



UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

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Efeitos do nitrito de sódio e sua associação ao
sildenafil na hipertensão gestacional
experimental em ratas

Botucatu, 2018

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Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Farmacologia e Biotecnologia, junto ao Programa de Pós-Graduação em Farmacologia e Biotecnologia, do Instituto de Biociências de Botucatu da Universidade Estadual Paulista —Júlio de Mesquita Filho, Campus de Botucatu.

Orientador:

Prof. Dr. Carlos Alan Candido Dias Junior

Botucatu, 2018

Ficha catalográfica

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP

BIBLIOTECÁRIA RESPONSÁVEL: ROSANGELA APARECIDA LOBO-CRB 8/7500

Rizzi, Victor Hugo Gonçalves.

Efeitos do nitrito de sódio e do sildenafil na hipertensão gestacional experimental em ratas / Victor Hugo Gonçalves Rizzi. - Botucatu, 2018

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências de Botucatu

Orientador: Carlos Alan Candido Dias Junior

Capes: 21001006

1. Citrato de Sildenafil. 2. Hipertensão Induzida pela Gravidez. 3. NG-Nitroarginina Metil Éster. 4. Nitrito de sódio. 5. Ratos Wistar.

Palavras-chave: Citrato de Sildenafil; Hipertensão gestacional; L-Name; Nitrito de sódio; Ratas.

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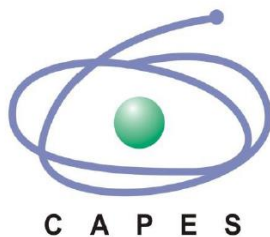
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Financiamento



(Bolsa capes de doutorado: Período de vigência 03/2014 – 05/2017)



Proc. 16/18782-3

(Auxílio a pesquisa regular: Prof. Dr. Carlos Alan Candido Dias Junior)

Proc. 12/21305-1

(Auxílio a pesquisa regular: Prof. Dr. Carlos Alan Candido Dias Junior)

Dedicatória

Aos meus pais, Luiz e Maria

*De quem Deus me deu a honra de ser filho,
responsáveis pela minha formação, meus exemplos e
meus maiores incentivadores.*

Meu amor e minha gratidão por vocês serão eternos.

AGRADECIMENTOS

... a **Deus**, que está sempre presente em minha vida,

... a minha eterna e fiel companheira **Mariana**, por ter sonhado esse sonho comigo, por sempre acreditar na minha capacidade, pelo incentivo em nunca desistir, pela paciência e compreensão de minha ausência,

... ao meu orientador **Prof. Dr. Carlos Alan Candido Dias Junior**, pela oportunidade, amizade, ensinamentos, comprometimento e acompanhamento do meu crescimento científico,

... a **Prof. Dra. Valéria Cristina Sandrim**, pela imensa colaboração durante todo o período do doutorado

...aos integrantes e ex-integrantes do Laboratório de Farmacologia Cardiovascular, **Zé, Regina, Gaby, Jomar, João, Tamiris, Maria Luiza, Jéssica e Thalita** pela grande amizade, por compartilhar conhecimentos, sonhos e até frustrações nos experimentos. Desejo todo sucesso do mundo pra vocês. Podem contar comigo sempre !!

... aos amigos dos laboratórios vizinhos, **Kat, André, Rodrigo, Cristiane, Bia, Mayara, Nayara, Lilian, Alan** e todos os outros pela amizade, compartilhamento de equipamentos, vidrarias, reagentes e também pelo sagrado cafezinho,

... ao **Prof. Dr. Antônio Francisco Godinho** e por todos alunos e funcionários do CEATOX

... aos professores **Dra. Ana Angélica Henrique Fernandes, Dr. Luis Antonio Justulin Jr e Dr. Celso Acácio Rodrigues de Almeida Costa**, pelas contribuições na banca de qualificação,

... aos alunos do programa de Farmacologia e Biotecnologia, sem vocês os almoços, churrascos e festas não seriam os mesmos,

...a todos os técnicos e funcionários do departamento de farmacologia pelo auxílio profissional, **Paulão, Luiz, Cris, Janete** e principalmente ao **Hélio** pela grande ajuda nas análises bioquímicas,

...a todos grandes amigos que fiz nesses 5 anos morando em Botucatu,

...a todos da 21º Regional de Saúde, em especial ao pessoal da farmácia, **Rosana, Isabely, Cleusa, Elisângela, Érika e Marcelo** pela amizade, companheirismo e principalmente pela compreensão,

...aos funcionários da Seção de Pós Graduação do IBB, pela disposição em resolver vários problemas e esclarecer nossas dúvidas,

...ao Instituto de Biociências de Botucatu e a todos professores do departamento de Farmacologia, por todo suporte dado para a realização desse trabalho,

...a CAPES e FAPESP pelo auxílio financeiro.

“Quem acredita sempre alcança”
(Renato Russo)

Prefácio

A tese “Efeitos do nitrito de sódio e sua associação ao sildenafil na hipertensão gestacional experimental em ratas foi dividida em quatro partes.

A primeira parte refere-se a uma introdução sobre as desordens hipertensivas gestacionais como: suas principais diferenças e características, epidemiologia, fisiopatologia e principais medicamentos utilizados atualmente. Além disso, teve o objetivo de descrever os principais aspectos farmacológicos do nitrito de sódio e do citrato de sildenafil como possíveis estratégias terapêuticas baseadas no estudo da via NO-GMPc.

A segunda parte inclui dados do artigo “*Sodium nitrite attenuates hypertension-in-pregnancy and blunts increases in soluble fms-like tyrosine kinase-1 and in vascular endothelial growth factor*” publicado na revista “*Nitric Oxide*” onde se é possível observar os efeitos isolados do nitrito de sódio em um modelo de hipertensão gestacional induzido por L-NAME.

A terceira parte inclui dados do artigo “*Maternal hypertension and feto-placental growth restriction is reversed by sildenafil: evidence of independent effects of circulating nitric oxide levels*” publicado na revista “*European Journal of Pharmacology*” no qual apresenta resultados da associação entre sildenafil e o nitrito de sódio em um modelo de hipertensão gestacional induzido por L-NAME, sugerindo que o efeito do sildenafil não depende totalmente dos níveis circulantes de óxido nítrico e GMPc.

A quarta parte apresenta uma breve discussão sobre os achados dos dois artigos e a quinta parte descreve considerações finais da tese, concluindo os principais achados nos trabalhos desenvolvidos durante o doutorado.

Durante a execução desse projeto, outras atividades foram desenvolvidas com o objetivo de enriquecer a formação acadêmica.

Disciplinas cursadas

Disciplinas	Ano	Período Letivo	Créditos	Carga Horária	Freq	Conceito
Análise de Vesículas Extracelulares Contendo miRNAs e Proteínas	2014	1º semestre	2	30	100	A
Neurotoxicidade por Exposição a Metais Pesados e Pesticidas	2014	1º semestre	4	60	100	A

Tópicos Especiais em Farmacologia: Análise da variabilidade da frequência cardíaca e da pressão arterial e modulação autonômica vascular	2014	1º semestre	1	15	100	A
Farmacologia do Endotélio Vascular	2014	2º semestre	6	90	100	A
Prática de Ensino de Farmacologia	2014	2º semestre	4	60	92	A
Pré-eclâmpsia: da bancada ao leito	2014	2º semestre	4	60	100	A
Tópicos Avançados em Farmacologia e Biotecnologia	2014	2º semestre	3	45	93	A
Tópicos Avançados em Farmacologia e Biotecnologia	2015	1º semestre	3	45	84	A
Créditos aproveitados do mestrado			15	225		
Total de geral créditos			42	630		

Artigos publicados

- **GONÇALVES-RIZZI V.H.**; POSSOMATO-VIEIRA J.S.; NASCIMENTO R.A.; DIAS-JUNIOR C.A. Maternal hypertension and feto-placental growth restriction is reversed by sildenafil: evidence of independent effects of circulating nitric oxide levels. *European Journal of Pharmacology*. XX (2008)
- ZHU M.L.; ZHAO J.P.; CUI N.; **GONÇALVES-RIZZI V.H.**; POSSOMATO-VIEIRA J.S.; NASCIMENTO R.A.; DIAS-JUNIOR C.A. Cardiac myeloperoxidase activity is elevated in hypertensive pregnant rats. *J Huazhong Univ Sci Technolog Med Sci*. 37 (6): 904-909, (2017)
- POSSOMATO-VIEIRA J.S.; **GONÇALVES-RIZZI V.H.**; SALES T.U.; NASCIMENTO R.A.; DIAS-JUNIOR C.A. Hydrogen sulfide donor prevents hypertension and both increases in vascular endothelial growth factor (VEGF) and its receptor soluble form (sFlt-1) induced by L-NAME in pregnant rats. *Naunyn schmiedebergs*. 389 (12): 1325-1332, (2016).

- **GONÇALVES-RIZZI V.H.**; SENE L.B.; FERNANDEZ C.B.; GONTIJO J.A.; BOER P.A. Impact of long-term high-fat diet intake gestational protein-restricted offspring on kidney morphology and function. *Journal of Developmental Origins of Health and Disease* (2016).

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- CHAGURI JL, GODINHO AF, HORTA DF, **GONÇALVES-RIZZI VH**, POSSOMATO-VIEIRA JS, NASCIMENTO RA, Dias-Junior CA. Exposure to fipronil elevates systolic blood pressure and disturbs related biomarkers in plasma of rats. *Environmental Toxicology and Pharmacology*. 42:63-68, (2015).

- **GONÇALVES-RIZZI VH**; NASCIMENTO, REGINA APARECIDA ; POSSOMATO-VIEIRA, JOSE SERGIO; DIAS, CARLOS A. Sodium Nitrite Prevents both Reductions in Circulating Nitric Oxide and Hypertension in 7-Day Lead-Treated Rats. *Basic & Clinical Pharmacology & Toxicology*. 118(3):225-30, (2015).

- ROCHA, THALITA L. A. ; DIAS-JUNIOR, CARLOS A. ; POSSOMATO-VIEIRA, JOSE S.; **GONÇALVES-RIZZI, VICTOR H.** ; NOGUEIRA, FLÁVIA R. ; DE SOUZA, KÁTINA M.; BRAZ, LEANDRO G. ; BRAZ, MARIANA G. Sevoflurane Induces DNA Damage Whereas Isoflurane Leads to Higher Antioxidative Status in Anesthetized Rats. *BIOMED RES INT*. 264971, (2015).

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Participação em eventos

- Simpósio de Farmacologia e Biotecnologia – SIMFARTEC (Unesp/Botucatu), 2017
- 48º Congresso da Sociedade Brasileira de Farmacologia e Terapêutica Experimental (2016) – Foz do Iguaçu - PR
- Experimental Biology (2015) - Boston - EUA
- V Simpósio de Farmacologia da UNESP - SIMFAR (UNESP/Botucatu), 2015.
- XIII Workshop da Pós Graduação (UNESP/Botucatu), 2014.

Resumos em evento

- 49º Congresso da Sociedade Brasileira de Farmacologia e Terapêutica Experimental (2017)- Ribeirão Preto – SP

Nascimento RA.; Possomato-Vieira JS.; **Gonçalves-Rizzi VH.**; Dias-Jr CA. Doxycycline Reduces Blood Pressure and Reestablishes the Antioxidant Capacity without Changes in Feto - Placental Restriction in Hypertensive Pregnant Rats. 2017.

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Possomato-Vieira JS.; Chimini JS.; Santos-Silva ML.; **Gonçalves-Rizzi VH.**; Dias-Jr CA. Hydrogen Sulfide (H₂S) Attenuates Hypertension in Pregnancy and Blunts Fetal Growth Restriction through an Increase in Placental Efficiency in Hypertensive Pregnant Rats. 2017.

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Gonçalves-Rizzi VH.; Possomato-Vieira JS.; Nascimento RA.; Silva KP.; Caldeira-Dias M.; Sandrim VC.; Dias-Jr CA. Placental-fetal interface is affected positively by sodium

nitrite and sildenafil and concomitantly shows reductions in hypertension-in-pregnancy. 2016

- 48° Congresso da Sociedade Brasileira de Farmacologia e Terapêutica Experimental (2016) – Foz do Iguaçu - PR

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- 48° Congresso da Sociedade Brasileira de Farmacologia e Terapêutica Experimental (2016) – Foz do Iguaçu - PR

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- 7 Simposio Internacional de Graduação e Pesquisa (SinposPq) – Ribeirão Preto/SP, 2016

Nascimento RA; Possomato-Vieira JS; **Gonçalves-Rizzi VH**; Dias-Junior CA. Doxycycline reduces blood pressure without changes in fetal weight in hypertensive pregnant rats. 2016

- 18° Encontro Nacional de Biomedicina (ENBM) - Botucatu/SP, 2015

CHAGURI, J. L. ; **RIZZI, V. H. G.** ; GODINHO, A. F. ; DIAS JUNIOR, C. A. C. Efeitos da exposição ao pesticida fipronil nas alterações pressóricas em ratos acordados. 2015

- V Simpósio de Farmacologia da Unesp (Simfar)

RIZZI, V.H.G. Efeito do nitrito de sódio e sua associação ao sildenafil na pré-eclâmpsia experimental. 2015

- Experimental biology - Boston - EUA, 2015

RIZZI, V. H. G.; POSSOMATO-VIEIRA, J. S. ; MENDES, G. ; NASCIMENTO, R. A. ; SILVA, K. P. ; PUPO, A. S. ; DIAS JUNIOR, C. A. C. . Sodium nitrite reduces systolic

blood pressure in preeclampsia. In: Experimental Biology, 2015, Boston - MA. The FASEB journal, 2015. v. 29

- Experimental biology - Boston - EUA, 2015

POSSOMATO-VIEIRA, J. S.; **RIZZI, V. H. G.**; SILVA, K. P. ; NASCIMENTO, R. A. ; MENDES, G. ; PUPO, A. S. ; DIAS JUNIOR, C. A. C. Hydrogen Sulfide (H₂S) Donor Attenuates Systolic Blood Pressure and Enhances Placentas Weights in Preeclampsia. In: Experimental Biology, 2015, Boston - MA. The FASEB journal, 2015.

- IV Simpósio de Farmacologia da Unesp (Simfar)

RIZZI, V.H.G. Efeito do nitrito de sódio e sua associação ao sildenafil na pré-eclâmpsia experimental induzida por redução da pressão de perfusão uterina em ratas. 2014

- VI Simpósio Internacional de Pós-Graduação e Pesquisa (Ribeirão Preto), 2014.

NASCIMENTO, R. A. ; POSSOMATO-VIEIRA, J. S. ; **RIZZI, V. H. G.** ; GRACA, T. U. S. ; DIAS JUNIOR, C. A. C. Doxycycline abolished both reduced adrenomedullin plasma levels and hypertension caused by lead in rats. 2014. (Apresentação de Trabalho/Simpósio)

- 17º Encontro Nacional de Biomedicina (Botucatu), 2014

NASCIMENTO, R. A. ; POSSOMATO-VIEIRA, J. S. ; **RIZZI, V. H. G.** ; GRACA, T. U. S. ; DIAS JUNIOR, C. A. C. Doxicilina reverte a hipertensão e o comprometimento da adrenomedulina circulante causados pelo chumbo em ratos. 2014. (Apresentação de Trabalho/Simpósio)

Aulas/palestra/cursos ministrados

Palestra

- Farmacoterapia da pré-eclâmpsia: implicações para o profissional farmacêutico (IX Jornada de farmácia da USC/Bauru), 2014

- Farmacologia das desordens hipertensivas (Disciplina de Tópicos em Biologia Geral e Aplicada)

Aula na Pós Graduação

- Farmacologia e farmacoterapia dos fármacos que atuam no trato gastrointestinal. 2015 - Especialização em assistência farmacêutica - Universidade do Sagrado Coração (USC - Bauru)
- Efeitos do nitrito de sódio e de sua associação ao sildenafil na pré-eclâmpsia experimental induzida pela redução da pressão de perfusão uterina em ratas. 2014 - (disciplina toxicologia)

Aulas na Graduação

- Professor substituto da disciplina de fisiologia humana para o curso de Farmácia nas Faculdades Integradas de Ourinhos durante o 2º semestre de 2016
- Estágio de docência realizado na disciplina de Farmacodinâmica no 1º semestre de 2015
- Professor Bolsista do departamento de Farmacologia no 2º semestre de 2015 ministrando tópicos de farmacologia para os cursos de Biologia, Nutrição, Biomedicina, Medicina Humana, Medicina Veterinária, Enfermagem e tópicos de toxicologia para o curso de Biomedicina.

Mini-Curso ministrado

- Desordens hipertensivas gestacionais com ênfase em pré-eclâmpsia. Minicurso de curta duração ministrado no Workshop da Pós graduação (2015).

Lista de abreviaturas e siglas

ANOVA – Análise de Variância
BH4 – Tetrahydrobiopterina
CYP3A4 – Citocromo P450 3A4
EDTA - Ácido etilenodiamino tetra-acético
ELISA – Ensaio de imunoadsorção enzimática
eNOS – Óxido nítrico sintase endotelial
EROs – Espécies reativas de oxigênio
FAD – Flavina adenina dinucleotídeo
FMN – Flavina mononucleotídeo
GCs – Guanilato ciclase solúvel
GMPc – Guanosina monofosfato cíclico
HIF1 α – Fator induzido por hipóxia 1
HUVECS – Células endoteliais da veia umbilical humana
iNOS - Óxido nítrico sintase induzível
iPDE5 – Inibidores da fosfodiesterase 5
L-NAME - N(G)-Nitro-L-arginine methyl ester
L-NMMA - L-N^G-monomethyl Arginine
L-NNA - L-N^G-Nitroarginine
MPO – mieloperoxidase
MTT - Brometo de [3-(4,5-dimetiltiazol-2yl)-2,5-difenil tetrazolium]
NADPH - Fosfato de dinucleótido de nicotinamida e adenina
NaNO₂ – Nitrito de sódio
nNOS – Óxido nítrico sintase neuronal
NO – Óxido nítrico
NO⁻² – Nitrito
NO⁻³ – Nitrato
NOS – Óxido nítrico sintase
O²⁻ – Superóxido
ONOO⁻ - Peroxinitrito
PDE – Fosfodiesterase
PE – Pré-eclâmpsia

PGI2 – Prostaciclina

RUPP – Redução da pressão de perfusão uterina

sFlt-1 – Tirocína quinase solúvel 1

SRAA – Sistema renina angiotensina aldosterona

TBARS – Substâncias reativas ao ácido tiobarbitúrico

TEAC – Trolox equivalent antioxidant capacity

TXA2 – Tromboxano A2

VCAM-1 – Molécula de adesão celular vascular 1

VEGF – Fator de crescimento vascular endotelial

XO – Xantina oxidase

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Resumo

As desordens hipertensivas gestacionais são complicações que acometem em torno de 5-10% das gestações. Essas desordens são as maiores causas de morbidade e mortalidade tanto materna quanto fetal. Estudos demonstram redução da biodisponibilidade do óxido nítrico (NO) em doenças hipertensivas gestacionais, tornando uma das condições responsáveis por agravar a disfunção endotelial durante o curso desta doença. Neste sentido, trabalhos demonstram que a administração oral de nitrito de sódio (NaNO_2) e a administração de citrato de sildenafil podem reverter a redução da biodisponibilidade de NO e potencializar a via de sinalização NO-GMPc respectivamente. Para realização dos trabalhos foram utilizadas ratas Wistar, no qual, após confirmação da prenhez receberam L-NAME i.p para indução da hipertensão e tratamentos com nitrito de sódio e/ou citrato de sildenafil via oral entre os dias 14-21 de prenhez. A pressão arterial sistólica foi aferida pelo método de pletismografia de cauda. Parâmetros materno-fetais como: peso fetal e placentário, número de fetos viáveis e reabsorvidos foram realizados após a morte das ratas no 21º dia gestacional. O plasma das ratas foram armazenados para dosagens dos níveis plasmáticos de nitrito+nitrato, guanosina monofosfato cíclico (GMPc), mieloperoxidase (MPO), peroxidação lipídica (TBARS), TEAC, MTT sFlt-1 e VEGF. Além disso o plasma das ratas foram incubadas com células endoteliais de cordão umbilical humano (HUVECS) para avaliar a produção de NO endotelial. Em ambos os trabalhos, nós encontramos redução da pressão arterial sistólica com nitrito e sildenafil, tanto isolado quanto em associação. Somente o sildenafil foi capaz de aumentar o peso fetal, enquanto o peso placentário foi melhorado por ambas as drogas. Tanto nitrito quanto sildenafil foram capaz de reduzir a reabsorção, possivelmente por aumentar a viabilidade fetal. Foi encontrado aumento de NO plasmático em todos os grupo que receberam nitrito, enquanto curiosamente a síntese de NO em HUVECS incubadas com plasma das ratas foi aumentada em ambas as drogas. Ainda em relação a cultura celular, nós encontramos aumento da viabilidade celular em ratas hipertensas tratadas com sildenafil. A concentração plasmática de GMPc estava aumentada em ratas normotensos que receberam sildenafil. Ambas as drogas apresentam efeito antioxidante, mas somente o sildenafil foi capaz de reduzir a atividade plasmática da MPO. Fatores angiogênicos (VEGF) e anti-angiogênicos (sFlt-1) presentes em doenças hipertensivas gestacionais estavam aumentados em ratas que receberam L-NAME, porém o nitrito de sódio foi capaz de reduzir esses fatores. Nosso dados sugerem que o nitrito e o sildenafil, tanto isolado

quanto em associação apresentam efeitos anti-hipertensivos e antioxidantes. Portanto, nossos resultados sugerem que a ativação da via NO-GMPc aumentou o fluxo sanguíneo na interface materno-fetal e protegeu contra a hipertensão e a restrição do crescimento fetal induzidas pelo L-NAME.

Palavras-chaves: Hipertensão gestacional, citrato de sildenafil, nitrito de sodio, L-NAME, ratas

Abstract

Gestational hypertensive disorders are complications that affect around 5-10% of pregnancies. These disorders are the major causes of both maternal and fetal morbidity and mortality. Studies have demonstrated a reduction in the bioavailability of nitric oxide (NO) in gestational hypertensive diseases, making it one of the conditions responsible for aggravate the endothelial dysfunction during the course of this disease. In this sense, studies demonstrate that oral administration of sodium nitrite (NaNO₂) and administration of sildenafil citrate may reverse the reduction of NO bioavailability and potentiate the NO-cGMP signaling pathway, respectively. Wistar rats were used to perform the study, in which, after confirmation of pregnancy, they received L-NAME i.p for induction of hypertension and treatments with sodium nitrite and/or sildenafil citrate orally between days 14-21 of pregnancy. Systolic blood pressure was measured by the tail plethysmography method. Maternal-fetal parameters such as: fetal and placental weight, number of viable and reabsorbed fetuses were performed after the death of the rats on the 21st gestational day. Plasma of the rats were stored for plasma levels of nitrite + nitrate, cGMP, myeloperoxidase (MPO), lipid peroxidation (TBARS), TEAC, MTT sFlt-1 and VEGF. In addition the plasma of the rats were incubated with human umbilical vein endothelial cells (HUVECS) to evaluate endothelial NO production. In both studies, we found reduction of systolic blood pressure with nitrite and sildenafil, both alone and in combination. Both nitrite and sildenafil were able to reduce reabsorption, possibly by increasing fetal viability. No increase in plasma NO was found in all groups receiving nitrite, while curiously NO synthesis in HUVECS incubated with plasma from rats was increased in both drugs. Still in relation to cell culture, we found increased cell viability in hypertensive rats treated with sildenafil. Plasma cGMP concentration was increased in normotensive rats receiving sildenafil. Both drugs have an antioxidant effect, but only sildenafil was able to reduce plasma myeloperoxidase activity (MPO). Angiogenic (VEGF) and anti-angiogenic (sFlt-1) factors present in gestational hypertensive diseases were increased in rats receiving L-NAME, but sodium nitrite was able to reduce these factors. Our data suggest that both nitrite and sildenafil, both alone and in combination, have antihypertensive and antioxidant effects. Therefore, our results suggest that activation of the NO-cGMP pathway increased blood flow at the maternal-fetal interface and protected against hypertension and fetal growth restriction induced by L-NAME.

Keywords: Gestational hypertension, sildenafil citrate, sodium nitrite, L-NAME, rats

1 – Introdução

1.1 – Gestação e desordens hipertensivas gestacionais

A gestação é caracterizada por adaptações cardiovasculares que visam acomodar o suprimento constante de nutrientes e metabólitos ao feto, sem comprometer as necessidades maternas. Estas alterações são resultantes de interações hormonais, fatores vasoativos e a angiogênese (Mandala e Osol, 2012).

Durante o período gestacional há um aumento de 45% do volume sanguíneo total, em decorrência dos aumentos do volume plasmático, da massa total de eritrócitos e leucócitos, na circulação materna (Hyttén, 1985; Mandala e Osol, 2012). Estas adaptações fisiológicas gestacionais são então acompanhadas também do aumento de 30% a 50% do débito cardíaco e elevação da frequência cardíaca que são fisiologicamente contrabalanceadas pela angiogênese e redução da resposta a vasoconstritores, que conduzem a diminuição da resistência vascular periférica. No entanto, durante algumas doenças hipertensivas gestacionais parece ocorrer o contrário, ou seja, prevalece a vasoconstrição e compromete a angiogênese e ambas podem explicar a gênese da hipertensão (Hyttén, 1985; Faupel-Badger, 2007).

As desordens hipertensivas gestacionais são complicações que acometem em torno de 5-10% das gestações (Lo et al., 2013; Jim e Karumanchi, 2017). Essas desordens são as maiores causas de morbidade e mortalidade tanto materna quanto fetal (Uzan et al., 2011). Estas alterações hipertensivas podem ser classificadas em:

- Hipertensão crônica/pré-existente – hipertensão (sistólica \geq 140 ou diastólica \geq 90mmHg) diagnosticada antes da gestação ou anterior a 20ª semana gestacional, ou diagnosticada durante a gestação e que não regride após o parto;
- Hipertensão gestacional – hipertensão diagnosticada após a 20ª semana gestacional sem presença de proteinúria ou complicações sistêmicas

características da pré-eclâmpsia, regressão do quadro hipertensivo em aproximadamente 12 semanas após o parto;

- Pré-eclâmpsia – hipertensão (sistólica \geq 140 ou diastólica \geq 90mmHg) diagnosticada após 20^a semana de gestação com presença de proteinúria (\geq 0,3g/24h) ou complicações hematológicas, hepáticas, neurológicas;
- Hipertensão crônica/pré-existente sobreposta por pré-eclâmpsia – mulheres com hipertensão crônica que desenvolvem proteinúria ou outras alterações sistêmicas características da pré-eclâmpsia;

1.2 - Fisiopatologia

Embora a etiologia de algumas síndromes hipertensivas não esteja totalmente compreendida, está demonstrado que sua fisiopatologia é influenciada diretamente pela placenta, sendo a isquemia placentária um dos fatores primordiais (Robillard, 2002; Redman e Sargent, 2005). Além da isquemia placentária, algumas síndromes hipertensivas parecem ser acompanhada por outros fatores, como: angiogênese insuficiente (Levine et al., 2006), adaptação imunológica alterada, estresse oxidativo (Gupta et al., 2005; Redman e Sargent, 2005), resposta inflamatória excessiva, disfunção endotelial (Sedeek et al., 2008) e ativação do sistema renina-angiotensina-aldosterona (Palei et al., 2013).

Por volta da 20^a semana de gestação, os citotrofoblastos migram em direção as arteríolas uterinas espiraladas onde sofrem diferenciação em células com fenótipo endotelial (Palei et al., 2013). Os trofoblastos são as células precursoras da placenta humana, cujas funções são críticas para o sucesso da gravidez (Li et al., 2012).

No processo de invasão dessas células ocorre remodelamento gradual da camada endotelial destes vasos com destruição do tecido elástico-muscular das artérias e

arteríolas. Esse remodelamento das artérias espiraladas faz com que elas fiquem mais dilatadas e conseqüentemente ocorra um aumento acentuado do fluxo sanguíneo para o desenvolvimento do feto (Granger et al., 2001; Li et al., 2012). Esta migração/diferenciação dos citotrofblastos deve-se a alterações nos perfis de expressão de certas citocinas, moléculas de adesão, constituintes da matriz extracelular, metaloproteinases e o antígeno de histocompatibilidade. A ação citotrofbástica combinada (vascular e intersticial) assegura suprimento sanguíneo adequado ao crescimento fetal, por aumentar o calibre das artérias maternas (Myatt e Webster, 2008).

No quadro de pré-eclampsia (PE) por exemplo, parece ocorrer uma inadequada invasão trofoblástica, gerando uma falha no remodelamento vascular levando à modificação incompleta das artérias espiraladas maternas e, conseqüentemente, à redução da perfusão sanguínea placentária (Granger et al., 2001; Reedman e Sargent, 2005; Myatt e Webster, 2008). O diâmetro médio das artérias espiraladas em mulheres com PE é menor que o diâmetro dos vasos de mulheres sem essa complicação e conforme o avanço gestacional, a placenta vai se tornando cada vez mais isquêmica (Figura 1) (Granger et al., 2001).

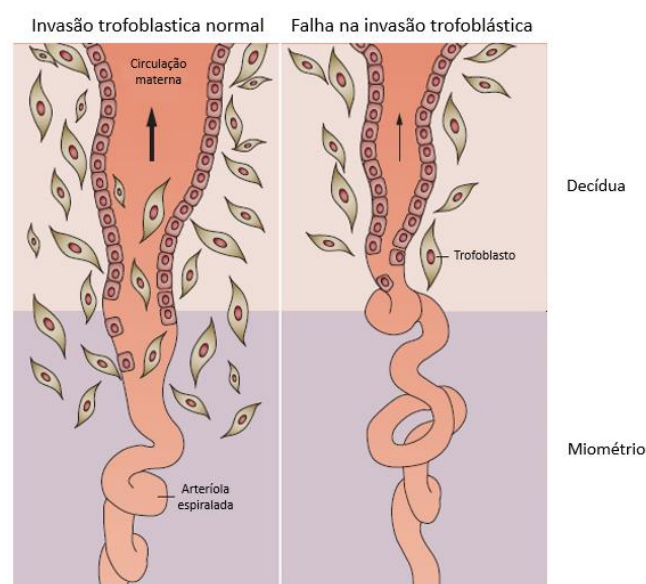


Figura 1. Diferenças no diâmetro das arteríolas uterinas em mulheres grávidas saudáveis com remodelamento efetivo atingindo o miométrio (painel à esquerda) e gestantes com pré-eclâmpsia com remodelamento ineficiente (painel à direita). O remodelamento ocorre com a invasão pelos trofoblastos extravilosos que adquirem fenótipo endotelial (trofoblastos endovasculares) (Reproduzido e adaptado de Moffett-King, A. et al. 2002).

A isquemia reduz o aporte sanguíneo materno gerando um quadro de hipóxia e consequentemente, o estresse oxidativo (Zhou et al., 2013). A placenta de mulheres com PE se caracteriza pelo aumento de estresse oxidativo (Burton e Jauniaux, 2004; Myatt e Webster, 2008). O aumento do estresse oxidativo é representado pela liberação de mediadores da disfunção da célula endotelial como peróxidos lipídicos e citocinas pró-inflamatórias (Sedeek et al., 2008; Palei et al., 2013). Além do estresse oxidativo, o estado de hipóxia também se caracteriza pela produção de fatores anti-angiogênicos, entre eles a endostatina, que é um potente inibidor endógeno de angiogênese e crescimento de células endoteliais e tumorais (Aref et al., 2008; Thissier-Levy et al., 2013). Sendo assim, a endostatina inibe o crescimento das células endoteliais e neutraliza as ações vasculares protetoras mediadas pelo VEGF (*Vascular Endothelial Growth Factor*) (Wikstrom et al., 2009). O VEGF é um fator angiogênico, que participa da manutenção endotelial de órgãos como rim, fígado e cérebro (Di-Marco et al., 2009). Estudos realizados por George e colaboradores (George et al., 2013) demonstraram que diante da isquemia placentária há um aumento de sFlt-1 (soluble fms-like tyrosine kinase 1), o qual é um receptor solúvel para o VEGF. Dessa forma, o sFlt-1 se liga às moléculas de VEGF circulantes e impede que esses fatores angiogênicos se liguem aos seus receptores comuns na membrana celular (Di-Marco et al., 2009). Embora ainda não se conheçam os mecanismos exatos da regulação do sFlt-1, estes, parecem estar associados à hipóxia, já que, pesquisas mostram uma secreção do sFlt-1 em tecido placentário *in vitro* exposto à baixas concentrações de oxigênio, provavelmente pela regulação do HIF-1 α (*hypoxia-inducible factor-1 α*) (George et al., 2011).

1.3 – Disfunção endotelial nas doenças hipertensivas gestacionais

O endotélio tem funções importantes como, controlar o tônus da musculatura lisa dos vasos sanguíneos através da liberação de substâncias vasodilatadoras e vasoconstritoras e controlar a anticoagulação através da redução da agregação plaquetária. (Goulopoulou e Davidge, 2015). Esse equilíbrio é fundamental para determinar a resistência vascular periférica e conseqüentemente tem influência direta na pressão arterial. Quando há desequilíbrio entre esses fatores com predominância de vasoconstritores e redução de vasodilatadores temos um quadro de disfunção endotelial, no qual está relacionado a doenças cardiovasculares, incluindo as desordens hipertensivas durante a gestação (Goulopoulou e Davidge, 2015; Possomato-Vieira e Khalil, 2016).

Dentre os principais vasodilatadores destaca-se o NO e as prostaglandinas. Além da redução de NO também já foi evidenciado uma redução de prostaglandinas em gestantes com pré-eclampsia sendo descrita a diminuição da síntese de produtos vasodilatadores – como a prostaciclina – associada ao aumento da produção de vasoconstritores como o tromboxano A₂. Além do tromboxano A₂, outros vasoconstritores que participam do controle do tônus vascular foram encontrados em excesso como a endotelina, componentes do sistema renina angiotensina aldosterona (SRAA) e os mediadores do sistema nervoso autonômico simpático (Possomato-Vieira e Khalil, 2016).

O estresse oxidativo característico nas síndromes hipertensivas levam a produção placentária de grandes quantidade de fatores anti-angiogênicos como o sFlt-1 o qual favorece ainda mais a disfunção do endotélio por interferir com fatores angiogênicos que melhorariam o fluxo sanguíneo (Levine et al., 2004). Além disso o aumento da atividade de enzimas pró-oxidantes leva o aumento da produção de O₂- reage com NO e assim elevando e favorecendo a formação de peroxinitrito (ONOO⁻) e conseqüentemente

diminuição da biodisponibilidade de NO, podendo agravar os quadros hipertensivos (Lowe, 2000).

Romero e colaboradores, demonstraram níveis elevados de proteínas de adesão celular do tipo 1 (VCAM-1) e a molécula de adesão intracelular do tipo 1 (CAM-1) em pacientes com PE, sugerindo a dosagem dessas moléculas como marcador de lesão endotelial (Romero et al., 2008).

1.4 – Papel do óxido nítrico (NO) na regulação da pressão sanguínea gestacional

O NO é um gás solúvel com papéis fisiológicos importantes, destacando-se a regulação da pressão arterial sanguínea e outros efeitos benéficos no sistema vascular: inibição da endotelina e produção tromboxano (TXA₂), tem a capacidade de estimular a produção de prostaciclina (PGI₂), inibe a agregação plaquetária, inibe a produção de VCAM –1 (*Vascular Cell Adhesion Molecule 1*) e fibronectina, inibe a oxidação LDL e proliferação da musculatura lisa (Lowe, 2000).

Essa biossíntese ocorre durante a transformação do aminoácido L-arginina em L-citrulina e óxido nítrico em uma reação mediada pela enzima óxido nítrico sintase (NOS). Para que essa reação ocorra a NOS necessita de cofatores como: NADPH, tetrahydrobiopterina (BH₄), flavina adenina dinucleotídeo (FAD) e flavina mononucleotídeo (FMN) (Moncada et al., 1991; Bredt, 1999).

A síntese de NO, pode ser inibida por análogos da L-arginina tais como N^G-monometil-L-arginina (L-NMMA), N^G-nitro-L-arginina (L-NNA) e N^G-nitro-L-arginina-metil-éster (L-NAME). Estes inibidores da síntese de NO têm importância na pesquisa experimental devido os efeitos biológicos importantes do NO (Moncada et al., 1991)

Foram descritas 3 isoformas da NOS, sendo 2 constitutivas (nNOS - neuronal e a eNOS - endotelial) e 1 isoforma denominada induzível (iNOS). A eNOS e a nNOS produzem NO em pequenas quantidades. A iNOS não é expressa sob condições normais, é induzida

por citocinas e outras células como macrófagos, linfócitos T, neutrófilos, essa isoforma requer algumas horas para ser expressa, porém sintetiza quantidades maiores de NO comparada com as isoformas constitutivas (Moncada et al, 1991; Bredt, 1999).

Como apresentado na Figura 2, o óxido nítrico difunde-se da célula endotelial para a célula muscular lisa vascular, ativando a enzima guanilato ciclase solúvel (GCs). A GCs sintetiza o segundo mensageiro GMPc (guanosina monofosfato cíclico). O aumento nos níveis de GMPc ativa a proteína quinase G (PKG), no qual resulta em aumento da abertura dos canais cálcio sensível ao potássio, havendo hiperpolarização da membrana com inibição do influxo de cálcio através dos canais de cálcio tipo-L, ocasionando a relaxamento da musculatura lisa vascular e consequentemente redução da resistência vascular periférica (Shekerdemian et al., 2002; Francis et al., 2012).

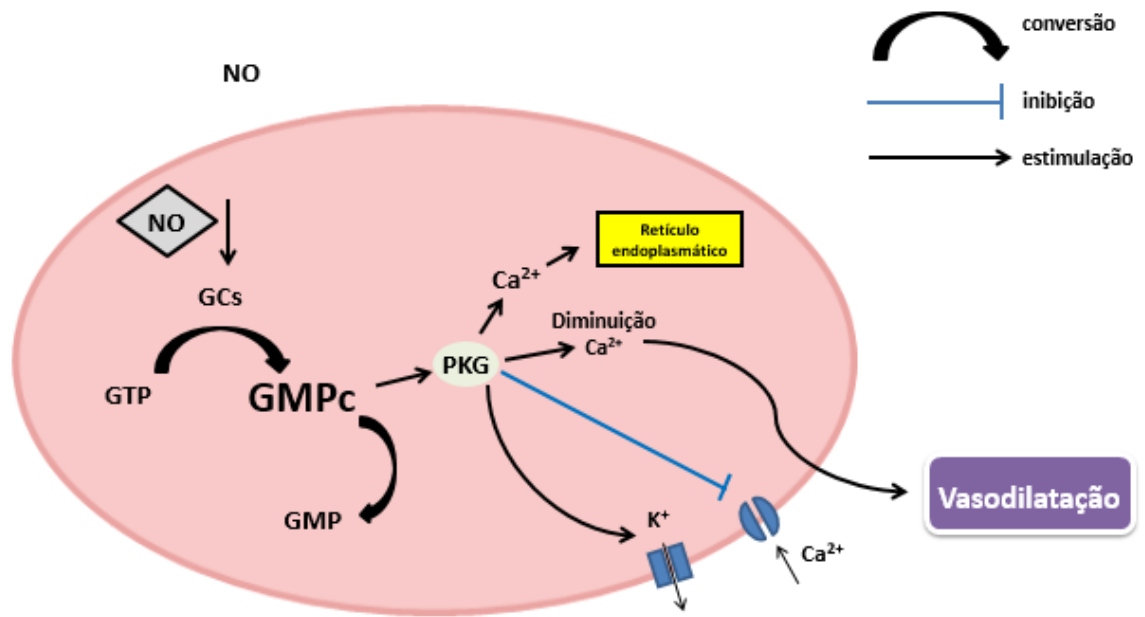


Figura 2. Esquema do mecanismo de ação vasodilatador do óxido nítrico na célula do músculo liso vascular.

O NO pode desempenhar um papel importante na regulação da pressão arterial sanguínea durante a gestação. Há evidências, demonstrando que há um aumento do NO durante a gestação normal e que o comprometimento da biodisponibilidade de NO pode estar associado aos prováveis fatores desencadeantes e agravantes da PE, tais como: (1) a disfunção da invasão trofoblástica, (2) o surgimento de áreas isquêmicas acompanhadas de (3) reperfusão, levando a (4) geração de espécies reativas de oxigênio (EROs) e (5) a ativação de neutrófilos e leucócitos durante a PE (Myatt e Webster, 2008).

Trabalhos realizados por Lyall e colaboradores (Lyall et al., 1995) mostraram redução dos níveis de metabólitos do NO (nitrito e nitrato) no líquido amniótico e na veia umbilical de gestantes com pré-eclâmpsia quando comparado com gestações normais. Portanto, a diminuição da biodisponibilidade de NO pode contribuir para a disfunção endotelial, que é uma alteração evidenciada na PE.

1.5 - O nitrito de sódio, uma estratégia para reverter a redução do NO

O desenvolvimento de algumas condições patológicas como hipertensão arterial, aterosclerose, doença coronariana, hipertensão pulmonar e PE tem relação com a redução na produção de NO (Sandrim et al., 2011). Em partes, o comprometimento na biodisponibilidade de NO pode ser devida a variações na atividade da enzima sintase endotelial do óxido nítrico (eNOS), a qual produz NO a partir do aminoácido L-arginina, NADPH, tetrahydrobiopterina e oxigênio molecular (Moncada e Higgs, 1993). Esta cascata enzimática clássica de formação de NO resulta ainda nos principais produtos do metabolismo do NO, os íons nitrato (NO_3^-) e nitrito (NO_2^-) (Lauer et al., 2001).

Embora os íons nitrito e nitrato sejam formados endogenamente como consequência do metabolismo das sintases do NO, a maior parte desses íons são provenientes da dieta, uma vez que são encontrados em altas concentrações nos alimentos

de origem vegetal. O nitrato proveniente da dieta é convertido a nitrito por enzimas comensais da flora bucal, e uma vez ingerido junto com a saliva, se depara com o baixo pH do suco gástrico estomacal e reduzindo-se em NO (Lundberg e Weitzberg, 2005).

Foi demonstrado que a infusão intravenosa de nitrato não apresenta efeito hipotensor, no entanto, a infusão de nitrito de sódio (NaNO₂) promoveu vasodilatação de forma dose-dependente em ratos anestesiados, por mecanismos independentes do endotélio (Vleeming, 1997; Kanematsu et al., 2008). Estudos demonstraram que o nitrito de sódio possui propriedades anti-hipertensivas e antioxidantes decorrentes do aumento da biodisponibilidade de NO e redução da atividade da NADPH oxidase, respectivamente (Montenegro et al., 2011; Montenegro et al., 2012; Pinheiro et al., 2012). Além disso, o nitrito confere significativo efeito protetor sobre as alterações induzidas por isquemia/reperfusão no miocárdio, fígado, rim, pulmões e cérebro (Gladwin et al., 2005; Triptara et al., 2007). Há evidências que a isquemia e/ou hipoxemia são condições favoráveis à conversão do nitrito em NO e alguns mecanismos foram propostos: (1) a conversão de nitrito em NO é por meio da ação da enzima xantina oxidoreductase (Webb et al., 2004), deoxihemoglobina (Gladwin et al., 2005), deoximioglobina (Shiva et al., 2007) (Figura 3) ou via acidificação química no estômago, por meio da circulação entero-salivar (Lundberg et al., 2008). Sendo assim, o nitrito pode ser convertido a NO, abrindo a possibilidade de formação de NO por uma via independente das sintases do NO, conferindo valor biológico ao íon nitrito como uma possível alternativa terapêutica no manejo de doenças onde a redução da biodisponibilidade do NO se torna um dos fatores agravantes (Gladwin et al., 2005).

Embora estudos demonstrem efeito benéfico do nitrito de sódio em vários modelos experimentais de hipertensão, a literatura até o momento não apresentava estudos que demonstrasse que o nitrito poderia ser reciclado a NO em modelos de

hipertensão gestacional. Sabe-se que elevados níveis de sFlt-1 estão relacionados a disfunção endotelial por sequestrar moléculas angiogênicas. Além disso trabalhos demonstram que a redução de NO é inversamente proporcional ao aumento de sFlt-1 (Sandrim et al. 2008; Pimentel et al., 2013; Amaral et al., 2015) e a terapia atual para o manejo das doenças hipertensivas gestacionais pouco influência na concentração de sFlt-1. (Noris et al., 2005; Palei et al., 2013; Uzan et al., 2011)

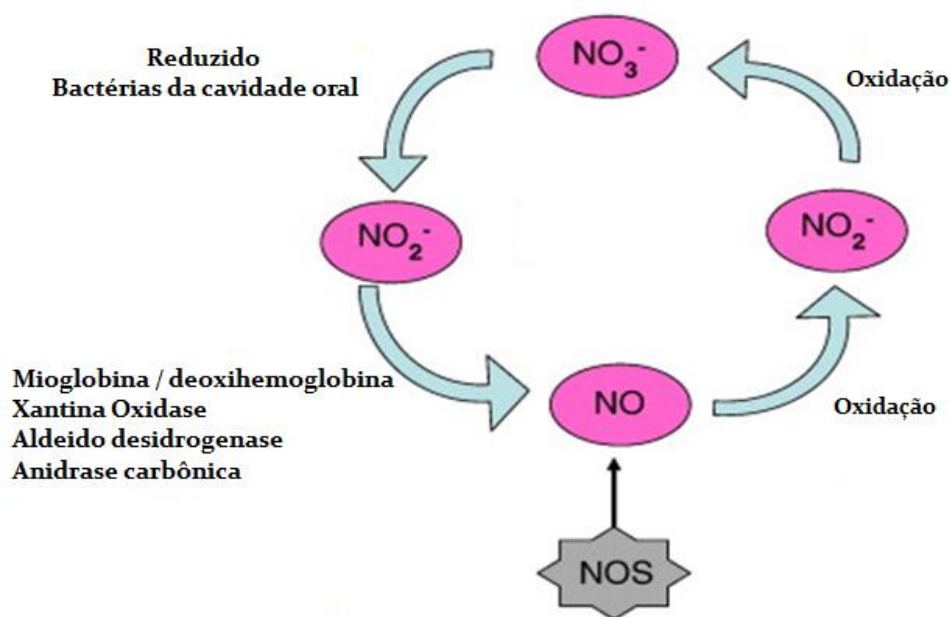


Figura 3. Representação esquemática do ciclo nitrito-nitrato-NO. (Reproduzido e adaptado de: Parthasarathy e Bryan, 2012).

1.6 – Sildenafil

O Sildenafil é um inibidor potente e seletivo da fosfodiesterase tipo 5 (iPDE 5), presente em células musculares lisas dos corpos cavernosos do pênis, células musculares lisas de vasos periféricos arteriais e venosos, bem como na circulação coronária e pulmonar, e nas plaquetas, sendo atualmente usado no tratamento da disfunção erétil por potencializar as ações do NO endógeno nos corpos cavernosos do pênis, maximizando as ações do GMPc. A seletividade do sildenafil para a PDE5 é cerca de 1000 vezes para esta

isoenzima humana em comparação com a PDE2, PDE3 e PDE4 e moderada seletividade (cerca de 80 vezes) sobre a PDE1 (Gupta et al., 2005).

Em relação a farmacocinética do Sildenafil, estudos indicam que doses entre 25-100 mg quando administradas via oral são absorvidos rapidamente, com concentrações plasmáticas máxima de 30–120 minutos após administração e tempo de meia vida ($t_{1/2}$) de eliminação de cerca de 3 – 5 horas. Sua absorção via oral sofre influência de alimentos, sendo melhor absorvido em jejum. Sildenafil sofre metabolismos hepático pelas enzimas CYP3A4 e sua excreção é predominantemente nas fezes (73-88%) e em menor quantidade na urina (6-15%) (Gupta et al., 2005; Rang et al., 2012).

Muitos dos efeitos adversos dos iPDE 5 são causados pela vasodilatação de outros leitos vasculares; esses efeitos incluem hipotensão, rubor, cefaleia e congestão nasal. (Rang et al., 2012).

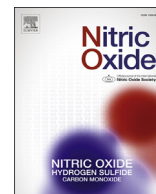
Com base no mecanismo de ação do sildenafil na potencialização da vasodilatação induzida pelo NO, pode-se considerar que como um fármaco dependente de NO, o sildenafil não teria potencial para alcançar objetivos terapêuticos, considerando que em doenças hipertensivas pode haver redução de NO, ou experimentalmente a síntese de NO pode ser reduzida por inibidores da síntese endógena como o L-NAME. (Motta et al., 2005). No entanto, estudos prévios mostraram que o sildenafil atenua a hipertensão e a restrição de crescimento fetal em ratos tratados com L-NAME (Ramesar et al., 2010; Nassar et al., 2012) bem como em camundongos hipertensos pela deficiência da síntese endógena de NO (Roberts et al., 2016). Juntos esses achados sugerem que os efeitos do sildenafil podem não depender totalmente dos níveis circulantes de NO (Chrysant e Chrysant, 2012). No entanto, mais estudos mecanísticos são necessários para explicar esses efeitos do sildenafil e para determinar seu potencial terapêutico em transtornos

hipertensivos durante a gestação (Trapani et al., 2016), mesmo com níveis reduzidos de NO (Sandrim et al., 2008).

Outros trabalhos demonstraram uma redução da pressão arterial com o sildenafil em ratas submetidas a redução da pressão de perfusão uterina (RUPP), além disso estes pesquisadores encontraram um aumento placentário de sFlt-1 e VEGF e pela primeira vez foi observado uma maior expressão medular renal de PDE5 em ratas prenhes submetidos a cirurgia de isquemia placentária (George et al., 2013).

A PDE5 pode ser encontrada no tecido uterino e placentários de ratas (Buhimschi et al., 2004). O uso do Sildenafil foi relacionado a diminuição a contratilidade uterina durante o trabalho de parto, sugerindo seu uso para situações de parto prematuro, onde a diminuição da contração uterina é capaz de prolongar o parto (Coppage et al., 2005). Recentemente o sildenafil apresentou efeito vasodilatador *in vitro* em pequenas artérias do endométrio (Wareing et al., 2005), fatores esses que fortalecem as evidências que seu uso pode melhorar a restrição de crescimento intrauterino (Wareing et al., 2005).

2 - Capítulo 1



Sodium nitrite attenuates hypertension-in-pregnancy and blunts increases in soluble fms-like tyrosine kinase-1 and in vascular endothelial growth factor



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ARTICLE INFO

Article history:

Received 6 March 2016

Received in revised form

5 May 2016

Accepted 11 May 2016

Available online 12 May 2016

Keywords:

Hypertension-in-pregnancy

Sodium nitrite

N(G)-nitro-L-arginine methyl ester

Rats

ABSTRACT

Preeclampsia is a pregnancy-associated disorder characterized by hypertension with uncertain pathogenesis. Increases in antiangiogenic soluble fms-like tyrosine kinase-1 (sFlt-1) and reductions in nitric oxide (NO) bioavailability have been observed in preeclamptic women. However, the specific mechanisms linking these detrimental changes to the hypertension-in-pregnancy are not clearly understood. In this regard, while recent findings have suggested that nitrite-derived NO formation exerts antihypertensive and antioxidant effects, no previous study has examined these responses to orally administered nitrite in hypertension-in-pregnancy. We then hypothesized restoring NO bioavailability with sodium nitrite in pregnant rats upon NO synthesis inhibition with N(omega)-nitro-L-arginine methyl ester (L-NAME) attenuates hypertension and high circulating levels of sFlt-1. Number and weight of pups and placentae were recorded to assess maternal-fetal interface. Plasma sFlt-1, vascular endothelial growth factor (VEGF) and biochemical determinants of NO formation and of antioxidant function were measured. We found that sodium nitrite blunts the hypertension-in-pregnancy and restores the NO bioavailability, and concomitantly prevents the L-NAME-induced high circulating sFlt-1 and VEGF levels. Also, our results suggest that nitrite-derived NO protected against reductions in litter size and placental weight caused by L-NAME, improving number of viable and resorbed fetuses and antioxidant function. Therefore, the present findings are consistent with the hypothesis that nitrite-derived NO may possibly be the driving force behind the maternal and fetal beneficial effects observed with sodium nitrite during hypertension-in-pregnancy. Certainly further investigations are required in preeclampsia, since counteracting the damages to the mother and fetal sides resulting from hypertension and elevated sFlt-1 levels may provide a great benefit in this gestational hypertensive disease.

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1. Introduction

Preeclampsia and related hypertensive pregnancy disorders affect 5–8% of all births in the United States, resulting in 15–20% of maternal deaths worldwide [1]. These disorders present serious complications to the mother and the baby, and the mechanisms involved are not clearly understood [2]. Currently, despite intense investigation, definitive treatment is limited to preterm delivery of

the baby and placenta, suggesting that the causative symptoms of preeclampsia may be dependent on the presence of the placenta [3]. In fact, placental ischemia is thought to be an initiating event, leading to the release of circulating biomarkers of inflammatory response, oxidative stress and antiangiogenic factors in the maternal circulation [4,5].

There may be an imbalance among the pro- and antiangiogenic factors, in which the circulating antiangiogenic protein sFlt-1 (soluble fms-like tyrosine kinase-1) binds and sequesters the vascular endothelial growth factor (VEGF), causing endothelial dysfunction and producing preeclampsia-like symptoms [6]. Accordingly, the extracorporeal removal of circulating sFlt-1 in preeclamptic patients may improve the symptoms [7], thus, confirming the key role of sFlt-1 in the maternal side of the disorder.

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However, it has been shown that the high levels of circulating sFlt-1 and uteroplacental circulation are not affected by the most commonly antihypertensive drugs used in clinic to treat preeclampsia [2,4,6]. In addition, reductions on the formation of nitric oxide (NO) may be inversely related to serum levels of sFlt-1, highlighting that hypertension-in-pregnancy during preeclampsia may be explained, at least in part, by reductions of NO bioavailability [8–10].

Importantly, accumulated experimental evidences have showed a potential role for the anion nitrite, being more than a simple biomarker of NO formation. Nitrite is recycled back to NO as a physiological alternative to NO formation independent of NO synthase (NOS)-related pathways, restoring the vasodilator actions of the NO [11–13]. In this context, recent studies have suggested that NO generation from nitrite may occur in conditions such as hypoxia and that sodium nitrite may selectively deliver NO to ischemic/hypoxic tissues [12,13]. Therefore, if uteroplacental ischemia is thought to play a major role in preeclampsia [4,5], we hypothesized that these conditions would create the ideal biochemical environment for the *in vivo* reduction of nitrite to NO, thus, attenuating hypertension (mother side) and concomitantly improving fetal detrimental changes caused by hypertension-in-pregnancy.

In order to confirm this hypothesis, pregnant rats were treated with N(omega)-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthesis, during mid-to late gestation, in which hypertension, reductions of litter size [14] and placental weight [15] are manifested. We have also examined the circulating levels of sFlt-1 and VEGF, and biochemical determinants of oxidative stress [16], and if the attenuation of hypertension with sodium nitrite would be associated with reduced levels of sFlt-1 and antioxidant effects [11].

2. Materials and methods

2.1. Animals and experimental protocol

Wistar rats (200–250 g) were housed in cages at 22 ± 2 °C on a 12-hr light/dark cycle and given free access to water and rat chow. Each female rat was separately mated overnight. Day 0 of pregnancy was defined as the day when spermatozoa were found in a vaginal smear.

On pregnancy day 14, each pregnant rat mother was first placed into a single cage and randomized to one of the four treatment groups ($n = 10$ per group, total of 40 rats): Norm-Preg, Preg + Sodium nitrite, HTN-Preg and HTN-Preg + Sodium nitrite groups. Pregnant rats received daily 0.9% saline solution by gavage and by via intraperitoneal (i.p.) in **Norm-Preg group**; or sodium nitrite by gavage (Sodium nitrite; Sigma, St. Louis, MO, #S2252; 15 mg/kg/day for 7 days) and saline injections by via i.p. in **Preg + Sodium nitrite group**; or i.p. injections of N(G)-nitro-L-arginine methyl ester (L-NAME; Sigma, St. Louis, MO, # 5751; 60 mg/kg/daily [17]) and saline solution by gavage in **HTN-Preg group**; or i.p. injections of L-NAME (60 mg/kg/daily) and sodium nitrite by gavage (15 mg/kg/day for 7 days) in **HTN-Preg + Sodium nitrite group**.

The dose of sodium nitrite (15 mg/kg or 0.217 mmol/kg; by gavage) was chosen with basis on previous studies showing that this dose exerts relevant antihypertensive and antioxidant effects in rats [11,18–21].

Rats were euthanized on gestation-day 21 under overdose of isoflurane followed by exsanguination. Blood samples were collected in lyophilised ethylenediaminetetraacetic acid (EDTA) (Vacutainer Becton-Dickinson, BD, Oxford, UK) and immediately centrifuged and plasma was separated and stored at -80 °C until use for biochemical analysis.

All procedures for animal experimentation were approved by

the Ethics Committee, Biosciences Institute of Botucatu, State University of Sao Paulo (Protocol #618/2014), which is complied with international guidelines of the European Community for the use of experimental animals.

2.2. Blood pressure measurements

Systolic blood pressure (mmHg) was measured on gestational day 13 (baseline with absence of gavage or i.p. injections) and days 14, 16, 18 and 20, 6 h after drugs administration, using tail-cuff plethysmography (Insight, Ribeirao Preto, Sao Paulo, Brazil, # EFF 306). Briefly, all pregnant rats were first acclimated in a quiet room, conditioned and restrained for 5–10 min in a warm box (Insight, Ribeirao Preto, Sao Paulo, Brazil, # EFF-307) to the measurements for 3 days before the pregnancy day 14 (these data were discarded) and then the baseline systolic blood pressure was determined as the average of the cuff inflation-deflation (3–6) cycles by a trained operator on pregnancy day 14 [22].

2.3. Effects on placenta and fetuses

On gestational-day 21, after euthanasia, animals were placed in supine position and cesarean section was performed. The averages of total number of viable fetuses, litter size, fetal weight and placental weight of each mother 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 [23].

2.4. Measurement of plasma NOx (nitrate + nitrite) concentrations

The plasma NOx concentrations were determined in duplicate by using the Griess reaction, as previously described [24]. Briefly, 40 μ L of plasma were incubated with the same volume of nitrate reductase buffer (0.1 M potassium phosphate, pH 7.5, containing 1 mM β -nicotinamide adenine dinucleotide phosphate and 2U of nitrate reductase/mL) in individual wells of a 96-well plate. Samples were allowed to incubate overnight at 37 °C in the dark; 8 μ L of freshly prepared Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride in 5% phosphoric acid) were added to each well and the plate was incubated, for 15 additional minutes, at room temperature. A standard nitrate curve was obtained by incubating sodium nitrate (0.2–200 μ M) with the same reductase buffer. The NOx levels in plasma were expressed in μ mol/L.

2.5. Determination of sFlt-1 and VEGF

Commercial enzyme immunoassay (ELISA) kits for sFlt-1 (R&D Systems Inc, Minneapolis, MN, USA #MVR100) and VEGF (R&D Systems Inc, Minneapolis, MN, USA #RRV00) were used to determine plasma levels. Assays were performed according to manufacturer's instructions. Plasmatic levels of sFlt-1 and VEGF were expressed in pg/mL.

2.6. Determination of myeloperoxidase (MPO) activity

Circulating plasma levels of MPO reflect the inflammatory response, as according to the method previously proposed by Suzuki [25]. Briefly, 30 μ L of centrifuged plasma samples received 100 μ L of TMB (tetramethyl benzidine) and 0.04% of H₂O₂. Posteriorly, microplate was incubated for 10 min at 37 °C, protected from light. The reaction was stopped with 100 μ L of H₂SO₄ (2N) and the absorbance at 450 nm with correction to 630 nm was read with the spectrophotometer (Synergy 4, BIOTEK, Winooski, VT, USA). The

results were expressed in $\Delta A_{630}/\text{min}/\text{mL}$).

2.7. Measurements of plasma antioxidant capacity

The trolox equivalent antioxidant capacity (TEAC) was performed as previously described [26]. Briefly, a standard curve was established using 100 μg of Trolox (6-hydroxy-2,5,7,8 - tetramethylchroman-2-carboxylic-acid, Sigma, St. Louis, MO, USA, catalogue# 238813) in 1 mL of sodium acetate buffer (0.4 M, $\text{C}_2\text{H}_3\text{NaO}_2 \cdot 3\text{H}_2\text{O}$) + glacial acetic acid (0.4 M). Firstly, 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). Secondly, 20 μL of sodium acetate buffer (0.03 M) and glacial acetic acid (0.03 M) + H_2O_2 + ABTS (2,2'-azino-bis(3-ethylbenzothiazolin-6 sulfonic acid, Sigma A 1888) was added to the samples and incubated for 5 min. Finally, a second spectrophotometer read was performed at 660 nm. The second reading values were subtracted from the values found in the first reading and the antioxidant activity of the sample was expressed as mmol of Trolox equivalent/L.

2.8. Evaluation of the antioxidant status of plasma

Direct reductions of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Sigma, St. Louis, MO, USA) were measured as previously described [27]. Briefly, 100 μL of plasma were mixed with 12.5 μL of dye solution (5 mg/mL in PBS); the final volume was adjusted to 200 μL with PBS and the mixture was incubated for 60 min at 37 °C. The reaction was terminated by the addition of 750 μL of 0.04 M hydrochloric acid in isopropanol. The tubes were centrifuged for 10 min at 1000 \times g and the supernatant collected and the absorbance were measured at 570 nm.

2.9. Statistical analysis

Using commercially available statistical software (Graph Pad Prism® 6.0 for Windows, San Diego, CA), a Shapiro-Wilk test was applied to verify normality of data distribution. Systolic blood pressure measurements were submitted to a two-way analysis of variance (ANOVA) with time and treatment defined as main effects, followed by Bonferroni's correction for multiple comparisons among groups to compare measurements on pregnancy days 16, 18 and 20, or one-way ANOVA followed by Bonferroni's correction for multiple comparisons were used to compare fetal and placental changes or sFlt-1, VEGF, TEAC, MPO, MTT and NOx levels. Since only fetal weight in Norm-Preg group presented no normal distribution, the Mann-Whitney test was used to compare the fetal weight among the four groups. Statistical significance was considered at $P < 0.05$. All values are expressed as mean \pm SEM.

3. Results

There were no significant differences in the systolic blood pressure values (116–123 \pm 4 mmHg) among the four groups on days 13 and 14 ($P > 0.05$, Fig. 1). Systolic blood pressure values were elevated in the HTN-Preg group on days 16, 18 and 20 (145 \pm 5; 149 \pm 3 and 140 \pm 4 mmHg, respectively, $^*P < 0.05$, Fig. 1). However, HTN-Preg + Sodium nitrite diminished systolic blood pressure values were significantly observed on days 16, 18 and 20 (126 \pm 3; 131 \pm 3 and 127 \pm 2.3 mmHg) compared to HTN-Preg group ($^{\#}P < 0.05$, Fig. 1).

Number of pups (litter size) was significantly lower in HTN-Preg (9.2 \pm 0.7) if compared to Norm-Preg, Preg + Sodium nitrite or HTN-Preg + Sodium nitrite groups (13 \pm 1; 11 \pm 0.6 or 12 \pm 0.5,

respectively), $^*P < 0.05$, Fig. 2A). Also, lower numbers of viable fetuses with higher number of resorbed fetuses were found only in HTN-Preg (7 \pm 0.7 and 1.20 \pm 0.29, respectively) and were compared to Norm-Preg, Preg + Sodium nitrite and HTN-Preg + Sodium nitrite groups (11 \pm 1.0 and 0.30 \pm 0.15; 11 \pm 0.7 and 0.20 \pm 0.13, and 11 \pm 0.6 and 0.40 \pm 0.16, respectively, $^*P < 0.05$, Fig. 2B and C). Surprisingly, the fetal weight was significantly higher only in the Preg + Sodium nitrite group (3.9 \pm 0.05 g) if compared to Norm-Preg, HTN-Preg and HTN-Preg + Sodium nitrite groups (2.9 \pm 0.1; 3.1 \pm 0.03 and 3.1 \pm 0.04 g, respectively, $^{\#}P < 0.05$, Fig. 2D). Lower placental weight was found only in HTN-Preg group (0.53 \pm 0.01 g) if compared to Norm-Preg, Preg + Sodium nitrite and HTN-Preg + Sodium nitrite groups (0.63 \pm 0.01; 0.6 \pm 0.01 and 0.6 \pm 0.01 g respectively, $^*P < 0.05$, Fig. 2E).

We evaluated NO bioavailability by measuring plasma nitrite and nitrate concentrations. We found significant lower NO bioavailability in HTN-Preg group (49 \pm 7 $\mu\text{mol}/\text{L}$) compared to those found in the Norm-Preg group (72 \pm 8 $\mu\text{mol}/\text{L}$, $^*P < 0.05$, Fig. 3A). However, the treatment with sodium nitrite enhanced the NO bioavailability in both Preg + Sodium nitrite and HTN-Preg + Sodium nitrite groups (138 \pm 10 and 131 \pm 11 $\mu\text{mol}/\text{L}$, respectively, $^{\#}P < 0.05$, Fig. 3A). Also, no significant differences were found in NO bioavailability between Preg + Sodium nitrite and HTN-Preg + Sodium nitrite groups ($P > 0.05$, Fig. 3A).

Sodium nitrite blunted both increases in free sFlt-1 and VEGF levels induced by L-NAME (both $^*P < 0.05$, Fig. 3B and C). Increased-free sFlt-1 levels were observed only in HTN-Preg group, if compared with Norm-Preg, Preg + Sodium nitrite and HTN-Preg + Sodium nitrite groups (368 \pm 48; 162.2 \pm 4.3; 136 \pm 9 and 190 \pm 22 pg/mL respectively, $^*P < 0.05$, Fig. 3B). Higher levels of VEGF in plasma were found only in HTN-Preg group, if compared to Norm-Preg, Preg + Sodium nitrite and HTN-Preg + Sodium nitrite groups (77 \pm 4.6; 58 \pm 6; 57 \pm 3 and 62 \pm 1 pg/mL respectively, $^*P < 0.05$, Fig. 3C).

Lower anti-oxidant capacity was found only in HTN-Preg group (0.04 \pm 0.01) if compare to Norm-Preg, Preg + Sodium nitrite or HTN-Preg + Sodium nitrite groups (0.11 \pm 0.01; 0.13 \pm 0.01 and 0.09 \pm 0.01, respectively, $^*P < 0.05$, Fig. 4A). Both Preg + Sodium nitrite and HTN-Preg + Sodium nitrite groups presented increases in antioxidant status of plasma (0.131 \pm 0.002 and 0.130 \pm 0.002, respectively) if compared to control Norm-Preg and HTN-Preg groups (0.11 \pm 0.009 and 0.11 \pm 0.006, respectively, $^{\#}P < 0.05$, Fig. 4B).

Also, HTN-Preg and HTN-Preg + Sodium nitrite groups presented increases in myeloperoxidase levels (2.06 \pm 0.10 and 1.9 \pm 0.15, respectively) if compared to Norm-Preg and Preg + Sodium nitrite groups (1.12 \pm 0.2 and 1.13 \pm 0.2, respectively, $^*P < 0.05$, Fig. 4C).

4. Discussion

This study shows that sodium nitrite, administered orally and once a day, diminishes systolic blood pressure in hypertension-in-pregnancy induced by L-NAME and concomitantly prevents the increases in circulating plasma sFlt-1 and VEGF levels. Also, our results suggest that increases in nitrite-derived NO has protected against reductions in number of viable fetuses, litter size and placental weight caused by L-NAME. In addition, we observed increases in MPO levels with concomitant reductions in antioxidant function of plasma induced by L-NAME, suggesting presence of oxidative stress in this rat model of hypertension-in-pregnancy. Also, sodium nitrite has maintained the antioxidant function in hypertensive pregnant rats. Therefore, the present findings are consistent with the idea that treatment with sodium nitrite attenuates the hypertension-in-pregnancy and that nitrite-derived

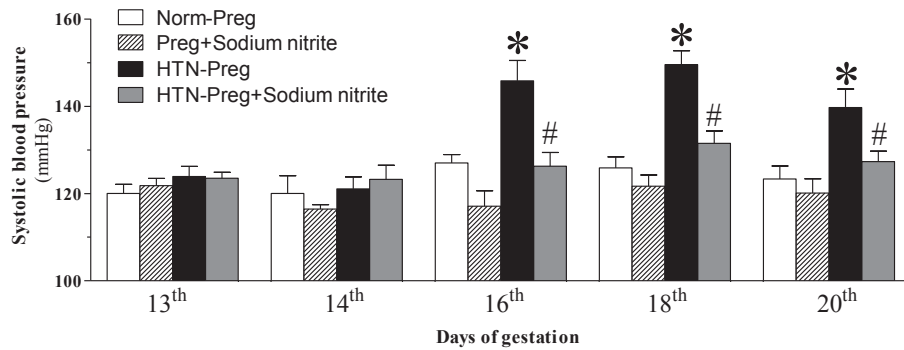


Fig. 1. Systolic blood pressure measured on days 13, 14, 16, 18 and 20 of gestation in Norm Preg, Preg + Sodium nitrite, HTN-Preg and HTN-Preg + Sodium nitrite groups. Values represent mean \pm SEM. Two-way ANOVA with Bonferroni's correction for multiple comparisons among groups were used to compare measurements on pregnancy days 13, 14, 16, 18 and 20. Significant increases were observed in pregnancy days 16, 18 and 20 and no significant differences among four groups were found in pregnancy days 13 and 14. * $P < 0.05$ for HTN-Preg vs. Norm Preg and Preg + Sodium nitrite groups. # $P < 0.05$ for HTN-Preg + Sodium nitrite vs. HTN-Preg group.

NO may possibly be the driving force behind the maternal and fetal beneficial effects observed here.

Pregnancy-induced hypertension results in a state of endothelial dysfunction in preeclampsia [28,29], in which the exact mechanisms leading to the hypertension remain obscure. However, recent studies have proposed that there may be a pro/anti-angiogenic imbalance in the pathogenesis of this disorder, suggesting that high circulating levels of sFlt-1 in preeclamptic women may be associated with hypertension [30,31]. Nevertheless, the specific mechanisms linking high sFlt-1 levels to hypertension are unclear. Earlier findings have shown reductions of bioavailable NO in preeclamptic women [8,9,32–34] and that endogenous NO production was reduced in 70% in the hypertensive response caused by the infusion of sFlt-1 into pregnant rats [35] and other animal models of hypertensive-pregnancy [10]. Thus, the present findings are in accordance to the hypothesis that increased plasma levels of sFlt-1 may be due to reductions of NO, suggesting that there may be a crosstalk between reduced NO synthesis and increases in sFlt-1 levels.

To test this hypothesis, pregnant rats were treated with the NOS inhibitor L-NAME and received sodium nitrite (or saline) by gavage. Upon chronic NOS inhibition, sodium nitrite reduces systolic blood pressure and blunts the increases in circulating sFlt-1 levels. The present results are in accordance to previous studies suggesting that nitrite-derived NO after oral treatment with sodium nitrite may restore the vasodilator actions of the NO, resulting in antihypertensive effects in different rat models of hypertension [11–13,18,19,22,36–39]. Thus, our data suggests that nitrite-derived NO blunts the increases of sFlt-1 and attenuates the hypertension-in-pregnancy induced by L-NAME.

The formation of NO from nitrite may occur by enzymatic or non-enzymatic pathways. The enzymatic pathways involve deoxyhaemoglobin [38], xanthine oxidase [40], myoglobin [41], aldehyde oxidase [42], mitochondrial cytochromes [43] and carbonic anhydrase [44], while the non-enzymatic pathway comprises the intragastric formation through reduction of nitrite to NO in low pH in the stomach [13,37].

In our hands, following the treatment with sodium nitrite in pregnant rats without L-NAME (Preg + Sodium nitrite group) we observed increases in NO levels with no changes in systolic blood pressure, corroborating with previous studies that suggested compensatory mechanisms of normal blood pressure regulation [19,22,45]. Furthermore, we found increases in fetal weight with no changes in placental weight in the same Preg + Sodium nitrite group, which suggest nitrite-derived NO may afford better fetal maturational processes during late gestation [15,35,46].

While treatment with L-NAME (HTN-Preg group) produced reductions in litter size and viable fetuses with more resorbed fetuses and reductions in placental weights without affecting the fetal weight, treatment with sodium nitrite was able to prevent these detrimental changes to the fetuses and placenta. We then suggest that the antihypertensive effects with concomitant increases in litter size and placental weight, with more viable and less resorbed fetuses may reflect the improvement of the uteroplacental perfusion caused by nitrite-derived NO [47,48] and that NO may promote vasodilation of uteroplacental circulation [49,50]. Accordingly, earlier evidence has showed that the number of pups and placental weight are strongly correlated with uteroplacental blood flow and that reductions in these parameters may reflect the uteroplacental ischemia/hypoxia induced by L-NAME in rats [51]. Thus, we suggest that ischemic uteroplacental vasculature may be the ideal biochemical environment for the *in vivo* reduction of nitrite to NO.

We found higher circulating levels of VEGF in hypertension-in-pregnancy induced by L-NAME than in normotensive pregnant rats treated with (or without) sodium nitrite (Norm-Preg and Preg + Sodium nitrite groups). Supporting our results, recent reports have demonstrated that increases in circulating levels of VEGF may result in adverse maternal and fetal side effects [31,52–55]. Accordingly, the exogenous VEGF administration in pregnant rats caused hypertension and hypercoagulation in the placental circulation, similar to human preeclampsia [56,57]. Additionally, mice with VEGF-overexpression revealed increases in maternal serum levels of sFlt-1, which resulted in pregnancy losses, placental vascular defects, increases in the number of resorption sites and decreases in the number of viable fetuses and preeclampsia-like symptoms including hypertension in the mother [56]. Thus, we may suggest that VEGF production could be elevated due to an increase in vascular shear stress, mainly in uteroplacental circulation [58]. Importantly, treatment of hypertensive pregnant rats with sodium nitrite (HTN-Preg + Sodium nitrite group) resulted in a reduction in VEGF levels. This may occur due to the fact that treatment with sodium nitrite may result in vasodilation [59], leading to decreases in vascular shear stress, and consequently reducing the production of VEGF [58], and that along with reductions of sFlt-1 could be responsible for protective effects in mother and fetal side of hypertension-in-pregnancy induced by L-NAME.

Additionally, the antioxidant effect of sodium nitrite demonstrated previously [11,19] is further supported by our results showing that sodium nitrite restored the plasma antioxidant capacity (higher TEAC in HTN-Preg + Sodium nitrite compared to HTN-Preg group). However, although there was no reduction of

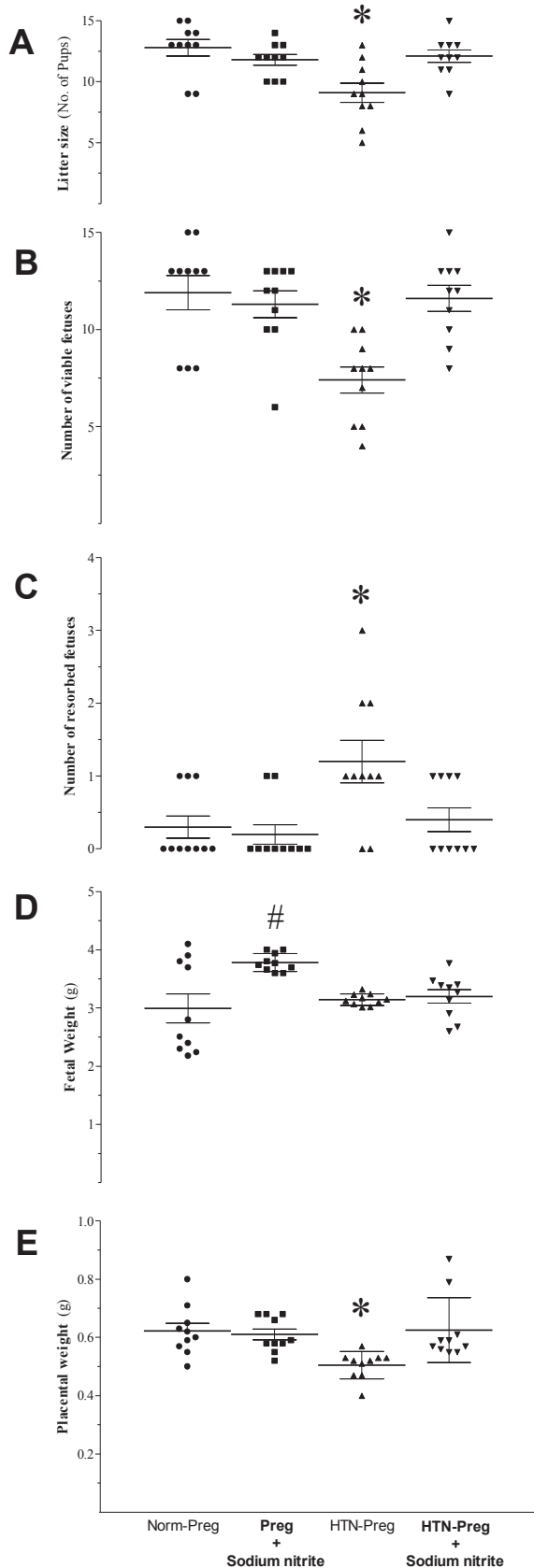


Fig. 2. Average of fetal and placental parameters were recorded on gestational-day 21: litter size (A), number of viable (B), resorbed (C) fetuses, fetal (D) and placental (E) weights in Norm Preg, Preg + Sodium nitrite, HTN-Preg and HTN-Preg + Sodium nitrite groups. Values represent mean \pm SEM. One-way ANOVA followed by Bonferroni's

plasma antioxidant status in HTN-Preg compared to Norm-Preg group (presenting MTT assay with similar values), sodium nitrite treated (Preg + Sodium nitrite and HTN-Preg + Sodium nitrite) groups presented MTT assay with higher values than those obtained with Norm-Preg and HTN-Preg groups. In this regard, while we found these results with MTT assay to be intriguing, the sensitivity of methods used may be related to these apparently conflicting data. In fact, although the MTT assay is widely used to measure antioxidant status of plasma, the reference range and linearity for plasma antioxidant capacity measured with TEAC assay may be better than those found with MTT method [26,27,60]. Accordingly, a previous study showed that human plasma albumin is responsible for only 40–60% of the total MTT reduction observed and the platelets were not involved [27]. So, it is not entirely known which of the remaining plasma components might be responsible for the further direct reduction of MTT [27]. On the other hand, TEAC assay can determine the antioxidative effects of bilirubin, vitamin C, uric acid, polyphenols, and several proteins, including albumin; thus, the range for plasma antioxidant capacity measured with TEAC seems to be higher than that for the MTT method, since the antioxidative effect of proteins is accounted for, providing more sensitivity for determining the global antioxidant function [26]. Furthermore, it has been suggested that an oxidant/antioxidant imbalance may be associated with pregnancy complications [61] and that reductions of availability of endogenous NO during hypertension may be due to oxidative stress [61]. In fact, reactive oxygen species may result in the inactivation/sequestration of NO [62]. For these reasons, we then have decided to assess the circulating levels of MPO in our rat model of hypertension-in-pregnancy, since MPO is a possible source of reactive oxygen species that is also elevated in preeclamptic women [63,64], thus, helping us to clarify the interaction between oxidative stress and hypertension-in-pregnancy induced by L-NAME.

We have found that L-NAME increased the circulating levels of MPO in hypertensive pregnant rats treated with (or without) sodium nitrite (HTN-Preg + Sodium nitrite and HTN-Preg groups). This is particularly important due to the fact that MPO is an enzyme abundantly expressed in neutrophil granulocytes and that may become sequestered in the sub-endothelial space and accumulate in the cell matrix, releasing reactive oxygen species into circulation [65]. Corroborating these findings, studies have shown that members of the family heme-peroxidases, in which MPO is prototype, are able to consume NO, affecting its vasodilatory response and exacerbating endothelial dysfunction [66]. Together, the protective (antihypertensive and antioxidant) effects of sodium nitrite treatment were found even under presence of increased MPO levels (similar levels in both HTN-Preg and HTN-Preg + Sodium nitrite groups), suggesting that the antioxidant effects of sodium nitrite presented no relation with MPO; however, the protective effects may result of inhibition of NADPH oxidase and xanthine oxidoreductase activities by sodium nitrite, preventing enzymes-dependent reactive oxygen species production, as previously suggested [11,19].

The oxidative stress may be a common feature, even during healthy pregnancies, since there is an increase in the metabolic demand requested by maternal-fetal interface, which is offset by increases in antioxidant capacity [62,67]. However, upon

correction for multiple comparisons were used to compare measurements among four groups. Since only fetal weight in Norm-Preg group presented no normal distribution, the Mann-Whitney test was used to compare the fetal weight among the four groups. * $P < 0.05$ for HTN-Preg vs. Norm Preg, Preg + Sodium nitrite and HTN-Preg + Sodium nitrite groups. # $P < 0.05$ for Preg + Sodium nitrite vs. Norm Preg, HTN-Preg and HTN-Preg + Sodium nitrite groups.

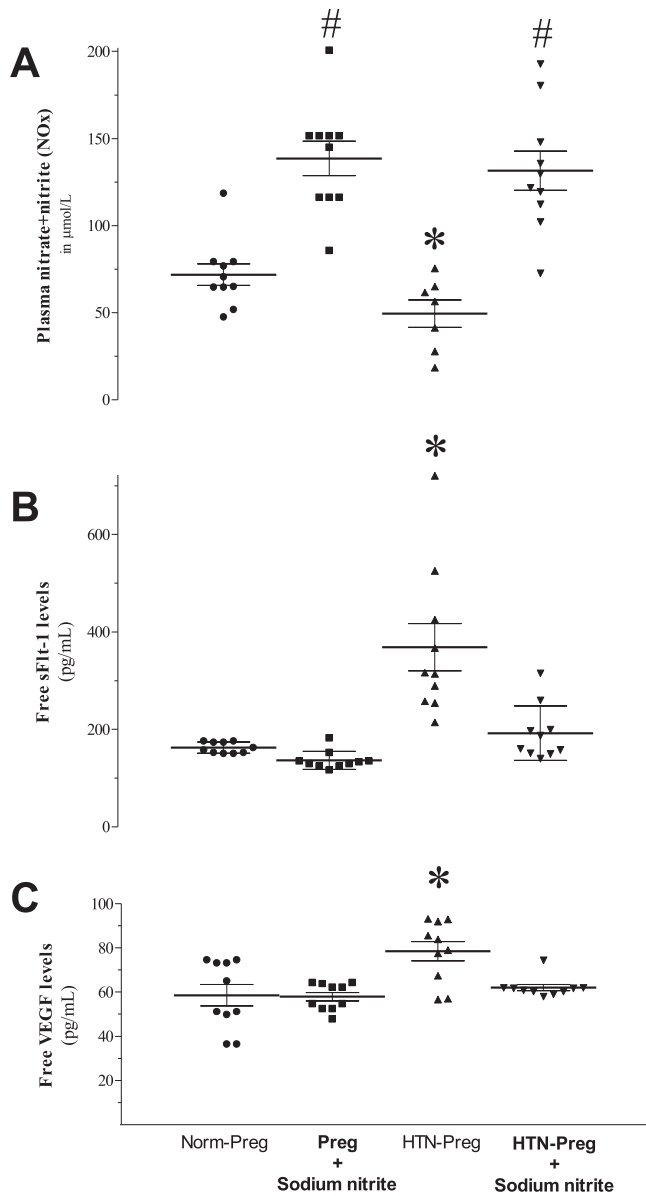


Fig. 3. Plasma NOx (A), circulating free sFlt-1 (B) and VEGF (C) levels in Norm Preg, Preg + Sodium nitrite, HTN-Preg and HTN-Preg + Sodium nitrite groups. Values represent mean \pm SEM. One-way ANOVA followed by Bonferroni's correction for multiple comparisons were used to compare measurements among four groups. * $P < 0.05$ for HTN-Preg vs. Norm Preg, Preg + Sodium nitrite and HTN-Preg + Sodium nitrite groups. # $P < 0.05$ for Preg + Sodium nitrite and HTN-Preg + Sodium nitrite vs. Norm Preg and HTN-Preg groups.

preeclampsia, antioxidant function has failed to prevent the damage promoted by excessive oxidative stress [68]. In this sense, maintaining antioxidant function may help in the prevention of injuries caused by oxidative stress during hypertension-in-pregnancy. Combined, these findings provide evidence that the oral administration of sodium nitrite consistently decreases the blood pressure in association with major antioxidant effects in experimental hypertension [11,19,45], including hypertension-in-pregnancy induced by L-NAME. Thus, the antioxidant function of plasma maintained with sodium nitrite treatment may highlight the importance of further investigation in preeclamptic women, since counterbalancing the excessive oxidative damage may provide a great benefit in this related hypertensive disorders of pregnancy.

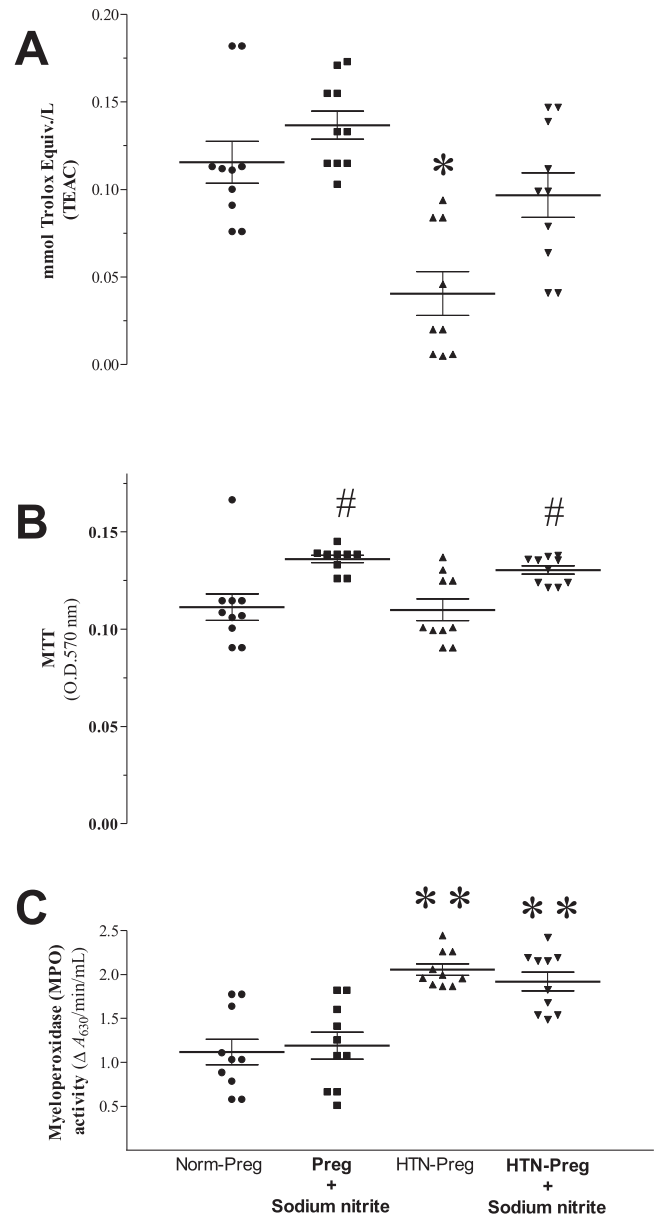


Fig. 4. Plasma antioxidant capacity (TEAC, A), antioxidant status (MTT, B) and MPO activity (C) in Norm Preg, Preg + Sodium nitrite, HTN-Preg and HTN-Preg + Sodium nitrite groups. Values represent mean \pm SEM. One-way ANOVA followed by Bonferroni's correction for multiple comparisons were used to compare measurements among four groups. * $P < 0.05$ for HTN-Preg vs. Norm Preg, Preg + Sodium nitrite and HTN-Preg + Sodium nitrite groups. # $P < 0.05$ for Preg + Sodium nitrite and HTN-Preg + Sodium nitrite vs. Norm Preg and HTN-Preg groups. ** $P < 0.05$ for HTN-Preg + Sodium nitrite vs. Norm Preg and Preg + Sodium nitrite groups.

The present study has some limitations that should be taken into consideration. Firstly, although previous findings have shown that 80 mg/day of sodium nitrite orally administered was safe and well tolerated in healthy adults with few side effects, and no difference in methemoglobin levels compared to placebo [69], the methemoglobin levels could be a reason for concern in patients as a result of sodium nitrite treatment, particularly when sodium nitrite is used at high doses. Secondly, as clinical studies in obstetric patients always remain a delicate ethical issue, animal models serve to answer specific questions regarding the pathophysiology of preeclampsia. Moreover, comparability of data from clinical studies and experimental models always remains speculative [70]. Thirdly,

the fetal weight should be interpreted with caution, since many factors may influence this parameter [71]. Finally, further studies are necessary to address the potential therapeutic of sodium nitrite during preeclampsia.

5. Conclusion

Taken together, our results suggest that nitrite-derived NO prevents hypertension-in-pregnancy induced by L-NAME and concomitantly reduces circulating plasma sFlt-1 and VEGF levels, improving the placental weight and litter size with more viable and less resorbed fetuses. In addition, our findings confirm the hypothesis that treatment with sodium nitrite attenuates the hypertension-in-pregnancy and that nitrite-derived NO may possibly be the driving force behind the maternal and fetal beneficial effects observed in the present study.

Author contributions

V.H.G.R., J.S.P.V., T.U.S.G. and R.A.N. 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.

Conflict of interest

There are no known conflicts of interest.

Acknowledgements

This study was supported by funding from the Fundacao de Amparo a Pesquisa do Estado de Sao Paulo (FAPESP, Brazil). The following are gratefully acknowledged: Valeria Cristina Sandrim and Helio Kushima for their co-operation and advice with technician issues.

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3 - Capítulo 2



Full length article

Maternal hypertension and fetoplacental growth restriction is reversed by sildenafil: Evidence of independent effects of circulating nitric oxide levels

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ARTICLE INFO

Keywords:

Hypertension
Pregnancy
Sildenafil citrate
Sodium nitrite
N(G)-nitro-L-arginine methyl ester
Rats

ABSTRACT

Sildenafil has shown nitric oxide (NO)-independent pleiotropic effects, however the mechanisms involved are unclear. We investigated the protective effects of sildenafil against hypertension in pregnancy and fetoplacental growth restriction induced by NO inhibition, and if sodium nitrite-derived NO formation influences sildenafil effects. We evaluated the plasmatic levels of NO metabolites, cyclic guanosine monophosphate (cGMP), oxidative stress and myeloperoxidase, which are involved in endothelial dysfunction during hypertension in pregnancy. Also, we performed in vitro experiments to examine cell viability and NO synthesis in human umbilical vein endothelial cells (HUVECs) cultures incubated with plasma from healthy or hypertensive pregnant rats treated (or not) with both drugs, either alone or in association. Sildenafil blunted hypertension in pregnancy and protected against fetoplacental growth restriction induced by NO inhibition and these effects of sildenafil alone were similar to those presented by its association with sodium nitrite. Protective effects of sildenafil were observed even with low plasmatic NO levels and were not followed by increases in cGMP levels. Also, sildenafil, but not sodium nitrite, blunted the increases in myeloperoxidase activity. Both drugs (isolated or in association) presented antioxidant effects. Plasma from hypertensive pregnant rats treated with sildenafil, but not sodium nitrite alone, increased the viability of HUVECs. NO synthesis in HUVECs cultures was increased with plasma from rats treated with both drugs. We conclude that sildenafil effects are not dependent of circulating NO levels in hypertension and fetoplacental growth restriction. These findings may reflect a protection against myeloperoxidase and pro-oxidant activation in hypertension in pregnancy.

1. Introduction

Hypertensive disorders of gestation complicate about 5–10% of pregnancies, including gestational hypertension that could progress to preeclampsia (Jim and Karumanchi, 2017; Lo et al., 2013). If untreated, these disorders are major causes of maternal and fetal morbidity and mortality (Uzan et al., 2011). Preeclampsia is also associated with intrauterine fetal growth restriction, accounting for 10–15% of preterm births (Mitani et al., 2009); however, the underlying mechanisms of this disorder are unclear. The initiating event is widely believed to be the impaired spiral artery remodeling that, in turn, leads to a stage of poor placentation with posterior ischemia/hypoxia (Roberts, 2014). Ischemic placenta releases soluble factors into maternal circulation, resulting in the secondary stage of the disorder featured by endothelial dysfunction (Possomato-Vieira and Khalil, 2016).

Physiological blood pressure during pregnancy may rely greatly on the vasodilatory action of nitric oxide (NO) (Leiva et al., 2016). NO also seems to influence the cytotrophoblast invasion and mediates the spiral artery remodeling to allow an adequate supply for the growing fetus (Velicky et al., 2016). In fact, circulating levels of nitrite, a NO metabolite, are increased in normal pregnant women compared to both healthy non-pregnant and preeclamptic women (Cadenapaphornchai et al., 2001). Pregnant rats develop hypertension and fetoplacental growth restriction if NO formation is pharmacologically reduced by N^o-Nitro-L-arginine methyl ester (L-NAME), an agent that effectively inhibits endothelial, neuronal and inducible NO synthases (Ramesar et al., 2010).

Sildenafil is clinically used to treat erectile dysfunction (Hatzimouratidis, 2006). The known mechanism of sildenafil's action is the inhibition of phosphodiesterase type 5 (PDE5), which lengthens the NO–cyclic guanosine 3',5'-monophosphate (cGMP) signaling by preventing the degradation of cGMP (Francis et al., 2010). Hence, based

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on this canonic mechanism of sildenafil in potentiating NO-induced vasodilation, one may consider that as a NO-dependent drug, sildenafil would have no potential to achieve therapeutic goals, considering there may be reduction of NO in preeclampsia or when the endogenous NO synthesis is reduced by L-NAME in pregnant rats (Motta et al., 2015). However, previous studies showed that sildenafil attenuates hypertension and feto-placental growth restriction in L-NAME-treated rats (Nassar et al., 2012; Ramesar et al., 2010) as well as in hypertensive pregnant mice deficient in endothelial NO synthase (Roberts et al., 2016). Together, these preclinical findings suggest that sildenafil effects may not depend of circulating NO levels (Chrysant and Chrysant, 2012). However, mechanistic studies are needed to explain these sildenafil effects and to determine its potential efficacy in hypertensive disorders of gestation complicated by fetal growth restriction (Trapani et al., 2016), even with reduced levels of NO (Sandrim et al., 2008).

The main hypotheses tested in the present study were that sildenafil, independently of NO levels into maternal circulation, attenuates hypertension-in-pregnancy and feto-placental growth restriction and that these effects could be associated with endothelial cells protection against oxidative stress.

2. Materials and methods

2.1. Animals and experimental protocol

Wistar rats (200–250 g) were housed in cages at 22 ± 2 °C on a 12-hr light/dark cycle and given free access to water and rat chow. Each female rat was separately mated overnight. Day 1 of pregnancy was defined as the day when spermatozoa were found in a vaginal smear.

On pregnancy day 14, each pregnant rat mother was first placed into a single cage and randomized to one of the eight treatment groups ($n = 8$ – 10 per group): Normal Pregnant (NP), Normal Pregnant + Sildenafil (NP + S), Normal Pregnant + Nitrite (NP + N), Normal Pregnant + Sildenafil + Nitrite (NP + S + N), Hypertensive Pregnancy (HP), Hypertensive Pregnancy + Sildenafil (HP + S), Hypertensive pregnancy + Nitrite (HP + N) and Hypertensive Pregnancy + Sildenafil + Nitrite group (HP + S + N). In hypertensive pregnant groups (HP groups), rats received intraperitoneal (i.p.) injections of L-NAME (Sigma, St. Louis, MO, #5751) 60 mg/kg/daily from 15th – 21st gestational day (Yang et al., 2011). Sildenafil citrate (Pfizer, UK-92480-10) was administered by gavage at a dose of 10 mg/kg/day from 15th – 21st gestational day (Baijnath et al., 2014). Sodium nitrite was administered by gavage at dosage of 15 mg/kg/day (Sigma, St. Louis, MO, #S2252) from 15th – 21st gestational day. The dose of sodium nitrite was chosen with basis on previous studies showing that this dose exerts relevant antihypertensive and antioxidant effects in rats (Gonçalves-Rizzi et al., 2016; Montenegro et al., 2011; Pinheiro et al., 2014, 2015).

Rats were euthanized on gestation-day 21 under overdose of isoflurane followed by exsanguination. Blood samples were collected in lyophilized ethylenediamine tetraacetic acid (EDTA, Vacuntainer Becton-Dickinson, BD, Oxford, UK), immediately centrifuged and plasma was separated and stored at -80 °C until use for biochemical analysis.

All procedures for animal experimentation were approved by the Ethics Committee, Biosciences Institute of Botucatu, São Paulo State University (Protocol #618/2014), which is complied with international guidelines of the European Community for the use of experimental animals.

2.2. Blood pressure measurements

Systolic blood pressure (mmHg) was measured on gestational day 14 (baseline with absence of gavage or i.p. injections) and days 16, 18 and 20, before drugs administration, using tail-cuff plethysmography (Insight, Ribeirao Preto, Sao Paulo, Brazil, # EFF 306). Briefly, all

pregnant rats were first acclimated in a quiet room, conditioned and restrained for 5–10 min in a warm box (Insight, Ribeirao Preto, Sao Paulo, Brazil, # EFF307) to the measurements for 3 days before the pregnancy day 14 (these data were discarded) and then the baseline systolic blood pressure was determined as the average of the cuff inflation-deflation 3–6 cycles by a trained operator on pregnancy day 14 (Gonçalves-Rizzi et al., 2015).

2.3. Effects on placenta and fetuses

On gestational day 21, after euthanasia, animals were placed in supine position and cesarean section was performed. The number of viable fetuses, litter size, fetal weight and placental weight 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).

2.4. Determination of myeloperoxidase activity

Myeloperoxidase activity was determined by measuring tetramethylbenzidine (TMB) oxidation in an end-point colorimetric assay. For that, 30 μ l of plasma (1:100) were incubated with 20 μ l of phosphate buffer and 100 μ l of liquid substrate system, composed by TMB (Sigma, St. Louis, MO, USA) and hydrogen peroxide 0.04%, at 37 °C for 10 min, protected from light. After incubation, the reaction was stopped with 100 μ l of H_2SO_4 (2 N) and the absorbance at 450 nm with correction to 630 nm was read with the spectrophotometer (Synergy 4, BIOTEK, Winooski, VT, USA). The results were expressed in Myeloperoxidase activity (U/L) (Suzuki et al., 1983).

2.5. Measurements of plasma antioxidant capacity

The trolox equivalent antioxidant capacity (TEAC) was performed as previously described (Erel, 2004). Briefly, a standard curve was established using 100 μ g of Trolox (6-hidroxy-2,5,7,8 - tetramethylchroman-2-carboxylic-acid, Sigma, St. Louis, MO, USA, catalogue# 238813) in 1 ml of sodium acetate buffer (0.4 M, $C_2H_3NaO_2 \cdot 3H_2O$) + glacial acetic acid (0.4 M). Firstly, 20 μ l of plasma samples were added to 200 μ l of sodium acetate buffer + glacial acetic acid and the absorbance at 660 nm was read with the spectrophotometer (Synergy 4, BIOTEK, Winooski, VT, USA). Secondly, 20 μ l of sodium acetate buffer (0.03 M) and glacial acetic acid (0.03 M) + H_2O_2 + ABTS (2,2'-azino-bis-3-ethylbenz-thiazolin-6 sulfonic acid, Sigma A 1888) was added to the samples and incubated for 5 min. Finally, a second spectrophotometer read was performed at 660 nm. The second reading values were subtracted from the values found in the first reading and the antioxidant activity of the sample was expressed as mmol of Trolox equivalent/L.

2.6. Assessment of lipid peroxidation

Plasma lipid peroxide levels were determined by measuring thiobarbituric acid-reactive substances (TBARS) (Perico et al., 2015). In test tubes, 100 μ l of distilled water, 50 μ l of 8.1% sodium dodecyl sulfate (SDS), 375 μ l of 20% acetic acid pH 3.5, and 375 μ l of 0.8% 2-thiobarbituric acid (TBA) diluted in 20% acetic acid were added to 100 μ l of sample. For the standard curve, the test tubes contained 25 μ l of a malondialdehyde solution of known concentration, 175 μ l of distilled water, 50 μ l of 8.1% sodium dodecyl sulfate, 375 μ l of 20% acetic acid pH 3.5, and 375 μ l of 0.8% 2-thiobarbituric acid (TBA) diluted in 20% acetic acid. The test tubes were incubated in water bath at 95 °C for 1 h and centrifuged at 1792 g for 10 min. A 200 μ l aliquot of each sam-

plasma was transferred to a 96-well plate. The malondialdehyde formed by the sample reacts with the TBA to produce a colorimetric reaction that was measured using a spectrophotometer (Synergy 4, BIOTEK, Winooski, VT, USA) at 532 nm. The lipoperoxide levels were expressed in terms of malondialdehyde (nmol/ml).

2.7. Measurement of plasma cyclic guanosine monophosphate (cGMP) concentration

Arterial blood samples were drawn in tubes containing EDTA and stored at -80°C until the determination of plasmatic cGMP levels using commercial enzyme immunoassay (ELISA) kit (Cayman Chemical no 581021). Assays were performed according to manufacturer's instructions. Plasmatic cGMP levels were expressed in pmol/ml.

2.8. Endothelial cell culture and plasma incubation

The human umbilical vein endothelial cells (HUVECs) were cultured in DMEM medium (Gibco, CA, USA) supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco), 50 $\mu\text{g/ml}$ penicillin, 50 $\mu\text{g/ml}$ streptomycin and 0.5 $\mu\text{g/ml}$ amphotericin B (Gibco) at 37°C in 5% CO_2 incubator, as described previously (Roberts et al., 2006). After reaching 80% confluence, HUVECs were re-suspended in DMEM medium and re-plated in 96-well tissue culture plates (Corning), where they were grown to 80% confluence for incubation experiments. Then, the medium was removed and cells were washed twice in PBS. Cells were incubated in medium, without FCS, with 10% (v/v) plasma from rats treated with saline (NP group), sildenafil (NP + S group), nitrite (NP + N group), sildenafil + nitrite (NP + S + N group), L-NAME (HP group), L-NAME + sildenafil (HP + S group), L-NAME + nitrite (HP + N) and L-NAME + sildenafil + nitrite (HP + S + N) for 24 h. The cell supernatant was collected and stored at -80°C to determine the nitrate + nitrite (NOx) concentrations.

2.9. HUVECs viability assay

The toxicity for plasma from each pregnant rat was assessed and used to determine the cell viability by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (Mosmann, 1983). Briefly, after 24 h of plasma incubation in HUVECs (line CRL 2873 obtained from American Type Culture Collection, ATCC, Manassas, VA, USA), the medium was carefully removed and cells were washed twice in PBS. Then, MTT solution (0.5 mg/ml PBS) (Sigma-Aldrich) was added and the plate was placed in the incubator (37°C , 5% CO_2). MTT is reduced to blue formazan crystals by metabolically active cells. After 3 h, MTT-formazan crystals were dissolved in DMSO (Sigma-Aldrich) for 10 min and absorbance was measured at 570 nm on a multifunctional plate reader (Synergy 4, BIOTEK, Winooski, VT, USA). Viability was compared to control (untreated cells, 100% viability).

2.10. Measurement of nitrate + nitrite (NOx) concentrations

The NOx concentrations were determined in duplicate in plasma and cell supernatant, by Griess reaction, as previously described (Dias-Junior et al., 2010). Briefly, 50 μl of samples were incubated with the same volume of nitrate reductase buffer (0.1 M potassium phosphate, pH 7.5, containing 1 mM β -nicotinamide adenine dinucleotide phosphate and 2U of nitrate reductase/ml) in individual wells of a 96-well plate. Samples were allowed to incubate overnight at 37°C in the dark; 8 μl of freshly prepared Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride in 5% phosphoric acid) were added to each well and the plate was incubated, for 15 additional min, at room temperature. A standard nitrate curve was obtained

by incubating sodium nitrate (0.2–200 μM) with the same reductase buffer. The NOx levels in plasma were expressed in $\mu\text{mol/l}$.

2.11. Statistical analysis

Using commercially available statistical software (Graph Pad Prism® 6.0 for Windows, San Diego, CA), a Shapiro-Wilk test was applied to verify normality of data distribution. Systolic blood pressure measurements were submitted to a two-way analysis of variance (ANOVA) followed by Bonferroni's correction for multiple comparisons among groups to compare measurements on pregnancy days 14, 16, 18 and 20, or one-way ANOVA followed by Bonferroni's correction for multiple comparisons were used to compare fetal and placental changes, and oxidative stress analysis, NOx and cGMP levels in plasma, and viability and NOx levels of cells. Statistical significance was considered at $P < 0.05$. All values are expressed as mean \pm S.E.M.

3. Results

3.1. Antihypertensive effects of sildenafil citrate and sodium nitrite in hypertensive pregnant rats

Baselines systolic blood pressures were similar in all experimental groups $116\text{--}125 \pm 4$ mmHg) on day 14 of gestation ($P > 0.05$, Fig. 1). Pregnant rats from NP + S, NP + N and NP + S + N groups showed no significant changes in systolic blood pressure values throughout the study period, on days 16, 18 and 20 of gestation (NP + S, 120 ± 3 ; 124 ± 3 and 118 ± 2.8 mmHg; NP + N, 120 ± 3 ; 122 ± 1.8 and 122 ± 1.7 mmHg; NP + S + N, 125 ± 4.5 ; 120 ± 2.6 and 121 ± 2 mmHg; $P > 0.05$, Fig. 1). However, pregnant rats that received L-NAME presented elevated systolic blood pressure values on days 16, 18 and 20 of gestation (HP group, 145 ± 5 ; 149 ± 3 and 146 ± 4 mmHg, respectively, $^{\#}P < 0.05$, Fig. 1). However, the pregnant rats that received L-NAME and treated with sildenafil citrate (HP + S group, 129 ± 3 ; 128 ± 2.3 ; 129 ± 1 mmHg), sodium nitrite (HP + N group, 126 ± 3 ; 131 ± 3 ; 127 ± 2 mmHg), and their combination (HP + S + N group, 131 ± 2 ; 130 ± 2.5 ; 123 ± 5 mmHg) showed significant and similar lower systolic blood pressure values on days 16, 18 and 20 of gestation when compared to HP group (145 ± 5 ; 149 ± 3 and 146 ± 4 mmHg, respectively, $^{\#}P < 0.05$, Fig. 1).

3.2. Sildenafil citrate, but not sodium nitrite, reversed fetal growth restriction

The fetal weight was significantly lower in HP group (2.9 ± 0.08 g) when compared to normal pregnant groups: NP; NP + S; NP + N and NP + S + N (3.6 ± 0.2 ; 3.9 ± 0.2 ; 3.6 ± 0.08 and 3.8 ± 0.04 g, respectively, $^{\#}P < 0.05$, Fig. 2A). Sildenafil reversed fetal growth restriction in HP + S group (3.9 ± 0.2 g) and in HP + S + N group (3.7 ± 0.06 g, $^{\#}P < 0.05$, Fig. 2A). However, sodium nitrite alone showed no significant changes in fetal weight (HP + N group, 3.4 ± 0.1 g; $P > 0.05$, Fig. 2A).

Placental weight was significantly lower in HP group (0.49 ± 0.01 g) when compared to normal pregnant groups: NP; NP + S; NP + N and NP + S + N (0.61 ± 0.02 ; 0.60 ± 0.01 ; 0.60 ± 0.01 and 0.59 ± 0.02 g, respectively, $^{\#}P < 0.05$, Fig. 2B). However, treatment with sildenafil citrate (HP + S), sodium nitrite (HP + N), and their combination (HP + S + N) reversed placental growth restriction (0.61 ± 0.01 ; 0.59 ± 0.03 ; and 0.63 ± 0.02 g, respectively, $^{\#}P < 0.05$, Fig. 2B).

Number of pups (litter size) was significantly lower in HP group (9.3 ± 0.7) when compared to normal pregnant groups: NP; NP + S; NP + N and NP + S + N (11.9 ± 1 ; 11.1 ± 0.6 ; 11.4 ± 0.6 ; and 11.8

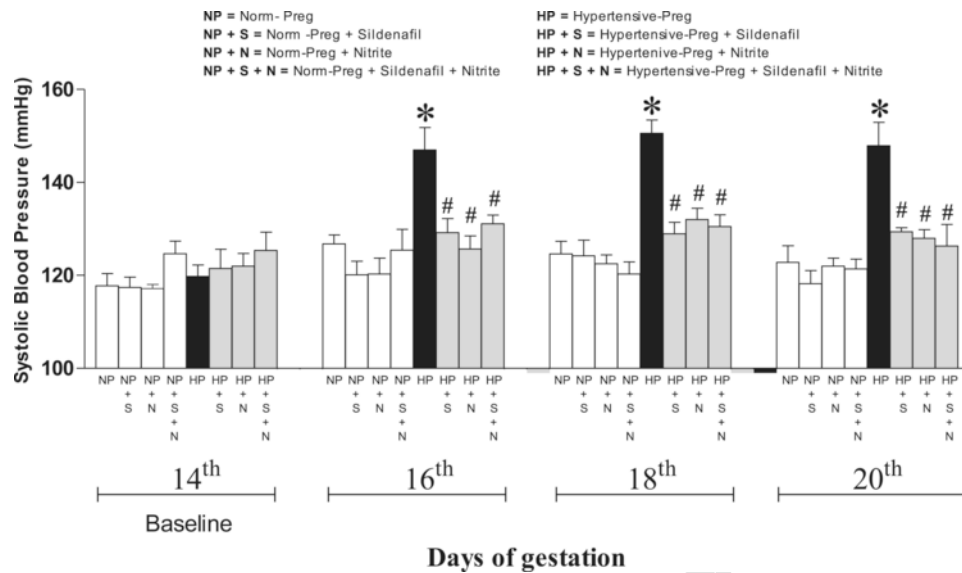


Fig. 1. Systolic blood pressure measured by tail cuff plethysmography on days 14, 16, 18 and 20 of gestation in Normal Pregnant (NP), Normal Pregnant + Sildenafil citrate (NP + S), Normal Pregnant + Sodium nitrite (NP + N), Normal Pregnant + sildenafil citrate + sodium nitrite (NP + S + N), Hypertensive pregnancy + Sildenafil citrate (HP + S), Hypertensive pregnancy + Sodium nitrite (HP + N), and Hypertensive pregnancy + sildenafil citrate + sodium nitrite group (HP + S + N). Values represent mean \pm S.E.M. Two-way ANOVA with Bonferroni's correction for multiple comparisons among groups was used to compare measurements on pregnancy days 14, 16, 18 and 20. Significant increases were observed in HP group on pregnancy days 16, 18 and 20. Treatment with sildenafil citrate, sodium nitrite and their association reduced SBP in HP groups. * $P < 0.05$ versus NP group. # $P < 0.05$ versus HP group.

± 0.5 , respectively, * $P < 0.05$, Fig. 2C). Sildenafil citrate (HP + S, 12.2 ± 0.5), sodium nitrite (HP + N, 12.1 ± 0.5) and their combination (HP + S + N, 11.8 ± 0.5) improved litter size (* $P < 0.05$, Fig. 2C).

Lower numbers of viable fetuses (Fig. 2D) with higher number of reabsorbed fetuses (Fig. 2E) were found only in HP group (7 ± 0.7 and 1.3 ± 0.29 , respectively) when compared to normal pregnant groups: NP (11 ± 1.0 and 0.30 ± 0.15), NP + S (11.3 ± 0.7 and 0.25 ± 0.16); NP + N (10.5 ± 0.7 and 0.33 ± 0.13) and NP + S + N (11.3 ± 0.6 and 0.28 ± 0.18 , * $P < 0.05$, Fig. 2D and E, respectively). The treatment with sildenafil, nitrite and their combination increased viable fetuses and concomitantly reduced number of reabsorbed fetuses in HP + S (11.6 ± 0.4 and 0.5 ± 0.18), HP + N (11.6 ± 0.6 and 0.4 ± 0.16) and HP + S + N (11 ± 0.5 and 0.5 ± 0.26), * $P < 0.05$, Fig. 2D and E, respectively).

3.3. Sodium nitrite, but not sildenafil citrate, increases plasmatic NOx levels in normotensive and hypertensive pregnant rats

L-NAME treatment significantly reduced NO bioavailability, which was evaluated by measuring plasmatic NOx concentrations in HP group ($45 \pm 7 \mu\text{mol/l}$) compared to those found in NP group ($76 \pm 9 \mu\text{mol/l}$, * $P < 0.05$, Fig. 3A). As expected, pregnant rats that received (or not) L-NAME and treated with sodium nitrite showed increases in NO bioavailability (NP + N group, 118 ± 27 ; NP + S + N group, 108 ± 14 ; HP + N group, 124 ± 10 ; HP + S + N group, $100 \pm 12 \mu\text{mol/l}$; *, # $P < 0.05$, Fig. 3A). However, pregnant rats treated with sildenafil showed no changes in NOx concentrations (NP + S group, 63 ± 3.7 ; HP + S group, $50 \pm 3.5 \mu\text{mol/l}$; Fig. 3A).

3.4. Sildenafil citrate (or sodium nitrite) alone did not increase plasmatic cGMP levels in hypertensive pregnant rats

Similar plasmatic cGMP levels were found in NP group ($3.1 \pm 0.32 \text{ pmol/ml}$) and NP + N group ($3.5 \pm 0.9 \text{ pmol/ml}$) and HP group ($4.7 \pm 0.39 \text{ pmol/ml}$). As expected, normal pregnant rats treated with sild-

nafil alone (or in association with nitrite) presented higher plasmatic cGMP levels (NP + S, 11.9 ± 2.3 ; NP + S + N, $10.3 \pm 2.3 \text{ pmol/ml}$, respectively) compared to NP and HP groups (*, # $P < 0.05$, Fig. 3B). However, pregnant rats that received L-NAME and treated with sildenafil (or nitrite) alone presented no changes in plasmatic cGMP levels (HP + S group, 3.3 ± 0.7 ; HP + N group, $4.1 \pm 0.45 \text{ pmol/ml}$, respectively) compared to NP and HP groups. The sildenafil in association with nitrite presented higher plasmatic cGMP levels (HP + S + N group, $10.3 \pm 1.9 \text{ pmol/ml}$) compared to NP and HP groups (*, # $P < 0.05$, Fig. 3B).

3.5. Sildenafil citrate, but not sodium nitrite, decreases myeloperoxidase activity

HP and HP + N groups presented increases in myeloperoxidase activity (16.9 ± 1.8 and $17 \pm 1.8 \text{ U/L}$, respectively, * $P < 0.05$, Fig. 3C) when compared to normal pregnant groups: NP ($10 \pm 1 \text{ U/L}$), NP + S ($7.5 \pm 0.9 \text{ U/L}$), NP + N ($9.9 \pm 2.3 \text{ U/L}$), and NP + S + N ($9.4 \pm 1.5 \text{ U/L}$). Interestingly, sildenafil prevented increases in myeloperoxidase activity (HP + S group, $10.6 \pm 1.1 \text{ U/L}$; HP + S + N group, $10.1 \pm 1.3 \text{ U/L}$, respectively, # $P < 0.05$, Fig. 3C).

3.6. Sildenafil citrate and sodium nitrite reduce lipid peroxides levels in hypertensive pregnant rats

To evaluate the effects of sodium nitrite and sildenafil citrate on hypertension-in-pregnancy-induced increase in oxidative stress, lipid peroxides levels were evaluated by thiobarbituric acid-reactive substances (expressed in malondialdehyde levels). Lipid peroxidation in HP group was elevated ($148 \pm 19 \text{ nmol/ml}$) compared to normal pregnant groups: NP ($88 \pm 9 \text{ nmol/ml}$), NP + S ($80 \pm 5 \text{ nmol/ml}$), NP + N ($83 \pm 3 \text{ nmol/ml}$), and NP + S + N ($76 \pm 5 \text{ nmol/ml}$, * $P < 0.05$, Fig. 3D). The treatment with sildenafil citrate, sodium nitrite and their combination prevented the increases in lipid peroxidation (HP + S group, $87 \pm 7 \text{ nmol/ml}$; HP + N group, $103 \pm 9 \text{ nmol/ml}$ and HP + S + N group, $74 \pm 6 \text{ nmol/ml}$, # $P < 0.05$, Fig. 3D).

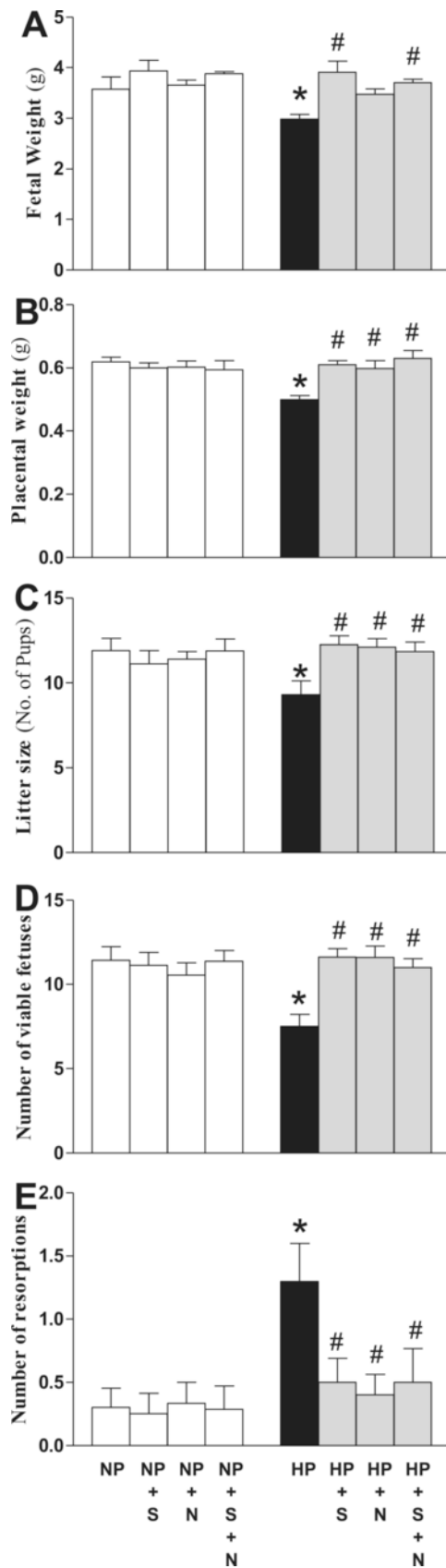


Fig. 2. Fetal and placental parameters were recorded on gestational-day 21: Fetal weight (A), placental weight (B), litter size (C), number of viable fetuses (D) and number of resorptions (E) in Normal Pregnant (NP), Normal Pregnant + Sildenafil citrate (NP +

S), Normal Pregnant + Sodium nitrite (NP + N), Normal Pregnant + sildenafil citrate + sodium nitrite (NP + S + N), Hypertensive Pregnancy (HP), Hypertensive pregnancy + Sildenafil citrate (HP + S), Hypertensive Pregnancy + Sodium nitrite (HP + N), and Hypertensive Pregnancy + sildenafil citrate + sodium nitrite group (HP + S + N). Values represent mean ± S.E.M. One-way ANOVA followed by Bonferroni's correction for multiple comparisons were used to compare measurements among eight groups. *P < 0.05 versus NP group. #P < 0.05 versus HP group.

3.7. Sildenafil citrate and sodium nitrite improve antioxidant capacity

Lower antioxidant capacity was found in HP group (0.16 ± 0.006 mmol of Trolox equivalent/L) when compared to normal pregnant groups: NP (0.21 ± 0.008 mmol of Trolox equivalent/L) and NP + S (0.21 ± 0.10 mmol of Trolox equivalent/L) *P < 0.05, Fig. 3E). Although hypertensive pregnant rats treated with sildenafil alone (HP + S group, 0.19 ± 0.10 mmol of Trolox equivalent/L) or sodium nitrite alone (HP + N group, 0.20 ± 0.10 mmol of Trolox equivalent/L) showed trends to reverse the reduction of the antioxidant capacity, no statistical differences were observed. However, sildenafil associated with sodium nitrite, surprisingly, significantly increased the antioxidant capacity (HP + S + N group, 0.25 ± 0.21 mmol of Trolox equivalent/L, #P < 0.05, Fig. 3E).

3.8. Plasma from hypertensive pregnant rats treated with sildenafil citrate, but not with sodium nitrite, increases endothelial cell viability

The HUVECs showed higher cell viability when incubated with plasma from hypertensive pregnant rats treated with sildenafil citrate (HP + S group, $58.6 \pm 5\%$; HP + S + N group, $58.1 \pm 5\%$) when compared to all other groups: NP ($17 \pm 2\%$), NP + S ($23 \pm 5\%$), NP + N ($27 \pm 3\%$), NP + S + N ($16 \pm 5\%$), HP ($30 \pm 7\%$) and HP + N ($23 \pm 5\%$), *P < 0.05, Fig. 4A). However, hypertensive pregnant rats treated with sodium nitrite alone (HP + N group) showed no changes in HUVECs viability (P > 0.05, Fig. 4A).

3.9. Plasma from normotensive and hypertensive pregnant rats treated with sildenafil citrate and sodium nitrite (alone or in association) increase NO production in endothelial cells

The HUVECs incubated with plasma from normal pregnant rats treated with sodium nitrite showed higher NOx concentrations in cells supernatants (NP + N group, 78 ± 1 ; NP + S + N group, 72 ± 0.7 $\mu\text{mol/l}$) compared to NP group (54 ± 0.9 $\mu\text{mol/l}$, *P < 0.05, Fig. 4B). Interestingly, HUVECs incubated with plasma from normal pregnant rats treated with sildenafil also showed significant increases in NOx concentrations in cells supernatants (NP + S group, 62 ± 1 $\mu\text{mol/l}$) compared to NP group (53 ± 0.9 $\mu\text{mol/l}$, *P < 0.05, Fig. 4B). Also, HUVECs incubated with plasma from hypertensive pregnant rats treated with sildenafil, sodium nitrite isolated or in combination presented increases in the NOx concentrations in cells supernatants (HP + S group, 58 ± 1 ; HP + N group, 61 ± 1 ; HP + S + N group, 60 ± 3 $\mu\text{mol/l}$) when compared to HP group (49 ± 0.7 $\mu\text{mol/l}$, #P < 0.05, Fig. 4B).

4. Discussion

The main findings in the present study were that (1) sildenafil attenuated hypertension and fetoplacental growth restriction caused by L-NAME in pregnant rats; (2) both maternal and fetoplacental beneficial sildenafil effects observed here were not dependent of NO and cGMP bioavailability, because similar sildenafil effects were found in the presence of increased or decreased circulating NO levels caused by sodium nitrite or L-NAME, respectively; and were not dependent of increases in cGMP levels (3) sildenafil (but not sodium nitrite) protected

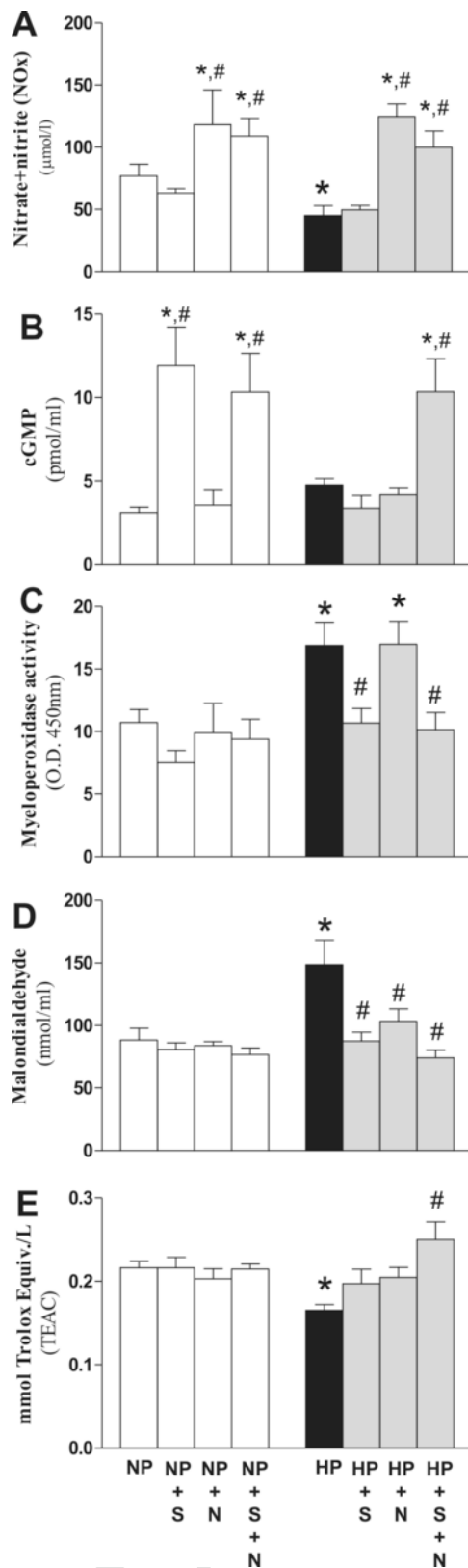


Fig. 3. Plasmatic NOx levels (A), plasmatic cGMP levels (B), myeloperoxidase activity in plasma (C), malondialdehyde levels in plasma (D), plasma antioxidant capacity (TEAC, E) in Normal Pregnant (NP), Normal Pregnant + Sildenafil citrate (NP + S), Normal Pregnant + Sodium nitrite (NP + N), Normal Pregnant + sildenafil citrate + sodium nitrite (NP + S + N), Hypertensive Pregnancy (HP), Hypertensive pregnancy + Sildenafil citrate (HP + S), Hypertensive Pregnancy + Sodium nitrite (HP + N), and Hypertensive

Pregnancy + sildenafil citrate + sodium nitrite group (HP + S + N). Values represent mean ± S.E.M. One-way ANOVA followed by Bonferroni's correction for multiple comparisons were used to compare measurements among eight groups. *P < 0.05 versus NP group. #P < 0.05 versus HP group.

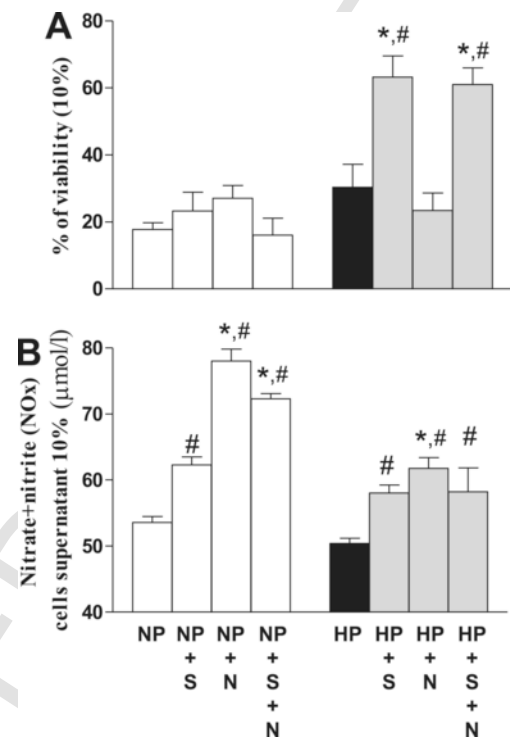


Fig. 4. Cell viability (A) and NOx cell supernatant (B) in Normal Pregnant (NP), Normal Pregnant + Sildenafil citrate (NP + S), Normal Pregnant + Sodium nitrite (NP + N), Normal Pregnant + sildenafil citrate + sodium nitrite (NP + S + N), Hypertensive Pregnancy (HP), Hypertensive pregnancy + Sildenafil citrate (HP + S), Hypertensive Pregnancy + Sodium nitrite (HP + N), and Hypertensive Pregnancy + sildenafil citrate + sodium nitrite group (HP + S + N). Values represent mean ± S.E.M. One-way ANOVA followed by Bonferroni's correction for multiple comparisons were used to compare measurements among eight groups. *P < 0.05 versus NP group. #P < 0.05 versus HP group.

against fetal growth restriction as well as increases in myeloperoxidase activity caused by L-NAME; (4) while both drugs isolated blunted increases in lipid peroxidation and reductions in antioxidant capacity caused by L-NAME, only association enhanced the plasma antioxidant capacity; (5) higher HUVECs viability was found after incubation with plasma from L-NAME-induced hypertensive pregnant rats treated with sildenafil; (6) HUVECs cultures incubated with plasma from healthy or from L-NAME-induced hypertensive pregnant rats treated with both drugs (either isolated or combined) showed higher NO levels in cells supernatants compared with controls.

Studies have shown that reductions in circulating NO levels by pharmacological inhibition of NOS synthesis with L-NAME resulted in maternal hypertension, fetoplacental growth restriction and increases in oxidative stress, which are features of hypertension-in-pregnancy-like state (Gonçalves-Rizzi et al., 2016; Possomato-Vieira et al., 2016; Ramesar et al., 2010). In the present study, we found that sildenafil treatment resulted in immediate and sustained attenuation of hypertension and, sildenafil, but not sodium nitrite, also protected against fetal growth restriction caused by L-NAME. To expand these findings and to confirm that sildenafil effects may not depend of circulating NO levels, we examined whether sildenafil effects could be enhanced by sodium nitrite, a drug with the capability to increase the circulating NO levels upon L-NAME-induced inhibition of NO synthesis in pregnant (Gonçalves-Rizzi et al., 2016) and male rats (Kanematsu et al., 2008; Montenegro et al., 2014; Pinheiro et al., 2014). In our hands, neither synergistic nor additive effects of sildenafil combined with sodium ni-

trite were found. Similar effects on blood pressure and on placental growth were found, independently if sildenafil was used isolated or combined with sodium nitrite. Importantly, sildenafil, but not sodium nitrite, reversed fetal growth restriction. Also, while similar circulating NO levels were found in groups treated with sildenafil or saline, sodium nitrite treatment (alone or combined with sildenafil) resulted in significant increases in circulating NO levels. Therefore, these results indicate that sildenafil effects are not dependent of circulating NO levels.

Particularly, sildenafil is known to prolong the effects of NO signaling by inhibiting the breakdown of the second messenger of NO, cGMP. However, NO bioavailability had not been evaluated in previous studies reporting that sildenafil treatment ameliorates the maternal syndrome of hypertension-in-pregnancy and rescues the fetal growth in different animal models, including the reduced uterine perfusion pressure (RUPP) rat model, Dahl salt-sensitive rat, suramin-treated rat and catechol-O-methyl transferase knockout mouse (George et al., 2013; Gillis et al., 2016; Stanley et al., 2012; Turgut et al., 2008).

Our study accessed the circulating NO and cGMP levels and indicated that maternal and feto-placental protective effects with sildenafil treatment are not dependent of circulating NO and cGMP levels, i.e. sildenafil could present beneficial effects even under NO synthesis inhibition with L-NAME and upon increases of NO bioavailability with sodium nitrite.

In our hands, treatment with sildenafil alone or in combination with nitrite increased plasmatic cGMP levels in normotensive animals, while only combined treatment increased plasmatic cGMP levels in hypertensive animals. As expected, treatment with sildenafil in normotensive animals (NP + S and NP + S + N groups) increased plasmatic cGMP levels, as this is the canonic mechanism by which sildenafil exerts its effects (Francis et al., 2010; Hatzimouratidis, 2006). However, NO derived from sodium nitrite did not promoted increases in plasmatic cGMP levels (NP + N and HP + N groups). We suggest that an increase in activity of cGMP-specific PDE5 in pregnancy (Ni et al., 2004) inhibited increase in cGMP in NP + N and HP + N groups.

Interestingly, beneficial effects of sildenafil alone in hypertensive pregnant rats were not followed by increases in plasmatic cGMP levels (HP + S group), because in the presence of reduced NO bioavailability induced by L-NAME, there may be not sufficient NO to stimulate soluble guanylate cyclase (sGC) and therefore to increase cGMP levels. This supports our overall observation that sildenafil effects are not dependent of circulating NO levels. Moreover, plasmatic cGMP levels were found to be elevated in HP + S + N group, suggesting that nitrite-derived NO stimulated sGC and increased cGMP levels, which remained higher due to PDE5 inhibition by sildenafil citrate. Thus, increases in the signaling of NO-cGMP pathway by nitrite in association with sildenafil may also be involved in the effects observed in HP + S + N group. Taken together, these results show that the combined administration of nitrite and sildenafil is not advantageous compared with sildenafil alone, because sildenafil alone protected against hypertension and fetal growth restriction without increase cGMP levels in HP + S group, thus, suggesting that sildenafil effects in hypertension in pregnancy were not dependent of NO and cGMP. Thereby, our present results provide pre-clinical evidences to support the use of sildenafil in women with hypertension-in-pregnancy with features of preeclampsia (Trapani et al., 2016), even though preeclamptic women have shown reduced NO formation compared to healthy pregnant women (Ehsanipoor et al., 2013; Eleuterio et al., 2013; Schiessl et al., 2006; Tranquilli et al., 2004; Wang et al., 2015).

In order to explain the findings with sildenafil, biochemical determinants of oxidative stress were determined, because sildenafil has revealed antioxidant effects (Gillis et al., 2016; Milani et al., 2005; Ozdegirmenci et al., 2011; Semen et al., 2016; Soobryan et al., 2017). However, this potential and beneficial pleiotropic effect of sildenafil, that counteracts the endothelial dysfunction caused by oxidative stress (Chrysant and Chrysant, 2012) needs to be confirmed in hypertension-

in-pregnancy. Regarding to oxidative stress, it is believed that under stress conditions, there are elevated levels of myeloperoxidase, increases in lipid peroxidation and concomitant reduction of plasma antioxidant capacity in maternal circulation that may trigger or maintain widespread maternal endothelial dysfunction in hypertensive disorders of gestation (Gupta et al., 2009; Rocha-Penha et al., 2017). Our present results are in line with these suggestions and we also found that sildenafil (but not sodium nitrite) treatment blunted the increases in myeloperoxidase activity. Furthermore, lower lipid peroxidation (assessed by TBARS) concentrations and rescues of antioxidant capacity were found with both drugs (alone or in association). Interestingly, synergic effects were observed in plasma antioxidant capacity when drugs were used in association.

Given the fact that antihypertensive effects of sildenafil were associated with reductions in both myeloperoxidase activity and TBARS concentrations and that sildenafil restored the plasma antioxidant capacity, our data suggest that sildenafil protected the maternal vasculature against the endothelium dysfunction caused by oxidative stress induced by L-NAME. In addition, similar protective effects against lipid peroxidation observed with both drugs (alone and combined) probably resulted in antioxidant mechanisms shared by both drugs, as previously reported (Amaral et al., 2015; Gillis et al., 2016; Guimarães et al., 2013; Montenegro et al., 2011). Therefore, to clarify the pleiotropic effects of sildenafil involving the endothelium protection, experiments *in vitro* were performed.

We then assessed the cell viability in HUVECs cultures incubated with plasma from healthy or from L-NAME-induced hypertensive pregnant rats without treatment or treated with sildenafil and nitrite (both alone and in association). Interestingly, higher cell viability was found in HUVECs incubated with plasma from L-NAME-induced hypertensive pregnant rats treated with sildenafil alone or combined with nitrite compared with other groups. However, HUVECs incubated with plasma from L-NAME-induced hypertensive pregnant rats treated with sodium nitrite alone presented no increases in HUVECs viability. Therefore, these findings *in vitro* suggest that sildenafil, but not sodium nitrite, could protect the endothelial cells against the endogenous pro-oxidants agents present in plasma, allowing pro-proliferative machinery of HUVECs.

We suggest that sildenafil reduced reactive oxygen species production, because we found that myeloperoxidase activity and lipid peroxidation were reduced by sildenafil. Although the mechanism is not completely elucidated, studies have shown that sildenafil has antioxidant effect by increasing activity of antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutase (Celik et al., 2014; Perk et al., 2008). In addition, sildenafil reduces the activity of NADPH oxidase and vascular levels of nitrotyrosines (Guimarães et al., 2013).

Increased myeloperoxidase activity has a role in endothelial dysfunction (Rocha-Penha et al., 2017), which is a hallmark of maternal hypertensive syndromes (Roberts, 1998, 2014). An important fact in gestational hypertensive disorders is ischemia/hypoxia (George et al., 2013). During ischemic conditions there is accumulation of neutrophils in the endothelium, in which activated neutrophils release cytotoxic substances that interact with the endothelium and cause tissue damage (Suzuki et al., 1993). In our hands, sildenafil, but not nitrite, reduced myeloperoxidase activity, which is in accordance with previous studies that reported that lower myeloperoxidase activity indicates lower neutrophil activation (Suzuki et al., 1993) and that drugs that reduce myeloperoxidase activity lead to increases in endothelial cell viability (Tian et al., 2017). In line with these reports, reduction of myeloperoxidase activity by sildenafil may be responsible for increasing in endothelial cell viability in our study.

Considering that sildenafil protects the endothelial cells *in vivo* and *in vitro* against damages caused by oxidative stress, and that oxidative stress impairs the NO formation by endothelial cells and further aggravates endothelial dysfunction (Förstermann, 2010), we then assessed

the NO availability in vitro in cell supernatant of HUVECs incubated with plasma from healthy or from L-NAME-induced hypertensive pregnant rats without treatment or treated with sildenafil and sodium nitrite (alone or combined). The NO levels in supernatant of HUVECs cultures incubated with plasma from pregnant rats treated with both drugs (either alone and combined) were significantly higher compared to respective controls (pregnant rats treated with saline or L-NAME).

Taken together, our present results are in accordance with the idea that in vivo sildenafil treatment produces beneficial effects independent of circulating NO levels, while in vitro sildenafil may protect the healthy endothelial cells against possible damages caused by pro-oxidants agents present in plasma, enabling the HUVECs ability to proliferate, and to synthesize and to release NO. Accordingly, antioxidant mechanisms activated by both drugs (sildenafil and sodium nitrite) may reduce the reactive oxygen species in plasma (Amaral et al., 2015; Gillis et al., 2016; Guimarães et al., 2013; Montenegro et al., 2011), preventing the impairment of NO availability caused by oxidative stress. Alternatively, other mechanisms have been proposed to explain our present results showing the increases in NO availability in HUVECs incubated with plasma from rats treated with sildenafil. Previous reports have suggested that sildenafil directly triggers a signaling cascade in endothelial cells, through the action of kinases, resulting in the phosphorylation of endothelial NO synthase, which provides NO synthesis (Kukreja et al., 2004). However, it is important to make clear that further studies are needed to clarify the mechanisms responsible by the effects observed with sildenafil in this study.

Based on protective effects on endothelial cells observed here, we suggest that sildenafil can be compared with soluble fms-like tyrosine apherisis (Thadhani et al., 2016) for the treatment of preeclampsia (Villanueva-García et al., 2007). Furthermore, the pleiotropic and beneficial effects of sildenafil (Karasu et al., 2012) could be more advantageous in cost, access and risks than NO donors (Trapani et al., 2016), including sodium nitrite treatment used here, which may results in side effects such as methemoglobinemia-induced hypoxemia (DeVan et al., 2016) and/or in peroxynitrite formation after superoxide reaction with sodium nitrite-derived NO, leading to vasoconstriction instead of vasodilation (Schulz et al., 2011).

Importantly, since the most commonly used antihypertensive drugs have shown to cause systemic vasodilatation, but have no significant clinical effects on increasing placental blood flow and protection of fetal growth, additional trials should be conducted to address the efficacy and safety of sildenafil in hypertensive pregnant women, particularly, the potential therapeutic role of sildenafil in pregnancies complicated by intrauterine growth restriction (Trapani et al., 2016).

5. Conclusion

We concluded that sildenafil effects may not be dependent of circulating NO levels in hypertension in pregnancy, because protective sildenafil effects against hypertension and fetal growth restriction were found in the presence of L-NAME and were independently of sodium nitrite-derived NO. Moreover, these same sildenafil effects were not followed by increases in plasmatic cGMP levels. Also, sildenafil may protect endothelial cells against myeloperoxidase and pro-oxidant activations in hypertension in pregnancy, suggesting pleiotropic sildenafil effects. Clinical studies should be carried out to validate the beneficial effects exerted by sildenafil during hypertension in pregnancy associated with fetal growth restriction.

Acknowledgements

This study was supported by funding from the Fundacao de Amparo a Pesquisa do Estado de Sao Paulo (FAPESP, Brazil). The following is gratefully acknowledged: Dr Helio Kushima for his co-operation and advice with technician issues.

Conflict of interest

There are no known conflicts of interest.

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4 – Discussão

As desordens hipertensivas quando não controlada, trazem grandes consequências tanto para a mãe quanto para o bebê. Sabendo que o NO tem grandes influências na fisiopatologia dessas condições, a exploração da via NO-GMPc se apresenta como uma potencial opção terapêutica para o manejo dessas doenças. O modelo experimental utilizando L-NAME mimetiza várias condições encontradas nas desordens hipertensivas gestacionais como: aumento de pressão sistólica, redução do número de filhotes, redução do peso placentário e fetal, aumento da reabsorção fetal, aumento de marcadores do estresse oxidativo e redução da capacidade antioxidante, além de aumento de fatores anti-angiogênicos como sFlt-1, demonstrando a importância do NO na manutenção da homeostase do sistema cardiovascular durante a gestação.

Nossos resultados demonstram que a administração oral de nitrito de sódio é capaz de reestabelecer os níveis de NO após inibição da síntese endógena, sugerindo uma maior vasodilatação de artérias responsáveis pela perfusão útero-placentária, resultando em aumento de peso placentário e maior viabilidade fetal pela menor taxa de reabsorção. Foi demonstrado que o nitrito é convertido a NO em condições de hipóxia, possivelmente por uma tentativa do organismo em manter a homeostase durante baixas tensões de oxigênio (Cosby et al., 2003). Evidências anteriores mostraram que os parâmetros materno-fetais estão fortemente correlacionados com o fluxo sanguíneo uteroplacentário e que reduções nesses parâmetros podem refletir em isquemia/hipóxia induzidas por L-NAME em ratos (Kaya et al., 2011). Assim, sugerimos que a vasculatura uteroplacentária isquêmica pode ser um dos ambientes ideais para a redução química de nitrito para NO.

A formação de NO a partir do ânion nitrito tem sido objeto de dezenas de estudos. Até recentemente o ânion nitrito era considerado apenas um metabólito da degradação do NO (Lauer et al., 2001). O aumento de NO causado pelo nitrito também resultou em

redução da pressão arterial sistólica. Foi observado inicialmente que a administração concomitante de nitrito diminui a hipertensão induzida por L-NAME, além de reduzir lesão renal e proteinúria decorrentes dos agravos causados pela hipertensão (Tsuchiya et al., 2005; Tsuchiya et al., 2010). O estômago é o principal órgão que se observa a redução química de nitrito a NO, devido a acidez do pH (Ingelfinger, 2006). Apesar da acidez gástrica se mostrar como um fator importante na redução de nitrito a NO, estudos demonstram que a presença de antioxidantes na cavidade gástrica como, flavonóides e ácido ascórbico elevam a capacidade de formação de NO a partir do nitrito em meio ácido. (Benjamin et al., 1994; Lundberg et al., 1994; McColl et al., 2009; Rocha et al., 2009). A via gástrica se mostra importante devido ao fato do nitrito após ser deglutido formar também espécies nitrosiladas, que são capazes de liberar NO de uma maneira mais lenta, visto que a meia vida do nitrito embora muito variável é relativamente curta (entre 5 – 40 minutos) (Dejan et al., 2005, Dejan et al., 2007; Van Faassen et al., 2009). Ainda sobre as espécies nitrosiladas, Leclerc e colaboradores sugerem que outro mecanismo anti-hipertensivo do nitrito se dá pela nitrosilação do receptor AT1, responsável pelos efeitos vasopressores da angiotensina II (Ang II). Essa nitrosilação, resultaria em diminuição da capacidade de ligação da Ang-II ao seu receptor AT1, com consequente menor resposta a Ang-II (Leclerc et al., 2006).

Em relação ao estresse oxidativo, atualmente sabe-se que em doenças hipertensivas gestacionais há um desequilíbrio entre moléculas oxidantes e antioxidantes, com predominante aumento de pró-oxidantes e redução das substâncias com caráter antioxidante (Matsubara et al., 2015). Além disso, reduções na biodisponibilidade de NO durante quadros hipertensivos podem estar envolvidas no estresse oxidativo (Matsubara et al., 2015).

Embora em nosso trabalho não observamos redução da atividade plasmática da mieloperoxidase, outros autores já demonstraram que o nitrito de sódio exerce efeito antioxidante por inibir a atividade da NADPH oxidase (Montenegro et al., 2011) e também da xantina oxidase (Montenegro et al., 2014).

Sabe-se que elevados níveis de sFlt-1 estão relacionados a disfunção endotelial por sequestrar moléculas angiogênicas. Além disso trabalhos demonstram que a redução de NO é inversamente proporcional ao aumento de sFlt-1 (Sandrim et al. 2008; Pimentel et al., 2013; Amaral et al., 2015). Nitrito de sódio foi capaz de reduzir níveis plasmáticos de sFlt-1 e VEGF, demonstrando efeito benéfico, visto que o aumento de sFlt-1 se encontra elevado em doenças hipertensivas gestacionais por reduzir a biodisponibilidade de NO e contribuir para agravo da disfunção endotelial. O aumento de VEGF também encontrado após administração de L-NAME foi relacionado com hipertensão e hipercoagulação na circulação materna (Murakami et al., 2005; Fan et al., 2014).

Sabendo que a redução de NO é um fator importante nas desordens hipertensivas gestacionais e que o nitrito de sódio tem a capacidade de restaurar os níveis de NO melhorando vários parâmetros, inserimos nesse cenário o citrato de sildenafil, no qual exerce seu efeito principal inibindo a PDE5 e potencializando a via NO-GMPc. Entretanto, curiosamente trabalhos demonstram efeitos benéficos do sildenafil mesmo em condições que a síntese endógena de NO é inibida por L-NAME (Ramesar et al., 2010; Nassar et al., 2012; Soobryan et al., 2017). Além disso poucos trabalhos utilizando sildenafil em doenças hipertensivas gestacionais apresenta dados da atividade da PDE5 e níveis plasmáticos de GMPc, mesmo a literatura mostrando que a atividade fosfodiesterásica é maior na pré-eclâmpsia, o que poderia estar associado com a degradação do GMPc e assim reduzindo a ação do NO (Pinheiro et al., 2006).

Portanto as principais hipóteses testadas no presente estudo foram que o sildenafil atenuasse a hipertensão na gestação e evitaria a restrição de crescimento fetal e placentário independentemente dos níveis de NO na circulação materna, exercendo seus efeitos por outro mecanismo.

Em nosso trabalho nós também demonstramos que mesmo com a redução da síntese de endógena de NO com o L-NAME, o sildenafil apresentou efeitos benéficos, como redução da pressão arterial sistólica, melhora nos parâmetros maternos e fetais e aumento da viabilidade celular, apresentando ação protetora celular contra a atividade de pró-oxidantes em doenças hipertensivas. As evidências de que os efeitos do sildenafil não são totalmente dependes dos níveis circulantes de NO e nem dos níveis de GMPc ganharam força em nosso trabalho devido os resultados demonstrando que a associação entre sildenafil e nitrito (capaz de reestabelecer os níveis de NO) em ratas tratadas com L-NAME não apresentavam efeitos aditivos ou sinérgicos comparado ao tratamento isolado com sildenafil.

Outro possível mecanismo que poderia explicar os efeitos do sildenafil, se refere à capacidade antioxidante. Na pré-eclâmpsia, por exemplo, devido à falha da invasão trofoblástica, ocorre uma redução da perfusão sanguínea, o que acarreta em áreas de isquemia e hipóxia que aumentam a geração de espécies reativas de oxigênio (EROs). A EROs mais comum gerada nas células pela NADPH oxidase, mitocôndria e xantina oxidase é o ânion superóxido ($O_2^{\cdot-}$) (Sánchez-Aranguren et al., 2014). O aumento de superóxido se torna importante nas doenças cardiovasculares devido à capacidade de se complexar com o NO, formando então o peroxinitrito ($ONOO^-$), um forte agente pró-oxidante capaz de causar nitratação de proteínas, oxidação de lipídios e danos no DNA. Recentemente tem sido demonstrado que o $ONOO^-$ pode causar desacoplamento da eNOS pela capacidade de oxidar o BH_4 , um dos cofatores importantes na síntese de NO.

Esse desacoplamento faz com que a eNOS produza mais superóxido contribuindo para a disfunção endotelial (Sánchez-Aranguren et al., 2014).

Em nosso trabalho demonstramos que o sildenafil impede o aumento da atividade plasmática da MPO, sendo uma das enzimas responsáveis por grande produção de superóxido e também reduz a peroxidação lipídica, demonstrando papel na disfunção endotelial. Estudos realizados por Santos e colaboradores demonstraram que o sildenafil reduz a atividade da MPO possivelmente pela capacidade de reduzir moléculas de adesão leucocitária (Santos et al., 2005). Além disso há evidências de que o sildenafil aumente a atividade de enzimas antioxidantes como, superóxido dismutase e catalase (Perk, 2008). Sabendo que o estresse oxidativo tem papel importante nas doenças hipertensivas gestacionais, nós acreditamos que os efeitos benéficos do sildenafil nesse modelo experimental ocorrem devido ao efeito antioxidante.

5 – Conclusão

Tanto o nitrito de sódio quanto o citrato de sildenafil, apresentam efeitos anti-hipertensivos e antioxidantes. O nitrito de sódio é capaz de reestabelecer os níveis de NO e impedir o aumento de sFlt-1 e VEGF, enquanto o sildenafil melhora a viabilidade do endotélio, sugerindo que seus efeitos podem não depender dos níveis circulantes de NO na hipertensão gestacional induzida pelo L-NAME. Nós acreditamos que houve uma maior vasodilatação resultando em uma melhora na perfusão útero-placentária, no qual resultou em uma melhora nos parâmetros maternos e fetais. Embora o uso isolado de ambas as drogas tenha demonstrado efeito benéficos nos parâmetros analisados, a associação entre as drogas não demonstrou efeito aditivo. Entretanto mais estudos clínicos são necessários para demonstrar a eficácia e segurança do nitrito e sildenafil durante quadros hipertensivos na gestação.

6 – Referências

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