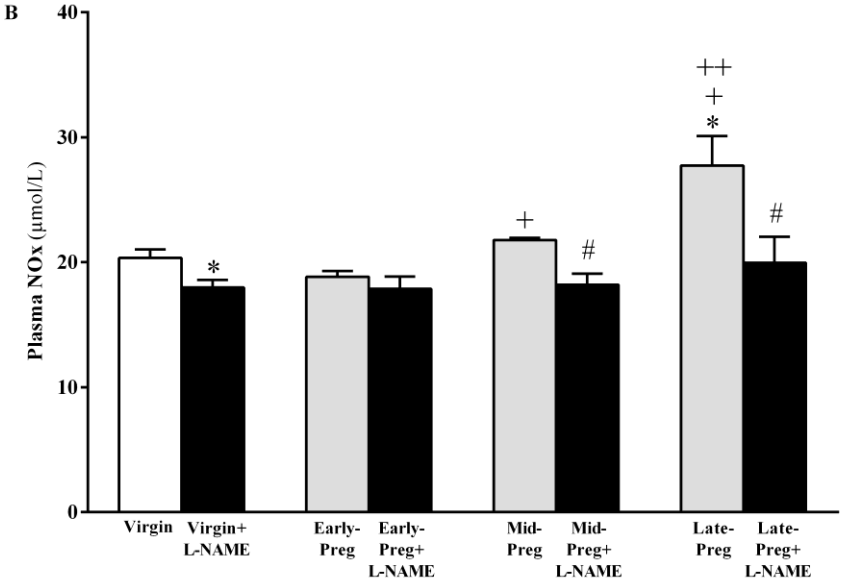
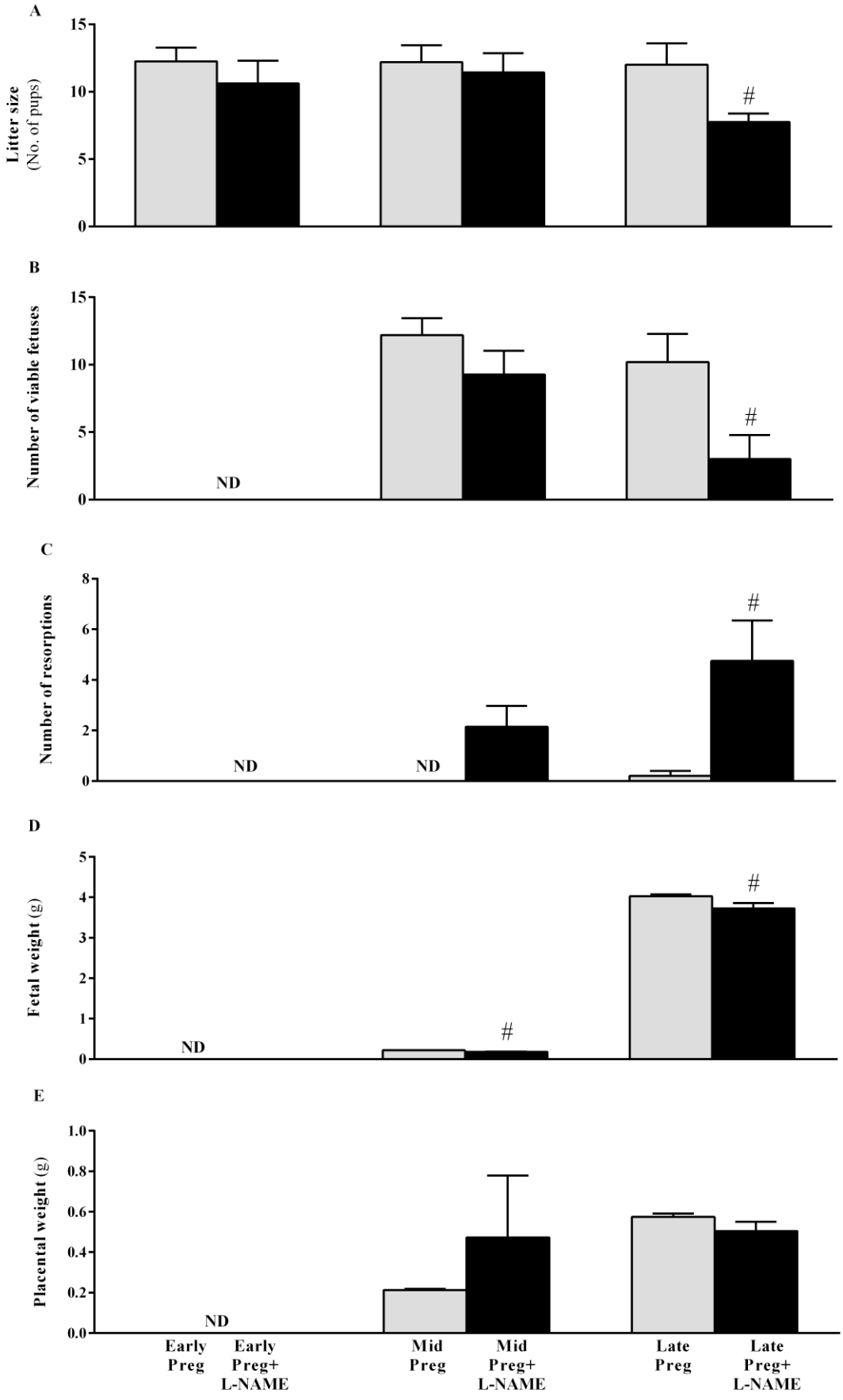
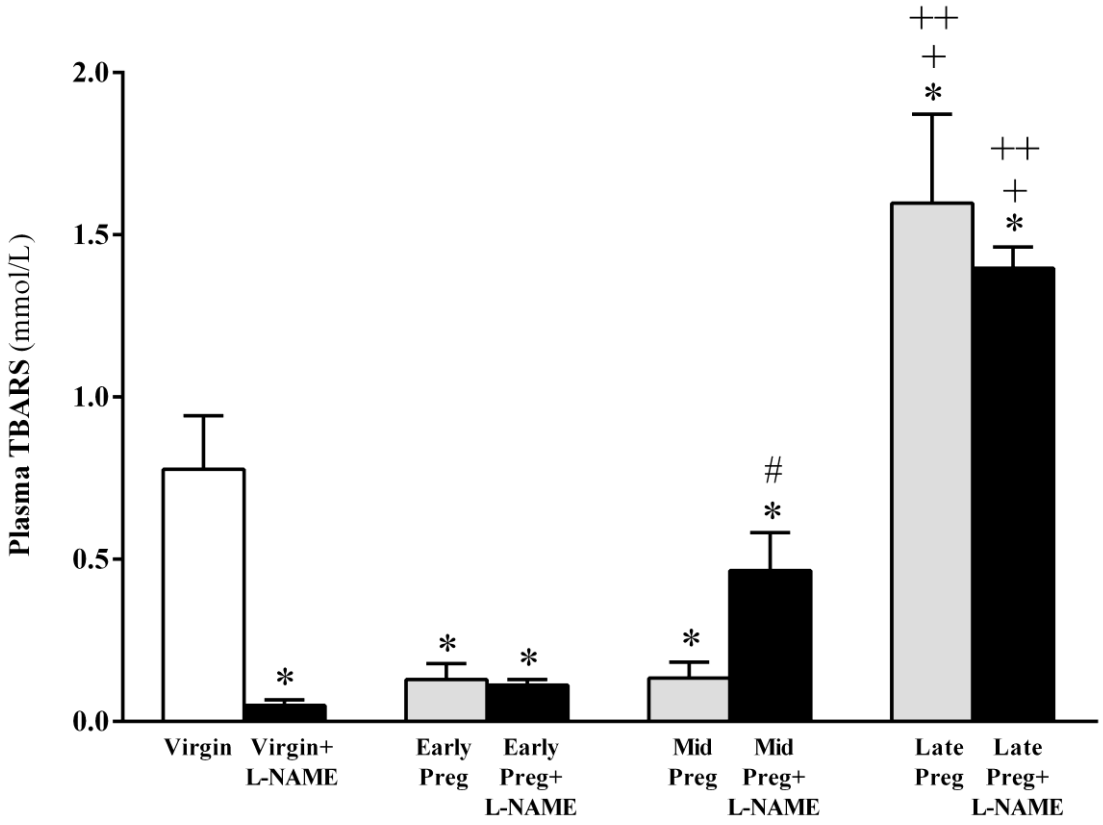
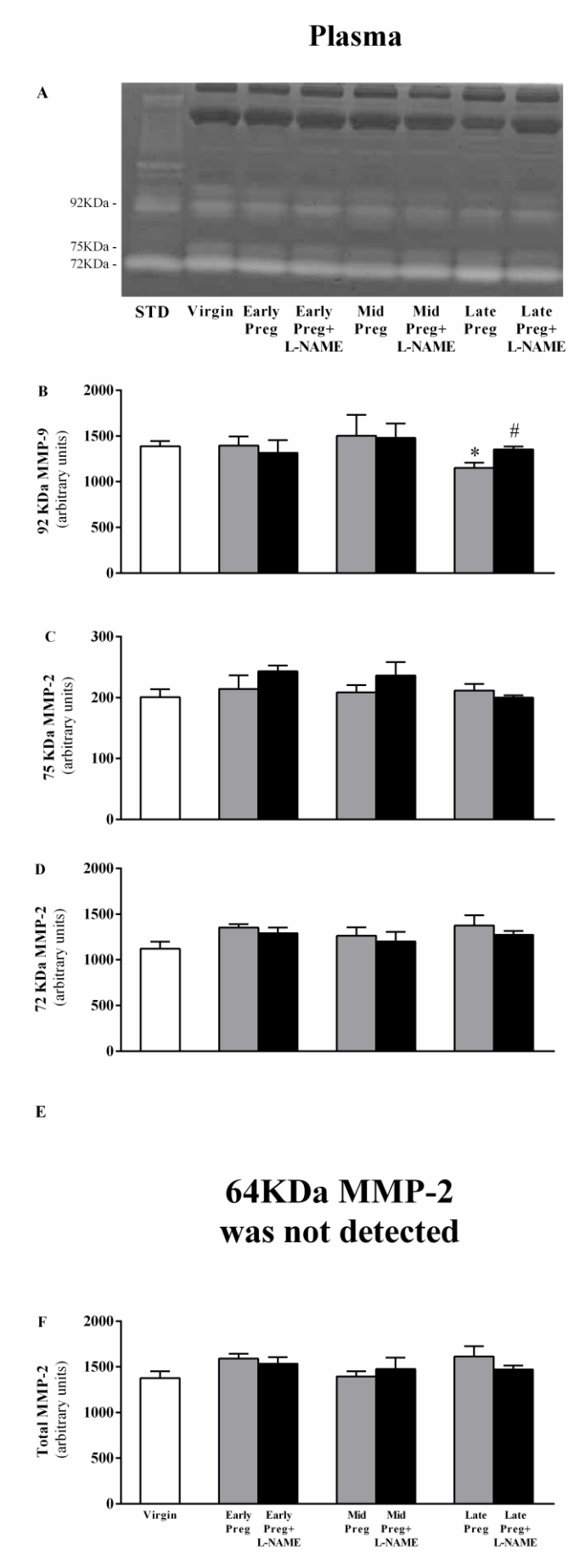
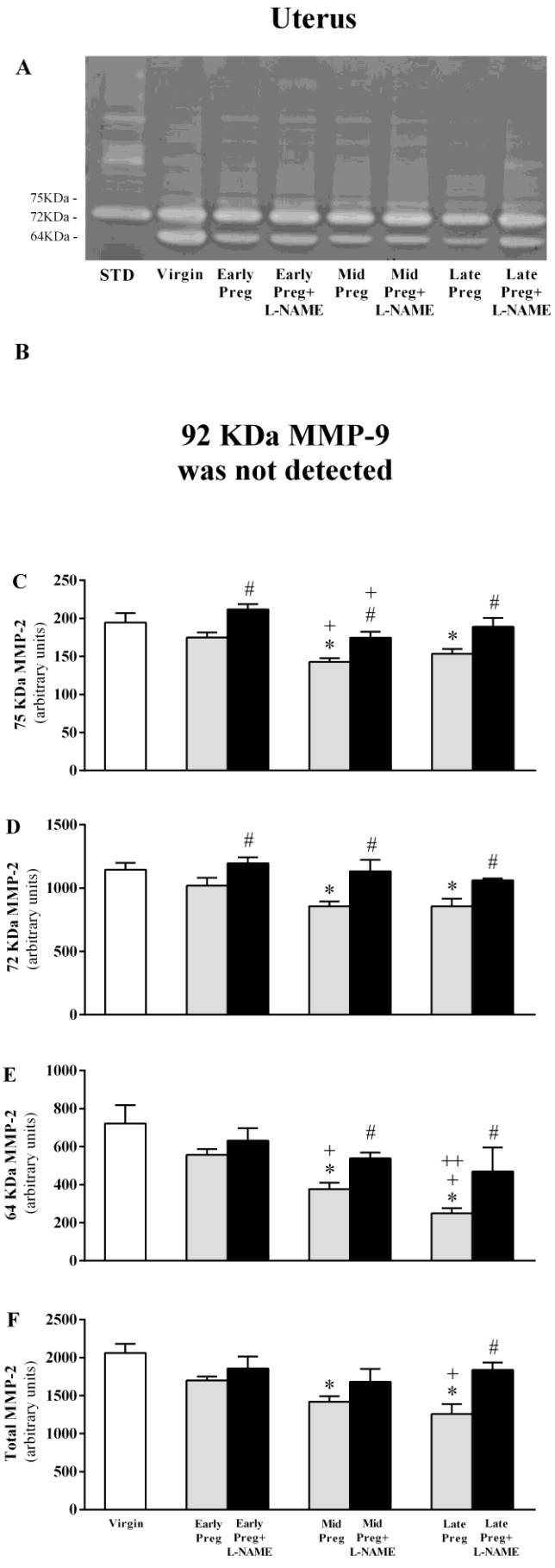

A
Virgin
Early-Pregnacy
Mid-Pregnancy
Late-Pregnancy
Systolic Blood Pressure (mmHg)
180
170
160
150
140
130
120
110
1 2 3 4 5 6
BL
3 4 6 8
BL
9 11 13 14 15 16 18 20
BL
Days
Gestational days
Virgin Basal
Virgin
Virgin+L-NAME
Early-Preg Basal
Early-Preg
Early-Preg+L-NAME
Mid-Preg Basal
Mid-Preg
Mid-Preg+L-NAME
Late-Preg Basal
Late-Preg
Late-Preg+L-NAME

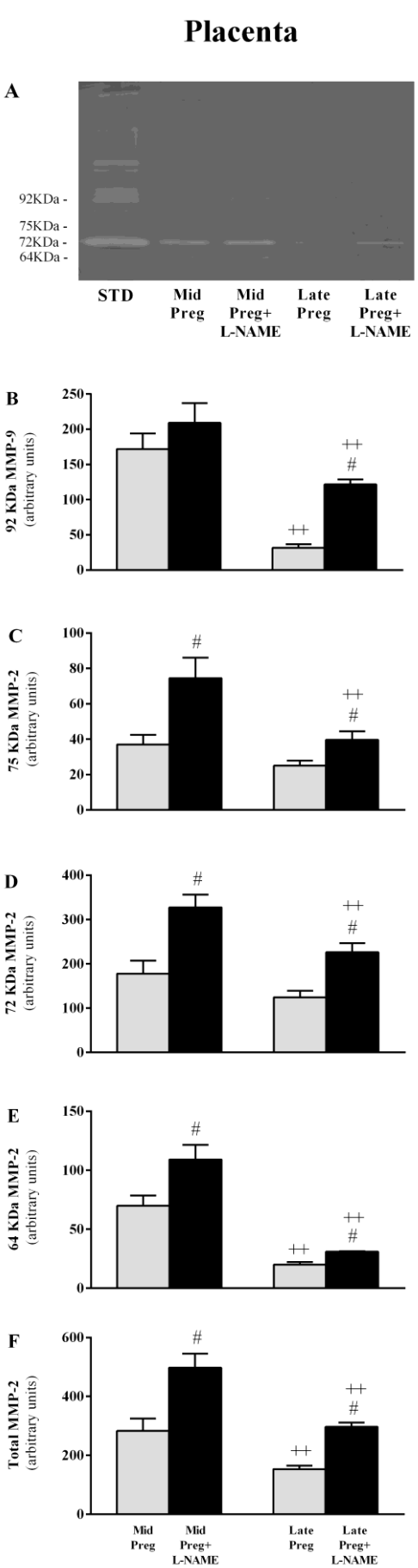

B
Plasma NOx (µmol/L)
40
30
20
10
0
Virgin
Virgin+ L-NAME
Early- Preg
Early- Preg+ L-NAME
Mid- Preg
Mid- Preg+ L-NAME
Late- Preg
Late- Preg+ L-NAME


A
Litter size
(No. of pups)
15
10
5
0
#
B
Number of viable fetuses
15
10
5
0
ND
#
C
Number of resorptions
8
6
4
2
0
ND
ND
#
D
Fetal weight (g)
5
4
3
2
1
0
ND
#
#
E
Placental weight (g)
1.0
0.8
0.6
0.4
0.2
0.0
ND
Early Preg
Early Preg+ L-NAME
Mid Preg
Mid Preg+ L-NAME
Late Preg
Late Preg+ L-NAME


Plasma TBARS (mmol/L)
2.0
1.5
1.0
0.5
0.0
Virgin
Virgin+ L-NAME
Early Preg
Early Preg+ L-NAME
Mid Preg
Mid Preg+ L-NAME
Late Preg
Late Preg+ L-NAME


Plasma
A
92KDa -
75KDa -
72KDa -
STD
Virgin
Early Preg
Early Preg+ L-NAME
Mid Preg
Mid Preg+ L-NAME
Late Preg
Late Preg+ L-NAME
B
92 KDa MMP-9 (arbitrary units)
2000
1500
1000
500
0
*
#
C
75 KDa MMP-2 (arbitrary units)
300
200
100
0
D
72 KDa MMP-2 (arbitrary units)
2000
1500
1000
500
0
E
64KDa MMP-2 was not detected
F
Total MMP-2 (arbitrary units)
2000
1500
1000
500
0
Virgin
Early Preg
Early Preg+ L-NAME
Mid Preg
Mid Preg+ L-NAME
Late Preg
Late Preg+ L-NAME


Uterus
A
75KDa -
72KDa -
64KDa -
STD
Virgin
Early Preg
Early Preg+ L-NAME
Mid Preg
Mid Preg+ L-NAME
Late Preg
Late Preg+ L-NAME
B
92 KDa MMP-9
was not detected
C
75 KDa MMP-2
(arbitrary units)
D
72 KDa MMP-2
(arbitrary units)
E
64 KDa MMP-2
(arbitrary units)
F
Total MMP-2
(arbitrary units)


Placenta
A
92KDa -
75KDa -
72KDa -
64KDa -
STD
Mid Preg
Mid Preg+ L-NAME
Late Preg
Late Preg+ L-NAME
B
92 KDa MMP-9 (arbitrary units)
C
75 KDa MMP-2 (arbitrary units)
D
72 KDa MMP-2 (arbitrary units)
E
64 KDa MMP-2 (arbitrary units)
F
Total MMP-2 (arbitrary units)
Mid Preg
Mid Preg+ L-NAME
Late Preg
Late Preg+ L-NAME

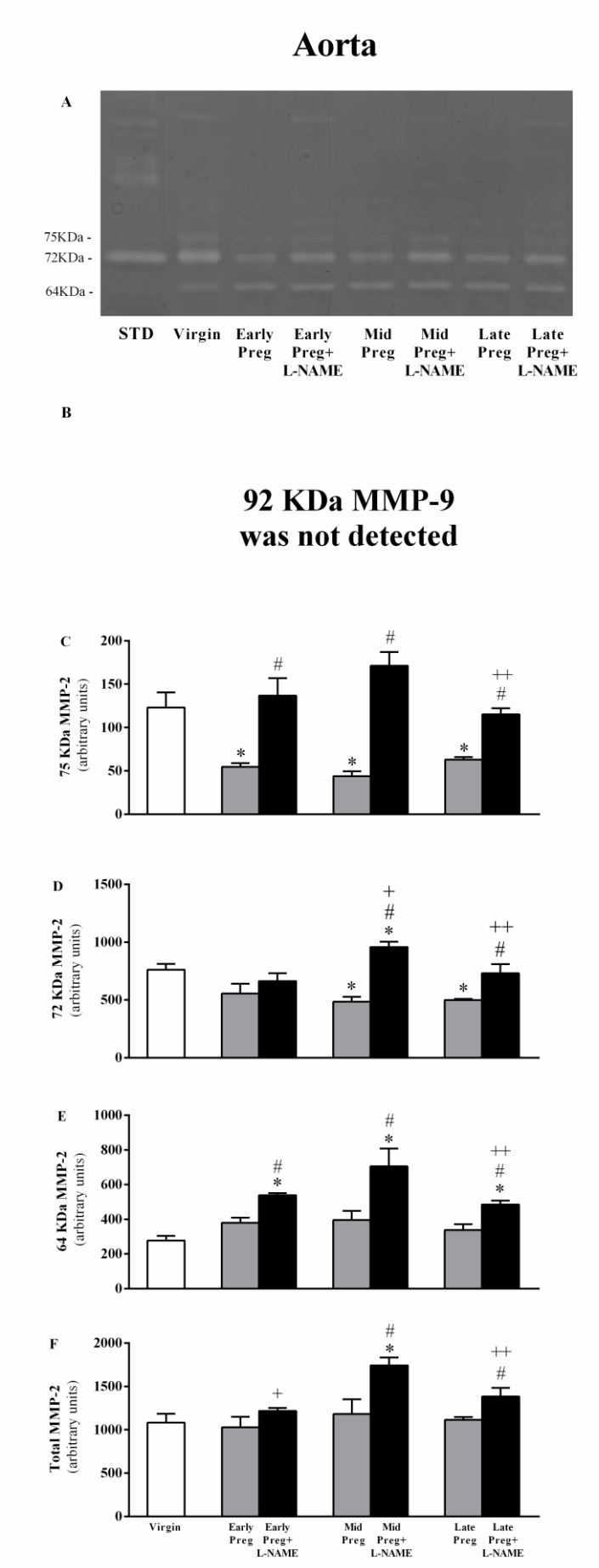

Aorta
A
75KDa -
72KDa -
64KDa -
STD
Virgin
Early Preg
Early Preg+ L-NAME
Mid Preg
Mid Preg+ L-NAME
Late Preg
Late Preg+ L-NAME
B
92 KDa MMP-9 was not detected
C
75 KDa MMP-2 (arbitrary units)
D
72 KDa MMP-2 (arbitrary units)
E
64 KDa MMP-2 (arbitrary units)
F
Total MMP-2 (arbitrary units)
Virgin
Early Preg
Early Preg+ L-NAME
Mid Preg
Mid Preg+ L-NAME
Late Preg
Late Preg+ L-NAME

Fig. 1A. Systolic blood pressure measurements. Systolic blood pressure measured by tail-cuff plethysmography for five days in control virgin and hypertensive virgin rats. The following are measurements in the groups of pregnant rats: earlypregnant on days 3, 4, 6 and 8 of gestation, middle pregnant on days 9, 11, 13 and 15 of gestation, late pregnant on days 13, 14, 16, 18 and 20 of gestation. Baseline blood pressure represents a measure of systolic blood pressure in all animals prior to randomization into normotensive or hypertensive groups: virgin rats (Virgin), virgin rats treated with L-NAME (virgin+L-NAME), early period of pregnancy (Early Preg), early period of pregnant rats treated with L-NAME (Early Preg+L-NAME), middle period of pregnancy (Mid Preg), middle period of pregnant rats treated with L-NAME (Mid Preg+L-NAME), late period of pregnancy (Late Preg), late period of pregnant rats treated with L-NAME (Late Preg+L-NAME).◇represents virgin, △ represents Early Preg, □ represents Mid Preg and ○represents Late Preg. Open symbols means basal measurement, grey symbols means animals that did not receive L-NAME, black symbols means animals that received L-NAME *P < 0.05 *versus* Basal for the same period; $^{\#}$P < 0.05 *versus* normotensive for the same period. **Fig. 1B.** Plasma NOx µmol/L were evaluated among the groups: virgins rats (Virgin), virgins rats treated with L-NAME (Virgin+L-NAME), early period of pregnancy (Early Preg), early period of pregnant rats treated with L-NAME (Early Preg+L-NAME), middle period of pregnancy (Mid Preg), middle period of pregnant rats treated with L-NAME (Mid Preg+L-NAME), late period of pregnancy (Late Preg), late period of pregnant rats treated with L-NAME (Late Preg+L-NAME). Values represent mean ± SEM. *P < 0.05 *versus* Virgin, $^{\#}$P < 0.05 *versus* Preg (from the same period), $^{+}$P < 0.05 *versus* early period and $^{++}$P < 0.05 *versus* middle period in the same condition (with or without L-NAME).

Fig 2. Fetal and placental parameters: **A)** Litter size, **B)** Number of viable fetuses, **C)** Number of reabsorption, **D)** Fetal weight and **E)** Placental weight. Parameters were evaluated in 8th gestationalday between the groups early period of pregnancy (Early Preg) and early period of pregnant rats treated with L-NAME (Early Preg+L-NAME); 15thgestational day between the groups middle period of pregnancy (Mid Preg) and middle period of pregnant rats treated with L-NAME (Mid Preg+L-NAME);

20^{th}gestational day between the groups late period of pregnancy (Late Preg) and late period of pregnant rats treated with L-NAME (Late Preg+L-NAME). Values represent mean ± SEM. $^{\#}P < 0.05$ *versus* Preg (from the same period).

Fig. 3. Plasma levels of MDA levels were evaluated among the groups: virgins rats (Virgin), virgins rats treated with L-NAME (Virgin+L-NAME), early period of pregnancy (Early Preg), early period of pregnant rats treated with L-NAME (Early Preg+L-NAME), middle period of pregnancy (Mid Preg), middle period of pregnant rats treated with L-NAME (Mid Preg+L-NAME), late period of pregnancy (Late Preg), late period of pregnant rats treated with L-NAME (Late Preg+L-NAME). Values represent mean ± SEM. $*P < 0.05$ *versus* virgin, $^{\#}P < 0.05$ *versus* Preg (from the same period), $^{+}P < 0.05$ *versus* early period and $^{++}P < 0.05$ *versus* middle period in the same condition (with or without L-NAME).

Fig. 4. Representative zymography gel of plasma (**A**), Gelatinase activity of plasma (**B – F**) MMP-2 and -9 isoforms were assessed among the groups: virgin rats, early period of pregnancy (Early Preg), early period of pregnant rats treated with L-NAME (Early Preg+L-NAME), middle period of pregnancy (Mid Preg), middle period of pregnant rats treated with L-NAME (Mid Preg+L-NAME), late period of pregnancy (Late Preg), late period of pregnant rats treated with L-NAME (Late Preg+L-NAME). Values represent mean ± SEM. $*P < 0.05$ *versus* virgin, $^{\#}P < 0.05$ *versus* Preg (from the same period).

Fig. 5. Representative zymography gel of tissue homogenate from the uterus **(A)**. Gelatinase activity of uterine (**B – F**), MMP-2 and -9 isoforms were assessed among the groups:virgin rats, early period of pregnancy (Early Preg), early period of pregnant rats treated with L-NAME (Early Preg+L-NAME), middle period of pregnancy (Mid Preg),

middle period of pregnant rats treated with L-NAME (Mid Preg+L-NAME), late period of pregnancy (Late Preg), late period of pregnant rats treated with L-NAME (Late Preg+L-NAME). Values represent mean ± SEM. *P < 0.05 *versus* virgin, $^{\#}$P < 0.05 *versus* Preg (from the same period), $^{+}$P < 0.05 *versus* early period and $^{++}$P < 0.05 *versus* middle period in the same condition (with or without L-NAME).

Fig. 6. Representative zymography gel of tissue homogenate from the placenta **(A)**. Gelatinase activity of placental (**B – F**) MMP-2 and -9 isoforms were assessed among the groups: middle period of pregnancy (Mid Preg), middle period of pregnant rats treated with L-NAME (Mid Preg+L-NAME), late period of pregnancy (Late Preg), late period of pregnant rats treated with L-NAME (Late Preg+L-NAME). Values represent mean ± SEM. $^{\#}$P < 0.05 *versus* Preg (from the same period) and $^{++}$P < 0.05 *versus* middle period in the same condition (with or without L-NAME).

Fig. 7. Representative zymography gel of tissue homogenate from the aorta (**A**), Gelatinase activity of aorta (**B – E**) MMP-2 isoforms were assessed among the groups: virgin rats, early period of pregnancy (Early Preg), early period of pregnant rats treated with L-NAME (Early Preg+L-NAME), middle period of pregnancy (Mid Preg), middle period of pregnant rats treated with L-NAME (Mid Preg+L-NAME), late period of pregnancy (Late Preg), late period of pregnant rats treated with L-NAME (Late Preg+L-NAME). Values represent mean ± SEM. *P < 0.05 *versus* virgin, $^{\#}$P < 0.05 *versus* Preg (from the same period),$^{+}$P < 0.05 *versus* early period and $^{++}$P < 0.05 *versus* middle period in the same period (with or without L-NAME)..

Discussão

4 Discussão

As desordens hipertensivas, quando não tratadas, causam grandes prejuízos tanto para a mãe, quanto para o bebê. Conforme estudos anteriores (Valdes *et al.*, 2009; Cavalli *et al.*, 2012; Hodzic *et al.*, 2017) o óxido nítrico (NO), um gás produzido por células endoteliais possuem papel essencial para promover a vasodilatação, sobretudo durante a época gestacional, contribuindo, dessa maneira, para evitar aumento exacerbado da resistência vascular sistêmica, pois durante esse período, aumenta-se o volume sanguíneo, o débito cardíaco e a frequência cardíaca (Lopes Van Balen *et al.*, 2013)

Quando há disfunções endoteliais, reduz-se a biodisponibilidade de NO, provocando vasocontrição e esta consequentemente aumenta atividade das MMPs (Metzger, Sandrim e Tanus-Santos, 2012). Essa queda do NO e aumento das MMPs culminam em desordens hipertensivas gestacionais (Matsubara *et al.*, 2015; Sutton, Harper e Tita, 2018).

Sabendo que o NO e as metaloproteinases têm grande influência na fisiopatologia dessas condições (Santos e Couto, 2018), a exploração da via do NO e estudo das alterações das metaloproteinases (MMP-2 e MMP-9) se apresentam como uma potencial opção terapêutica para o manejo dessas doenças.

O nosso modelo experimental de hipertensão gestacional utilizando L-NAME mimetiza as principais características encontradas nas desordens hipertensivas gestacionais, como: aumento de pressão arterial sistólica, redução do peso fetal, redução da capacidade antioxidante, além de aumento de fatores anti-angiogênicos como sFlt-1, e de redução de fatores pró-angiogênicos como o PLGF, demonstrando a importância do NO na circulação sistêmica e uteroplacentária durante a gestação.

Em nossa pesquisa descrita no artigo presente no Capítulo I, há os resultados do tratamento de ratas prenhes hipertensas tratadas com um inibidor não seletivo de metaloproteinases: a doxiciclina. Em nossos estudos, o uso dessa droga preveniu o aumento

da pressão arterial sistólica desses animais. Esse achado corrobora com os achados de outros estudos que mostraram o efeito anti-hipertensivo da doxiciclina, em diferentes modelos de hipertensão (Rizzi *et al.*, 2010; Antonio *et al.*, 2014; Nascimento *et al.*, 2015).

O tratamento com doxiciclina preveniu a hipertensão gestacional, não afetou a prole e também não diminuiu o peso dos fetos. É importante ressaltar que o baixo peso, ao nascer, é uma característica marcante das desordens gestacionais hipertensivas (Chisholm *et al.*, 2018) e sua melhoria seria um benefício, durante o tratamento da hipertensão durante a gravidez. Estudos experimentais mostraram que esses parâmetros materno-fetais estão correlacionados com o fluxo sanguíneo uteroplacentário e que suas reduções podem ocorrer devido à isquemia/hipóxia induzidas por L-NAME em ratos (Kaya *et al.*, 2011).

Conforme a teoria dos dois estágios da PE, fatores bioativos liberados pela placenta promovem danos às células endoteliais (Roberts, 1998). Posteriores estudos têm demonstrado que elevados níveis de sFlt-1 estão relacionados à disfunção endotelial por sequestrar moléculas angiogênicas, como VEGF e PLGF, em modelos animais de hipertensão gestacional (Shah e Khalil, 2015; Goncalves-Rizzi *et al.*, 2016) e em mulheres com PE (Maynard *et al.*, 2003).

Estudos anteriores demonstraram que o PLGF, um fator pró-angiogênico, esteve diminuído em mulheres com PE (Bian, Shixia e Duan, 2015) e em modelos animais de hipertensão (Gilbert, Babcock e Granger, 2007; Agunanne *et al.*, 2010). Posteriores estudos demonstraram a importância do fator anti-angiogênico sFlt-1 durante a hipertensão gestacional (Goncalves-Rizzi *et al.*, 2016; Possomato-Vieira *et al.*, 2016; Baijnath *et al.*, 2017). Por esse motivo, procuramos investigar os efeitos do tratamento com doxiciclina no balanço anti-angiogênico / pró-angiogênico.

Em seguida, observamos um aumento dos níveis plasmáticos de sFlt-1 em ratos HTN-Preg. Além disso, os níveis plasmáticos de PLGF estavam diminuídos no grupo HTN-Preg, o

que corrobora com achados anteriores (De Vivo *et al.*, 2008; Agunanne *et al.*, 2010; Baijnath *et al.*, 2017). É importante ressaltar que o tratamento com doxiciclina reduziu sFlt-1 e aumentou os níveis de PLGF no grupo HTN-Preg + Doxy. Portanto, o inverso do desequilíbrio antiangiogênico / pró-angiogênico promovido pela doxiciclina sugere que o equilíbrio angiogênico pode melhorar o fluxo sanguíneo da circulação uteroplacentária.

Além disso, durante a gravidez, a redução da resistência vascular sistêmica é uma importante adaptação vascular para garantir a manutenção da pressão sanguínea (Ouzounian e Elkayam, 2012). Portanto, aumentos do fluxo sanguíneo da circulação uteroplacentária devido à angiogênese aumentada poderiam contribuir ainda mais para a redução da pressão arterial sistólica, caso seguida do tratamento com doxiciclina.

Quanto ao estresse oxidativo, sabe-se que em doenças hipertensivas gestacionais é comum a existência de um aumento de moléculas oxidantes e redução das substâncias antioxidantes em condições patológicas, como a hipertensão (Matsubara *et al.*, 2015). Por causa disso, avaliamos a capacidade antioxidante plasmática e os níveis plasmáticos de MDA, os quais podem indicar a presença de estresse oxidativo (Bakacak *et al.*, 2015; Atiba *et al.*, 2016).

No entanto, não observamos alterações significativas nos níveis de MDA entre os quatro grupos experimentais do primeiro estudo. Apesar disso, o tratamento com doxiciclina promoveu aumento da capacidade antioxidante plasmática em ratas prenhas hipertensas, uma vez que os níveis da capacidade antioxidante total medidos com o ensaio equivalente ao trolox (TEAC) esteve aumentados no grupo de ratas prenhas hipertensas tratadas com doxiciclina, quando comparados aos níveis de TEAC presentes no grupo de ratas prenhas hipertensas não tratadas e no grupo de ratas normais. Portanto, sugerimos que a doxiciclina pode exercer efeitos antioxidantes e contrabalançar o estresse oxidativo. E esses achados estão em conformidade com trabalhos anteriores (Antonio *et al.*, 2014; Tilakaratne e Soory, 2014).

O nosso estudo demonstrou também que a administração de doxiciclina é capaz de restabelecer a capacidade antioxidante plasmática, após inibição da síntese endógena de NO, sugerindo uma proteção contra o estresse oxidativo, que pode ter acarretado uma maior vasodilatação de artérias responsáveis pela perfusão útero-placentária. O aumento da capacidade antioxidante do plasma pode proteger as células contra reações de peroxidação, preservar a integridade da membrana celular e, assim, proteger a vasculatura dos danos causados pelas espécies reativas de oxigênio, mantendo a função vascular (Raijmakers, Dechend e Poston, 2004; Matsubara *et al.*, 2015).

Além disso, em nosso trabalho, especulamos que esses níveis reduzidos de NO podem induzir uma maior vasoconstrição e, consequentemente, aumentar essas forças hemodinâmicas em vasos de interface uteroplacentária. Esse mecanismo contribuiria, pelo menos em parte, para o aumento da atividade de MMPs, como ocorreu em nossa pesquisa.

Durante a gestação, esse aumento excessivo da atividade das MMPs pode contribuir para a fisiopatologia da hipertensão gestacional (Myers *et al.*, 2005; Martinez-Fierro *et al.*, 2018). A inibição das MMPs pela doxiciclina pode estar relacionada com os efeitos benéficos observados em nosso estudo. MMPs são enzimas envolvidas na degradação de muitos componentes da matriz extracelular (Li *et al.*, 2014).

As MMP-2 e -9 são encontradas em vários tecidos e órgãos (Mayer *et al.*, 2018), como útero (Lombardi *et al.*, 2018), placenta (Sahay *et al.*, 2018a) e células miometriais (Cardozo *et al.*, 2018) em ratas e humanas prenhas normais, entretanto, sua atividade aumenta quando há a hipertensão durante a gravidez. É esse o motivo de relacionarmos os benefícios promovidos pela doxiciclina às ratas prenhas hipertensas tratadas com esse inibidor não seletivo de metaloproteinases, uma vez nossos resultados mostraram que as MMP-2 e -9 placentárias e uterinas estavam inibidas nesse grupo, quando comparadas ao grupo de ratas prenhas hipertensas não tratadas com doxiciclina .

Apesar da inibição geral das MMP-2, houve a ausência de mudanças significativas dessa metaloproteinase em algumas isoformas. Os níveis de MMP-2 placentárias no 72 KDa, 64 KDa e total KDa do grupo de ratas hipertensas tratado com doxiciclina não mostrou diferença significativa em relação aos níveis do grupo de ratas prenhas hipertensas que não receberam doxiciclina. Esses resultados muito provavelmente se relacionam com o que Springman et al. (1990) nos sugere em seus estudos.

Conforme Springman et al. (1990), durante a técnica de zimografia, a MMP latente sofre modificações na estrutura da membrana devido à ação do Duodecil Sulfato de Sódio. Esse reagente possui efeito surfactante, muda a estrutura da membrana e expõe o resíduo de cisteína de seu habitat na enzima latente onde normalmente ela reside, formando um complexo com o Zn^{2+}. Essa modificação confere um desequilíbrio conformacional à enzima, tornando-a parcialmente ativa. A parcialidade da ativação pode ter causado a ausência de mudança significativa dessas isoformas de MMP-2 placentárias 72 KDa, 64 KDa e total KDa grupo de ratas prenhas hipertensas tratadas com doxiciclina em relação ao grupo de ratas prenhas hipertensas não tratadas com esse inibidor de metaloproteinas.

Tem sido demonstrado que forças hemodinâmicas excessivas, como tensão de cisalhamento e forças hidrostáticas, podem levar a aumentos do estiramento mecânico do músculo liso vascular e, portanto, aumentar a atividade das MMPs. (Seo *et al.*, 2013; Seo *et al.*, 2015). Tendo conhecimento que a redução de NO é um fator importante das desordens hipertensivas gestacionais (Goncalves-Rizzi *et al.*, 2018) , propomos avaliar as atividades de MMP-2 e -9 em diferentes períodos da gestação e mudanças das atividades de MMPs, com diminuição da biodisponibilidade do NO ao longo da gravidez.

Embora não tenhamos relatado diferenças dos níveis plasmáticos de NO em ratas virgens e prenhas no período inicial da gravidez, nossos resultados em relação aos níveis

plasmáticos de NO durante a gestação média e tardia, indicam que a demanda por esse gás deve aumentar nos últimos estágios da gravidez saudável.

Esses resultados estão de acordo com estudos anteriores os quais mostram que a excreção de nitrato / nitrito urinário aumenta ao longo da gestação em ratos, atingindo níveis máximos durante os estágios finais da gravidez (Alexander *et al.*, 1999). Estudos também mostraram que a pressão arterial aumenta em camundongos prenhes nocautes para eNOS, enquanto a pressão arterial permanece inalterada em camundongos virgens nocautes para eNOS (Kusinski *et al.*, 2012).

Quando comparado à latência observada, é importante ressaltar que, em nossa pesquisa, o aumento da pressão arterial ocorreu de forma mais abrupta em ratas prenhes tardias do que no grupo de ratas virgens e grupo de ratas no início da prenhes e grupo de ratas no tempo intermediários da prenhes, com hipertensão induzida por L-NAME. Isso indica que a demanda por NO deve ter aumentado nas fases finais da gravidez.

Além disso, observamos mudanças do peso dos fetos, tamanho da ninhada e número de fetos viáveis ou reabsorvidos apenas durante os estágios mais avançados da gravidez, enquanto que esses parâmetros não se alteraram na gravidez precoce e média (a exceção de somente um: peso fetal, alterado no estágio intermediário da gravidez), o que confirma um maior envolvimento do NO, durante o estágio final da gestação.

No artigo apresentado no segundo capítulo desta tese, procurou-se investigar a participação das MMPs circulantes, aórticas, placentárias e uterinas como mediador fisiológico normal, durante a gestação saudável, como também a alteração da atividade dessas enzimas em diferentes períodos da hipertensão gestacional, induzida pela inibição da síntese de NO.

Em geral, observamos que, durante a gravidez saudável, há um aumento do NO, seguido por diminuições da atividade da MMP-2 no útero, placenta e aorta e MMP-9 no

plasma e placenta no final da prenhes. Embora algumas isoformas não tenham apresentado mudanças em suas atividades na prenhes comparadas a ratas virgens, nossos dados mostram que uma redução da atividade das MMPs pode estar sujeita a pelo menos parte do processo fisiológico envolvido nas adaptações útero-placentárias e vasculares da gravidez (Chen *et al.*, 2017).

É importante observar que no útero não há alterações na atividade da MMP-2 durante o início da gravidez, mas há diminuições da atividade da MMP-2 uterina em gestações intermediárias e tardias em comparação com ratas virgens, o que pode estar relacionado à reduzida atividade fisiológica dessas enzimas para que haja o remodelamento adequado da matriz extracelular, invasão trofoblástica e remodelamento vascular das artérias espirais.

Por outro lado, o L-NAME reduziu a biodisponibilidade do NO e esse efeito foi associado ao aumento da atividade da MMP-2 no útero, placenta e aorta e MMP-9 no plasma e placenta de ratas hipertensas comparados com ratas normais prenhes. Importante: um aumento excessivo da atividade de MMPs (Myers *et al.*, 2005; Eleuterio *et al.*, 2015) pode estar associado à redução da biodisponibilidade de NO (Schiessl *et al.*, 2006; Matsubara *et al.*, 2010; Sandrim *et al.*, 2010; Ehsanipoor *et al.*, 2013; Pimentel *et al.*, 2013; Mazzuca *et al.*, 2014; Amaral *et al.*, 2015), contribuindo, desta forma, para a fisiopatologia da hipertensão gestacional.

As sugestões desses trabalhos estão em conformidade com as descobertas apresentadas no artigo presente no primeiro capítulo, mostrando que a ativação de MMPs induzida por L-NAME foi atenuada pela doxiciclina, um inibidor não-seletivo de MMPs, em ratas prenhes com hipertensão tardia (Nascimento *et al.*, 2018).

Nossos resultados sugerem que o NO regula negativamente a MMP-2 e a MMP-9 durante a gravidez normal, porque o aumento da atividade das MMPs ocorreu juntamente com a redução da biodisponibilidade de NO causada pelo L-NAME. Por esse motivo, isso

pode representar um importante mecanismo envolvido em desordens hipertensivas gestacionais, uma vez que reduções dos níveis de NO foram encontradas nos distúrbios relacionados a prenhes (Myatt e Webster, 2009; Sandrim *et al.*, 2010).

Em conjunto, nossos resultados indicam que pode haver uma demanda maior por aumento de NO com o avanço da gestação saudável, enquanto reduções na biodisponibilidade de NO induzido por L-NAME durante os períodos intermediário e tardio da gestação podem resultar em maior atividade das MMPs e contribuições para a hipertensão gestacional.

Também observamos que o L-NAME aumentou o estresse oxidativo em prenhas de período intermediário e tardio, mas não no início da prenhes, em comparação com ratas virgens. Tem sido proposto que o estresse oxidativo pode desencadear a ativação de MMPs (Mori *et al.*, 2018) e, portanto, sugerimos que o aumento do estresse oxidativo também pode contribuir para a ativação de MMPs, como observado em ratas prenhes hipertensas. É importante ressaltar que embora o estresse oxidativo elevado tenha sido semelhante em gestantes normais e hipertensas no final da gestação, seu aumento contrabalançado por defesas antioxidantes abundantes pode ocorrer mesmo em gestações saudáveis devido às maiores demandas metabólicas exigidas para o desenvolvimento fetal no final da gestação (Sanchez-Aranguren *et al.*, 2014; Ali e Khalil, 2015). Portanto, sugerimos que o sistema antioxidante prejudicado associado com aumento do estresse oxidativo causado por L-NAME (Goncalves-Rizzi *et al.*, 2018) também poderia contribuir para desencadear a ativação de MMPs durante a hipertensão na gestação (Yu *et al.*, 2018).

Conclusão

5 Conclusão

Os nossos achados estão de acordo com as nossas hipóteses de que reduções na formação de NO podem estar correlacionadas ao aumento das atividades de MMP-2 e -9, sugerindo que pode haver um *crosstalk* entre redução da síntese de NO e aumento da atividade de MMPs na hipertensão durante a gravidez. Além disso, nossos estudos sugerem também que após a inibição da NOS, a doxiciclina reduz a pressão arterial sistólica e atenua os aumentos das atividades de MMP-2 e -9.

Em resumo, nossos dados indicam que a demanda por NO pode ser maior durante os períodos finais da gestação e que o NO pode modular negativamente as MMPs durante a gestação saudável, uma vez que, quando a biodisponibilidade de NO é reduzida, observamos aumentos na atividade das MMP-2 e -9. Portanto, nossos resultados estão de acordo com a hipótese de que o NO modula a atividade das MMPs durante a gestação.

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