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MECANISMOS MOLECULARES DA FIBROSE RENAL EM RATOS SUBMETIDOS À INFLAMAÇÃO DURANTE O DESENVOLVIMENTO: POSSÍVEIS ESTRATÉGIAS TERAPÊUTICAS

Recife
2019

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Tese apresentada para cumprimento parcial das exigências para obtenção do título de Doutor em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco.

Área de concentração: Fisiologia renal

Orientadora: Profª. Drª. Ana Durce Oliveira da Paixão

Coorientador: Prof. Dr. Leucio Duarte Vieira Filho

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À minha família que desde cedo investiu na minha formação e me apoiou em todas as decisões tomadas ao longo da minha trajetória acadêmica,

Dedico

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(Bíblia N.T. Romanos, 2009)

RESUMO

A endotoxemia materna durante a gestação pode programar doença renal crônica (DRC) na prole adulta, além de torná-la mais sensível a insultos pós-natais. A fibrose renal é a principal característica da DRC e é resultante do desequilíbrio entre a produção/degradação de matriz extracelular (MEC). A terapia com antioxidantes e com células-tronco mesenquimais (MSCs) podem ser importantes na prevenção/tratamento da fibrose. Dessa forma, investigou-se os impactos da endotoxemia materna durante a gestação sobre as vias regulatórias da deposição de MEC na lesão renal na vida adulta da prole, bem como sobre a função protetora de MSCs obtidas de animais adultos. Para isso, ratos machos Wistar adultos foram obtidos de mães controles (C) ou submetidas à administração de lipopolissacarídeo (LPS - L) durante a gestação. No 1º protocolo, as mães também foram tratadas com α-tocoferol (T) durante a gestação, para obtenção dos grupos C, L, CT e LT, nos quais foi avaliado a programação de fibrose renal na prole adulta. No 2º protocolo, a prole adulta foi submetida a obstrução ureteral unilateral (OUU) e tratada ou não com apocinina (A), cujos grupos foram designados C, L, CA e LA. Nestes grupos foi avaliado o papel do tratamento pré-natal no desenvolvimento de dano renal produzido pela OUU. No 3º protocolo, foram isoladas MSCs da prole adulta que foram administradas em um grupo de ratos controles submetidos à OUU, surgindo os grupos Sham, OUU, O+MSC-C, O+MSC-L, nos quais foi avaliado a capacidade protetora das MSCs sobre a fibrose renal. A endotoxemia materna induziu, na prole adulta, proteinúria e elevação da pressão arterial sistólica, bem como aumentou a deposição de colágeno intersticial no tecido renal simultaneamente à elevação da expressão da isoforma 2 da NADPH oxidase, do TGF-β e da atividade da metaloproteinase de matriz-2 (MMP-2). Essas alterações foram prevenidas pelo tratamento materno com α-tocoferol. Além disso, a OUU elevou a deposição de colágeno no rim contralateral apenas na prole L que foi acompanhada de uma maior atividade da NADPH oxidase. Adicionalmente, a deposição de colágeno foi normalizada diante da administração de apocinina, inibidor desta enzima. As MSCs obtidas de ratos submetidos à endotoxemia materna apresentaram efeitos protetores na lesão renal induzida pela OUU de maneira semelhante as MSCs controles. Esses efeitos incluem prevenção da diminuição do clearance de creatinina, redução da deposição colágeno no cortéx renal, e diminuição da expressão proteica do TGF-β e

IL-6. No rim contralateral, as MSCs de ambas origens preveniram a elevação da NADPH oxidase, contudo apenas as MSCs controles diminuiram a expressão da MMP-2. Esses dados nos permitem concluir que a endotoxemia materna induz desenvolvimento de fibrose renal na vida adulta, aumenta danos renais produzido pela obstrução ureteral unilateral, bem como, diminui a capacidade de células-tronco mesenquimais modular metaloproteinase de matriz-2 na fibrose renal.

Palavras-chave: α-Tocoferol. Células-tronco mesenquimais. Endotoxemia materna. Estresse oxidativo. Fibrose renal. Obstrução ureteral unilateral.

ABSTRACT

Maternal endotoxemia during pregnancy may induce chronic kidney disease (CKD) in adult offspring, in addition to making it more susceptible to postnatal injury. Renal fibrosis is a hall mark of CKD and results from imbalance between the production and degradation of extracellular matrix (ECM). Antioxidant therapy and mesenchymal stem cells (MSCs) may have an important role in the prevention/treatment of fibrosis. Thus, we investigated the impact of maternal endotoxemia during pregnancy on regulatory pathways correlated to ECM deposition in renal injury of adult offspring, as well as, the protective function of MSCs obtained from adult animals. For this, adult male Wistar rats were obtained from Control (C) dams or mothers submitted to lipopolysaccharide (LPS - L) administration during gestation. In the first protocol, to evaluate renal fibrosis, dams were also treated with α-tocopherol (T) during gestation, to obtain adult offspring designated C, L, CT and LT. In a second protocol, to evaluate the role of prenatal LPS treatment on renal damage induced by unilateral ureteral obstruction (UUO), adult offspring were submitted to UUO and additionally were treated with apocynin (A), comprising the groups designated C, L, CA and LA. Finally, in a third protocol, to assess the protective ability of MSCs on renal fibrosis, MSCs were obtained from Control and LPS-exposed adult rats to be administered in rats submitted to UUO, emerging groups Sham, UUO, O+MSC-C and O+MSC-L. Maternal endotoxemia induced proteinuria and elevation of systolic blood pressure, as well as increased deposition of interstitial collagen in renal tissue of adult offspring. Simultaneously it was observed elevation of NADPH oxidase isoform 2 and TGF-β expressions, as well as elevation in Matrix metalloproteinases-2 (MMP-2) activity. These changes were prevented by maternal treatment with α-tocopherol. Additionally, it was observed that UUO increased collagen deposition in the contralateral kidney only in L group, which presented elevated NADPH oxidase activity. It was also observed that NADPH oxidase inhibition prevented collagen deposition UUO-induced. In addition, we have identified that MSCs obtained from rats submitted to intrauterine endotoxemia have protective effects on UUO-induced renal injury similarly to control CTMs. These effects included improved creatinine clearance, reduced renal cortical collagen deposition and downregulated TGF-β and IL-6 expressions. In addition, it was observed that in the contralateral kidney, MSCs from both origins were able to prevent elevation of NADPH oxidase activity, however

only the control MSCs were able to decrease MMP-2 expression. These data indicate that maternal endotoxemia induces renal fibrosis in adult life, increases renal damage due to unilateral ureteral obstruction, as well as decreases the ability of mesenchymal stem cells to modulate matrix metalloproteinase 2 in renal fibrosis.

Keywords: Maternal Endotoxemia. α-Tocopherol. Mesenchymal stem cells. Oxidative stress. Unilateral ureteral obstruction. Renal fibrosis.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

AGEs	Produtos finais da glicação avançada, do inglês: *Advanced glycation end products.*
Ang II	Angiotensina II
Bcl	Linfoma de células b, do inglês: *B-cell lymphoma*
Casp	Caspase
CAN	Nefropatia de aloenxertos crônica
CMs	Células mesangiais
CTMs	Células-tronco mesenquimais
DCV	Doenças cardiovasculares
DICs	Doenças isquêmicas do coração
DRC	Doença renal crônica
DT2	Diabetes mellitus do tipo 2
ECA	Enzima conversora de angiotensina
EGF	Fator de crescimento epidermal, do inglês: *Epidermal growth factor*
EMT	Transição epitélio-mesênquima, do inglês: *epithelial-mesenchymal transition*
FGF	Fator de crescimento de fibroblasto
Flt-1	Do inglês: *receptor fms-like tyrosine kinase-1*
GSH	Glutationa reduzida
HGF	Fator de crescimento de hepatócito, do inglês: *Hepatocyte growth factor*
HLA	Antígeno leucocitário humano, do inglês*: human leukocyte antigen*
IGF	Fator de crescimento ligado a insulina, do inglês: *Insulin-like growth fator*
IGFR	Receptor do fator de crescimento ligado a insulina, do inglês: *Insulin-like growth fator receptor*
IL	Interleucina
IMC	Índice de massa corporal

LPS	Lipopolissacarídeo
MCP	Proteína quimiotática de monócitos, do inglês: *Monocyte chemoattractant protein*
MEC	Matriz extracelular
MMP	Metaloproteinases de matriz
MPO	Mieloperoxidase
MV-CTMs	Microvesículas obtidas de CTMs
NADPH oxidase	Nicotinamida adenina dinucleotídeo fosfato oxidase
NF- kB	Fator nuclear kappa B
NO	Óxido nítrico, do inglês: *nitric oxide*
NOX	NADPH oxidase
OUU	Obstrução ureteral unilateral
PCNA	Antígeno nuclear de proliferação celular, do inglês: *Proliferating cell nuclear antigen*
PKC	Proteína kinase C, do inglês: *protein kinase C*
RFG	Ritmo de filtração glomerular
ROS	Espécies reativas de oxigênio, do inglês: *reactive oxygen species*
SNGFR	Ritmo de filtração glomerular no single-néfron
SNS	Sistema nervoso simpático
SOD	Superóxido dismutase
SRA	Sistema renina-angiotensina
TGF-β	Fator de crescimento transformante β, do inglês: *transforming growth factor β*
TIMP	Inibidor tecidual da metaloproteinase
VEGF	Fator de crescimento do endotélio vascular, do inglês: *vascular endothelium growth factor*
α-SMA	α-Actina de músculo liso, do inglês: *Alpha-smooth muscle actin*

SUMÁRIO

1 INTRODUÇÃO

Insultos ambientais que ocorrem no período gestacional podem promover alterações no desenvolvimento fetal e aumentar os riscos de desenvolvimento de doenças crônicas tardiamente (QUILTER et al., 2014; HESHMATI; KOUPIL, 2014; TAIN; HSU, 2017; RASYID; BAKRI, 2016). Essa correlação de eventos é conhecida pelo termo programação intrauterina (SIMON; LANGLEY-EVANS, 2006) e o estresse oxidativo está envolvido no mecanismo de programação (PAIXÃO; ALEXANDER, 2013; VIEIRA-FILHO et al., 2014). A inflamação durante a gestação é um dos fatores que pode perturbar a mãe e o ambiente intrauterino (HOSSEINI-CARROLL et al., 2015). Alterações funcionais renais são achados comuns nas investigações de programação intrauterina (YOU-LIN; CHIEN-NING, 2017). Alterações no desenvolvimento fetal, além de desencadearem o surgimento de doenças crônicas na vida adulta, também tornam o indivíduo mais sensível a qualquer insulto enfrentado após o nascimento (RIZZI et al., 2017).

O desenvolvimento de fibrose renal é uma característica comum na maioria dos pacientes com doença renal crônica (DRC) (LIU, 2004), e é induzida por diversas situações, inclusive pela hipertensão (GRIFFIN, 2006). A fibrose renal é caracterizada pelo acúmulo exagerado de matriz extracelular (MEC), o que, por sua vez, pode decorrer de sua maior produção ou menor degradação (VISSE E NAGASE, 2003). A obstrução ureteral unilateral (OUU) é o modelo animal mais comum para investigar a progressão da fibrose renal (CHEVALIER et al., 2009).

O estresse oxidativo além de participar do mecanismo de programação intrauterina, também pode participar da gênese das doenças tardiamente (ENTRINGER et al., 2012). A NADPH oxidase (NOX) é a principal fonte de produção de espécies reativas do oxigênio (RASTOGI et al., 2017), o aumento de sua expressão foi co-localizado em sítios de fibrose renal (ZHAO et al., 2008), bem como foi observado em modelos de programação de lesão renal (VIEIRA-FILHO et al., 2014).

Assim, antioxidantes são ferramentas importantes na prevenção e tratamento da DRC. O α-tocoferol, uma isoforma da vitamina E, reprograma alterações renais e hipertensão na prole adulta de ratos submetidos à desnutrição materna (VIEIRA-FILHO et al., 2014). Além disso, a inibição da NOX, pela apocinina, apresenta

efeitos benéficos em resposta a danos renais que acontecem na vida adulta (ABDELRAHMAN et al., 2017;CHOI et al., 2015). Outra estratégia importante para a terapia renal é a administração de células-tronco mesenquimais (CTMs), demonstrando que a sua utilização melhora a DRC através da modulação de fatores responsáveis pela fibrose (BAI et al., 2013; DA SILVA et al., 2015; SUN et al., 2013). Porém, a funcionalidade das CTMs mediante a programação intrauterina ainda não foi investigada.

1.1 JUSTIFICATIVA

Com base nessas evidências, alterações renais podem levar ao desenvolvimento de DRC, através da formação de tecido fibrótico no rim. Portanto, é fundamental a compreensão de eventos que possam programar o desenvolvimento da fibrose, bem como investigar os componentes responsáveis pela sua formação e regulação. Além disso, tendo em vista que eventos adicionais ao longo da vida podem promover doenças renais, é importante investigar se a programação intrauterina intensifica as lesões renais provocadas por esses eventos. Devido à grande possibilidade da participação do estresse oxidativo tanto na programação, quanto no estabelecimento da DRC, o uso do α-tocoferol ainda no período intrauterino pode prevenir a formação de fibrose no rim da prole adulta, assim como, o uso da apocinina em paralelo a OUU pode evidenciar o papel da NOX no desenvolvimento e progressão da DRC. A investigação de alterações nas CTMs de indivíduos submetidos à programação fetal também é necessária, tendo em vista que o uso de CTMs é uma ferramenta promissora no tratamento da DRC.

1.2 OBJETIVOS

1.2.1 Objetivo geral

Investigar os impactos da endotoxemia materna durante a gestação sobre mecanismos de lesão renal na prole, em ratos Wistar.

1.2.2 Objetivos específicos

→ Investigar se o aumento da deposição de colágeno induzido por endotoxemia materna envolve a produção de ROS, mediada pela NADPH oxidase e produção de matriz extracelular reguladas por TGF-β e MMPs.

→ Investigar se alterações na deposição de matriz extracelular induzida pela endotoxemia materna são sensíveis ao tratamento materno com o antioxidante α-tocoferol.

→ Investigar se a inibição da NADPH oxidase, por apocinina, na vida adulta é capaz de prevenir fibrose renal induzida por obstrução ureteral unilateral.

→ Investigar se a endotoxemia materna altera a capacidade protetora de células-tronco mesenquimais da medula óssea, sobre lesão renal induzida por obstrução ureteral unilateral.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 PROGRAMAÇÃO INTRAUTERINA

O ambiente intrauterino é muito sensível a insultos que acontecem durante a gestação, devido à alta taxa de replicação e diferenciação celular (RASYID; BAKRI, 2016). Condições precárias de oxigenação e nutrição fetal, alterações hormonais, tabagismo, exposição ao etanol, exposição a fármacos e enfermidades maternas são alguns insultos que prejudicam o desenvolvimento fetal (FOWDEN et al., 2006). O baixo peso ao nascimento é um importante indicador de restrição do crescimento e estado nutricional do feto. Indivíduos com baixo peso ao nascimento possuem maiores riscos de apresentar hipoglicemia, hipotermia, hipotensão, síndromes respiratórias, acidose arterial umbilical, e um aumento de 20 vezes no risco de morte neonatal (NEGRATO; GOMES, 2013). A restrição do crescimento fetal também foi correlacionada com desenvolvimento de doenças crônicas na vida adulta (FOWDEN et al., 2006).

Ainda em 1986, através de estudos demográficos, Barker demonstrou uma correlação entre o aumento da taxa de mortalidade por doenças isquêmicas do coração (DICs) no período de 1968–1978 e o aumento da morbimortalidade infantil ocorrido na geração anterior, entre os anos de 1921 e 1925, período em que houve um alto índice de desnutrição em mulheres na idade fértil (BARKER; OSMOND, 1986). Com base nas proposições de Barker, a hipótese de programação intrauterina foi definida como adaptações durante a fase crítica do crescimento e desenvolvimento para garantir a sobrevivência fetal em decorrência de insultos no ambiente intrauterino. Porém, essas adaptações promovem mudanças em longo prazo na estrutura e função tecidual aumentando os riscos de doenças crônicas (SIMON; LANGLEY-EVANS,2006), como diabetes do tipo 2 (DT2) (QUILTER et al., 2014), doenças isquêmicas do coração (DICs) (HESHMATI; KOUPIL, 2014), DRC (TAIN; HSU, 2017) e hipertensão (RASYID; BAKRI, 2016).

A epigenética é apontada como o principal mecanismo de desenvolvimento da programação intrauterina. As modificações epigenéticas envolvem mudanças na expressão gênica sem mudanças na sequência do DNA, isso acontece através da regulação da cromatina por diversas moléculas. (COSTA; PACHECO, 2013). A

cromatina é um complexo nuclear formado por DNA, histonas e RNA. Mudanças na estrutura da cromatina modificam a acessibilidade dos genes à maquinaria transicional, determinando quais genes serão transcritos durante o desenvolvimento e diferenciação celular (TORAÑO et al, 2016). Em mamíferos, os mecanismos epigenéticos mais conhecidos são a metilação do DNA e a modificação de histonas, como mostra a Figura 1. A metilação do DNA consiste na adição de grupamentos metila a base citosina, promovendo o silenciamento de genes. Já a modificação de histonas consiste em modificações reversíveis na ramificação N-terminal das histonas, tais como acetilação, metilação, fosforilação e ubiquitinação, estabelecendo um mecanismo de afinidade proteína-DNA, regulando assim, a expressão gênica. Anormalidades nesses processos epigenéticos resultam na desregulação de importantes vias de sinalização e função celular, levando ao desenvolvimento de doenças (TORAÑO et al, 2016). Já foi demonstrado experimentalmente que situações associadas à restrição do crescimento intrauterino, como pré-eclampsia e insuficiência placentária, induzem alterações na metilação do DNA (REID et al., 2010). Recentemente, Wang e colaboradores demonstraram que a endotoxemia materna aumenta a expressão de IL-6 e TNF-α no cótex renal da prole em paralelo ao aumento da expressão da DNA metiltransferase, esse efeito combinatório pode silenciar ou alterar a expressão de genes-chaves ligados ao desenvolvimento da hipertensão na prole adulta, no entanto, esses genes ainda não foram identificados (WANG et al 2017).

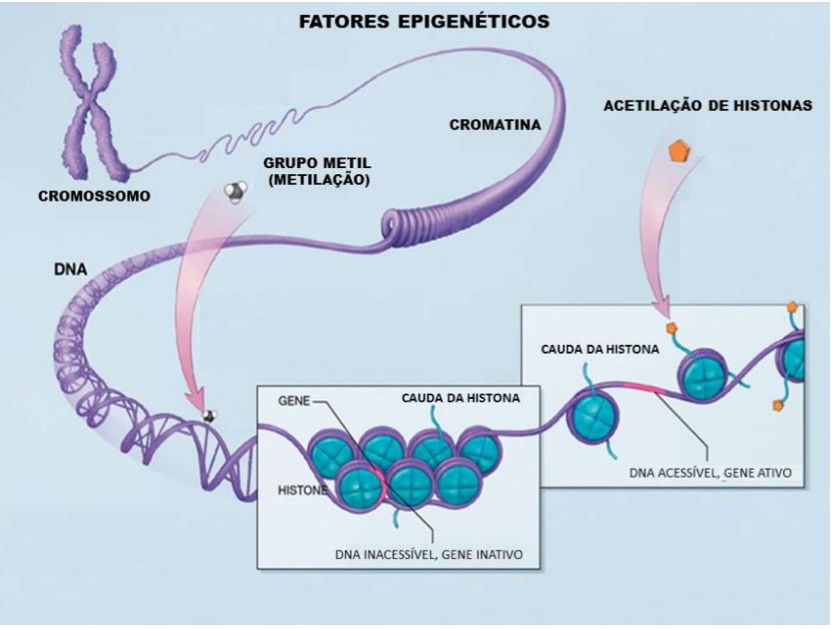

Figura 1. Modificações epigenéticas na programação intrauterina. Metilação do DNA e acetilação das histonas (VALDES, 2017)

.

2.2 INFLAMAÇÃO E A GESTAÇÃO

A inflamação consiste em uma resposta protetora do nosso organismo que é iniciada por uma lesão celular e tem como objetivo final livrar o organismo da causa e consequências desta lesão (ABBAS, 2010). O processo inflamatório é ativado pelas células imunes residentes gerando uma cascata de eventos que envolve a liberação de citocinas pró-inflamatórias, como o fator de necrose tumoral alfa (TNF-α) e anti-inflamatórias, como a interleucina 10 (IL-10) (HENKIN et al., 2009). Porém, a resposta inflamatória pode ser prejudicial em algumas situações como em reações de hipersensibilidade secundária a efeitos de picaduras de insetos, fármacos e outras substâncias tóxicas (ABBAS, 2010).

Durante a gestação, a inflamação traz complicações para a mãe e para o feto (HOSSEINI-CARROLL et al., 2015). O estado gestacional apresenta uma rede imunorregulatória complexa que participa do desenvolvimento e manutenção do feto, além de manter a tolerância materno-fetal. As citocinas possuem um papel importante nesse processo, participando da implantação, placentação, ativação uterina e amadurecimento cervical (VIANNA, 2009). A endotoxemia materna pode interferir nessa rede imunorregulatória e alterar a dinâmica entre essas citocinas, induzindo uma maior produção de citocinas pró-inflamatórias maternas, o que comprometeria o desenvolvimento da gestação (ORSI; TRIBE, 2008). Além disso, apesar do patógeno em si não ultrapassar a barreira uteroplacentária, os leucócitos maternos ativados em resposta a esse patógeno, principalmente as células T, são transferidos para o feto (WEGORZEWSKA et al., 2014), comprometendo ainda mais o desenvolvimento fetal.

Os prejuízos da endotoxemia materna acontecem antes mesmo da concepção, diminuindo a taxa de fertilidade da mulher. Após o estabelecimento da gestação, os prejuízos da inflamação para a mãe incluem desnutrição, tromboembolismo e hemorragias pré-parto. Além disso, os riscos do parto cesário são aumentados. Os prejuízos da endotoxemia materna para o feto incluem abortos, baixo peso ao nascimento e parto prematuro (HOSSEINI-CARROLL et al., 2015).

Experimentalmente, o componente molecular mais utilizado para desencadear a resposta inflamatória e imune é o lipopolissacarídeo (LPS). O LPS é um componente importante da membrana externa da maioria das bactérias gram-negativas. Já foi visto que pequenas quantidades de LPS no sangue, proveniente de infecções bacterianas, são suficientes para induzir uma potente resposta inflamatória (RHEE, 2014). Dependendo da dose e do tipo de bactéria, a administração do LPS em animais gestantes pode promover reabsorção embrionária (AISEMBERG *et al*., 2007), partos prematuros (OLGUN et al., 2014), prejuízos ao desenvolvimento cerebral do feto (OSKVIG et al., 2012), doenças fetais intrauterinas (XU *et al*., 2007), bem como, induzir efeitos teratogênicos (ZHAO et al., 2008). Prejuízos tardios da prole também foram observados após a administração materna do LPS. Comprometimentos na memória espacial e nas habilidades de aprendizagem (THANGARAJAN et al., 2015), disfunção ventricular esquerda (VELTEN et al., 2011) e hipertensão (WEI et al., 2007) na prole adulta foram associados a inflamação intrauterina induzida pelo LPS.

Estudos já demonstraram que doenças fetais e partos prematuros provocados pela endotoxemia materna estavam correlacionados com o aumento do estresse oxidativo, tanto placentário, como fetal (XU et al. 2007; ZHAO et al., 2008), deixando claro o envolvimento do estresse oxidativo nos prejuízos fetais induzidos pela endotoxemia materna.

2.3 ESTRESSE OXIDATIVO

O estresse oxidativo é caracterizado por um desequilíbrio entre a produção de moléculas oxidantes e a defesa antioxidante (VASCONCELOS et al., 2014). As substâncias oxidantes possuem átomos de oxigênio com elétrons desemparelhados, conhecidos como espécies reativas de oxigênio (em inglês: *reactive oxygen species*, ROS) e incluem os ânions superóxidos (O_2^-), radical hidroxila (OH), péroxido de hidrogênio (H_2O_2), radicais hidroperoxila (O_2H) e o oxigênio singleto ($^1O_2$) (ALZOGHAIBI, 2013). As moléculas antioxidantes são as responsáveis pela doação de elétrons com objetivo de neutralizar a ação de ROS, como mostra a Figura 2 (VASCONCELOS et al., 2014). Assim, podemos afirmar que o estresse oxidativo pode acontecer de 3 formas: i) quando há um aumento da produção de ROS, sem

alteração nas defesas antioxidantes; ii) quando há uma diminuição nas defesas antioxidantes, sem alteração na produção de ROS; ou iii) quando acontece simultaneamente um aumento da produção de ROS e uma diminuição nas defesas antioxidantes.

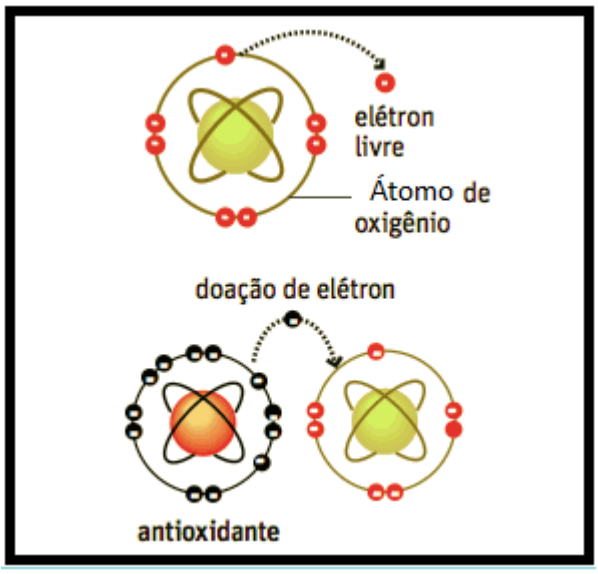

Figura 2. Mecanismo de ação das defesas antioxidantes (Adaptado de VASCONCELOS et al., 2014).

Em condições de equilíbrio, a produção de ROS tem papel importante na proteção celular de infecções por patógenos invasores, na regulação da função de células cardíacas e vasculares, e também na regulação intracelular da concentração de cálcio (SALIM, 2014). No entanto, o aumento de ROS, pode causar danos celulares através da sua ação em ácidos graxos insaturados presentes na membrana, processo conhecido como peroxidação lipídica, e também por danificar a estrutura e função de proteínas e de ácidos nucléicos (ALZOGHAIBI, 2013), sendo de grande importância a ação antioxidante para a prevenção desses danos.

As moléculas antioxidantes são divididas usualmente em enzimáticas e não enzimáticas. As enzimáticas são produzidas pelo nosso organismo e incluem a superóxido dismutase (SOD), a catalase e a glutationa peroxidase. Nesse mecanismo, radicais superóxidos (O_2^-) são convertidos a peróxido de hidrogênio pela superóxido dismutase, cujo metabolismo é continuado pela glutationa peroxidase ou pela catalase (BIRI et al., 2007). As não enzimáticas podem ou não serem produzidas pelo organismo e incluem glutationa reduzida (GSH), vitaminas,

flavanoides, licopeno e bilirrubina. O sistema de defesa antioxidante pode atuar de duas maneiras, a principal é neutralizando o agente oxidante antes que ele cause a lesão, mas também pode atuar como reparador da lesão ocorrida pelo agente oxidante (VASCONCELOS et al., 2014). Enquanto isso, a produção de ROS pode ser resultante da atividade de diversas enzimas que participam do metabolismo celular e mitocondrial (BOUEIZ et al., 2008; LOOR et al.,2011).

2.3.1 Estresse oxidativo e a gestação

No fim do primeiro trimestre de gestação, a concentração de oxigênio placentário e fetal triplica, devido a maturação placentária, provocando um aumento exponencial na concentração de ROS. A elevação fisiológica do ROS fetal e placentária contribui para o desenvolvimento normal do feto. Após o primeiro trimestre de gestação, ROS induz a regressão das vilosidades que se formaram sobre toda a superfície do saco coriônico para deixar a placenta discoide e está envolvido no processo de desintoxicação placentária, protegendo o embrião da teratogênese (TORRES-CUEVASA, et al., 2017). ROS também tem papel fisiológico de atuar como molécula de sinalização para induzir a transcrição de genes responsáveis pela diferenciação e proliferação de vários tipos de células durante o desenvolvimento fetal (BURTON, 2009). Apesar disso, o feto apresenta uma capacidade antioxidante muito baixa (THOMPSON; AL-HASAN, 2012), e sob condições de estresse no período gestacional, o excesso de ROS é um fator determinante para o surgimento de complicações fetais. A elevação do estresse oxidativo durante a gestação também pode programar doenças na vida adulta. Já foi demonstrado a correlação entre a elevação do estresse oxidativo, baixo peso ao nascimento e o desenvolvimento de hipertensão (PAIXÃO; ALEXANDER, 2013) e diabetes (SIMMONS, 2006) na vida adulta.

O prejuízo do desenvolvimento fetal induzido pelo estresse oxidativo envolve o comprometimento da circulação sanguínea por diversos fatores: i) ROS inativa biomacromoléculas essenciais para o metabolismo celular, induzindo aumento da taxa de apoptose de células trofoblásticas e disfunção endotelial (S´ANCHEZ-ARANGUREN et al., 2014); ii) ROS aumenta a expressão de fatores anti-angiogênicos como Flt-1 solúvel (do inglês, Fms-like tyrosine kinase) e endoglina

solúvel, moléculas que são responsáveis por se ligar e neutralizar fatores angiogênicos como VEGF (do inglês, vascular endothelial growth fator) e TGF-β1 (do inglês, transforming growth factor-β1), respectivamente (WU et al., 2015); iii) ROS promove vasoconstrição placentária por provocar o desequilíbrio entre tromboxano e prostaciclina, estimulando a síntese de tromboxano, que é um vasoconstritor, e em paralelo, inibindo a síntese de prostaciclina, que é um vasorrelaxante (WALSH, 2004), e, adicionalmente, iv) ROS também diminui a biodisponibilidade de óxido nítrico (NO) na circulação placentária (YU et al., 2012), amplificando o quadro de vasoconstrição.

No feto, o estresse oxidativo pode promover modificações epigenéticas responsáveis pela programação de doenças na vida adulta. A acetilação de resíduos de histona, induzida por ROS, foi correlacionada com o aumento da expressão do fator de transcrição NF- kB (fator nuclear kappa B), em células endoteliais aórtica, ativando assim, vias inflamatórias responsáveis pela programação de doenças relacionadas com a síndrome metabólica na vida adulta (EL-OSTA et al., 2008). Também foi demonstrado que a elevação do estresse oxidativo, decorrente da exposição pré-natal ao LPS, promove modificações epigenéticas relacionadas ao “upregulation” da ECA 1 (enzima conversora de angiotensina 1) no córtex renal da prole (WANG et al., 2016), aumentando os riscos de desenvolvimento de hipertensão e doença renal crônica.

A diminuição da perfusão placentária induzida pelo aumento do estresse oxidativo e retardo do crescimento fetal (WU et al., 2015) está associada, pelo menos parcialmente à ativação da NOX2 (XIAO et al., 2013). Em nosso laboratório, já foi demonstrado que a inibição da NADPH oxidase, através da administração de apocinina, está envolvida com a prevenção do retardo do crescimento intrauterino induzido pela endotoxemia materna (COSTANTINO, 2018). Além disso, complicações intrauterinas relacionadas ao estresse oxidativo estão associadas a ativação da via produtora de superóxido mediada pela NADPH oxidase na vida adulta (VIEIRA-FILHO et al., 2014; VIEIRA et al., 2018).

2.3.2 NADPH oxidase

A nicotinamida adenina dinucleotídeo fosfato (NADPH) oxidase (NOX) tem como única função a produção de ROS. A NOX é uma família de complexos enzimáticos presente em todo o organismo. Classicamente a NOX, quando ativada, atua em células fagocíticas gerando ânions superóxidos (O_2^-) a partir de moléculas de oxigênio com objetivo de destruir o patógeno fagocitado, atuando assim, como um anti-microbiano. Em outros tipos celulares a NOX participa do processo de biossinalização e regulação celular. Contudo, a superativação da NOX também está envolvida em um grande número de patologias (RASTOGI et al., 2017).

O complexo enzimático da NADPH oxidase inclui subunidades catalíticas e eletrotransportadoras. Sua família é constituída por 7 diferentes membros que incluem as NOXs 1–5, e a dual oxidases 1–2, que são expressas em tecidos diferentes e também reguladas de diversas formas (COSO et al., 2012). A maior parte do conhecimento sobre a NOX é baseado em estudos sobre o complexo enzimático da NOX2, subunidade catalítica produtora de ROS também conhecida como $gp91^{phox}$. Em humanos, a NOX2 é altamente glicosilada possuindo um peso molecular entre 70 e 90 KDa, contendo 6 domínios transmembranares, e suas regiões aminoterminal e carboxiterminal estão inseridas no citoplasma (BEDARD; KRAUSE, 2007).

A ativação da NOX2 é iniciada pela fosforilação da subunidade $p47^{phox}$ que está acoplada as subunidades $p67^{phox}$ e $p40^{phox}$no citoplasma. Após a ativação da $p47^{phox}$, o complexo citoplasmático ($p47^{phox}$, $p67^{phox}$ e $p40^{phox}$) é deslocado para a membrana e interage com a subunidade membranar $p22^{phox}$. Em paralelo a fosforilação da $p47^{phox}$, ocorre a ativação da GTPase, a RAC1 também é deslocada para a membrana e se acopla ao complexo. Uma vez que o complexo é formado, a $p67^{phox}$ ativa o domínio catalítico da NOX2 ($gp91^{phox}$), gerando superóxidos através da transferência de elétrons para o citosol, e a RAC1 fornece a energia necessária para a produção desses superóxidos (BEDARD; KRAUSE, 2007), como mostra a Figura 3.

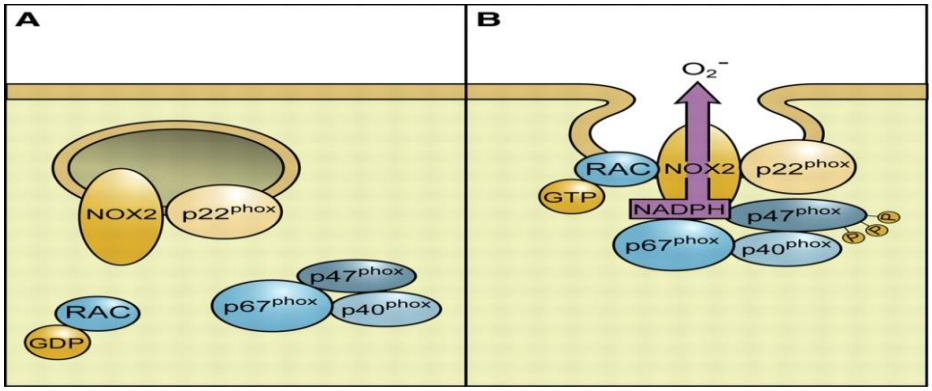

Figura 3. Esquema representativo da ativação da NOX2. A. NOX2 inativa: complexo membranar, formado pela NOX2 e $p22^{phox}$ desacoplado ao complexo citoplasmático, formado pela $p47^{phox}$, $p67^{phox}$ e $p40^{phox}$. **B.** Formação do complexo e ativação da NOX2: iniciado pela fosforilação da $p47^{phox}$ que traz para o complexo as subunidades citoplasmáticas e ativa a NOX2 através da $p67^{phox}$. (BEDARD; KRAUSE, 2007).

A ativação e/ou “upregulation” da NOX2 foi associada a diversos processos patológicos, tais como hiperglicemia (MOHAMMEDE; KOWLURU, 2013), câncer (MARULLO et al., 2015) e disfunção vascular (TROIANO et al., 2016). No sistema nervoso central, sua ativação participa da fisiopatologia de doenças neurodegenerativas, como as deficiências cognitivas relacionadas ao envelhecimento, doença de Alzheimer e doença de Parkinson (CAHILL-SMITH; LI, 2014). A síndrome cardiorenal pode ser iniciada pelo aumento do estresse oxidativo proveniente da ativação da NOX2 no rim e no coração (RUBATTU et al., 2013). O processo inflamatório induzido por LPS é capaz de ativar a NOX2 e outras NOXs, contribuindo para o desenvolvimento de doenças associadas a inflamação (DOLUNAY et al., 2017).

2.4 DOENÇA RENAL CRÔNICA

A DRC é conceituada como uma desordem heterogênea que afeta a estrutura e função do rim. Os riscos de desenvolvimento da DRC incluem fatores de susceptibilidade sócio-demográficos e genéticos, assim como a exposição a fatores indutores da doença. Os estágios iniciais da DRC são geralmente assintomáticos. A constatação de falhas na função renal pode ocorrer após alguns meses, porém na maioria dos pacientes a progressão da doença leva décadas e, em alguns casos, a doença não progride (LEVEY; CORESH, 2012).

A DRC é detectada pela redução do ritmo de filtração glomerular (RFG) (<60 mL/min/1.73m² por >3 meses) ou avaliação de marcadores de danos renais, tais como, a taxa de excreção da albumina (>30 mg/dia), anormalidades no sedimento urinário, alterações eletrolíticas relacionada a distúrbios tubulares ou anormalidades estruturais detectadas por histologia ou imagiologia (LEVEY et al., 2015). O modelo conceitual para o desenvolvimento, progressão e complicações da DRC é ilustrado na Figura 4.

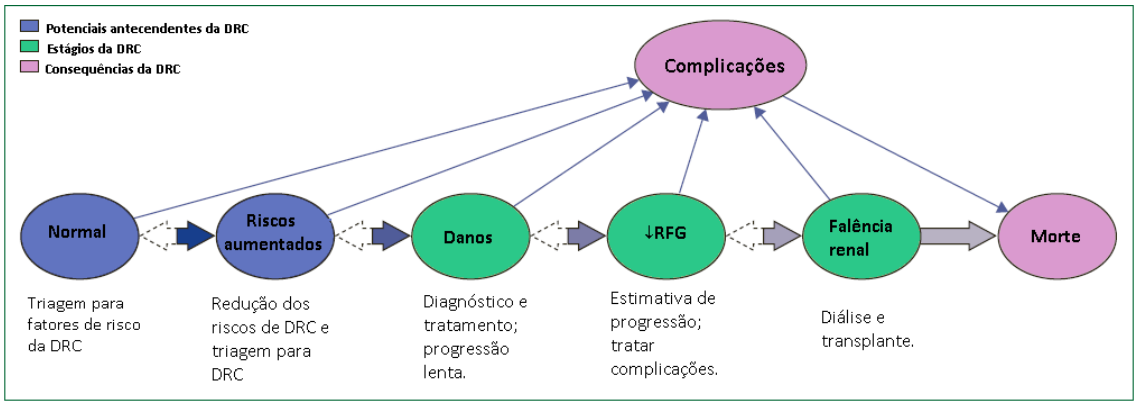

Figura 4. Modelo conceitual da doença renal crônica. Desenvolvimento, progressão e complicações da DRC. Setas grossas entre os círculos representam desenvolvimento e progressão. Setas tracejadas indicam remissão menos frequente do que progressão. Setas ligadas a complicações estão relacionadas a complicações no RFG e doenças cardiovasculares (Adaptado de LEVEY; CORESH, 2012).

Os principais fatores de riscos para a DRC são obesidade, diabetes *mellitus* e hipertensão. A obesidade tem um mecanismo multifatorial e é considerado como um fator independente na DRC. Estudos relacionados à obesidade revelaram que o índice de massa corporal (IMC) tem relação diretamente proporcional com o aumento da DRC. Os impactos diretos da obesidade no rim incluem hiperfiltração, elevação da pressão glomerular e injúria em podócitos (FELIZARDO et al., 2014). A diabetes mellitus induz disfunção renal em cerca de 20 a 30% dos pacientes. A hiperglicemia provoca perturbações no crescimento celular e induz um aumento na produção de matriz extracelular. A hiperglicemia também pode induzir a formação de produtos finais da glicação avançada (AGEs) que levam a lesão celular, e assim a complicações renais e microvasculares (SHAHBAZIAN; REZAII**,** 2013)**.** Por outro lado, a hipertensão arterial é tanto causa quanto consequência da DRC. O aumento da pressão arterial pode ocasionar lesão renal de natureza microvascular conhecida como nefrosclerose hipertensiva **(**BORTOLOTTO, 2008)**.** Outros fatores de riscos da

DRC incluem doenças de origem infecciosas (HAN et al, 2006), desordens relacionadas direta ou indiretamente com volume corpóreo (TIESSEN et al 1997), assim como, desordens que ocorrem ainda no período de nefrogênese (KAZANCIOĞLU et al., 2013) e obstruções nas vias ureterais (CHEVALIER et al., 2009). A DRC promove um desequilíbrio na homeostase corporal e pode levar até a morte. Algumas consequências da DRC são: alterações no metabolismo mineral, como hipocalcemia e hipofosfatemia que levam a deficiências na produção de vitamina D; acidose metabólica e anemia (BASTOS et al., 2010).

A DRC também está relacionada com o risco aumentado para outras doenças. A injúria renal aguda (IRA) tem como um dos principais fatores de riscos a DRC (HSU et al, 2008). Por sua vez, o uso intenso de medicamentos utilizados na DRC, como anti-inflamatório não-esteroidal, antibióticos, inibidores da enzima conversora de angiotensina são umas das principais causas para a ocorrência da IRA (JHA; PARAMESWARAN, 2013). As doenças cardiovasculares (DCV) também podem estar associadas ao declínio da função renal. A DRC é um fator de risco independente para várias DCV como, hipertrofia ventricular esquerda e doenças na artéria coronária. Fatores já bem estabelecidos na DRC como, inflamação, estresse oxidativo e hipertensão são os responsáveis pelo aumento dos riscos de DCV (ALANI et al., 2014). Além disso, esses fatores também estão envolvidos na formação do tecido fibrótico renal (YIU et al.,2014; ZHAO et al., 2008), sugerindo que a fibrose renal, pode estar envolvida no aumento dos riscos de DCV.

2.4.1 Fibrose renal

A fibrose é o resultado patológico de reações inflamatórias crônicas, caracterizada por intensa deposição de colágeno. Embora a deposição de colágeno seja essencial para a reparação tecidual, se a lesão for intensa ou repetitiva, o próprio processo de cicatrização pode ser desregulado e evoluir para uma resposta fibrótica progressivamente irreversível. O acúmulo de matriz extracelular (MEC) em torno do tecido lesionado causa mau funcionamento ou até perda de função de determinados órgãos, como acontece, por exemplo, nas doenças de fígado em fase terminal, fibrose pulmonar idiopática, insuficiência cardíaca e doença renal (WYNN; RAMALINGAM, 2013). Independente dos achados histológicos originais, a maioria

dos pacientes com doença renal crônica apresentam o aumento da MEC como principal fator responsável pela insuficiência renal. Quando esse processo ocorre no glomérulo, ele é chamado de glomerulosclerose e quando ocorre no espaço túbulo-intersticial são caracterizados como fibrose túbulo-intersticial (LIU, 2004).

Em animais, o modelo mais comum de DRC para investigar a progressão da fibrose renal é o modelo de OUU. Nesse modelo, o ureter esquerdo do animal é obstruído e as consequências nas primeiras 24hs são hidronefrose, infiltração de células inflamatórias e apoptose, caracterizando uma lesão renal aguda. No entanto, a persistência da hidronefrose severa, em torno de 7 a 14 dias, causa uma injúria renal irreversível, caracterizada pela progressão da fibrose renal (CHEVALIER et al., 2009).

No rim, as células predominantes na síntese fisiológica do colágeno são as células mesangiais no glomérulo e os fibroblastos no interstício. Esses dois tipos de células respondem similarmente a estímulos pró-fibróticos durante a doença renal, se diferenciando em miofibroblastos que produzem fibronectina, lamininas e colágeno tipo IV ativamente, além de colágeno tipo I e III (BARNES; GORIN, 2011). Além disso, podócitos e células epiteliais tubulares podem sofrer transição epitélio-mesenquimal (epitelial-mesenchymal transition, EMT), onde as células perdem marcadores de diferenciação epitelial como a E-caderina e citoqueratina, e adquirem proteínas específicas de células produtoras de MEC, como a α-actina de músculo liso (α-SMA) (BARNES; GORIN, 2011). A ativação de fibroblastos, células mesangiais e a EMT é regulada por vários fatores de crescimento, citocinas, hormônios e outras moléculas, porém a mais importante delas é o fator de crescimento transformante-β (TGF-β) (GOUMENOS et al., 2002). O TGF-β é um fator de crescimento que atua de forma autócrina e parácrina ligando-se a receptores de membrana específicos, e é regulado em condições normais por um mecanismo de “feedback”. Em condições patológicas, a expressão do TGF-β está correlacionada com uma acentuada produção de MEC (GOUMENOS et al., 2002). Outros fatores como, o fator de crescimento epidermal (EGF) e o fator de crescimento de fibroblastos 2 (FGF-2) também podem desencadear a deposição de MEC, porém em menor proporção, mas também podendo atuar sinergicamente a ação do TGF-β. Já foi demonstrado que a baixa ingestão protéica materna durante a gestação programa uma maior EMT na prole de ratos adultos via aumento da

expressão do TGF-β (SENE et al., 2013). Já foi visto também que a inibição farmacológica do TGF-β em camundongos submetidos à OUU diminui significativamente a expressão de α-SMA, colágeno e fibronectina, prevenindo assim, a fibrose renal (HU et al., 2016).

Além de alterações na síntese de MEC, a fibrose renal também pode originar de alterações no sistema de degradação da MEC. As enzimas mais importantes no remodelamento da MEC são endopeptidases dependentes de zinco conhecidas como metaloproteinases de matriz (MMPs). Essas enzimas são primeiramente expressas como pró-forma latente, chamadas zimogênios ou pró-MMPs. Para que sejam ativadas, precisam que seus domínios pró-peptídeos sejam clivados por outras proteases, como a plasmina ou a MT-MMPs (NAGASE; WOESSNE, 1999). A família das MMPs abrange cerca de 23 membros, porém, no rim, a degradação da MEC é feita sobretudo pelas MMP-2 e 9, conhecidas como gelatinases. A MMP-2 é produzida por fibroblastos, células epiteliais e células mesangiais (CMs), enquanto que a MMP-9 é produzida pelas células epiteliais glomerulares e células mesangiais (KEELING; HERRERA, 2008).

As MMPs desempenham um papel importante em muitos processos biológicos, tais como embriogênese, remodelamento tecidual normal, cicatrização de feridas, angiogênese entre outros. Porém, a perda do controle de sua atividade pode resultar em doenças tais como artrite, esclerose múltipla, ulceração de tecidos, cancro, inflamação, nefrite e fibrose (KEELING; HERRERA, 2008). A regulação da atividade das MMPs envolve alterações na expressão gênica, ativação de suas pró-formas latentes e regulação da expressão proteica dos seus inibidores teciduais, os TIMPS (tissular inhibitor of matrix metalloproteinases). O TGF-β pode influenciar a atividade e expressão das MMPs e dos TIMPs (ZHAO et al., 2008). Estudos mostram que o espessamento da membrana basal glomerular e da matriz mesangial estão relacionados com o aumento do TGF-β associado ao aumento dos níveis de MMP-2, sugerindo que o TGF-β influencia a produção das MMPs pelas células mesangiais (KEELING; HERRERA, 2008).

Portanto, alterações na modulação da degradação/produção da MEC são responsáveis pela formação do tecido fibrótico no rim, o TGF-β apresenta um papel central na regulação desse processo, porém essa modulação sofre influência de diversos fatores tais como citocinas, angiotensina II, aldosterona, estresse de

cisalhamento e estresse oxidativo (VISSE E NAGASE, 2003). O estresse oxidativo estimula a produção de fatores pró-fibróticos, incluindo o TGF-β, o que favorece o desenvolvimento de fibrose renal (ZHAO et al., 2008).

2.4.2 DRC e estresse oxidativo

A elevação do estresse oxidativo está relacionado tanto com a causa, quanto com a consequência da DRC. Karamouzis e colaboradores observaram que pacientes com DRC possuíam níveis elevados de estresse oxidativo em comparação a indivíduos saudáveis, além disso, o estresse oxidativo aumentava progressivamente com o avanço da DRC, sendo os pacientes com DRC terminal os que apresentaram os maiores níveis de estresse oxidativo (KARAMOUZIS et al., 2008). Considerando que a fibrose renal é a principal característica da doença renal crônica (LIU, 2004), ROS, por ser uma molécula pró-fibrótica, tem papel importante no desenvolvimento e progressão dessa doença.

Os fatores indutores da fibrose (infecções, lesões, produtos químicos tóxicos e radiação) além de ativarem miofibroblastos e células inflamatórias também induzem o aumento da produção de ROS. A elevação de ROS está interligada com a produção e ativação de vários fatores de crescimento, citocinas e quimiocinas através de um mecanismo de feedback. Em condições normais (resposta não fibrótica), a ativação transitória da inflamação por ROS é seguida por uma regeneração tecidual. Contudo, níveis elevados de ROS contribuem direta e indiretamente (via aumento da inflamação) para o desenvolvimento da fibrose. Além disso, a própria fibrose induz uma maior produção de quimiocinas, fatores de crescimento e ROS, contribuindo para a progressão da fibrose (RICHTER et al., 2015), como mostra a Figura 5.

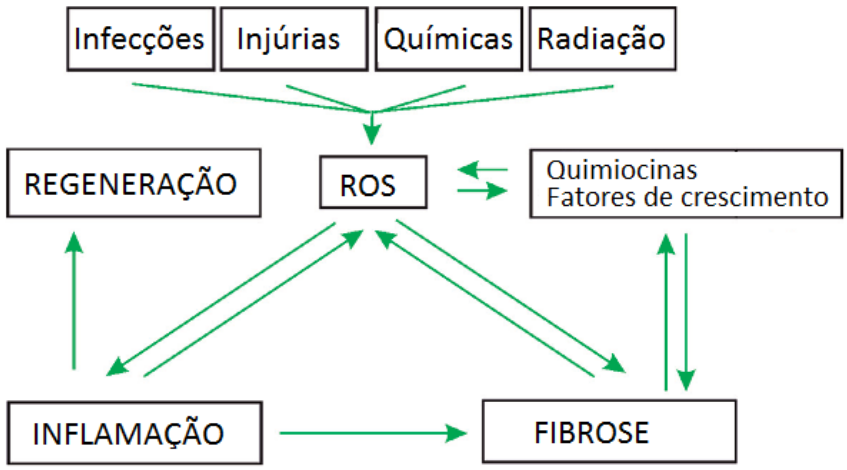

Figura 5. Mecanismo de feedback induzido pelo aumento da produção de ROS para o desenvolvimento e progressão da fibrose (Adaptado de RICHTER et al., 2015).

No rim, o excesso de ROS promove um “upregulation” de Ang II e TGF-β, medeia a ativação de inflamassomas, além de diminuir a biodisponibilidade de óxido nítrico (NO) (WAN et al., 2016). Por sua vez, a elevação de Ang II além de promover o feedback positivo estimulando a produção de ROS pela NOX2, também promove a migração e proliferação de fibroblastos, estimula a produção de interleucinas, colágeno do tipo 1, fibronectina, α-SMA (WANG et al., 2017), e TGF-β (SHEN et al., 2016). Portanto, a associação ROS/Ang II promove eventos como: i) diminuição no fluxo sanguíneo renal, pelo aumento da Ang II e diminuição da biodisponibilidade do NO; 2) inflamação tecidual, pela ativação de inflamassomas e iii) aumento da síntese de MEC, pelo aumento da expressão do TGF-β.

O estresse oxidativo também é capaz de interferir nas MMPs. ROS pode modular a atividade das MMPs-2 e -9 através de reações com grupos tiol que sevem como mecanismo comum de ativação das MMPs (RAJAGOPALAN et al., 1996). Além disso, Ben Yosef e colaboradores mostraram a influência do estresse oxidativo em mecanismos transcricionais sobre a expressão do RNA da MMP-2 e do RNA da MT1-MMP, enzima que ativa a MMP-2 (BEN YOSEF et al, 2002). Karanovc e colaboradores demonstraram em um modelo de ratos hipertensos que a elevação do estresse oxidativo promoveu aumento da expressão da MMP, inflamação, e lesão renal (KARANOVC et al., 2016), mostrando assim, que a hipertensão pode induzir a DRC através do estresse oxidativo.

2.5 DRC, HIPERTENSÃO E PROGRAMAÇÃO INTRAUTERINA

A hipertensão é uma doença crônica grave, sendo a principal causa de morbimortalidade em todo o mundo (CHOBANIAN, 2011). Sua natureza é complexa e multifatorial, envolvendo fatores genéticos, fisiológicos e ambientais. Diversas alterações funcionais contribuem para a elevação da pressão arterial incluindo, a elevação da resistência vascular e do débito cardíaco (CHAN et al., 2016), diminuição da produção e/ou responsividade de vasodilatadores (GILES et al., 2012), inflamação e reações imunológicas (RODRIGUEZ-ITURBE et al., 2017), ativação do sistema nervoso simpático (SNS) (VOGIATZAKIS et al., 2017), ativação do sistema renina-angiotensina (SRA) (WANG et al., 2017), elevação do estresse oxidativo (SIMPLICIO et al., 2017) e disfunção renal (JOHNSON et al., 2015).

Diversos fatores de origem renal podem contribuir para o desenvolvimento da hipertensão, tais como:. i) mecanismos genéticos, principalmente relacionados a modificações nos transportadores de sódio; ii) alterações na hemodinâmica renal, como a vasoconstrição renal, principalmente a pré-glomerular e a ativação do SRA, que tem um papel importante na vasoconstrição renal e na retenção de água e sódio; e iii) redução do número de néfrons, proveniente de um mal desenvolvimento fetal (JOHNSON et al., 2015).

Em humanos, a nefrogênese inicia por volta da 9ª semana, porém, 60% dos néfrons são formados no terceiro trimestre de gestação, em torno da 36ª semana os néfrons estão completamente formados e permanecem durante toda a vida (CARMODY; CHARLTON, 2013). Já nos roedores, a nefrogênese continua a ocorrer após o nascimento, até aproximadamente 2 semanas de vida (LASAITIENE et al.,2006). Portanto, insultos maternos que ocorrem principalmente no terceiro trimestre de gestação provocam alterações no desenvolvimento renal e diminuem a quantidade de néfrons. Inicialmente, a redução do número de néfrons não prejudica o ritmo de filtração glomerular (RFG). O déficit de néfrons, aumenta a pressão no capilar glomerular que leva a expansão da área de superfície glomerular dos néfrons (hipertrofia glomerular) e promove hiperfiltração glomerular (LUYCKX; BRENNER, 2005). Contudo, essa resposta adaptativa torna-se prejudicial com o tempo, tendo em vista que a hiperfiltração glomerular perturba o mecanismo de autorregulação renal, gera hipertensão intraglomerular, ativa processos inflamatórios e fibróticos,

induz lesão da barreira de filtração glomerular e proteinúria (HELAL et al., 2012). Adicionalmente, o aumento da pressão intraglomerular induz tardiamente uma maior perda de néfrons, promovendo um auto-estímulo, que por fim, acarreta em queda da filtração glomerular total (YOU-LIN; CHIEN-NING, 2017). Todos esses eventos além de tornarem o rim mais susceptível a influências ambientais e mais vulnerável a DRC, também elevam a pressão arterial, por aumentar a reabsorção de sódio (VIEIRA-FILHO et al., 2014).

No entanto, a redução do número de néfrons não é o único fator responsável pela programação intrauterina da DRC. A Figura 6 mostra que insultos maternos durante a gestação programam DRC através de fatores como: i) SRA, que na fase de desenvolvimento fetal está diminuída e no adulto sofre um efeito compensatório exacerbado, promovendo um "upregulation"; ii) transportadores de sódio, que tem sua expressão aumentada; iii) SNS renal, que está ativado e interferindo nas arteríolas do rim; iv) glicocorticóides, que participam da organogênese renal e estão diminuídos; v) Diferenças de sexo, onde machos são mais vulneráveis do que fêmeas; e vi) estresse oxidativo, como discutido anteriormente (YOU-LIN; CHIEN-NING, 2017).

Portanto, alterações renais programadas ainda no ambiente intrauterino deixam indivíduos mais vulneráveis ao desenvolvimento da DRC e hipertensão na vida adulta. Tendo em vista o papel fundamental do estresse oxidativo na programação de doenças cardiorrenais, estratégias terapêuticas antioxidantes podem ser ferramentas importantes na redução desses danos ou até mesmo na reprogramação fetal.

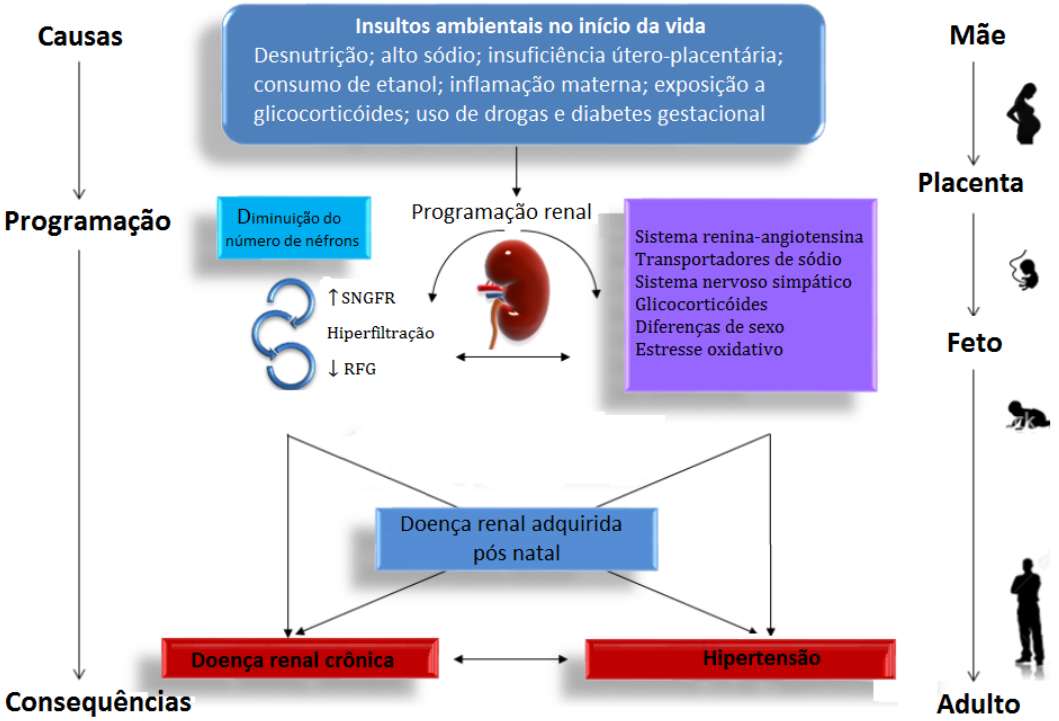

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Figura 6. Esquema representativo da programação da doença renal crônica (DRC) e hipertensão induzido por insultos no ambiente intrauterino. A DRC pode ser atribuída a diminuição do número de néfrons e a outros fatores que são alterados durante o desenvolvimento fetal (ex; estresse oxidativo). A programação de alterações renais também deixa o rim mais vulnerável a doenças que podem ser adquiridas ao longo da vida pós natal. SNGFR=Ritmo de filtração glomerular no único-néfron; RFG=Ritmo de filtração glomerular (adaptado de YOU-LIN; CHIEN-NING, 2017).

2.6 ESTRATÉGIAS TERAPÊUTICAS

Terapias antioxidantes têm sido bastante utilizadas para retardar a progressão da DRC e prevenir a elevação da pressão arterial (DING et al., 2015; SATIRAPOJ et al., 2015). No entanto, também é necessário a busca por mecanismos terapêuticos que amenizem os transtornos produzidos pela programação intrauterina. Foi demonstrado que prejuízos cardiorrenais induzidos por uma diminuição do número de néfrons em ratos jovens submetidos à uma dieta com alto sódio foram prevenidos pelo tratamento com o antioxidante alopurinol (CARLSTROM et al., 2013). Estratégias terapêuticas ainda no ambiente intrauterino são capazes de prevenir alterações renais (VIEIRA-FILHO et al., 2014), cardíacas (XIAO et al., 2016) e hipertensão (CARE et al., 2016) na vida adulta. Além disso, o tratamento com vitamina C em mães fumantes no período gestacional foi eficaz na prevenção de mudanças epigenéticas no feto (SHOREY-KENDRICK et al., 2017).

Esses estudos confirmam a hipótese de que a reprogramação fetal é possível e que agentes antioxidantes são ferramentas promissoras na prevenção e tratamento da DRC. Por outro lado, terapias baseadas em células tem sido largamente estudada no tratamento patologias relacionadas ao rim. A administração exógena de células-tronco mesenquimais (CTMs) é a terapia celular mais promissora no combate a estas patologias (IMAI et al., 2009), porém ainda é necessário uma maior compreensão sobre a biologia dessas células.

2.6.1 Vitamina E

A vitamina E é um elemento nutricional essencial para o corpo, sendo o principal antioxidante lipossolúvel no organismo (BOREL; DESMARCHELIER, 2016). Sementes e óleos vegetais, como amêndoas, germe de trigo, nozes, óleo de coco, entre outros, são boas fontes de vitamina E, além disso, frutas e legumes como o abacate e a azeitona também são fontes dessa vitamina, só que em menor quantidade. A ingestão diária recomendada de vitamina E é de 12 mg para mulheres e de 13-15 mg para homens, e a ingestão abaixo desses valores podem gerar complicações no organismo (CHMÖLZ et al., 2016).

O termo genérico da vitamina E engloba 8 isoformas naturais pertencentes a 2 grupos químicos. O primeiro grupo é derivado do tocol e apresenta uma cadeia lateral saturada contendo 16 átomos de carbono. Esse grupo inclui o α-tocoferol, β-tocoferol, γ-tocoferol e o δ-tocoferol. O segundo grupo é derivado do tocotrienol apresentando uma cadeia lateral insaturada contendo 16 átomos de carbono, e inclui o α-tocotrienol, o β-tocotrienol, o γ-tocotrienol e o δ-tocotrienol (WANKENNE, 2014). De todos esses compostos, o α-tocoferol é o que possui a maior atividade biológica (RIZVI et al., 2014).

A vitamina E localiza-se principalmente na membrana celular, onde exerce sua principal função sequestrando superóxidos de forma rápida e não enzimática, protegendo assim, a membrana da peroxidação lipídica (RIZVI et al., 2014). Além disso, sua ação antioxidante também consiste na diminuição da expressão da NOX e aumento da atividade da SOD (CHEN et al., 2001). No entanto, outras funções também podem ser atribuídas a vitamina E, como: i) sinalização celular relacionada a proteína kinase C (PKC) (COOK-MILLS, 2013); ii) participação na estabilidade

estrutural da membrana plasmática (SHARMA et al., 2016); e iii) participação funcional na cadeia transportadora de elétrons (GRUBER et al., 2014).

Vários estudos têm demonstrado a importância da vitamina E no período gestacional. Já foi visto que a deficiência de vitamina E foi associada ao baixo peso ao nascimento e à maiores riscos de pré-eclâmpsia (FARES et al., 2014). A suplementação materna com α-tocoferol apresenta efeitos benéficos sobre a angiogênese placentária (KASIMANICKAM et al., 2012). Em relação a programação intrauterina, dados do nosso laboratório mostraram que a suplementação materna com α-tocoferol no período de lactação, reprogramou alterações no transporte de sódio no túbulo proximal renal e hipertensão arterial na prole adulta, programadas pela desnutrição materna (VIEIRA-FILHO et al., 2014). Também já foi demonstrado que o tratamento materno com α-tocoferol previne a hipertensão induzida intrauterinamente pela endotoxemia materna, juntamente com prevenção de disfunções da hemodinâmica renal (VIEIRA et al., 2018). Esses dados sugerem que o tratamento com α-tocoferol ainda no período intrauterino pode ser eficaz na prevenção da fibrose e de outras alterações renais relacionadas a DRC na vida adulta.

2.6.2 Apocinina

A apocinina, também conhecida como acetovanilona, é um potente inibidor não tóxico da NADPH oxidase em células fagocíticas e não-fagocíticas. Sua fórmula molecular é a 4-hidroxi-3-metoxiacetofenona, composto pertencente a classe dos metóxi-catecois. A apocinina foi isolalada pela primeira vez em 1883 das raízes da planta *Apocynum cannabinum*, conhecida como maconha canadense, por Schmiedeberg, mas em 1971, foi isolada das raízes da planta nativa do himalaia, *Picrorhiza kurrooa,* que é utilizada na medicina tradicional indiana como um potente antioxidante e anti-inflamatório (KANEGAE, 2009).

O mecanismo de ação da apocinina ainda não está totalmente esclarecido, sabe-se que ela inibe a NADPH oxidase impedindo a translocação da $p47^{phox}$ do citosol para a membrana. Para que ocorra a inibição da NADPH oxidase, a apocinina, que inicialmente é uma pró-droga, precisa ser oxidadada pela mieloperoxidase (MPO) na presença de peróxido de hidrogênio e formar um dímero

que corresponde a sua forma ativa (STEFANSKA; PAWLICZAK, 2008), como mostrado na Figura 7. Em células que não possuem a MPO, a apocinina não é capaz de inibir a NADPH oxidase. No entanto, foi demonstrado nessas células que, mesmo na ausência de MPO, a apocinina atua como antioxidante através de outro mecanismo de ação, o sequestro de superóxidos (HEUMÜLLER et al., 2008).

Estudos demonstram os efeitos benéficos do tratamento com apocinina em diversos órgãos. Por exemplos, danos neurológicos (CRUZ-ALVAREZ et al., 2017) e hepáticos (RAHMAN et al., 2017) associados a elevação da produção de ROS podem ser prevenidos pelo tratamento com apocinina. Em vasos, a calcificação induzida por ANG II (FENG et al., 2016) e alterações na reatividade vascular (FAN et al., 2017) podem ser atenuadas pela apocinina. A apocinina também tem efeitos cardioprotetores, prevenindo a fibrose e o remodelamento cardíaco (LI et al., 2013). No rim, o uso da apocinina minimiza efeitos da nefrotoxicidade induzida por gentamicina (ABDELRAHMAN et al., 2017), bem como protege a elevação da creatinina e uréia sérica, elevação do estresse oxidativo e danos histológicos renais induzidos pela isquemia-reperfusão (CHOI et al., 2015). Além disso, já foi visto que a fibrose renal induzida pela OUU é atenuada pelo tratamento com apocinina (CHENG et al., 2016). No entanto, poucos estudos demonstraram o papel da apocinina no tratamento de doenças programadas intrauterinamente, principalmente doenças renais.

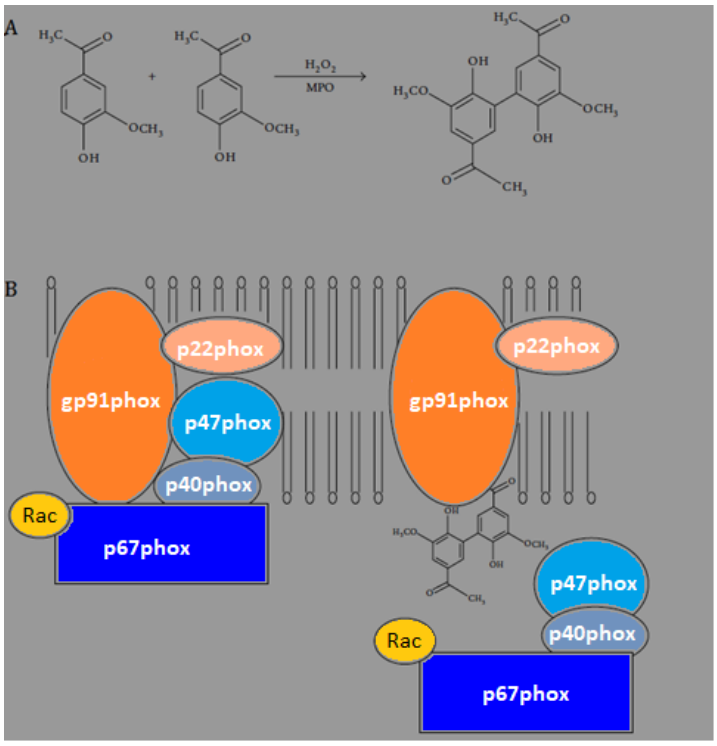

Figura 7. Mecanismo de inibição da NADPH oxidase pela apocinina. A. Ativação da apocinina pela mieloperoxidase na presença de peróxido de hidrogênio. **B**. A apocinina ativada impede a translocação da $p47^{phox}$ do citosol para a membrana, inibindo assim, a NADPH oxidase (STEFANSKA; PAWLICZAK, 2008).

2.6.3 Células-tronco mesenquimais

As células-tronco mesenquimais (CTMs), também conhecidas como células-tronco estromais, formam um grupo de células autorrenováveis, multipotentes, que podem ser isoladas de diversos tecidos. Essas células são identificadas *in vitro* pela sua capacidade de adesão ao plástico, pela expressão de marcadores típicos de superfície (CD44, CD73, CD90, CD105, CD166, CD271) juntamente com a ausência da expressão de CD14, CD34, CD45 e HLA-DR e pela diferenciação em células de origem mesodérmica incluindo condroblastos, adipócitos e osteoblastos (PEIRED et al., 2016). As células epiteliais de origem mesodérmica também são geradas a partir das CTMs, principalmente células endoteliais (ROOBROUCK; PADANILAM, 2011) e células tubulares renais (SINGARAVELU et al., 2009). A medula óssea marrom é a principal localização da CTMs e onde ela é melhor caracterizada, porém órgãos e tecidos tais como, sangue periférico, tecido conjuntivo, músculo esquelético, tecido

adiposo, polpa dentária, cordão umbilical, sangue do cordão umbilical, líquido amniótico e rim também possuem CTMs (PEIRED et al., 2016).

As CTMs extraídas do rim, polpa dentária, cordão umbilical, líquido amniótico, tecido adiposo e medula óssea, vem sendo utilizadas, com sucesso, no tratamento de diversas doenças renais (Figura 8) (PEIRED et al., 2016). Já foi visto que, ratos adultos, submetidos à nefrectomia 5/6 e tratados com CTMs no primeiro dia após a cirurgia, exibem um maior ganho de peso e uma menor proteinúria do que os ratos não tratados (CHOI et al., 2009). Hu e colaboradores reportaram uma deficiência de CTMs na medula renal de ratos hipertensos sensíveis ao sal, e que o transplante de CTMs para a medula renal inibiu a inflamação e melhorou a hipertensão induzida por uma dieta com alto sódio nesses animais (HU et al., 2014). A terapia com CTMs após o transplante renal mostrou um efeito protetor no desenvolvimento de proteinúria, glomerulosclerose e vasculopatia, características comuns da nefropatia crônica em aloenxertos (CAN) (FRANQUESA et al., 2012). O interessante é que, neste trabalho, a diminuição dos níveis de citocinas pró-inflamatórias e profibróticas ao longo do tempo, durante 24 semanas, foi associada ao aumento de citocinas anti-inflamatórias, apesar de nenhuma CTM injetada ter sido detectada após 7 dias do tratamento (FRANQUESA et al., 2012). Esses resultados sugerem que os efeitos benéficos do tratamento com CTMs não é exclusivamente pela diferenciação direta das CTMs, mas principalmente por mecanismos imunomodulatórios parácrinos.

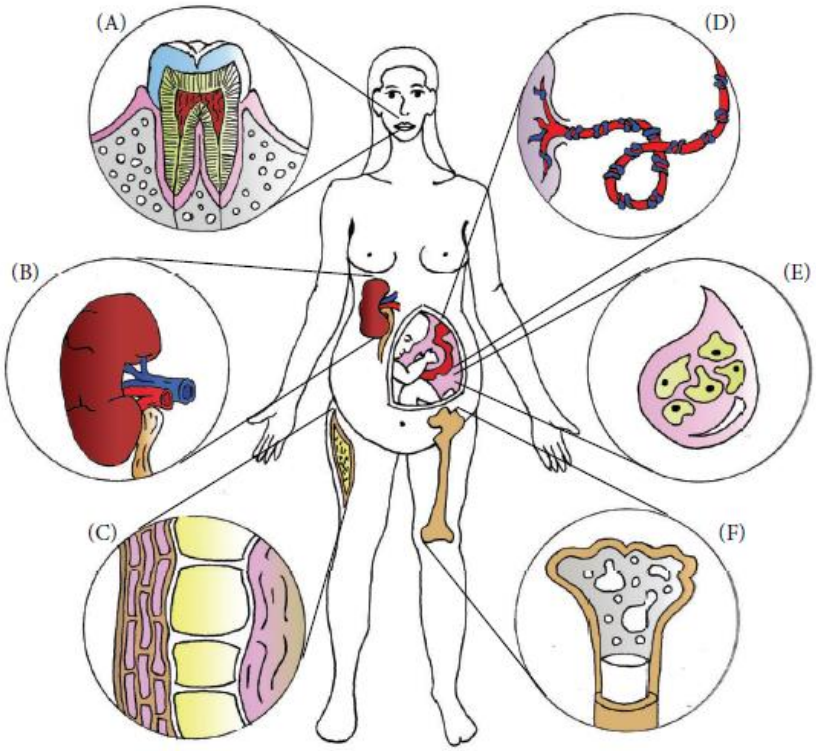

Figura 8. Fontes de CTM utilizadas em modelos experimentais de lesão renal. **A.** Polpa dentária. **B.** Rim. **C.** Tecido adiposo. **D.** Cordão umbilical. **E**. Líquido amniótico. **F.** Medula óssea. (PEIRED et al., 2016).

O mecanismo parácrino de regeneração exercido pelas CTMs vem sendo evidenciado por diversos estudos. Cheng e colaboradores mostraram que o tratamento com o meio condicionado das CTMs protege as células tubulares renais de forma semelhante ao tratamento com CTMs em modelos de lesão renal aguda induzida em camundongos (CHENG et al., 2013). Neste estudo, foram identificadas mais de 40 citocinas reguladoras no meio condicionado obtido a partir das CTMs (CHENG et al., 2013). Trabalhos mais recentes vêm mostrando que as CTMs podem induzir o reparo tecidual de células residentes através da secreção de microvesículas e exossomos que contêm fatores solúveis como citocinas, fatores de crescimento, mRNA e microRNA (FLEIG; HUMPHREYS, 2014). Esses fatores seriam os responsáveis pelos efeitos imumodulatórios (ex. aumento do fator de crescimento de hepatócito-HGF, aumento do IL-10 e diminuição do TGF-β); proliferativos (ex. aumento do fator de crescimento ligado a insulina-IGF, aumento do mRNA do receptor de IGF-IGFR-mRNA e aumento do fator de crescimento epidermal-EGF); anti-apoptóticos (ex. aumento do gene do linfoma de células B 2 –

Bcl 2 e diminuição do gene da caspase 1 – casp 1) e angiogênicos (ex. aumento do VEGF) das CTMs (Figura 9) (FLEIG; HUMPHREYS, 2014).

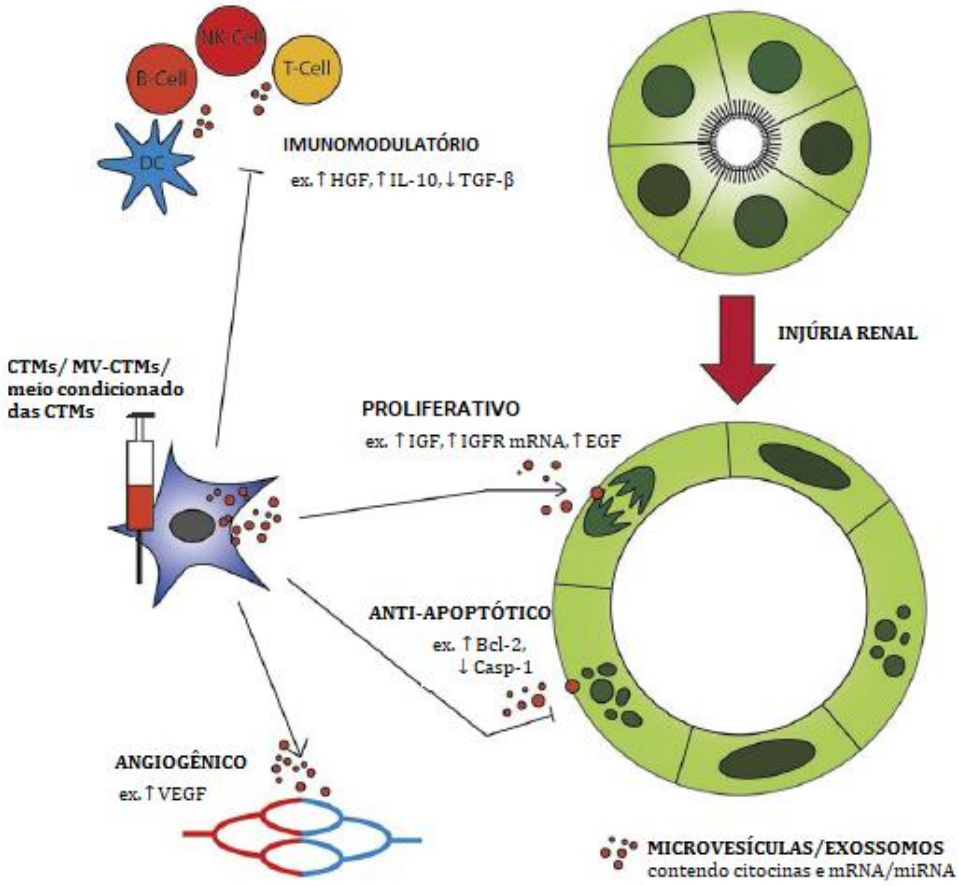

Figura 9. Modelo de ação parácrina das células-tronco mesenquimais (CTMs). Injeções de CTMs, microvesículas de CTMs (MV-CTMs) ou meio condicionado das CTMs acelera o reparo tecidual através da resposta imunomodulatória, proliferativa, anti-apoptótica e angiogênica, diante de uma injúria renal. As micovesículas secretadas contém citocinas e mRNA/microRNA (miRNA). IGF = fator de crescimento ligado a insulina; HGF = fator de crescimento de hepatócito; TGF- β = fator de crescimento transformador β; EGF = fator de crescimento epidermal; Bcl-2 = linfoma de células B-2; Casp-1 = caspase 1; VEGF = fator de crescimento do endotélio vascular (adaptado de FLEIG; HUMPHREYS, 2014).

Trabalhos recentes vem investigando a terapia de CTMs especificamente sobre a fibrose renal. Estudos que utilizam modelos de fibrose renal induzida pela OUU em ratos mostram que a administração exógena de CTMs melhora a função renal através da prevenção da EMT, diminuição do dano tubular, da deposição de colágeno, da expressão protéica do TGF-β, da proteína quimiotática de monócitos-1 e do TNF-α, bem como através do aumento da expressão protéica do VEGF e estimulação da proliferação celular (BAI et al., 2013; DA SILVA et al., 2015; SUN et al., 2013). No entanto, ainda não está claro o mecanismo específico pelo qual as

CTMs previnem lesões mais severas no rim e se essas células podem modular as MMPs e as TIMPs. Outra questão importante é se a capacidade regenerativa e renoprotetora da CTMs pode ser prejudicada pela programação intrauterina. Em um estudo clínico, Baker II e colaboradores mostraram que a obesidade materna alterava o metabolismo das CTMs do cordão umbilical, o que gerava alterações nos miócitos e adipócitos fetais, deixando a criança mais susceptível a obesidade (BAKER II et al., 2017). Porém, ainda são raros os estudos que correlacionam insultos na vida pré-natal com a funcionalidade das CTMs na vida adulta.

3 RESULTADOS

3.1 ARTIGO 1

MATERNAL ENDOTOXEMIA INDUCES RENAL COLLAGEN DEPOSITION IN ADULT OFFSPRING: ROLE OF NADPH OXIDASE/TGF-β1/MMP-2 SIGNALING PATHWAY

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ABSTRACT

Background. Maternal endotoxemia, during pregnancy, has been shown to increase collagen deposition in the kidneys of offspring. Renal fibrosis is a hallmark of progressive chronic kidney disease, which coexist with hypertension. It was investigated whether maternal reactive oxygen species (ROS) is leading to renal fibrosis or exacerbating unilateral ureteral obstruction (UUO)-induced renal fibrosis in the offspring of dams treated with to lipopolysaccharide (LPS). Furthermore, it was investigated whether TGF-β and matrix metalloproteinases (MMPs) could mediate ROS-induced renal fibrosis. **Methods.** Adults Wistar rats were obtained from dams submitted LPS administration through the third part of gestation. To evaluate the role of maternal ROS, part of the dams received α-tocopherol simultaneously with LPS. Part of the offspring in each group was submitted to UUO at adult life, when sub-groups was treated or untreated with apocynin, a NADPH oxidase inhibitor. TGF-β, NOX2 (one isoform of NADPH oxidase), MMP2, MMP9 and tissue inhibitor of metalloproteinases 1 and 2 (TIMP-1 and TIMP-2) were investigated by using Western blotting. **Results.** Maternal LPS administration induced higher proteinuria, systolic arterial pressure and renal collagen deposition in adult offspring. LPS offspring rats also presented increased MMP-2 activity in parallel to low renal cortical expression of its inhibitor TIMP-2. These changes were correlated to increased expression of TGF-β and NOX2. Maternal α-tocopherol treatment prevented collagen deposition and reduced arterial pressure in adult offspring. α-Tocopherol also inhibited maternal endotoxemia-induced changes in TGF-β1/NOX2/MMP-2 signalling. UUO led to increased collagen deposition in contralateral kidney of LPS offspring rats, which was correlated to increased NADPH oxidase activity and was prevented by NADPH oxidase inhibition. **Conclusions.** Maternal inflammation led to alterations in TGF-β1/NOX2/MMP-2 signalling pathway in renal tissue concomitant with collagen deposition, therefore contributing to hypertension in adult offspring.

Keywords: maternal inflammation, matrix metalloproteinase, NADPH oxidase, renal fibrosis, TGF-beta

INTRODUCTION

Inappropriate maternal environment during pregnancy impairs fetal development and increases risk of chronic diseases at adult life (Paixão and Alexander, 2013; Hanson and Gluckman, 2014,). Some diseases are clearly manifested when tardive disruptions of health conditions overlap with in utero adverse environment (Morton et al., 2016). Previously, we have pointed that placental oxidative stress is underlying lipopolysacharide (LPS)-induced hypertension at adult life (Vieira et al., 2018). Reactive oxygen species (ROS)-induced impairment of vascular reactivity and renal hemodynamics are especially pointed as responsible for offspring hypertension on this adverse maternal environment.

Besides hypertension (Wei et al., 2007; Vieira et al., 2018), maternal endotoxemia has been shown to increase collagen deposition in the offspring kidneys (Guo et al., 2016). Renal fibrosis is a hallmark of progressive chronic kidney disease (Genovese et al., 2014), which coexist with hypertension (Jager and Fraser, 2017) one being the cause and other consequence, or simply they feed each other (Johnson et al., 2015). However, it is not yet known by which mechanisms maternal endotoxemia induces renal fibrosis in adult offspring. Changes in matrix metalloproteinases (MMPs), and profibrotic cytokines, such as TGF-β1, have potential importance in renal fibrosis due to their pivotal role in regulation of extracellular matrix (ECM) content (Genovese et al., 2014).

Moreover, ROS may be protagonist in renal fibrosis programmed by maternal endotoxemia. First, ROS are also involved in ECM regulation (Lv et al., 2018) and NADPH oxidase can activate TGF-β1 and modulate its signaling pathway (Bondi et al., 2010; Samarakoon et al., 2013; Lee et al., 2015). Second, NADPH oxidase is upregulated in kidney from rats born from LPS-treated dams (Vieira et al., 2018). Finally, NADPH oxidase inhibition abrogates maternal endotoxemia-induced changes in mesenteric vascular reactivity and renal hemodynamic (Vieira et al., 2018).

Thus, we hypothesize that maternal endotoxemia induced by LPS lead to renal injury by promoting ECM deposition, through a mechanism involving NADPH oxidase activation and regulation of TGF-β1 and MMPs pathways. Taking into account that unilateral ureteral obstruction (UUO) is an effective renal injury to

investigate ECM deposition, overlapping UUO to maternal LPS-treatment could better shed light to mechanisms involved in renal fibrosis. Thus, the present study aimed to investigate whether maternal endotoxemia leads to renal fibrosis or exacerbates UUO-induced renal fibrosis in the offspring. Further, it was aimed to investigate whether NADPH oxidase, TGF-β and MMPs are underlying factors in maternal endotoxemia-induced renal fibrosis.

MATERIALS AND METHODS

Materials

Lipopolysaccharides from *Escherichia coli* 0111:B4 (LPS), α-tocopherol, apocynin (acetovanillone), phenylmethanesulfonyl fluoride (PMSF), anti-NOX2 antibody (SC-74514), β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), N,N′-Dimethyl-9,9′-biacridinium dinitrate (lucigenin), direct red and coomassie brilliant blue were purchased from Sigma-Aldrich (St. Louis, MO, USA). Colorimetric assays kits for creatinine and ureia measurement were from Labtest (Lagoa Santa, MG, Brazil) and sodium pentobarbital was purchased from Cristália (Produtos Químicos Farmacêuticos, Itapira, SP, Brazil). Polyvinylidene difluoride (PVDF) blotting membrane, Horseradish peroxidase-conjugated anti-rabbit IgG antibody (NA934V) and ECL Prime Western blotting system were purchased from GE Healthcare (Buckinghamshire, UK). Anti-TGF-β1 (SC-141) and anti-β-actin (SC-47778) antibodies were obtained from Santa-Cruz Biotechnologies (Dallas, TX, USA) and peroxidase-conjugated anti-mouse antibody (Ab6728) was purchased from Abcam (Cambridge, UK). Anti- MMP-2 (MAB3308), anti-MMP9 (MAB3309), anti-TIMP-1 (MAB3300) and anti-TIMP-2 (MAB13446) antibodies were obtained from Merck Millipore (Armstadt, Germany). All other reagents were of the highest purity available.

Animals

All protocols and procedures were carried out in accordance with Committee for Experimental and Animal Ethics of the Federal University of Pernambuco (nº

23076.060473/2014-91). Rats were maintained on 12h light/dark cycle at 23°C, and free access to water and commercial chow. Female Wistar rats, weighting 200–250 g, were mated and the beginning of gestation (gestation day 0) was confirmed by observation of spermatozoids in the vaginal smear. Pregnant females were allocated in individual cages and randomly assigned to experimental groups. At days 13, 15, 17 and 19 of gestation, dams were submitted to subcutaneous administration of LPS (0.5 mg/kg body weight; LPS group, L, n=34) or NaCl 0.9% (1 mL/kg; Control group, C, n=26). The chosen dosage leads to elevated placental oxidative stress and fetal growth retardation (Vieira et al., 2018), however at least 50% of gestations had litter size higher than 8 living pups. Control and LPS dams were daily treated with α-tocopherol (T, 350 mg/kg body weight by gavage; CT and LT, n=6 and 8, respectively) or corn oil (α-tocopherol vehicle, V; 1 mL/kg body weight by gavage; C and L, n=8 and 8, respectively) from gestation day 13 until parturition. At birth, offspring was culled up to 8 pups per dam, maintaining preferentially male rats. At 120 days of age, the male offspring rats (C, L, CT and LT; n=8, 8, 6 and 8, respectively) was submitted to evaluation of systolic blood pressure (SBP), creatinine clearance and proteinuria. Afterward, the rats were anesthetized (sodium pentobarbital, 60 mg/kg body weight, ip) to collect kidneys and, still under anesthesia, they were killed by rupturing the diaphragm. This procedure was always performed between 09-12 am. Kidney samples were used to evaluation of cortical collagen density. Protein expression and activity of MMP-2 and MMP-9 were evaluated in samples of isolated glomeruli, while protein expression of TIMP-1, TIMP-2, TGF-β1 and NOX2 was evaluated in renal cortex. In this set of experiments, it was used 1–2 rats per each litter.

To investigate whether prenatal LPS exposure exacerbate renal fibrosis induced by UUO, Control (n=12) and LPS (n=18) offspring, aging 120 days-old, were submitted to a surgical procedure. The UUO procedure was performed through laparotomy followed by permanent left ureter obliteration using a cotton suture under ketamine/xylazine (40/10 mg/kg body weight) anaesthesia. Then, abdominal musculature and skin were sutured and, after recovery from anaesthesia, the rats received one dose of acetaminophen (200 mg/kg bodyweight, by gavage). Control and LPS rats submitted to UUO (CO and LO groups, n=6 and 9, respectively) were compared to a group of control rats submitted to the same surgical procedure, except

to ureter obliteration (Sham group, n=5). Moreover, part of the rats were submitted to daily treatment with NADPH-oxidase inhibitor apocynin (A - 100 mg/kg body weight, in drinking water; COA and LOA, n=6 and 9, respectively). After 14 days of UUO, the groups were anesthetized (sodium pentobarbital, 60 mg/kg body weight, i.p.) and the kidney was collected to evaluate collagen density and NADPH oxidase activity. After biological samples collection, the rats were killed by rupturing the diaphragm while still under anesthesia. Before UUO and euthanasia, the groups were submitted to evaluation of SBP, blood urea nitrogen (BUN), creatinine clearance and proteinuria. In this set of experiments, it was also used only 1–2 rats per each litter from Control and LPS dams. The total number of rats, including dams and offspring was 120.

Measurement of BUN, creatinine clearance and proteinuria

To evaluate BUN, creatinine clearance and proteinuria, the rats were allocated in a metabolic cages to urine collection. After urine collection, serum samples were obtained from blood collected from tail vein. Urinary and serum creatinine levels were measured to allow creatinine clearance calculation. BUN and creatinine levels were measured using colorimetric assay kits (Labtest, Lagoa Santa, MG, Brazil). Proteinuria was calculated after measurement of urinary protein concentration by Folin phenol method (Lowry et al., 1951). The rats were always placed in metabolic cage at the same period of day.

Measurement of blood pressure

In both animal protocols, SBP was evaluated through tail-cuff plethysmography (IITC Life Science B60-7/16"; Life Science Instruments, Foster City, CA, USA) as described previously (Vieira et al., 2018). Before acquiring data, the animals were adapted during three consecutive days to experimental procedures. The data of each rat is represented by the average of five consecutive measurements, which were acquired always between 09–12 am. To avoid impact of experimental procedures in the others parameters, after SBP, the animals were allowed to a 24-hour period of resting without handling.

Measurement of renal cortical collagen deposition

Renal cortical collagen density was evaluated by histochemistry using Picro-Sirius staining. After euthanasia, kidneys were fixed in methacarn (methanol, chloroform and acetic acid; 6:3:1) during 24 hours and maintained in 70% ethanol until tissue paraffin-embedding. Sections of 6 µm were stained with Weigert's hematoxylin for 8 min, followed by one-hour Picro-Sirius staining (0.1% Direct Red 80 and saturated aqueous solutions of picric acid). Afterwards, the slides were washed in acidified water, and submitted to slide mounting steps. For each rat, collagen deposition was measured in 30 cortical fields (70,700 μm^2) using the Image Pro Plus software (version 4.5.1, Media Cybernetics, Bethesda, MD, USA). Cortical collagen density was expressed as percentage of collagen deposition in relation to cortical tubulointerstitial area. During measurement, the group that was being analyzed had been unadvertised to researcher. Images were obtained using light microscope (Nikon, Eclipse Ni-U, Shanghai, China) coupled to a camera (DS-i1C, Nikon).

Glomerular isolation

The activity and protein expression of MMP-2 and MMP-9 were evaluated in isolated glomeruli obtained using sieving technique. Renal cortex samples were macerated using a glass pistil over a 60 mesh sieve (0.297 mm pore diameter). The macerated samples were washed with Tris buffered saline containing 1% Triton X-100 and 1 mM PMSF. Then, the tissue was sequentially sieved through a 100 and 200 mesh sieves (0.149 and 0.074 mm pore diameter, respectively). Isolated glomeruli were obtained by aspiration of the remaining tissue on the 200 mesh sieve. The sample was then centrifuged for 5 min at 1,000 × *g* at 4ºC, ressuspended in 100µL buffered and stored at −80ºC.

Measurement of matrix metalloproteinase activities

MMPs activity was performed by gelatin zymography method according to Qin et al., (2009). Isolated glomeruli samples (50 µg protein) were submitted to electrophoresis in a 7.5% polyacrylamide gel containing sodium dodecyl sulfate

(SDS) and 0.1% gelatin under non-reducing conditions. The gels were washed 2x for 30min in 2.5% Triton X-100 solution, and then incubated for 40 hours in 50 mM Tris (pH 7.4), 200 mM NaCl and 10 mM $CaCl_2$ at 37°C. Following, the gels were stained by Coomassie Blue for 1 hour and discolored in a solution containing methanol, acetic acid and water (1:2:25). Gelatinase activities of MMPs were visualized as clear bands on a blue background and gel colorimetric images were acquired using ChemiDoc MP Imager (Bio-Rad, Hercules, CA, USA). The activities of MMP-2 and MMP-9 were identified according to their respective molecular weight and the bands density were quantified using Image Lab software (version 5.2.1, Bio-Rad).

Immunoblotting

MMP-2, MMP-9, TIMP-1, TIMP-2, TGF-β and NOX-2 protein expression were evaluated through immunoblotting of isolated glomeruli (MMP-2 and MMP-9) or renal cortex (TIMP-1, TIMP-2, TGF-β and NOX-2). Protein samples (80 µg) were separated by 10% (NOX-2, MMP-2 and MMP-9) or 15% (TIMP-1, TIMP-2 and TGF-β) SDS-PAGE and transferred onto PVDF membrane. After blocking non-specific binding, the membranes were probed overnight with primary antibodies at appropriated dilutions: anti-MMP-2 (1:500), anti-MMP-9 (1:500), TIMP-1 (1:500), TIMP-2 (1:500), anti-TGF-β (1:1,000) and anti-NOX-2 (1:1,000). The membranes were washed and probed for one-hour to appropriated peroxidase-conjugated secondary antibody. The blots were visualized using a chemiluminescence reagent (ECL Prime, GE Healthcare Life Sciences) and the images were acquired through ChemiDoc MP Imager (Bio-Rad). Bands densitometric analysis was performed using Image Lab software (Bio-Rad) and values were normalized according to protein load, by performing the ratio of densitometry of target proteins and β-actin immunoblotting.

Basal superoxide production and NADPH oxidase activity

Superoxide anion ($O_2^{\bullet-}$) production was assessed by chemiluminescence-derived from superoxide lucigenin oxidation. Renal cortical samples were homogenized (1 g/7 ml) in ice-cold RIPA solution (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Tritron X-100, 1% sodium deoxycholate, 0.1% sodium dodecylsulfate),

supplemented with protease inhibitor cocktail (2 mM AEBSF, 1 mM EDTA, 130 µM Bestatin, 14 µM E-64, 1 µM Leupeptin, 0.3 µM Aprotinin). Then, samples were centrifuged at 12,000 × *g* for 12 min at 4°C and the supernatant (1 mg/ml protein) was incubated for 2 minutes at 37°C in a reaction medium containing phosphate buffered saline (PBS, 0.02 M, pH 7.4) and 100 µM NADPH. After adding 10 µM lucigenin, luminescence was evaluated in 10 measurements, with 30 second interval between each other, at 37°C (Varioskan Flash, Thermo Scientific). The result was represented by the sum of the luminescence obtained in the 10 measurements and expressed as the relative unit of light (RLU) corrected for the amount of protein in the sample. The release of superoxide was also evaluated in the absence of NADPH, and represented the basal production of superoxide.

Statistical Analyses

Data are expressed as mean ± standard error of the mean (SEM). The distribution of each data set was evaluated using Shapiro-Wilk normality test, as well as by observing the distribution of the data of each biological parameter in histograms. Difference between groups was assessed using unpaired Student *t* test or using two-way ANOVA followed by the Bonferroni test. Differences of parameters analyzed before and after UUO, were assessed by paired Student *t* test. Statistical significance was considered for $P<0.05$. Statistical analysis and graphing were performed using the software GraphPad Prism 5 (version 5.01, La Jolla, CA, USA).

RESULTS

Prenatal LPS exposure decreased birth weight and led to proteinuria, increased SBP and increased cortical collagen deposition at adult life

Maternal LPS-induced intrauterine growth retardation was confirmed by the observation of decreased offspring birth weight in comparison to control rats (LPS = 4.9 ± 0.1 vs. C = 6.1 ± 0.1 g; $P<0.05$). The offspring from LPS dams treated with α-tocopherol presented similar birth weight to control rats (LT = 5.5 ± 0.4 vs. C = 6.1 ± 0.1 g; $P>0.05$). At adult life (120 days-old), the groups presented similar birth weight

(Table 1). Moreover, no differences were observed in water intake, food intake and diuresis. At 120 days, creatinine clearance was not affected by prenatal LPS exposure, however, proteinuria was increased by 60% ($P<0.01$) in comparison to control group. Rats from LPS exposed dams treated with α-tocopherol presented proteinuria values similar to control conditions. Prenatal LPS exposure also enhanced SBP (14%, $P<0.001$) at adult offspring, reinforcing that maternal endotoxemia has programming effects over arterial pressure (Figure 1). Moreover, maternal α-tocopherol treatment prevented the increased SBP in LPS-offspring, however, in control group, it induced higher (7%, $P<0.001$) SBP.

NADPH oxidase inhibition abolishes increased SBP programmed by maternal endotoxemia

Once we had previously observed a prominent upregulation of renal cortical NOX2 in LPS-treated rats (Vieira et al., 2018), we hypothesized that this pathway may be of great role in renal fibrosis programmed by maternal endotoxemia. Thus, we evaluated whether a renal fibrotic second hit would impact LPS-programmed rats in a higher extent than Control, and whether this could be prevented by NADPH oxidase inhibition.

At 120 days-old rats, Control and LPS-treated were submitted to UUO. After 14 days, we observed that UUO induced proteinuria increment at the same extent in Control and LPS offspring rats (Table 2), which was already 47% higher ($P<0.001$) in the later (Table 1 and 2). On the other side, UUO did not affect creatinine clearance (Table 2) and SBP (Figure 2). Apocynin treatment did not prevent UUO-induced proteinuria, however it increased (64-82%, $P<0.001$) creatinine clearance in both groups. Additionally, apocynin treatment lowered SBP in LPS offspring rats, which was already elevated even before UUO, reinforcing upregulation of this oxidative pathway programmed by maternal endotoxemia (Figure 2).

Maternal endotoxemia sensitizes renal collagen deposition through a NADPH-oxidase dependent mechanism

To investigate whether prenatal LPS-induced elevation of SBP and proteinuria were correlated to renal injury, we evaluate cortical collagen deposition through Picro-Sirius staining (Figure 3). In a similar profile of alterations as observed in proteinuria and SBP, rats which were exposed to prenatal LPS presented 12% higher ($P<0.05$) renal cortical collagen density. However, LPS group treated with α-tocopherol presented renal collagen density similar to control values. In the control group, α-tocopherol did not induce any change in Picro-Sirius staining.

Collagen deposition was also evaluated in obstructed and contralateral kidney after 14 days of performing UUO (Figure 4). In obstructed kidney, UUO increased in more than two times ($P<0.05$) collagen density in comparison to Sham group. However collagen deposition was similar in obstructed kidneys from LPS and Control rats (Figure 4A). Moreover, apocynin treatment prevented partially, in both groups, UUO-induced collagen deposition. In contralateral kidney from Control rats, collagen staining was similar to values observed in Sham-operated rats (Figure S2), evidencing that the UUO-induced renal contralateral overload was not able to promote renal fibrosis. However, in rats which were prenatally exposed to LPS, it was observed enhanced (31%, $P<0.01$) collagen deposition (Figure 4B) in contralateral kidney, and this was prevented by apocynin treatment.

LPS-exposed group presented 3 times higher ($P<0.001$) NOX2 protein expression than Control group (Figure 5). Maternal treatment with α-tocopherol prevented partially LPS-induced upregulation of NOX2 expression. In Control rats, the expression of this protein was unaffected by α-tocopherol. Besides UUO increased collagen deposition in obstructed kidney, it did not altered basal superoxide levels in comparison to Sham group (Figure S3). However, obstructed kidney from LPS group presented 48% higher ($P<0.01$) basal production of superoxide anions than Control rats (Figure 6A). This alteration was unaffected by apocynin treatment. In contralateral kidney, superoxide anions were unaffected by either UUO or prenatal LPS, however their levels were reduced (47 and 30%, $P<0.05$) by apocynin treatment (Figure 6B).

Albeit it did not affect basal superoxide anions, UUO induced 57% diminution ($P<0.01$) of NADPH-dependent superoxide production in obstructed kidneys versus Sham-operated kidney (Figure S3). On the other side, NADPH oxidase activity was higher (43 and 58%, $P<0.01$) in obstructed and contralateral kidneys from LPS offspring rats in comparison to Control group (Figure 6A and B). Apocynin treatment blunted the increased NADPH oxidase activity in contralateral kidney from rats exposed to LPS (Figure 6B).

Prenatal LPS exposure programmed changes in ECM regulatory signalling

NADPH oxidase may lead to ECM deposition through modulation of TGF-beta signalling, which, in part, is dependent of MMPs-TIMPs regulation (Tan and Liu, 2012). Then, we evaluated the impact of maternal LPS on protein expression of profibrotic cytokine TGF-β (Figure 7) and expression and activity of MMP-2 and 9, and expression of their inhibitors, TIMP-2 and TIMP-1, respectively (Figure 8). Maternal endotoxemia programmed elevation of TGF-β protein expression in renal cortex from adult rats, while maternal treatment with α-tocopherol prevented this alteration.

Moreover, prenatally LPS-treated rats presented higher (64%, $P<0.01$) MMP-2 activity at adult life, albeit its protein expression was only 52% ($P<0.05$) of the Control group (Figure 8A and C). On the other side, the upregulation of MMP-2 activity occurred in parallel to lowering (57%, $P<0.01$) of TIMP-2 protein expression (Figure 8E). Maternal treatment with α-tocopherol prevented entirely LPS-induced changes in MMP-2 activity and expression, and partially TIMP-2 expression. However, in Control rats, α-tocopherol treatment programmed higher MMP-2 activity (57%, $P<0.05$) and lower TIMP-2 expression (49%, $P<0.01$).

Although MMP-2/TIMP-2 system was affected in adult rats by maternal endotoxemia and/or α-tocopherol treatment, MMP-9 activity/expression and TIMP-1 expression were similar between experimental groups (Figure 8B, D and F).

COMMENT

The investigation of mechanisms that underlies renal matrix deposition regulation is seminal to the comprehension of pathophysiology of intrauterine programming of renal disease and hypertension. In the present study, we demonstrated that maternal endotoxemia impaired fetal development and programmed hypertension, proteinuria and increased renal collagen deposition in adult offspring. Moreover, it was shown that these changes were prevented by maternal α-tocopherol treatment. For the first time, it was reported that maternal LPS administration induces activation of MMP-2 activity, which may be determined by upregulation of both, NOX2 and TGF-β signaling pathway. It was also demonstrated that maternal treatment with LPS overlapping UUO increased renal collagen deposition that was inhibited by apocynin, an inhibitor of NADPH oxidase.

Maternal endotoxemia induces hypertension in adult offspring (Wei et al., 2007; Vieira et al., 2018) and increases collagen content in renal parenchyma (Guo et al., 2016). Recently, we reported that maternal treatment with α-tocopherol inhibits LPS-induced placental oxidative stress and prevents the onset of hypertension in adult offspring (Fig. 1; Vieira et al., 2018). The α-tocopherol is the most common form of vitamin E (Wang and Quinn, 1999) that has anti-oxidant activity. Moreover, we already reported that maternal treatment with α-tocopherol prevents nephrogenesis impairment induced by maternal malnutrition during gestation (Vieira-Filho et al., 2011) and renal dysfunctions and hypertension later in life (Vieira-Filho et al., 2014). Here, our data also suggest that maternal ROS induced by LPS is involved in the programming of higher renal collagen deposition, as it was evidenced for the first time that LPS exposed rats treated with α-tocopherol present collagen density similar to control levels (Fig. 2). This finding strengths the importance of maternal oxidative stress and renal fibrosis in the mechanism of hypertension onset.

Renal fibrosis is one of the main process involved in chronic renal failure, being ECM deposition highly correlated to the degree of renal injury (Kriz and LeHir, 2005; Genovese et al., 2014). This process leads to cellular crescents, diminution of filtration surface, compensatory hypertrophic growing of glomerulus, podocyte injury, and disruption of filtration barrier size selectivity (Kriz and LeHir, 2005). Proteinuria

observed in rats obtained from LPS-submitted dams also reinforces the programming of renal injury and the importance of collagen deposition. Furthermore, α-tocopherol simultaneously to maternal LPS treatment prevented proteinuria and collagen deposition indicating an association between these events (Vieira-Filho et al., 2018).

Additionally, the participation of oxidative stress in renal dysfunction induced by maternal LPS administration, is endorsed by increased renal NOX2 expression in offspring of LPS-dams as was also previously observed (Vieira et al., 2018). Thus, NADPH oxidase-dependent superoxide anion could be responsible for collagen deposition. When UUO was performed, it was observed increased collagen deposition in contralateral kidney of LPS-offspring concurrently with increased NADPH oxidase activity (Figures 4 and 6), while in control rats none of these alterations were observed (Supplementary Figures S2 and S3). Moreover, it was demonstrated that the alterations programmed by maternal-LPS administration were abrogated with NADPH oxidase inhibition, by apocynin treatment. These changes seems to be important in compensatory mechanisms in contralateral kidney, where function overload is occurring to preserve whole renal function (Li et al., 2003). In obstructed kidney, the increase in collagen deposition was similar between control and LPS-offspring groups and similarly reduced by apocynin treatment, indicating that NADPH oxidase has similar importance when these rats are submitted to more severe renal damage.

Previously we reported that NADPH oxidase inhibition prevents elevation of blood pressure in adult rats born from LPS-treated dams (Vieira et al., 2018). NADPH oxidase has been linked to impaired mesenteric vasorelaxation and impaired renal hemodynamics (Vieira et al., 2018). Data in the present study show further evidence of NADPH oxidase role in renal injury. First, its role can be stated from the higher contralateral kidney collagen deposition after UUO, as discussed previously. Secondly, its role is supported by the observation that apocynin prevents proteinuria induced by UUO on LPS-offspring (Table 2). ROS generation mediated by NADPH oxidase is linked to induction of numerous redox-sensitive transcription factors and upregulation of mitogen-activated protein kinases, including JNK and ERK1/2 (Ratliff et al., 2016), which represents important signaling related to inflammation, fibrosis and injury.

TGF-beta is a pivotal in the mechanism of ECM remodeling regulation (Lee et al., 2014), therefore it is present in renal fibrosis (Mansour et al., 2017). Maternal endotoxemia promoted elevation of TGF-beta expression in kidney from adult offspring, pointing to the participation of this profibrotic cytokine in the programming mechanism of renal fibrosis. TGF-beta upregulation could be underlying ECM deposition through enhanced collagen and fibronectin expression, by direct induction of protein expression and through stimulation of myofibroblast and mesangial cells activation and EMT (Lee, 2012). Moreover, TGF-beta-mediated fibrosis has been shown to be partially mediated by ROS production through NADPH oxidase (Bondi et al., 2010; Samarakoon et al., 2013; Lee et al., 2014; Rhyu et al., 2012). On the other hand, ROS are involved in activation of TGF-beta from its precursor form (Barcellos-Hoff and Dix, 1996) and in modulation of canonical Smad2/3 TGF-beta signaling pathway (Jiang et al., 2014). Taken together, NADPH oxidase and TGF-beta signaling seems to have a reciprocal regulation which culminates in enhanced ECM deposition and oxidative stress.

ECM content is regulated by its production and degradation rate (Tan et al., 2012; Genovese et al., 2014; Lv et al., 2018). MMP-2 and 9, particularly, play an important role in the modulation of renal collagen content (Tan et al., 2012; Genovese et al., 2014). The present results showed that maternal-LPS induced elevation of glomerular MMP-2 activity in adult offspring, while MMP-9 activity was not altered. This profile of MMPs change is also observed in ECM accumulation induced by mesangial cells irradiation (Zhao et al., 1999). However, it was already been demonstrated that renal fibrosis induced by UUO is associated with increased expression and activity of MMP-2 and MMP-9, nevertheless MMP-2 alone may be sufficient to induce epithelial-mesenchymal transition (EMT) (Du et al., 2012). Increased MMP-2 activity may be involved in activation of TGF-β and TNF-α by cleaving its inactive forms (Tan and Liu et al., 2012), which could, in turn, lead to paracrine stimulation of ECM production. Moreover, MMP-2 activity is also linked to stimulation of cell proliferation (Tan and Liu et al., 2012) and epithelial-mesenchymal transition (EMT) (Du et al., 2012).

Our data demonstrated a divergence between expression and activity of MMP-2. Albeit renal fibrosis induced by maternal endotoxemia was associated with increased MMP-2 activity, its expression was downregulated (see figure 3A and 3C).

The higher MMP-2 activity, even with diminished expression, can be justified by the decreased expression of TIMP-2 (see figure 3E). Tissue inhibitors of metalloproteinases (TIMPs) are secreted proteins related to MMPs inactivation (Sternlicht and Werb, 2001). There are four known isoforms of TIMPs (TIMP-1, 2, 3 and 4). The TIMP-2 is more selective to MMP-2, while TIMP-1 is more selective to MMP-9. In our study, both TIMP-1 and MMP-9 expressions were unaltered, as well as MMP-9 activity.

In maternal endotoxemia-induced renal fibrosis, the significance of NADPH oxidase/TGF-beta/MMP-2 signalling pathway is reinforced by the fact that maternal treatment with α-tocopherol prevented collagen deposition. The finding that α -tocopherol prevented increment of MMP-2 activity and collagen deposition in kidney also strengths the importance of placental oxidative stress in the programming renal dysfunction at later life. Indeed, it shows that intrauterine period is an important window to target a therapeutic approach. It is noteworthy that α -tocopherol administration to control dams induced some alterations in adult offspring, such as elevation of SBP and MMP-2 activity and decrease of TIMP-2 protein expression. Previously we reported that maternal α-tocopherol administration elevated arterial pressure in adult offspring (Vieira-Filho et al., 2014; Ribeiro et al., 2018; Vieira et al 2018). Alpha-tocopherol induced hypertension in control group had been correlated to altered angiotensin signaling and increased ATP-dependent Na+ transporters in the proximal tubule (Vieira-Filho et al., 2014; Ribeiro et al., 2018). We may speculate that in later life, these changes jointly could lead to renal fibrosis and renal injury.

In summary, our study shows that maternal endotoxemia induces alteration in TGF-beta/NOX2/MMP-2 signalling pathway in renal tissue that may lead to collagen deposition, therefore contributing to programming of hypertension at adult life. Furthermore, it highlights the importance of NADPH oxidase-induced oxidative stress in the programming renal dysfunction and hypertension. Overall, the present results show that inadequate placental environment make individuals more susceptible to renal diseases than healthy individuals that present adequate renal reserve.

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Table 1. Body mass and renal function data from offspring of dams submitted to LPS during gestation: effects of simultaneous α-tocopherol treatment

	C	L	CT	LT
Body mass at birth, g	6.1 ± 0.1	4.9 ± 0.3*	6.0 ± 0.1	5.5 ± 0.4
Body mass at weaning, g	43.5 ± 2.1	44.5 ± 2.2	49.0 ± 2.3	44.6 ± 1.9
Body mass at 120 days of age, g	330 ± 14	325 ± 20	324 ± 10	324 ± 13
Water intake, mL/100 g per 24 h	17.6 ± 2.3	18.9 ± 1.8	19.1 ± 2.8	18.4 ± 1.8
Food intake, g/100 g per 24 h	9.0 ± 1.3	9.1 ± 0.8	8.5 ± 0.4	8.1 ± 0.4
Diuresis, mL/100 g per 24 h	5.8 ± 1.7	5.7 ± 0.7	6.5 ± 1.1	6.8 ± 0.9
Creatinine clearance, mL/100 g per min	0.30 ± 0.05	0.26 ± 0.03	0.24 ± 0.02	0.29 ± 0.03
Proteinuria, mg/100g per 24 h	115 ± 7	185 ± 6***	125 ± 8	138 ± 16††

Results are presented as mean ± SEM. The groups were rats obtained from control dams (C) or from dams submitted to lipopolysaccharide (L; 0.5 mg/kg body mass, subcutaneous) during gestation in the absence or in the presence of simultaneous α-tocopherol treatment (350 mg/kg body mass, by gavage; CT and LT). Differences between experimental groups were compared by two-way ANOVA followed by Bonferroni test: *P<0.05 and ***P<0.001 vs. C; ††P<0.01 vs. L.

Table 2. Body mass and renal function data from offspring of dams submitted to LPS during gestation: effects of ureteral unilateral obstruction and apocynin treatment

	Before UUO		14 days after UUO			
	C	**L**	**C**	**L**	**CA**	**LA**
Body mass, g	340 ± 5	341 ± 5	333 ± 8	336 ± 5	328 ± 9	339 ± 4[a]
Water intake, mL/100 g per 12 h	9.1 ± 0.4	8.0 ± 0.3	12.6 ± 1.1	10.9 ± 0.4	11.8 ± 0.9	10.7 ± 0.1
Food intake, g/100 g per 12 h	3.9 ± 0.3	3.5 ± 0.2	4.4 ± 0.4	4.2 ± 0.3	5.7 ± 0.3	5.3 ± 0.4
Diuresis, mL/100 g per 12 h	2.5 ± 0.1	2.6 ± 0.1	5.4 ± 1.0	4.4 ± 0.5	4.3 ± 0.5	5.1± 0.5
BUN, mg/dL	39.5 ± 1.0	39.9 ± 0.3	51.3 ± 2.4	52.8 ± 0.9	53.1 ± 1.9	52.1 ± 1.8
Creatinine clearance, mL/100 g per min	0.44 ± 0.03	0.38 ± 0.03	0.32 ± 0.03	0.31 ± 0.02	0.52 ± 0.03***	0.43 ± 0.02††
Proteinuria, mg/100 g per 12 h	13.1 ± 0.7	16.6 ± 0.7**	19.3 ± 1.6	25.6 ± 1.4*	17.7 ± 1.9	18.6 ± 1.4†

Results are presented as mean ± SEM. The groups were rats obtained from control dams (C) or from dams submitted to lipopolysaccharide (L) during gestation. The parameters were evaluated at 120-days-old rats, before and 14 days after the animals underwent unilateral ureteral obstruction (UUO), in the absence or in the presence of daily apocynin treatment (A; 100 mg/kg body mass, in drink water). Means are compared within the same condition (before UUO or 12 days after UUO). Differences between experimental groups were analyzed by unpaired Student *t* test (before UUO) or two-way ANOVA followed by Bonferroni test (after UUO): *P<0.05, **P<0.01 and ***P<0.001 vs. C; †P<0.05 and ††P<0.01 vs. L.

FIGURE 1. Maternal α-tocopherol prevents programming of increased blood pressure at adulthood in rats obtained from dams submitted to lipopolysaccharide during gestation. Systolic blood pressure (SBP) was measured by tail-cuff plethysmography in conscious rats. The groups were 120-days-old rats obtained from control dams (C) or from dams submitted to lipopolysaccharide (LPS; 0.5 mg/kg body mass, subcutaneous) during gestation in the absence or in the presence of simultaneous α-tocopherol treatment (350 mg/kg body mass, by gavage; C+α-Toc and LPS+α-Toc. Differences between experimental groups were compared by two-way ANOVA followed by Bonferroni test: *******P<0.001 vs. C; †††P<0.001 vs. L, §§§P<0.001 vs CT.

FIGURE 2. Hypertension programmed by maternal inflammation is not potentiated by ureteral unilateral obstruction (UUO), however it is reversed by apocynin treatment in adulthood. The groups were rats obtained from control dams (C) or from dams submitted to lipopolysaccharide (L) during gestation. The systolic blood pressure (SBP) was evaluated at 120-days-old rats, before and 14 days after the animals underwent ureteral unilateral obstruction (UUO), in the absence or in the presence of daily apocynin treatment (A; 100 mg/kg body mass, in drink water). Continuous lines represent groups that were not treated with apocynin, while dashed lines represents apocynin treatment. Results are presented as mean ± SEM. Before UUO, differences between C and L groups were compared by unpaired *t* test: * P < 0.05. After UUO and apocynin treatment, differences between groups were compared by two-way ANOVA followed by Bonferroni test: *******P<0.001 vs. C; ††P<0.01 vs. L.

FIGURE 3. Evaluation of protecting effects of maternal α-tocopherol treatment on tubulointerstitial collagen deposition evaluated in renal cortex of 120-days-old rats submitted lipopolysaccharide-induced inflammation during intrauterine development. Collagen density was measured using Picro-Sirius staining method and the groups are the same as detailed in Figure 1. Upper panel shows the higher staining intensity in tubulointerstitial area of LPS compared to C and LT groups. Lower panel presents the quantification of collagen expressed as percentage of tubulointerstitial area. Results are presented as mean ± SEM. Differences between experimental groups were compared by two-way ANOVA followed by Bonferroni test: ******P<0.01 vs. C; ††P<0.01 vs. L.

FIGURE 4. Contralateral kidney from rats obtained from dams submitted to inflammation during gestation presents a higher sensibility to collagen deposition induced by ureteral unilateral obstruction (UUO) surgery that is not observed in obstructed kidney. Collagen density was measured using Picro-Sirius staining method in renal cortex of obstructed kidney (A) and contralateral kidney (B) from 120-days-old rats 14 days after UUO. The groups are the same as detailed in Figure 5. The upper part of each panel shows representative micrographs of Picro-Sirius staining observed in obstructed (A) and contralateral (B) kidney from all groups. The lower part of panels A and B presents the quantification of collagen expressed as percentage of tubulointerstitial area. Results are presented as mean ± SEM. Differences between experimental groups were analyzed by two-way ANOVA followed by Bonferroni test: *P<0.05 and ******P<0.01 vs. C; †P<0.05 and ††P<0.01 vs. L.

FIGURE 5. Maternal inflammation induces upregulation of NOX2 expression in renal cortex of adult offspring. The groups are the same as detailed in Figure 1. The protein expression was evaluated by immunoblotting and the upper part of panel shows representative images of a single gel. The results are mean ± SEM of densitometric evaluations expressed as percentage of C group (%).Differences between experimental groups were compared by two-way ANOVA followed by Bonferroni test: *P<0.05 vs.C; †P<0.05 vs. L.

FIGURE 6. Maternal inflammation induces increment of superoxide anions production in obstructed and contralateral kidney from 120-days-old rats submitted to ureteral unilateral obstruction (UUO). Superoxide anions production was evaluated in obstructed (A) and contralateral (A) kidneys 14 days after UUO, by measurement of luminescence emitted by lucigenin oxidation under basal conditions and in the presence of NADPH stimulus. The groups are the same as detailed in Figure 5. Results are presented as mean ± SEM of relative light units (RLU). Differences between experimental groups were analyzed by two-way ANOVA followed by Bonferroni test: *P<0.05, ******P<0.01 and *******P<0.001 vs. C; †P < 0.05 and †††P < 0.001 vs. L, §P < 0.05 and §§§P < 0.001 vs. CA.

FIGURE 7. Maternal endotoxemia upregulates TGF-β expression in renal cortex of adult offspring. The groups are the same as detailed in Figure 1. The protein expression was evaluated by

immunoblotting and the upper part of panel shows representative images of a single gel. The results are mean ± SEM of densitometric evaluations expressed as percentage of C group (%).Differences between experimental groups were compared by two-way ANOVA followed by Bonferroni test: **P<0.01 vs. C.

FIGURE 8. Maternal inflammation during gestation programs changes in key modulators of renal extracellular matrix degradation, which a prevented by simultaneous α-tocopherol treatment. The groups are the same as detailed in Figure 1. The activities of matrix metalloproteinase-2 and 9 (MMP-2 and -9) were measured by gelatin zymography (**A** and **B**). The expression of MMP-2 and MMP-9, and their tissular inhibitors, TIMP-2 and TIMP-1, respectively, were evaluated by immunoblotting (**C–F**). Zymography representative images of a single gel are presented in upper part of panels **A** and **B** in grey-scale. Upper part of panels **C–F** shows representative images from immunoblotting in a single gel. The results are mean ± SEM of densitometric evaluations expressed as percentage of C group (%). Differences between experimental groups were compared by two-way ANOVA followed by Bonferroni test: *P<0.05 and **P<0.01 vs. C; †P<0.05 vs. L, §P<0.05 vs. CT.

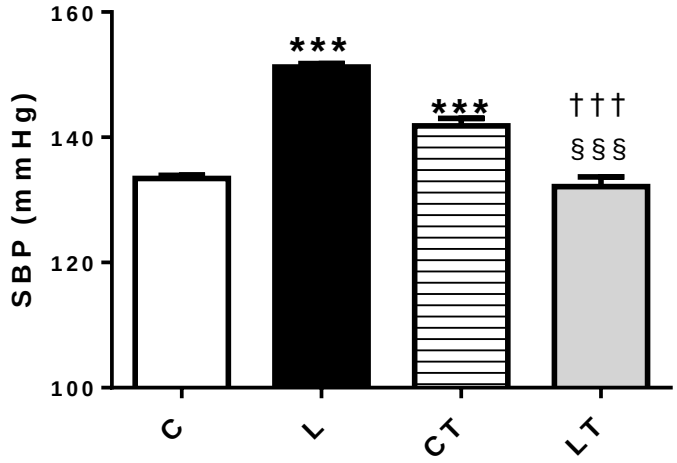
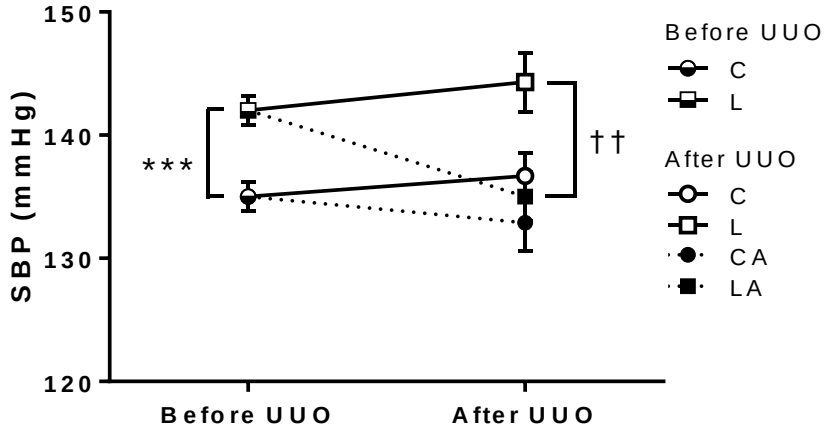


FIGURE 1. Farias *et al.*



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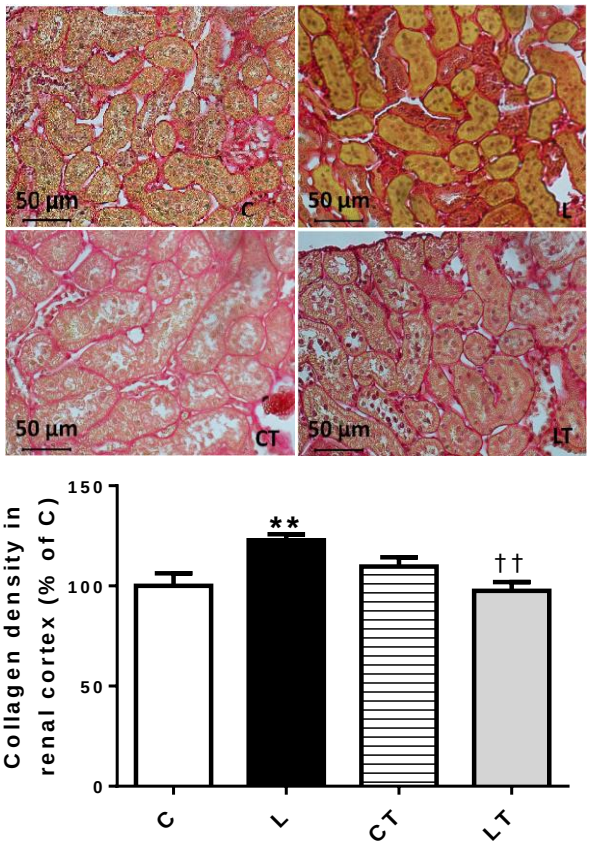

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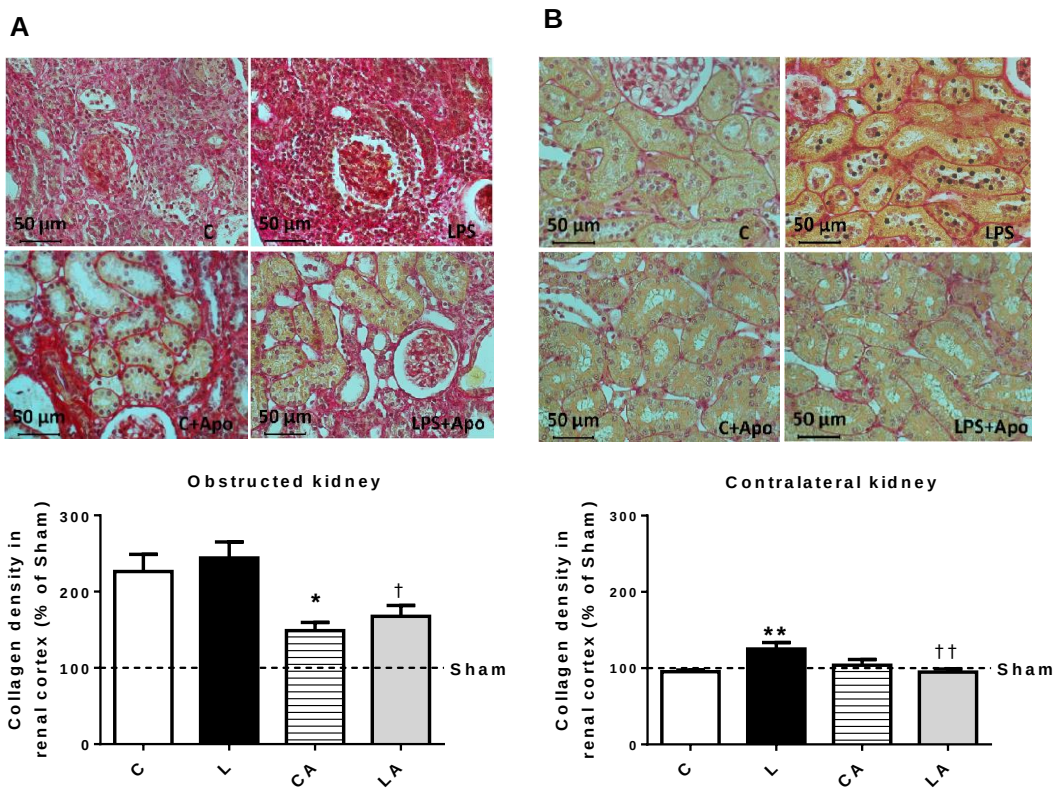

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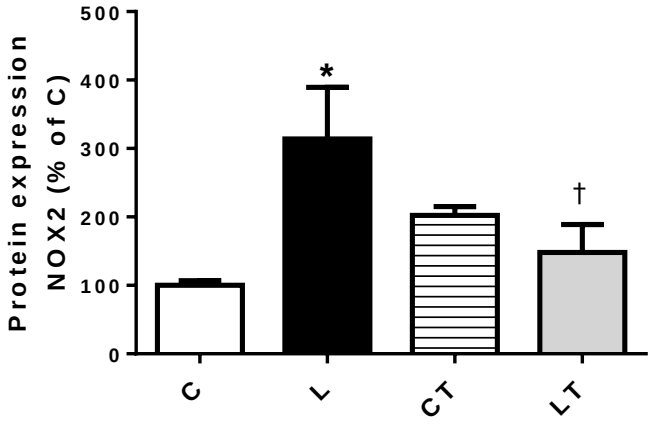

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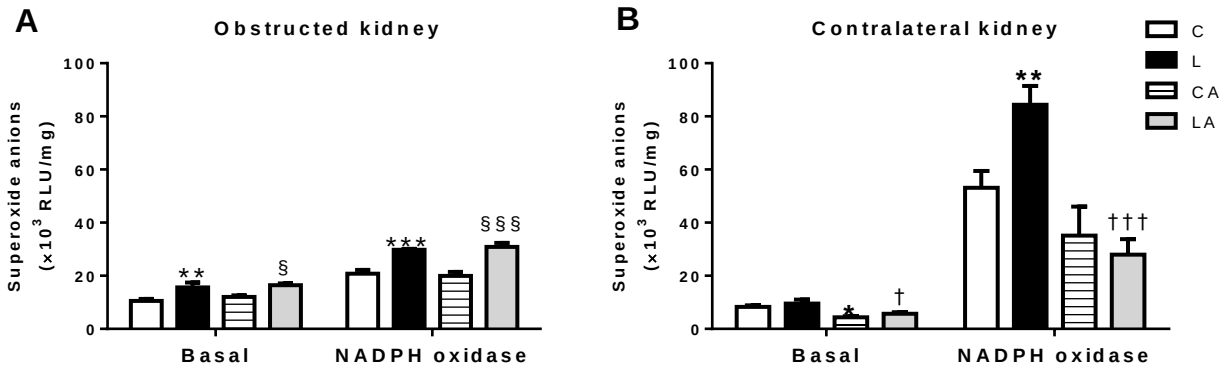

FIGURE 6. Farias *et al.*

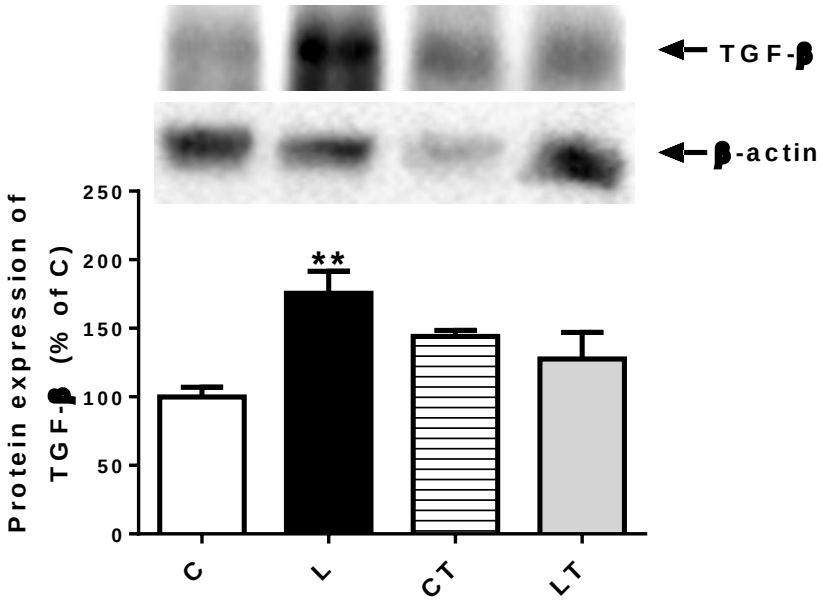

FIGURE 7. Farias *et al.*

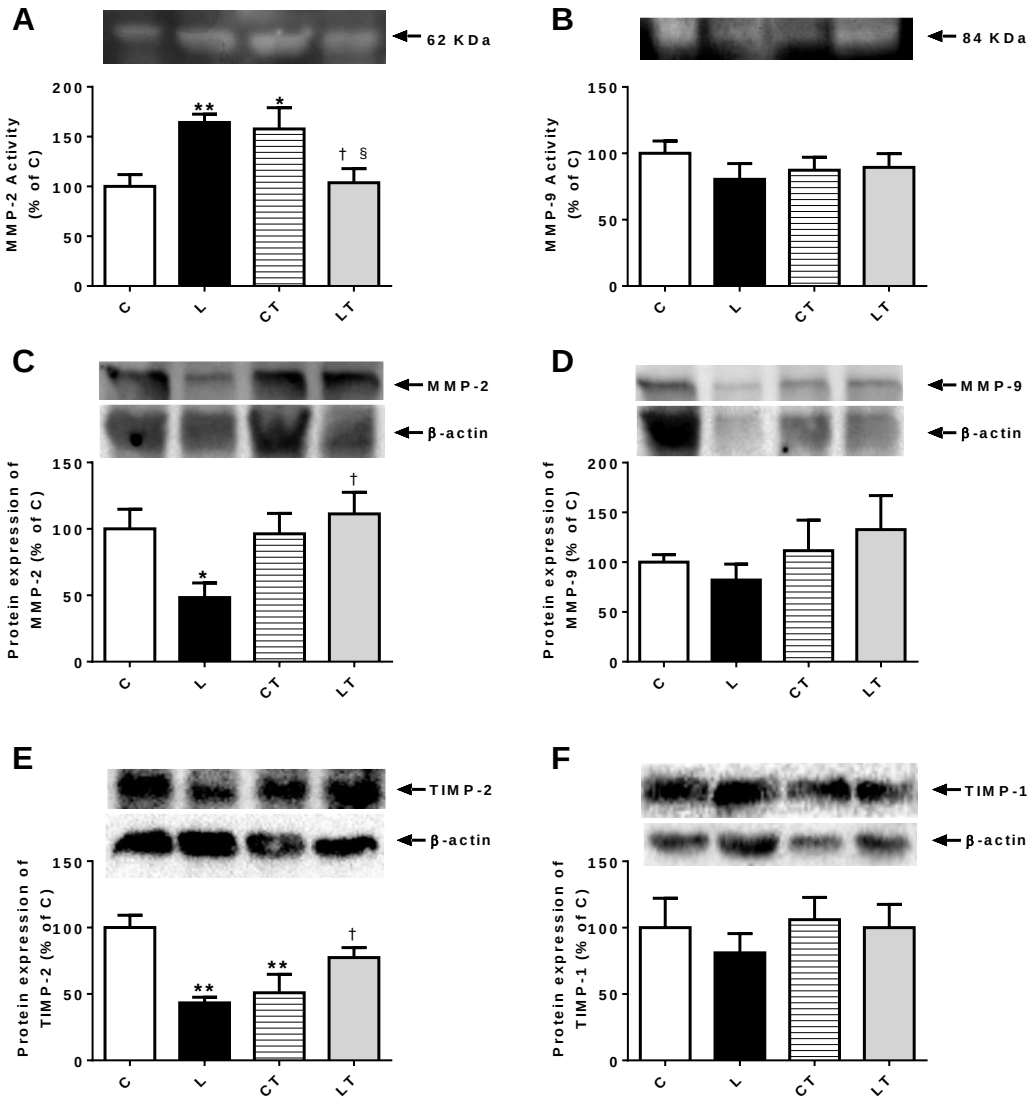

FIGURE 8. Farias *et al.*

3.1.1 Dados suplementares do artigo 1

SUPPLEMENTARY DATA

Maternal endotoxemia induces renal collagen deposition in adult offspring: role of NADPH oxidase/TGF-β1/MMP-2 signaling pathway

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Table S1. Effects of ureteral unilateral obstruction on body mass and renal function data from offspring of control dams

	Before UUO	Sham	UUO
Body mass, g	340 ± 5	331 ± 15	333 ± 8
Water intake, mL/100 g per 12 h	9.1 ± 0.4	12.4 ± 2.2	12.6 ± 1.1
Food intake, g/100 g per 12 h	3.9 ± 0.3	5.3 ± 0.4	4.4 ± 0.4
Diuresis, mL/100 g per 12 h	2.5 ± 0.1	3.7 ± 0.8	5.4 ± 1.0
BUN, mg/dL	39.5 ± 1.0	43.8 ± 1.8	51.3 ± 2.4*
Creatinine clearance, mL/100 g per min	0.44 ± 0.03	0.44 ± 0.03	0.32 ± 0.03*
Proteinuria, mg/100 g per 12 h	13.1 ± 0.7	8.7 ± 0.4	19.3 ± 1.6***

Results are presented as mean ± SEM. The groups were rats obtained from control dams, that were submitted to unilateral ureteral obstruction (UUO). The effect of UUO were compared to data obtained from rats submitted to a sham surgery. The parameters were evaluated at 120-days-old rats, before and 14 days after the animals underwent UUO/sham surgery. Results are presented as mean ± SEM. Differences between experimental groups were analyzed by Student *t* test: *P<0.05 and ***P<0.001 vs. Sham.

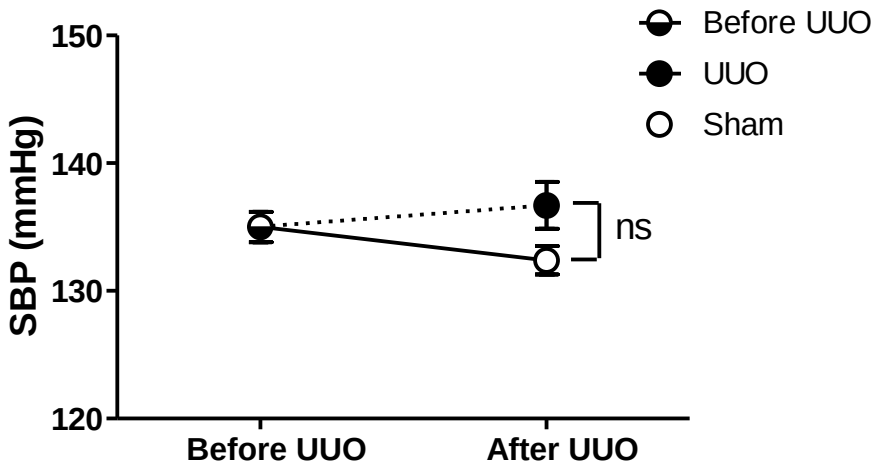

FIGURE S1. Unilateral ureteral obstruction (UUO) does not elevate systolic blood pressure (SBP) in control rats. SBP was measured by tail-cuff plethysmography in conscious rats. The groups were rats obtained from control dams, that were submitted to UUO (continuous line) or to sham surgery (dashed line) on 120-days-old rats. Results are presented as mean ± SEM. Differences between experimental groups were analyzed by Student *t* test; ns = not significant.

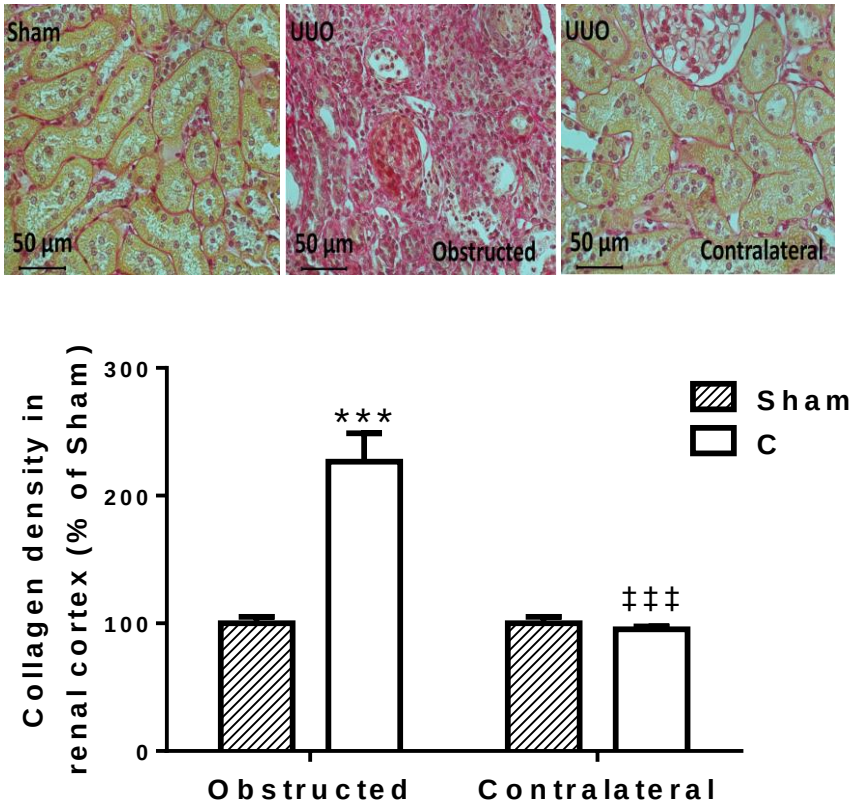

FIGURE S2. Tubulointerstitial collagen deposition induced by unilateral ureteral obstruction (UUO) evaluated in renal cortex on 120-days-old rats obtained from control dams. Collagen density was measured using Picro-Sirius staining method and the groups are the same as detailed in Figure S1. Upper panel shows the higher staining intensity in tubulointerstitial area of obstructed kidney from UUO rats compared to contralateral kidney or to Sham group. Lower panel presents the quantification of collagen expressed as percentage of Sham group (%). Results are presented as mean ± SEM. Differences between experimental groups were analyzed by Student *t* test: ***P<0.001 vs. Sham, ‡‡‡P < 0.001 vs. obstructed kidney.

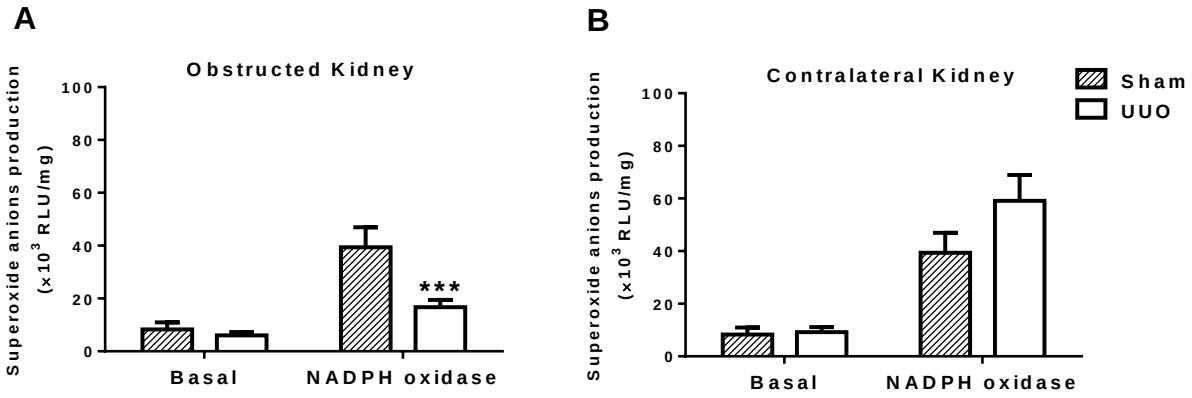

FIGURE S3. Superoxide anions production was evaluated on obstructed (A) and contralateral (A) kidneys 14 days after UUO, by measurement of luminescence emitted by lucigenin oxidation under basal conditions and in the presence of NADPH stimulus. The groups are the same as detailed in Figure S1. Results are presented as mean ± SEM. Differences between experimental groups were analyzed by Student *t* test: ***P<0.001 vs. Sham.

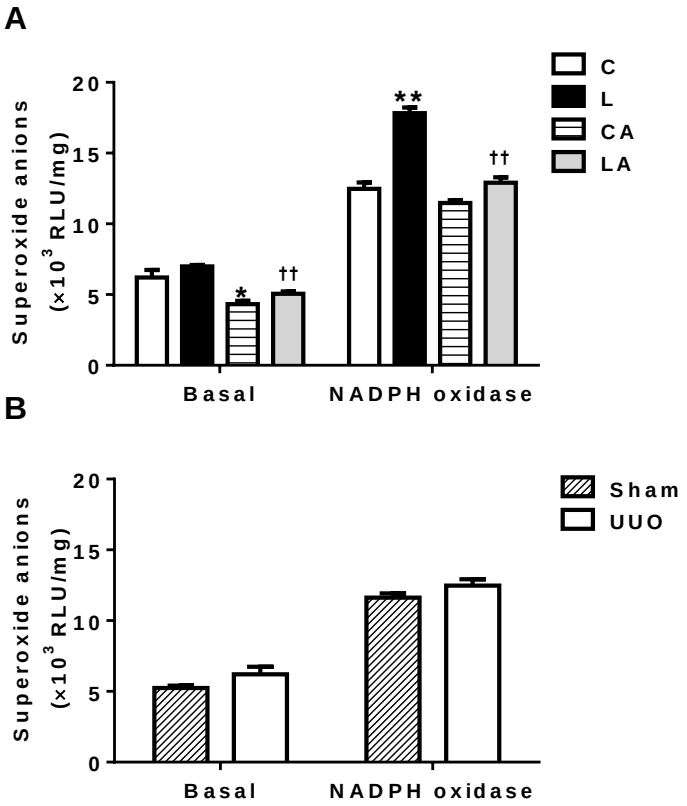

FIGURE S4. Maternal endotoxemia programs elevation of NADPH oxidase activity in the liver from adult offspring submitted to ureteral unilateral obstruction (UUO). A: Superoxide anions production was evaluated in the offspring obtained from control and lipopolysaccharide (L)-treated dams 14 days after they were submitted to UUO in the absence (C and L groups) or in the presence of apocynin (CA and LA groups). B: Effects of UUO in the hepatic superoxide levels. Superoxide anions production was evaluated by measurement of luminescence emitted by lucigenin oxidation under basal conditions and in the presence of NADPH stimulus. Maternal administration of LPS was performed (0.5 mg/kg body mass, subcutaneous) on days 13, 15, 17 and 19 of gestation. The apocynin was administrated daily in drinking water (100 mg/kg body mass). Results are presented as mean ± SEM. Painel A: differences between groups were compared by two-way ANOVA followed by Bonferroni test; Painel B: differences between C and Sham groups were compared by unpaired *t* test: *P<0.05 and **P<0.01 vs. C; ††P < 0.01 vs. L.

3.2 ARTIGO 2

MESENCHYMAL STEM CELLS (MSCS) MODULATE PRO-INFLAMMATORY, PRO-OXIDATIVE AND PRO-FIBROGENIC MEDIATORS IN RENAL INJURY: IMPACT OF THE MATERNAL ENDOTOXEMIA IN THE PROTECTIVE EFFECTS OF MSCS

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ABSTRACT

Background. Renal fibrosis is a hall mark of chronic kidney disease (CKD) and is correlated to progressive loss of renal function. Therapies that slow CKD progression are still limited, and due to their protective function, the use of mesenchymal stem cells (MSCs) could be a potential alternative. However, MSCs function may be affected by many adverse conditions. It was investigated whether MSCs could present protective effects upon fibrotic renal injury through modulation of extracellular matrix remodeling, and, additionally, whether maternal endotoxemia could impairs protective capacity of adult MSCs against renal injury. **Methods.** MSCs were isolated from femoral bone marrow mononuclear cells withdrawn from control adult rats and from adult rats obtained from dams submitted to lipopolysaccharide (LPS) during gestation. MSCs obtained from both groups were administered via tail vein in rats submitted to renal injury by unilateral ureteral obstruction (UUO). After 14 days, kidneys were removed to evaluate collagen deposition and the expression of pro-fibrotic, pro-oxidative and pro-inflammatory markers. **Results.** UUO decreased creatinine clearance and increased proteinuria. In the obstructed kidney, it was observed increased collagen deposition, superoxide production and upregulation of TGF-b1, TNF-a and IL-6 protein expression. On the other side, contralateral kidney did not present higher collagen density, but presented upregulation of TGF-b1, TNF-a and IL-6, in parallel to increased NAPDH oxidase activity. Moreover, both kidneys presented increased MMP-2 activity and protein expression. MSCs treatment, independent of their source, prevented creatinine clearance decrement, in parallel to prevention of collagen deposition and upregulation of TGF-b1, IL-6 and MMP-2 in obstructed kidney. In contralateral kidney, MSCs prevented increased NADPH oxidase activity and upregulation of TGF-b1, TNF-a and IL-6. On the other side, increased MMP-2 expression in contralateral kidney-induced by UUO was only prevented by MSCs obtained from control rats. **Conclusions.** MSCs administration may protect renal injury induced by UUO, through modulation of oxidative, inflammatory and fibrogenic pathways in both kidneys, leading to better preservation of renal function. Maternal endotoxemia induced subtle alterations of MSCs protective effects on renal injury, that do not seems to compromise their response capacity to acute injuries, but may be important in later events.

Keywords: inflammatory mediators, intrauterine inflammation, mesenchymal stem cells, oxidative stress, renal fibrosis

INTRODUCTION

Chronic kidney disease (CKD) is an important clinical issue, which prevalence is 8-16% worldwide (JHA et al., 2013). CKD is characterized by a pronounced reduction of glomerular filtration rate and increased urinary albumin excretion (JHA et al., 2013). Renal fibrosis is a hall mark of CKD, characterized by intense extracellular matrix (ECM) deposition that leads to loss of renal function (WYNN; RAMALINGAM, 2013). In turn, renal function impairment leads to compensatory glomerular hypertrophy of the remaining functional nephron, through glomerular ECM expansion and mesangial cell proliferation, which potentiates fibrosis and progressive feature of renal disease (LI et al., 2016).

ECM remodeling is linked to the appropriate balance between its production and degradation (BARNES; GORIN, 2011; KEELING; HERRERA, 2008). Renal ECM production depends of several cells types, such as mesangial cells, fibroblasts and pericytes, as wells as, of epithelial-mesenchymal transition (EMT) of podocytes and tubular cells (BARNES; GORIN, 2011; YU et al., 2016). The degradation of ECM in the kidney is mainly achieved through matrix metalloproteinases (MMP) activity (KEELING; HERRERA, 2008), and among the several isoforms, MMP-2 and MMP-9 importance is remarkable in renal injury (CAVDAR et al., 2017). TGF-β is a fibrogenic cytokine that has a pivotal role in ECM deposition through regulation of its production and degradation (HU et al., 2016; LI et al., 2014). Moreover, increased production of reactive oxygen species (ROS) is an underlying factor to altered ECM remodeling (BEN YOSEF et al, 2002). In the kidney, ROS is associated to TGF-β upregulation (WAN et al., 2016), as well as, altered MMPs expression (BEN YOSEF et al., 2002; KARANOVIC et al., 2016).

Therapies that slow the progression of CKD have been widely studied over the years, but in clinical practice they remain limited. Blood pressure control using angiotensin converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARBs) and glycemic control in diabetic patients are the most commonly used therapies to delay the progression of CKD (TURNER et al., 2012). These therapeutic approaches present anti-fibrotic effects, however, none of them are effective in stopping the progression of CKD (TURNER et al., 2012). Exogenous administration

of mesenchymal stem cells (MSCs) is one of the most promising therapy against progressive feature of CKD (DA SILVA et al., 2015). These cells present the hability of self-renewing and multipotent cells that can be isolated from several tissues. It had been reported that MSCs lowers renal damage, in parallel to a decrease of EMT, collagen deposition, TGF-β expression and pro-inflammatory mediators in renal tissue (BAI et al., 2013; DA SILVA et al., 2015; SUN et al., 2013).

Nevertheless, the regenerative and immunomodulatory capacity of MSCs may be impaired by some situations. It has been seen that MSCs isolated from patients with progressive multiple sclerosis present less the potential for expansion, premature senescence and reduction of their neuroprotective potential (REDONDO et al., 2018). Meng et al. showed that obesity alters mitochondrial function in MSCs and limits their functions (MENG et al 2018). However, it still unclear whether the regenerative and protective capacity of MSCs can be impaired by intrauterine adverse environment. Maternal inflammation had been clearly described as a factor that impairs renal function at adulthood (VIEIRA et al., 2018). We observed that adult rats obtained from dams submitted to endotoxemia presents increased renal collagen deposition, simultaneously to increased TGF-β1 expression, MMP-2 activity and ROS production (data not published).

Based on these data, it may be argued that protective effects of MSCs over renal fibrosis and renal injury occurs in parallel to modulation of oxidative stress, inflammation and MMPs function. In the present study, we investigated whether MSCs could protect renal injury induced by unilateral ureteral obstruction (UUO), and whether this treatment could prevent the changes provoked in TNF-α, interleukin-6 and MMP-2 and MMP-9 activities. Additionally, we investigated whether maternal endotoxemia could impairs protective capacity of adult MSCs against the renal injury.

MATERIALS AND METHODS

Ethical considerations

All protocols and procedures using animals were carried out in accordance with Committee for Experimental and Animal Ethics at Universidade Federal de Pernambuco (nº 23076.060473/2014-91).

Materials

Lipopolysaccharides from *Escherichia coli* 0111:B4, 1,1,3,3-tetraethoxy-propane, trypsin type II-S inhibitor, phenylmethanesulfonyl fluoride (PMSF), sodium duodecilsulphate (SDS), bovine serum albumin (BSA), Tris, Folin & Ciocalteu's phenol reagent, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), N,N′-Dimethyl-9,9′-biacridinium dinitrate (lucigenin), acrylamide, N,N′-Methylenebis(acrylamide), N,N,N′,N′-tetramethylethylenediamine, ammonium persulfate, glycine, triton x-100, direct red, Histopaque and trypan blue were purchased from Sigma-Aldrich (St. Louis, USA). Commercial kits for creatinine and urea measurement were from Labtest (Lagoa Santa, Brazil) and sodium thiopental was purchased from Cristália Produtos Químicos Farmacêuticos (Itapira, Brazil). EDTA, sodium chloride and potassium chloride were purchased from Vetec (Rio de Janeiro, Brazil). PVDF blotting membrane, horseradish peroxidase-conjugated anti-rabbit IgG antibody and ECL Prime Western blotting system were purchased from GE Healthcare (Buckinghamshire, UK). Anti-TGF-β and anti-β-actin antibodies were obtained from Santa-Cruz Biotechnologies (Dallas, USA) and peroxidase-conjugated anti- mouse was purchased from Abcam (Cambridge, UK). Anti TNF-α, Anti-IL-6. antibody were obtained from Immuny Biotechnology (Campinas, Brazil). Anti-MMP-2, anti-MMP9, anti-TIMP-1 and anti-TIMP-2 antibody were obtained from Merck Millipore (Armstadt, Germany). All other reagents were of the highest purity available. Tripsin-EDTA

(0,05%), Dulbecco's Modified Eagle Medium (DMEM) were obtained from Thermo Fisher (Waltham, USA).

Animals

Female Wistar rats, maintained in 12h cycle light/dark and at 23°C, were mated and pregnancy was determined by the presence of spermatozoids in the vaginal smear. After the beginning of gestation, the females were allocated in individual cages, with free access to water and food and randomly assigned to the experimental groups. In days 13, 15, 17 and 19 of pregnancy, dams were submitted to subcutaneous administration of lipopolysaccharide (LPS) (LPS – L, 0.5 mg/Kg of bodyweight) or 0.9% NaCl solution (Control – C, 1 mL/Kg) (GRACIARENA et al., 2010). After birth, the offsprings were culled up to 8 pups per mother, at 21 days of age, male rats were weaned to standard chow and maintained until adult life. At 120 days of age, male offspring (n=6 per group) was submitted to bone marrow withdrawal from the femurs, which were used to obtain mononuclear cells. Bone-marrow mononuclear cells (BMMCs) were maintained under in vitro cultivation to isolate MSCs.

Male Wistar rats ageing 120 days of life were submitted to renal fibrotic injury induced by unilateral ureteral obstruction (UUO). Immediately after the surgical procedure, the rats received caudal vein administration of 1×10^5 MSCs (viability of 92.8 ± 1.7%) obtained from Control (n=6) or LPS (n=6) rats. Part of the rats, were submitted to UUO and received only administration of phosphate buffered saline (PBS, 100 µL). Moreover, one group of rats were submitted to a simulation of UUO surgery with no ligation of ureter (Sham group, n=5). After 14 days of the UUO, the rats were submitted to evaluation of systolic blood pressure (SBP) and allocated in metabolic cage for collection of urine and blood samples. Subsequently, the rats were anesthetized with sodium thiopental (intraperitoneal, 60 mg/kg of body weight) to removal of obstructed and contralateral kidneys. In the renal cortex it was evaluated collagen density, basal and NOX-stimulated superoxide production plus protein expression of fibrogenic and inflammatory mediators. The rats were euthanized by exsanguination while still under anesthesia.

Extraction of Brown Marrow Mononuclear Cells (BMMCs) and isolation of MSCs

BMMCs from femurs were isolated using Histopaque® density gradient. Briefly, femurs were secctioned in both epiphysis regions, and bone marrow were withdraw by flushing low-glucose DMEM (Dulbecco's Modified Eagle's Medium; supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml)) into the bone. Then, the suspension of bone marrow cells were submitted to centrifugation (400 g) after addition of 1 volume of Histopaque®. After centrifugation, it was obtained a ring rich in mononuclear cells in the phase gradient, which was transferred to another tube, and washed 2 times in DMEM. Finally, the cells were placed in plastic bottles of culture containing DMEM with 20% fetal bovine serum (FBS), and cultured until reaching confluence of approximately 90%. At each confluence, the cells were detached from the culture bottles using 0.05% trypsin and transferred to another bottle. This procedure were carried out for 5 or 6 times to ensure isolated population of MSCs. Then, the cells were detached from the plastics bottles, diluted in PBS ($1x10^6$ cells/mL) and administered into the caudal vein of rats.

Evaluation of systolic blood pressure and renal functional parameters

Food and water intake, creatinine clearance, blood urea nitrogen (BUN), proteinuria and systolic blood pressure (SBP) were evaluated in experimental groups 14 days after surgical procedure. Rats was allocated in metabolic cages (Tecniplast Gazzada Sarl, Buguggiate, Italy) per 12 h for quantification of food and water consumption, urine collection and subsequent blood withdraw from renal vein. Protein concentration in urine was measured by folin method (LOWRY et al., 1951). Serum urea levels and serum and urine creatinine levels were measured through commercial kit.

SBP was measured by tail-cuff pletysmography (IITC Life Science B60-7/16'', Life Science Instruments, Woodland Wills, CA) in conscious rats. The animals were

acclimated to the experimental conditions to obtain tail-cuff SBP for two consecutive days. The results of each animal represent an average of 3–5 measurements.

Glomerular morphometry and measurement of tubulointerstitial collagen density

To evaluate glomerular tuft area and collagen depositon, sections of obstructed and contralateral kidneys were fixed in methacarn and then prepared to obtain histological blocks of paraffin. Then, 6 µm slices of kidney were stained with Weigert' hematoxylin for 8 min, followed by 1 hour Picro-Sirius staining (direct red 80 diluted in saturated aqueous solutions of picric acid). Afterwards, the slices were washed in acidified water, vigorously stirred and slide mounted. Cortical tubulointerstitial areas were obtained in a trinocular microscope (Nikon, Eclipse Ni-U, Shanghai, China) coupled to a camera (DS-i1C, Nikon). Images were obtained from thirty cortical fields measuring 70,700 μm^2. Collagen density in tubulointerstitial area was determined using Image Pro Plus software (version 4.5.1, Media Cybernetics, MD), by a researcher that was unaware of groups being evaluated.

Glomerular tuft area was determined by measurement of cross-sectional area of 30 glomerulus identified in non-overlapping fields obtained from one section per kidney stained by Picro-Sirius. Glomerular tufts image were acquired using 400× magnification fields and, then were outlined to determine cross-sectional area using Axiovision LE software (version 4.8.1.0, Carl Zeiss Imaging Solutions GmbH, Munich, Germany).

Superoxide anions production and NADPH oxidase activity

Superoxide anion (O_2^-) was measured by lucigenin-oxidation derived chemiluminescence. Cortical samples of renal tissue were homogenized in PBS and then centrifuged at 12.000×*g* at 4°C, for 12 min. The supernatant was added to a solution containing 10 µM lucigenin and 100 µM NADPH and chemiluminescence was measured during 5 minutes in a luminometer (Varioskan Flash, Thermo Scientific, Vantaa, Finland). The relative lights units were correct by protein content

of the sample and represent NADPH oxidase activity. The same assay was also performed in the absence of NADPH and represent basal superoxide production. Each measurement was performed in triplicate.

Measurement of matrix metalloproteinase activities

MMPs activity was performed by gelatin zymography, after separation of renal cortex samples (50 µg protein) in a 7.5% polyacrylamide gel containing 0.1% gelatin under non-reducing conditions. The gels were washed twice for 30min in 2.5% Triton X-100 solution, and then incubated for 40 hours (50 mM Tris (pH 7.4), 200 mM NaCl and 10 mM $CaCl_2$) at 37°C, and, finally, gels were stained with Coomassie Blue. Gelatinase acitivies of MMPs were represented by clear bands visualized in contrast to a blue background. Colorimetric images of the bands were acquired using an image acquisition system (ChemiDoc MP Imager, Bio-Rad, Hercules, CA, USA). The activities of MMP-2 and MMP-9 were identified according to their respective molecular weight and the bands density were quantified using Image Lab software (version 5.2.1, Bio-Rad).

Western blotting

The evaluation of protein expression of fibrogenic and inflammatory mediators was performed by Western Blotting in renal cortex samples maintained at –80ºC. Samples were thawed in an ice bath and homogenized (1 g:5 ml) in ice-cold radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris, pH 8.0; containing 2 mM AEBSF, 1 mM EDTA, 130 µM bestatin, 14 µM E-64, 1 µM leupeptin and 0.3 µM aprotinin). Following, protein samples (80 µg) were separated by electrophoresis in 10% (MMP-2 and MMP-9) or 15% (TIMP-1, TIMP-2, TGF-β, TNF-α and IL-6) SDS-PAGE, and transferred onto PVDF membrane. The membranes were immunoblotted with antibodies to MMP-2 (1:2,000 dilution), MMP-9 (1:2,000 dilution), TIMP-1 (1:2,000 dilution), TIMP-2 (1:2,000 dilution), TGF-β (1:2,000 dilution), TNF-α (1:2,000 dilution) or IL-6 (1:500 dilution). The membranes were probed overnight and exposed for 1h to peroxidase-conjugated secondary antibody (1:10,000 dilution) at room

temperature. The immunodetection was performed through chemiluminescence using Amershan ECL Prime Western Blotting Detection Reagent and an image acquisition system (ChemiDoc MP Imager). The densitometric quantification of the bands was performed by Image Lab software (version 5.2.1, Bio-Rad Laboratories Inc., USA). The control of protein load was performed by immunoblotting the membranes to β-actin.

Statistical Analyses

Statistical differences between the experimental groups were assessed by one-way ANOVA followed by Newman–Keuls test. Normal distribution of data was confirmed by using the Shapiro-Wilk test. The analyses were performed using GraphPad Prism 5 (Version 5.01, GraphPad Software, Inc.). Results are presented as mean ± SEM and were considered different when $P<0.05$.

RESULTS

After 24 hours of ureteral obstruction, we evaluated BUN and serum creatinine levels to confirm whether obstruction altered renal function (Table 1). It was observed that rats submitted to UUO presented 60% higher ($P<0.05$) BUN nearly three times higher ($P<0.01$) serum creatinine levels compared to Sham rats. Both groups of UUO rats treated with MSCs presented similar BUN to non-treated UUO rats. On the other side, serum creatinine was lower in MSCs-treated than non-treated rats, albeit this parameter remained higher than Sham group.

In Table 1, it is also presented metabolic and renal function data evaluated 14 days after UUO. The groups presented similar body weight, water and food intake and diuresis. Rats submitted to UUO presented increased serum creatinine (80%, $P<0.01$) and BUN (60%, $P<0.05$) 14 days after surgery. Additionally, it was observed that UUO elevated proteinuria at more than two times ($P<0.001$) and decreased by 40% ($P<0.001$) creatinine clearance. On the other side, both groups of MSCs-treated rats presented levels of serum creatinine and creatinine clearance similar to Sham group and lower ($P<0.05$) than UUO non-treated rats. Proteinuria of MSCs-treated

rats was nearly 20% lower ($P<0.05$) than UUO group, but it still remained higher ($P<0.001$) than Sham group. Furthermore, in MSCs-treated rats, BUN was similar to Sham group, but it was also similar to UUO non-treated rats. SBP was not affected by neither UUO either MSCs.

In addition to altered renal function, represented by the changes in urinary and serum biomarkers, UUO rats presented elevation of more than 50% ($P<0.001$) in obstructed kidney weight and of nearly 20% ($P<0.01$) in contralateral kidney (Table 2). Neither MSCs obtained from control rats, either MSCs obtained from LPS offspring, influenced kidney weight elevation-induced by UUO. Despite increased weight, it was observed that the cross-sectional area of glomerular tufts was reduced by 15% ($P<0.05$) in obstructed kidney of non-treated rats. On the other side, rats submitted to UUO and treated with control MSCs presented glomerular tuft area similar to sham group and higher than UUO group. However, rats treated with MSCs obtained from LPS-offspring presented glomerular tuft area similar to UUO group and lower than Sham group. Albeit there was hypertrophy in contralateral kidney of UUO rats, glomerular tuft area was similar between all experimental groups.

Renal injury induced by UUO presents collagen deposition as key feature. In the present study, obstructed kidney presented collagen deposition more than 2 times higher than Sham group (Figure 1C). The treatment with MSCs, independently of its origin, decreased by 40% ($P<0.05$) tubulointerstitial collagen staining in obstructed kidney. In contralateral kidney, tubulointerstitial collagen deposition was similar between groups (Figure 1D).

In the same way as UUO increased collagen deposition, it also increased ($P<0.01$) superoxide levels in obstructed kidney (Figure 2A). This change was not prevented by MSCs administration. On the other side, NADPH oxidase activity was decreased in obstructed kidneys, while MSCs prevented this alteration partially (Figure 2A). In the contralateral kidney, both basal superoxide production and NADPH oxidase activity were increased ($P<0.001$) in UUO group (Figure 2B), and these alterations were prevented by MSCs administration, independently whether the cells were from control or LPS rats.

Additionally to present ROS elevation, rats submitted to UUO presented increased protein expression ($P<0.05$) of TGF-β, TNF-α and IL-6 in both obstructed and contralateral kidney (Figure 3). Treatment with MSCs prevented the increased

expression of TGF-β and IL-6 in obstructed and contralateral kidney. Moreover, MSCs from both groups also prevented TNF-α upregulation in contralateral kidney, albeit it did not prevented increased TNF-α expression in obstructed kidney.

As ROS and TGF-β are likely to regulate collagen deposition by modulation of MMPs, MSCs effects on UUO-induced renal fibrosis could be partially due to regulation of the activity of these enzymes. It was observed that ureteral obstruction increased MMP-2 activity in renal cortex by nearly 4-times ($P<0.001$), while it lowered MMP-9 activity by 50% ($P<0.001$) (Figure 4A and C). MSCs administration, independently of the source, blunted approximately by 50% the elevation of MMP-2 activity induced by UUO in renal cortex, however their levels remained higher than that observed in sham group. MSCs treatment did not influence MMP-9 activity in the obstructed kidney. In contralateral kidney, UUO also induced elevation of MMP-2 activity by 30% ($P<0.01$), while that, in contralateral kidney from both MSCs-treated rats, MMP-2 activity was similar to Sham group and lower than non-treated rats (Figure 4B). The MMP-9 activity in contralateral kidney was not changed by UUO or by MSCs treatment (Figure 4D).

Further to the observed changes in MMP-2 activity, it was also observed that UUO induced upregulation of MMP-2 expression in both obstructed and contralateral kidney (Figure 5A and 5B). On the other side, UUO decreased protein expression of MMP-9 only in obstructed kidney (Figure 5C). MSCs obtained from control rats prevented MMP-2 upregulation in both kidneys, while MSCs from LPS rats prevented only the upregulation of MMP-2 in obstructed kidney. In a similar way to the observed effects in MMP-9 activity, none of the MSCs treatments prevented MMP-9 downregulation in the obstructed kidney (Figure 5C). In contralateral kidney, neither UUO either MSCs altered MMP-9 expression (Figure 5D).

We also evaluated TIMP-2 and TIMP-1 expression, once they are pivotal in regulation of MMP-2 and MMP-9 activity, respectively (Figure 6). In addition to elevation of MMP-2 expression, UUO also increased TIMP-2 protein expression in obstructed kidney (Figure 6A). The administration of MSCs from control and LPS offspring prevented also TIMP-2 upregulation (Figure 6A). The expression of TIMP-2 in contralateral kidney and the expression of TIMP-1 in obstructed and contralateral kidneys were not affected by UUO, even were influenced by MSCs administration (Figure 6B, C and D).

COMMENT

Our study demonstrated that MSCs therapy may protect renal injury induced by UUO, through modulation of oxidative, inflammatory and fibrogenic pathways. It was reported that MSCs lowered cortical collagen deposition in parallel to lowering of IL-6 and TGF-β expression in the obstructed kidney. In the contralateral kidney, MSCs also lowered IL-6, TNF-α and TGF-β expression, as well as lowered superoxide production and NADPH oxidase activity. Additionally, it was demonstrated that MSCs lowered MMP-2 expression and activity in the obstructed kidney and this could be an important effect on modulation of ECM remodeling. It is noteworthy wich MSCs of control and LPS rats presented similar protective effects among almost all the parameters evaluated, with exception to MMP-2 expression decrease in contralateral kidney, which was only induced by MSCs obtained from control rats.

Renal hypertrophy observed in the obstructed kidney may be correlated with increase tubulointerstitial area, although glomerular tuft is atrophic, as previously described (PENG et al., 2013). In contrast, UUO is linked to compensatory hypertrophy of the contralateral kidney that was not accompanied by alteration of glomerular tuft area. In the present study, we observed that compensatory hypertrophy of contralateral kidney was not able to maintain renal function. UUO rats presented elevation of serum creatinine and BUN, and proteinuria, while creatinine clearance was decreased, indicating the presence of glomerular damage and decreased glomerular filtration rate (Table 1). Although MSCs did not prevent renal hypertrophy, it prevented the UUO-induced proteinuria, lowering of creatinine clearance, and elevating BUN and serum creatinine. According to the present data, MSCs protection may be associated to the inhibition of tubulointerstitial fibrosis. Moreover, MSCs protective mechanisms had be associated to blunt of podocyte loss and glomerular capillary rarefaction, which leads to peritubular epithelial cell dysfunction and tubulointerstitial fibrosis (ROTA et al., 2018).

MSCs may also be associated to protection of renal injury through antioxidative mechanisms. Oxidative stress is a key factor for the development of

renal fibrosis (GUIMARÃES-SOUZA et al., 2015). It was observed that ROS formation was higher in contralateral kidney of UUO rats, and this change was prevented by MSCs. This effect of MSCs may be correlated to better maintenance of renal function due renal injury protection, as observed by creatinine clearance and proteinuria. It may be hypothesized also that in contralateral kidney NADPH oxidase presents an important role, once its activity was inhibited by MSCs. In the obstructed kidney, oxidative stress may also be an underlying mechanism of renal injury, however the protective effects of MSCs seems to be not related to antioxidant mechanisms, once basal superoxide levels were not impacted. NADPH oxidase-mediate ROS production may modulate ECM deposition by direct regulation of production and degradation of ECM components, as wells as, regulation of pro-fibrogenic signaling (BONDI et al., 2010; ZHAO et al., 2008).

Another possible explanation to protective effects of MSCs on obstructed kidney is the modulation of the pro-fibrogenic cytokine TGF-β and of the pro-inflammatory mediators TNF-α and IL-6. TGF-β has a pivotal role in ECM remodeling through mechanisms dependent of ROS (BONDI et al., 2010) but also acts through its canonical Smads-dependent signaling pathway (WANG et al., 2018). We observed that TGF-β upregulation induced by UUO was prevented by MSCs administration. It had been show that pharmacological inhibition of TGF-β in rats submitted to UUO decreases expression of α-SMA, collagen and fibronectin, thus preventing renal fibrosis (HU et al., 2016). Moreover, IL-6 may also be a stimulatory mediator of fibrosis through increased trafficking of TGF-β1 receptors and augmented TGF-β1 signaling (Zhang et al., 2005). In the contralateral kidney, we may speculate that the protective effects of MSCs also occurs in part through modulation of TNF-α expression, which is also known to be a modulator of TGF-β signaling (Khan et al., 2005).

Once TGF-β can also modulate ECM deposition through regulation of expression of MMPs and TIMPs (KEELING; HERRERA, 2008), we also investigated whether MSCs could regulate activity and protein expression of MMP-2 and MMP-9, and protein expression of their endogenous inhibitors, TIMP-2 and TIMP-1, respectively. These MMPs are critical to renal ECM remodeling due to their role in matrix degradation, as well as, their role in regulation of cell proliferation, EMT and in

the activation of pro-inflammatory and pro-fibrogenic mediators (TAN et al., 2012). In this study, it is shown that UUO-induced renal injury occurred in parallel to increased activity of MMP-2 and decreased activity of MMP-9, as previously reported in other animal models of renal disease (IIMURA et al, 2004;). Moreover, it was also observed that UUO induced elevation of MMP-2 activity in contralateral kidney. Albeit, it was not observed increased collagen deposition in contralateral kidney, it may be wondered that this alteration enhances the risk of renal injury later in life (ZHANG et al., 2004).

The increased activity of MMP-2 in obstructed and contralateral kidneys can be related to their increased protein expression (compare Figure 4A and 5A). In contrast, the elevation of MMP-2 activity may not be explained by the increased TIMP-2 expression observed in obstructed kidney (compare Figure 4A and 6A). It may speculated that this change represents a counter-regulatory mechanism to the increased MMP-2 activity or to the increased collagen deposition. Furthermore, lowered MMP-9 activity in obstructed kidney may also be correlated to decreased protein expression (compare Figures 4C and 5C).

The protective effects of control-MSCs seems to be related mainly to recovery of normal MMP-2 function, as MSCs administration prevented UUO-induced elevation of expression and activity of this protease in both obstructed and contralateral kidney, while had no effect on MMP-9 expression. Additionally, in parallel to regulation of MMP-2 activity and collagen, MSCs also prevented increased expression of TIMP-2 in obstructed kidney, which reinforces its role as counter-regulatory mechanism to renal fibrosis.

This study also aimed to investigate whether protective effects of MSCs could be influenced by intrauterine endotoxemia, in the same way as others cells and organs present increased risk to injury due to maternal adverse events (ZHAO, 2008). It has already been seen that maternal obesity induces changes in the metabolism of umbilical cord MSCs that are related to increased postnatal obesity (BAKER II et al., 2017). In addition, maternal obesity lowers glucose metabolism, promotes insulin resistance, and increases the senescence of osteogenic progenitor cells in the offspring, and can programmes osteoporosis and other chronic disorders (CHEN et al., 2016). Here, it was observed that MSCs obtained from control and LPS-rats presented similar protective effects in renal fibrosis and in several of pro-

inflammatory and pro-fibrogenic mediators studied. However, it was found some differences between the effects of the MSCs treatments. It was observed that control MSCs were able to prevent glomerular atrophy induced by ureteral obstruction, while LPS-offspring MSCs were not. In contralateral kidney, differently to the effects of control MSCs, it was observed that MSCs obtained from LPS-offspring were not able to prevent the increased MMP-2 expression induced by UUO. These differences apparently did not impact contralateral collagen deposition and whole renal function, however, it may be supposed that they increase the risk of later renal injury development.

In summary, it was observed that UUO increased oxidative stress and upregulated TGF-β, TNF-α, IL-6 and MMP-2 pathways in obstructed kidney. These alterations could be underlying factors in the increased collagen deposition and renal function loss. In contralateral kidney, albeit there were no elevation of ECM density, it was observed many molecular alterations related to renal fibrosis and elevated NADPH oxidase activity, which may impact renal function lately. Furthermore, it was observed that the protective effects of MSCs on UUO-induced renal fibrosis seems to converge to MMP-2 downregulation, and antioxidant, anti-inflammatory and anti-fibrogenic mechanisms. Finally, we observed that intrauterine endotoxemia may induce subtle alterations of regulatory effects of MSCs on kidney, that do not seems to compromise their capacity to respond to acute injuries, but that may be important in later events.

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FIGURE LEGENDS

FIGURE 1. Tubulointerstitial collagen deposition in renal cortex of rats 14 days after unilateral ureteral obstruction (UUO) treated with mesenchymal stem cells (MSCs). Collagen density was measured using Picro-Sirius staining method and evaluated in obstructed (**A and C**) and contralateral (**B and D**) kidney. Sham are rats that were submitted to simulated surgery of UUO; UUO are rats submitted to UUO; O+MSC-C are rats submitted to UUO that received administration of mesenchymal stem cells (MSCs) obtained from control rats; O+MSC-L are rats submitted to UUO that received MSCs obtained from rats that were offspring of dams administered lipopolysaccharide (L) during gestation. Upper panel shows the representative micrographs of Picro-Sirius staining of tubulointerstitial area in obstructed **(A)** and contralateral kidney **(B)**. Lower panel presents the quantification of Picro-Sirius staining expressed as percentage of tubulointerstitial area in the obstructed **(C)** and contralateral kidney **(D)**. Results are presented as mean ± SEM. Differences between experimental groups were analyzed by One-way ANOVA followed by Newman-Keuls: ***P<0.001 vs. Sham; ††P<0.01 vs. UUO.

FIGURE 2. Basal and NADPH-dependent superoxide production in renal cortex of rats 14 days after unilateral ureteral obstruction treated with MSCs from control or submitted to intrauterine inflammation rats. Superoxide anions levels were measured by luminescence produced by lucigenin oxidation and expressed as relative light units (RLU). Basal and NADPH-dependent superoxide production were quantified in obstructed **(A)** and contralateral kidney **(B)**. The groups are presented in detail in Figure 1 legend and in Material and Methods. Results are presented as mean ± SEM. Differences between experimental groups were analyzed by One-way ANOVA followed by Newman-Keuls: *P<0.05, **P<0.01 and ***P<0.001 vs. Sham; †P<0.05, ††P<0.01 and †††P<0.001 vs. UUO.

FIGURE 3. Protein expression of inflammatory markers (TGF-β, TNF-α and IL-6) in renal cortex of rats 14 days after unilateral ureteral obstruction treated with MSCs from control or submitted to intrauterine inflammation rats. The protein expression were evaluated by immunoblotting. Expression of TGF-β, TNF-α and IL-6 were quantified in obstructed **(A, C and E)** and contralateral kidney **(B, D and F).** Upper part of panels **A–F** shows representative images from immunoblotting in a single gel. The groups are presented in detail in Figure 1 legend and in Material and Methods. Results are presented as mean ± SEM. Differences between experimental groups were analyzed by One-way ANOVA followed by Newman-Keuls: *P<0.05 and ***P<0.001 vs. Sham; †P<0.05, ††P<0.01 and †††P<0.001 vs. UUO. TGF-β = Transforming Growth Factor beta; TNF-α = Tumor Necrosis Fator alpha; IL-6 = Interleukin 6.

FIGURE 4. MSCs effects on changes of matrix metalloproteinase-2 and 9 (MMP-2 and MMP-9) activities in renal cortex induced by unilateral ureteral obstruction (UUO) in rats. The MMP-2 and MMP-9 activities were evaluated after 14 days of UUO and MSCs administration in obstructed (**A and C**) and contralateral kidney (**B and D**). Upper panels show representative bands of gelatin zymographies. MMP-2 and MMP-9 bands were identified by their molecular weight. The groups are presented in detail in Figure 1 legend and in Material and Methods. Results are presented as mean ± SEM. Differences between experimental groups were analyzed by One-way ANOVA followed by Newman-Keuls: *P<0.05 and ***P<0.001 vs. Sham; †P < 0.05 and ††P < 0.01 vs. UUO.

FIGURE 5. Protein expression of matrix metalloproteinase-2 and 9 (MMP-2 and -9) in renal cortex of rats 14 days after unilateral ureteral obstruction treated with MSCs from control or submitted to intrauterine inflammation rats. Expression of MMP-2 and -9 were quantified in obstructed **(A and C)** and contralateral kidney **(B and D).** Upper part of panels **A–D** shows representative images from immunoblotting in a single gel. The groups are presented in detail in Figure 1 legend and in Material and Methods. Results are presented as mean ± SEM. Differences between experimental groups were analyzed by One-way ANOVA followed by Newman-Keuls: *P<0.05 and **P<0.01 vs. Sham; ††P<0.01 vs. UUO; §P<0.05 vs. O+MSC-C.

FIGURE 6. Evaluation of protein expression of tissular inhibitors of matrix metalloproteinase , TIMP-2 and TIMP-1, in renal cortex of rats 14 days after unilateral ureteral obstruction treated with MSCs from control or submitted to intrauterine inflammation rats. The protein expression were evaluated by immunoblotting. The groups are presented in detail in Figure 1 legend and in Material and Methods.

Expression of TIMP-2 and -1 were quantified in obstructed **(A and C)** and contralateral kidney **(B and D).** Upper part of panels **A–D** shows representative images from immunoblotting in a single gel. Results are presented as mean ± SEM. Differences between experimental groups were analyzed by One-way ANOVA followed by Newman-Keuls: *P<0.05 vs. Sham; ††P<0.01 vs. UUO.

TABLE 1
Renal function and metabolic parameters of rats after unilateral ureteral obstruction (UUO) and treatment with mesenchymal stem cells (MSCs)

	Sham	UUO	O+MSC-C	O+MSC-L
24 h after UUO				
BUN, mg/dL	32 ± 1	51 ± 4***	51 ± 2***	48 ± 2***
Serum creatinine, mg/dL	0.50 ± 0.02	1.41 ± 0.20***	1.05 ± 0.05** †	0.97 ± 0.07** †
14 days after UUO				
Body weight, g	398 ± 12	379 ± 14	351 ± 10	351 ± 12
Water intake, mL/100 g per 12 h	40.0 ± 1.9	41.1 ± 2.0	46.3 ± 3.2	44.2 ± 3.8
Food intake, g/100 g per 12 h	20.8 ± 0.7	19.8 ± 2.6	21.8 ± 2.2	21.0 ± 0.9
Diuresis, mL/100 g per 12 h	12.2 ± 1.0	18.1 ± 1.4	17.0 ± 1.8	16.1 ± 2.2
BUN, mg/dL	33.8 ± 1.3	54.0 ± 4.6**	43.4 ± 4.5	46.3 ± 2.7
Serum creatinine, mg/dL	0.63 ± 0.05	1.15 ± 0.15*	0.64 ± 0.03†	0.67 ± 0.07†
Creatinine clearance, mL/100 g per min	0.54 ± 0.05	0.31 ± 0.02*	0,42 ± 0.01†	0.45 ± 0.04†
Proteinuria, mg/100 g per 12 h	11.5 ± 0.6	29.2 ± 2.1*	24.5 ± 1.4*†	22.5 ± 1.4*†
SBP, mmHg	139 ± 2	141 ± 2.4	137 ± 3	134 ± 2

Sham are rats submitted to simulation of UUO surgery; UUO are rats submitted to UUO surgery; O+MSC-C are rats submitted to UUO that were treated with MSCs obtained from control rats; O+MSC-L are rats submitted to UUO that were treated with MSCs obtained from offspring of dams that received lipopolysaccharide (L) during gestation. Results are presented as mean ± SEM. Differences between experimental groups were analyzed by One-way ANOVA followed by Newman-Keuls: *P < 0.05, **P<0.01 and ***P<0.001 vs. Sham; †P<0.05 vs. UUO. BUN = blood urea nitrogen; SBP = systolic blood pressure

TABLE 2
Renal weight and glomerular tuft area after 14 days of unilateral ureteral obstruction (UUO) and treatment with mesenchymal stem cells (MSCs)

	Sham	**UUO**	**O+MSC-C**	**O+MSC-L**
Obstructed kidney weight, g	1.21 ± 0.04	1.94 ± 0.09***	1.87 ± 0.11***	1.79 ± 0.05***
Obstructed kidney index, % of BWg	0.31 ± 0.01	0.51 ± 0.03***	0.53 ± 0.02***	0.51 ± 0.01***
Contralateral kidney weight, g	1.25 ± 0.04	1.52 ± 0.06**	1.42 ± 0.02*	1.42 ± 0,02**
Contralateral kidney index, % of BWg	0.31 ± 0.01	0.40 ± 0.01***	0.41 ± 0.01***	0.41 ± 0.01***
Glomerular tuft area – obstructed kidney, $\times 10^3$ µm^2	4.42 ± 0.16	3.83 ± 0.04*	4.55 ± 0.12†	3.98 ± 0.10* §
Glomerular tuft area – contralateral kidney, $\times 10^3$ µm^2	4.42 ± 0.16	4.65 ± 0.11	4.66 ± 0.07	4.59 ± 0.04

Sham are rats submitted to simulation of UUO surgery; UUO are rats submitted to UUO surgery; O+MSC-C are rats submitted to UUO that were treated with MSCs obtained from control rats; O+MSC-L are rats submitted to UUO that were treated with MSCs obtained from offspring of dams that received lipopolysaccharide (L) during gestation. Results are presented as mean ± SEM. Differences between experimental groups were analyzed by One-way ANOVA followed by Newman-Keuls: *P<0.05, **P<0.01 and ***P<0.001 vs. Sham; †P<0.05 vs. UUO; §P<0.05 vs. O+MSC-C. BW = body weight.

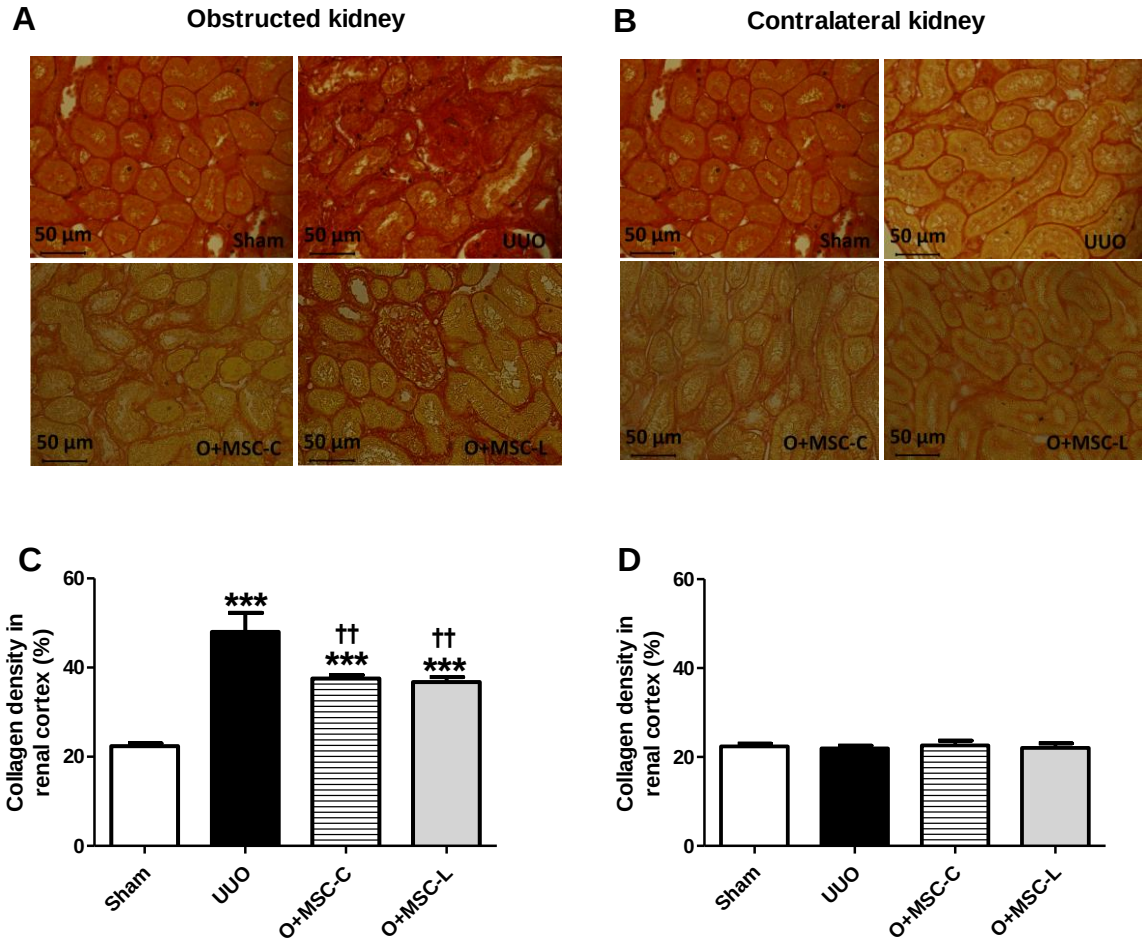


FIGURE 1. Farias *et al.*

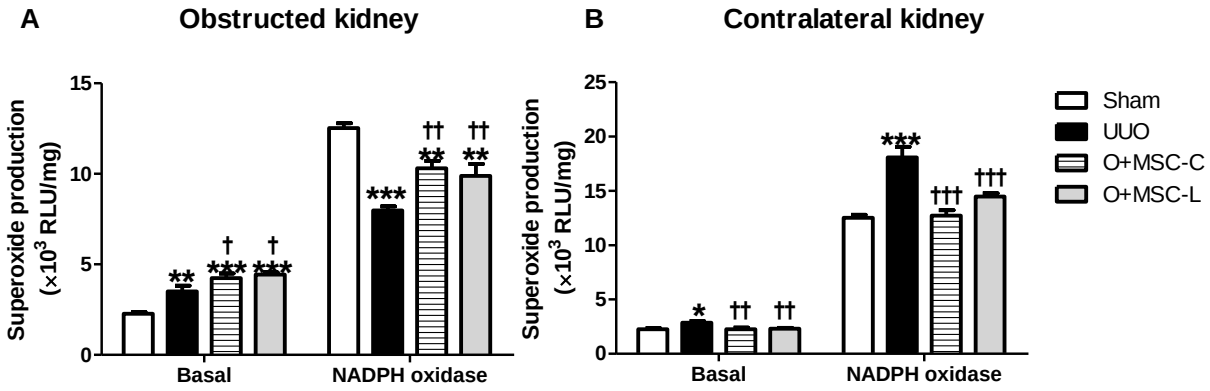


FIGURE 2. Farias *et al.*

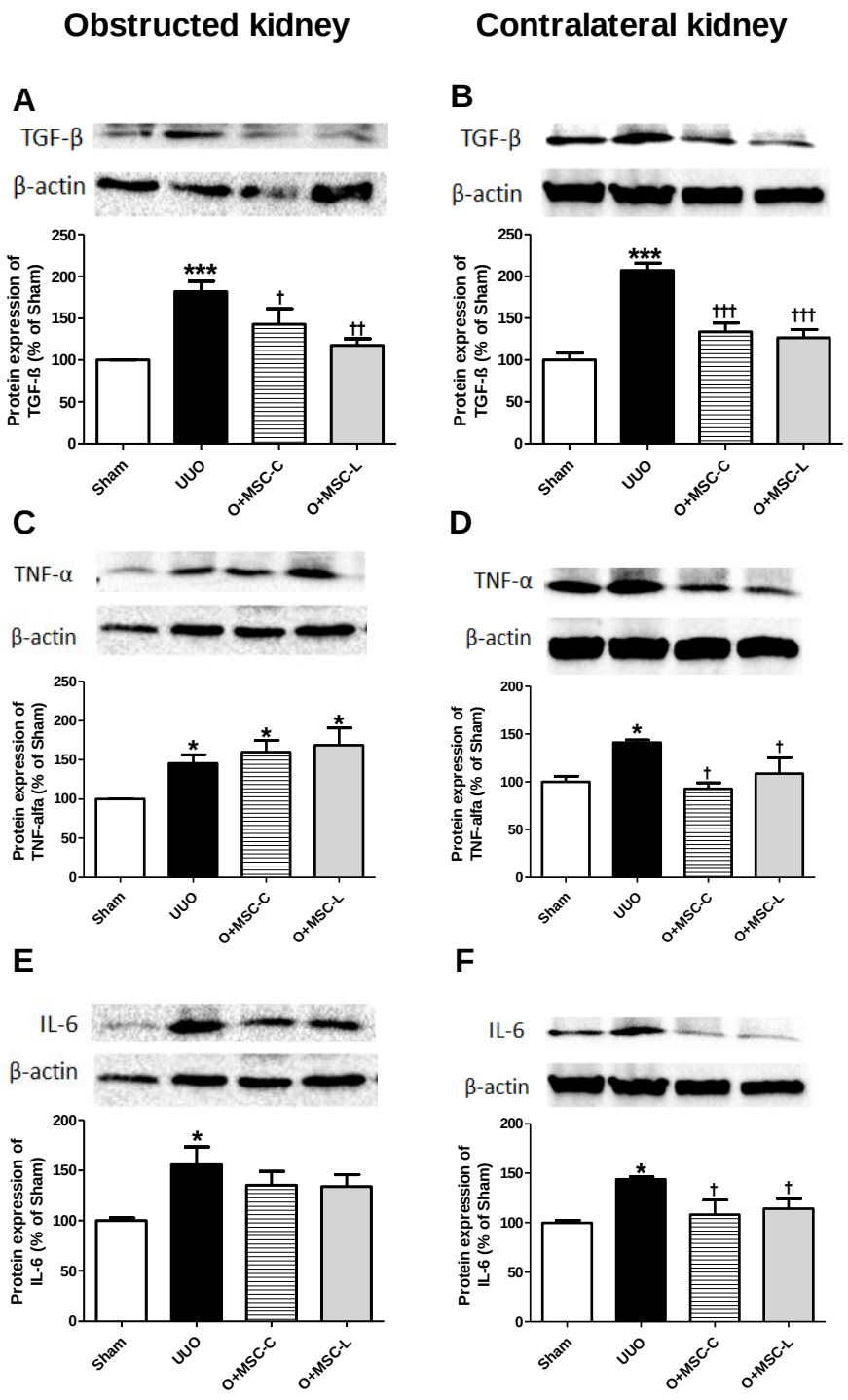
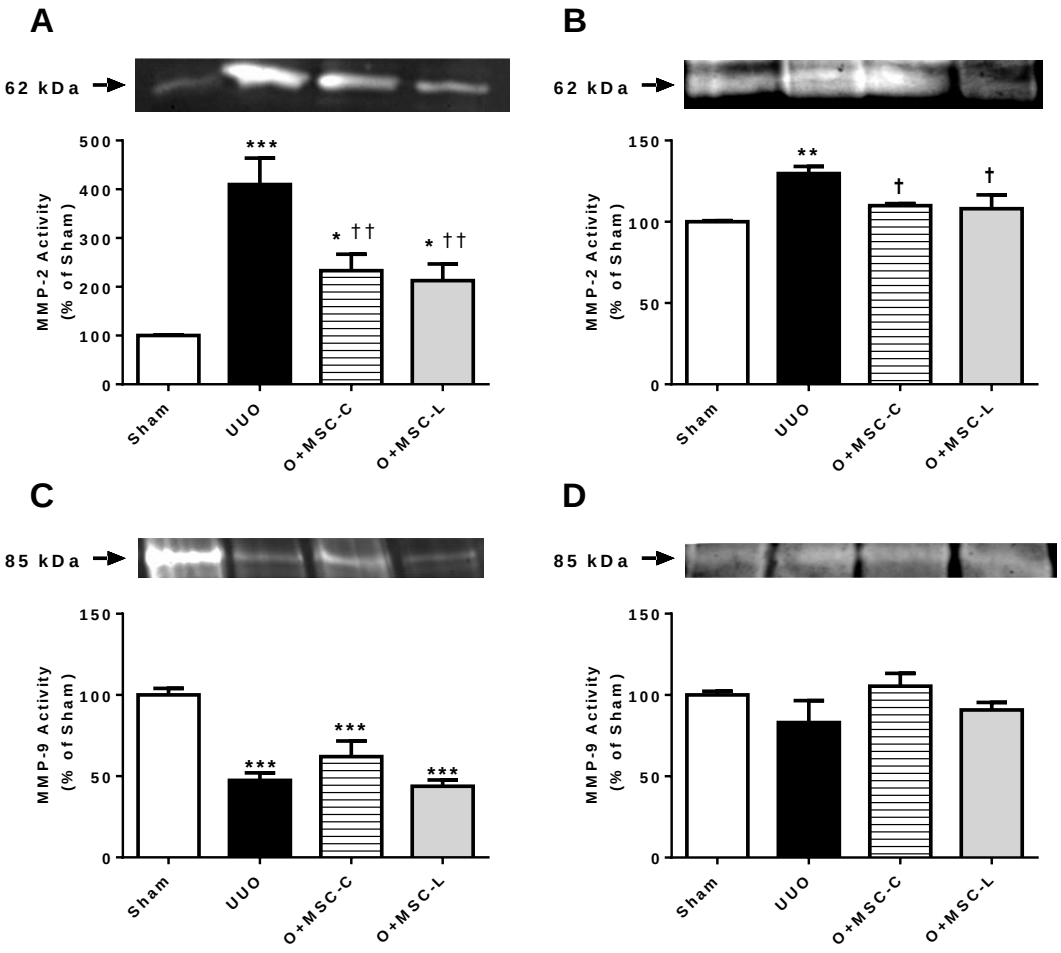


FIGURE 3. Farias *et al.*



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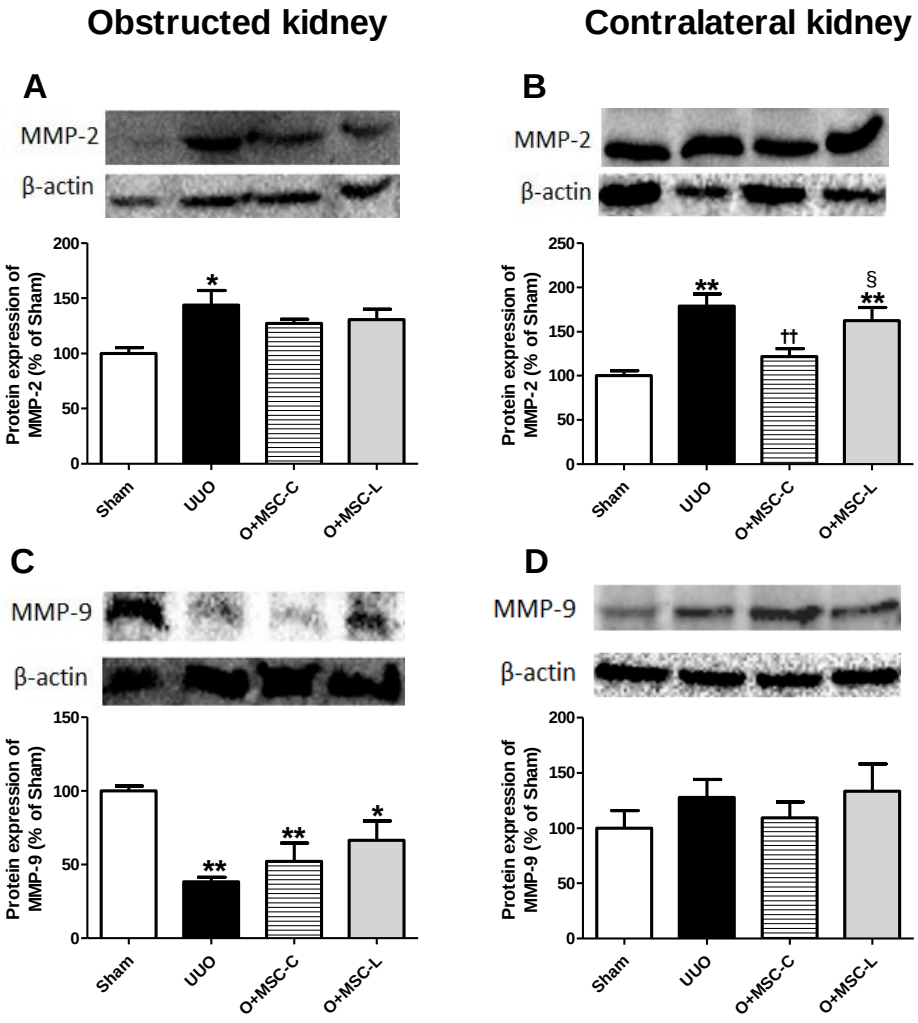

FIGURE 5. Farias *et al.*

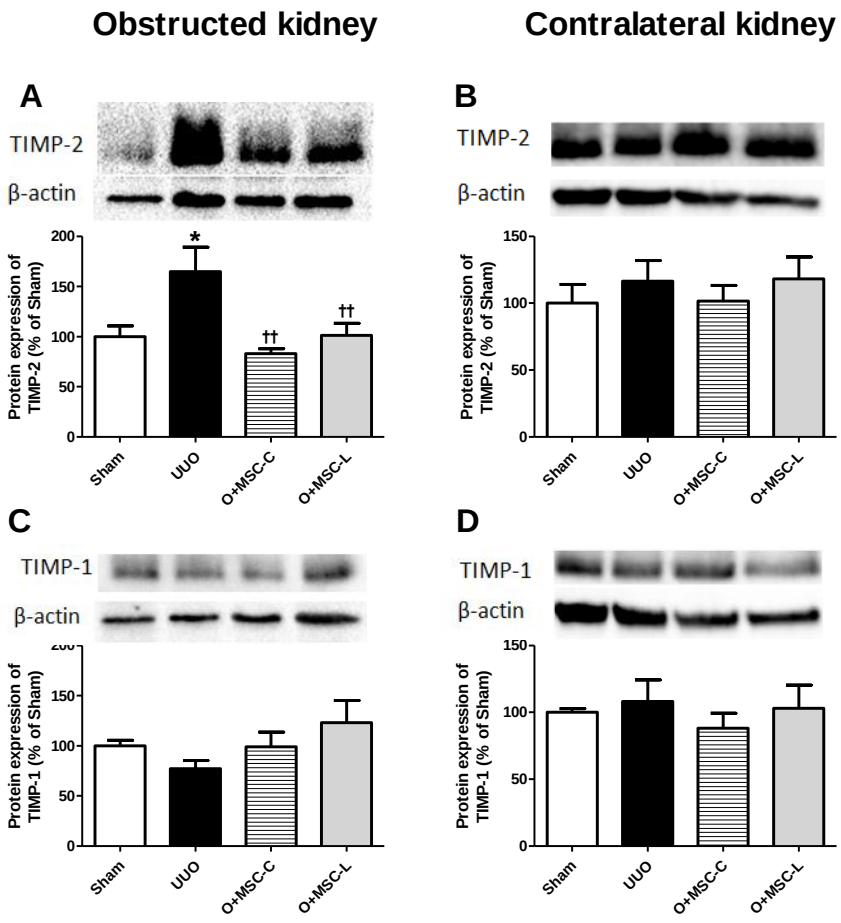

FIGURE 6. Farias *et al.*

4 CONCLUSÃO

Em conjunto, nossos resultados indicam que a endotoxemia materna induz alterações em vias moduladoras da deposição de matriz extracelular e inflamação no tecido renal. Essas alterações podem ser fundamentais para o aumento da susceptibilidade do órgão à injúria durante o envelhecimento ou na presença de quadros de sobrecarga funcional, e a NADPH oxidase parece apresentar um importante papel no mecanismo de susceptibilidade aumentada. Além disso, nossos resultados indicam que, além do tecido renal, as células-tronco mesenquimais da médula pode sofrer alterações funcionais induzidas no ambiente intrauterino, que prejudicam sua capacidade protetora. Embora as alterações observadas sejam suaves, elas podem contribuir cronicamente com uma maior sensibilidade ao desenvolvimento de doenças degenerativas.

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ANEXO A- APROVAÇÃO DO COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL

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Recife, 05 de maio de 2015.

Oficio nº 42/15

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Prof. Leucio Duarte Viera Filho
Departamento: Fisiologia e Farmacologia
Universidade Federal de Pernambuco
Processo nº 23076.060473/2014-91

Os membros da Comissão de Ética no Uso de Animais do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEUA-UFPE) avaliaram seu projeto de pesquisa intitulado, **"Mecanismo Moleculares da Fibrose Renal em Ratos Submetidos á Inflamação durante o Desenvolvimento."**

Concluímos que os procedimentos descritos para a utilização experimental dos animais encontram-se de acordo com as normas sugeridas pelo Colégio Brasileiro para Experimentação Animal e com as normas internacionais estabelecidas pelo National Institute of Health Guide for Care and Use of Laboratory Animals as quais são adotadas como critérios de avaliação e julgamento pela CEUA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 11.794 de 08 de outubro de 2008, que trata da questão do uso de animais para fins científicos e didáticos.

Diante do exposto, emitimos **parecer favorável** aos protocolos experimentais a serem realizados.

Origem dos animais: Biotério Setorial do Departamento de Fisiologia e Farmacologia da UFPE; Animais; Rato Heterogênico; Linhagem: Wistar; Idade: 90 dias; Peso; (200 - 250g); Sexo; mancho (72) e fêmea (16); Número total de Animais : 88

Atenciosamente,

Prof. Dr. Pedro V. Carelli
Presidente da CEUA / CCB - UFPE
SIAPE 1801584

CCB: Integrar para desenvolver