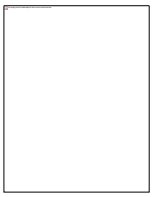
UNIVERSIDADE FEDERAL DE PERNAMBUCO

CENTRO DE CIÊNCIAS BIOLÓGICAS

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS



RAYANA LEAL DE ALMEIDA LUNA

INFLUÊNCIA DE CITOCINAS E FATORES DE CRESCIMENTO EM MODELOS MURINOS DE PRÉ-ECLÂMPSIA E DE PERDA GESTACIONAL: ALVOS-TERAPÊUTICOS ALTERNATIVOS NA PREVENÇÃO DO ABORTO E NA MÁ-FORMAÇÃO VASCULAR FETAL

Recife
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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas do Centro de Ciências Biológicas da Universidade Federal de Pernambuco para a obtenção do Título de Doutora em Ciências Biológicas na área de concentração de Biotecnologia.

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Orientadora: Drª. Christina Alves Peixoto

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DEDICATÓRIA

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RESUMO

O desenvolvimento de uma gestação saudável depende de uma adequada circulação uteroplacentária. Abortos recorrentes (AR), óbito fetal, partos prematuros, pré-eclâmpsia (PE) e trombofilia materna (TM) são algumas das patologias que interferem nessa rede vascular. São muitos os fatores que podem influenciar na tênue interface entre o parto de um feto saudável, um parto prematuro ou até mesmo um aborto. Uma variedade de moléculas como citocinas, proteínas de adesão e fatores de crescimento podem ter um papel importante durante o desenvolvimento fetal assim como em todo processo gestacional. Desta forma, desequilíbrio entre vias de sinalização assim como as vias inflamatórias têm sido relacionadas com o desencadeamento do processo abortivo. O fator de crescimento placentário (PGF) é um marcador importante para identificar deficiência placentária que pode ser ligada a PE. Mulheres com PE tendem a ter níveis inferiores de PGF circulantes quando comparadas a mulheres com gestações saudáveis. A inflamação aguda é comumente associada com injúria fetal assim como a inflamação crônica tem sido associada com a morte fetal e aborto. Estudos epigenéticos e epidemiológicos têm demostrado que indivíduos nascidos de mães que tiveram complicações durante a gravidez possuem maior risco de desenvolverem patologias diversas na vida adulta como acidente vascular cerebral, cardiopatias, obesidade e infertilidade. O Sildenafil, um inibidor de fosfodiesterase tipo-5 com características vasodilatadoras tem ajudado na terapia de restrição de crescimento fetal (RCF). Recentemente Sildenafil, tem sido demostrado por apresentar-se como anti-inflamatório em diversos modelos animais de patologias humanas como esclerose múltipla, hiperplasia prostática, doenças cardiovasculares, pulmonares entre outras. Por outro lado a Heparina de baixo peso molecular tem sido utilizada como tratamento para casos de AR quando o diagnóstico é associado ou não com TM, apesar deste tipo de esquema terapêutico não ser considerado eficaz em muitos casos. O presente estudo se propôs a investigar alvos-terapêuticos em modelos animais de AR e PE, através da avaliação do tratamento com Sidenafil ou/com Heparina para a prevenção do processo abortivo assim como a investigação da influência da sinalização via-PGF na formação da vascularização cerebral fetal. Tratamento com Sildenafil sozinho ou tratamento conjunto com Heparina protegeu contra a mortalidade fetal, diminuiu a sinalização inflamatória na placenta assim como melhorou a saúde dos fetos de camundongo expostos a altas doses de Lipopolissacarídeos (LPS) como modelo para perda gestacional. PGF foi identificado em áreas importantes do cérebro de fetos geneticamente normais, no período de gestação média assim como também foram identificados níveis compatíveis do receptor 1 para fator de crescimento vascular endotelial (VEGF): Flt-1. A via que envolve o PGF mostrou-se essencial para o desenvolvimento vascular cerebral saudável. A proteção placentária e por consequência manutenção da saúde do feto é de suma importância para que a gestação chegue a termo e resulte no parto de um indivíduo saudável e sem riscos à saúde da mãe. Esse trabalho traz informações detalhadas de como acontece a modulação durante a vascularização cerebral durante o desenvolvimento em animais geneticamente normais assim como em camundongos PGF-/-. Adicionalmente, demostra como a terapia utilizando Sildenafil isoladamente ou em combinação com Heparina pode diminuir o perfil inflamatório e/ou trombótico do sistema fisiológico gravídico e desta forma prevenir o processo abortivo, mantendo a saúde fetal.

Palavras- chave: Sildenafil, Heparina, PGF, VEGF, placenta.

ABSTRACT

The development of a healthy pregnancy depends on proper uteroplacental circulation. Recurrent miscarriages (RM), fetal death, pre-term labour, preeclampsia (PE) and maternal thrombophilia (MT) are some of the pathologies that may affect with arrangements of this circulation network. There are many factors that can influence the maternal-fetal interface and aberrant gene expression at the site of this interface can result in premature birth or even miscarriage. A variety of molecules including a variety of cytokines, adhesion proteins and growth factors play an important role during fetal development as well as throughout gestation. Indeed, an imbalance of signaling and inflammatory signaling pathways have been associated with the initiation of the abortive process, regardless of the origin of this event. Placental growth factor (PGF) is an important marker to identify placental defects that can be linked to PE. Women with PE tend to have lower levels of circulating PGF when compared to women with healthy pregnancies. Acute inflammation is often associated with fetal injury and chronic inflammation has been associated with abortion and fetal death. Additionally, epigenetic and epidemiological studies have shown that individuals born to mothers who have complications during pregnancy have a higher risk of developing various diseases in adulthood such as: stroke, heart disease, obesity and infertility. Sildenafil, a phosphodiesterase type-5 inhibitor with vasodilating characteristics has proved beneficial in fetal growth restriction (FGR) pathologies. Recent studies have shown that Sildenafil plays an anti-inflammatory agent in a wide range of pathologies in animal models of human diseases such as: multiple sclerosis, prostatic hyperplasia, cardiovascular, and pulmonary diseases among others. Low molecular weight heparin (LMWH) has been used as a treatment for RM cases when diagnosis is associated or not with MT. Despite these facts, recently published meta-analyses have demonstrated that treatments using LMWH are not superior. This study proposed to investigate new therapeutic approaches in animal models of RM and PE. This was accomplished by evaluation of the effectiveness of Sildenafil and/or Heparin in the prevention of fetal mortality/abortion as well as the investigation of the influence of PGF signaling pathway in the formation of fetal brain vasculature. Treatment with sildenafil; alone or in combination with heparin protected against fetal mortality and decreased inflammatory signaling in the placenta, as well as improved the health of the mouse fetuses exposed to high doses of lipopolysaccharide (LPS). PGF was detected in important areas of the brain in genetically normal fetuses and the average period of gestation was also identified as compatible to vascular endothelial growth factor (VEGF) receptor: Flt-1. The pathway involving PGF was found to be essential for healthy vascular development in the brain. Therefore, early placental protection and therefore maintaining the fetus' health is of paramount importance if the pregnancy comes to term and results in the birth of a healthy individual and without risks to health of the mother. This work provides detailed information into critical events that occur during the development of the cerebral vasculature both in genetically normal as well as in PGF -/- mice. Additionally, this work demonstrates how therapies with sildenafil alone or in combination with heparin can reduce the inflammatory status and/or thrombotic physiological systems of pregnancy and thereby prevent fetal mortality, thus maintaining fetal health.

Key-words: Sildenafil, Heparin, PGF, VEGF, placenta.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

AMPc - monofosfato cíclico de adenosina

AMPK - proteína quinase ativada por AMP

AR - aborto recorrente

AVC - acidente vascular cerebral

eNOS - sintase de óxido nítrico endotelial

Flt-1 - Receptor 1 para VEGF

GC - guanilato ciclase

GMPc - monofosfato cíclico de guanosina

HBPM - heparina de baixo peso molecular

Hep - heparina

IkBα - proteína inibitória IkBα

IL1β - interleucina 1β

iNOS - sintase de óxido nítrico induzível

JAK-STAT – via janus kinase

KDR - receptor 2 para VEGF

LPS - lipopolissacarídeo

MAPK – proteína ativada por mitógeno

NFκB - fator de transcrição nuclear kappa-B

NO - óxido nítrico

PDE5 - enzima fosfodiesterase tipo 5

PDEs - enzimas fosfodiesterases

PE - pré-eclâmpsia

PGF - fator de crescimento placentário

PKA - proteína quinase dependente de AMPc

PKG - Proteína quinases dependentes de GMPc

P-Sel - P-Selectina

RCF - Restrição de crescimento fetal

Sil - Sildenafil

SNC - Sistema nervoso central

TM - Trombofilia materna

TNFα - Fator de necrose tumoral-α

VEGF - Fator de crescimento vascular

VEGFR1 - Receptor 1 para VEGF

VEGFR2 - Receptor 2 para VEGF

SUMÁRIO

1. **INTRODUÇÃO**

A gestação é um evento fisiológico único no qual é necessário que haja equilíbrio entre hormônios, citocinas pró-inflamatórias, anti-inflamatórias, fatores de crescimento, ácidos graxos entre outras moléculas primordiais para o desenvolvimento embrionário e fetal (Clark 1999, Barker 2007). Estudos têm demostrado que o limiar tênue entre o sucesso e o fracasso de uma gestação é determinado principalmente por fatores imunológicos (Krieg, Fan et al. 2012). Por isso há um alto investimento em pesquisas utilizando anticorpos e terapia gênica para casos de deficiência, principalmente da vascularização durante a implantação completa do blastocisto na parede uterina (Cohen and Machupalli 2015).

Patologias associadas à gestação podem de fato trazer riscos para a mãe e para a criança, podendo ainda ocorer uma translação da patologia materna para o feto ainda no ambiente intrauterino (Hromadnikova, Kotlabova et al. 2015). Estudos envolvendo principalmente caracterização epigenética têm mostrado que filhos de mães obesas, tendem a ter Diabetes tipo II, o mesmo acontece com filhos de mulheres que fizeram uso de álcool durante a gestação, pois seus filhos podem possuir uma tendência fisiológica ao vício (Abate, Hernandez-Fonseca et al. 2014, Kruse, Keyhani-Nejad et al. 2015). Pouco ainda se sabe sobre quais tipos de interações irão influenciar diretamente na saúde do indivíduo em desenvolvimento de forma permanente (Rogers and Velten 2011). De fato tem sido descrito que alguns fatores podem acarretar em dano imediato ao feto, causando a morte e do mesmo o aborto.

Tem sido estabelecido que o aumento da inflamação está associada ao início da injúria fetal, a mesma é seguida de ativação da cascata de coagulação que inicia também a ativação do sistema complemento caracterizando o início do sofrimento fetal (Abumaree, Chamley et al. 2012, Sharma 2014). Essas etapas se não sanadas seja por medida medicamentosa, cirúrgica ou paliativa podem resultar em primeiramente morte celular, seguida da parada de órgãos e sistemas do feto e por fim morte fetal e aborto (Kwak-Kim, Yang et al. 2009). Alguns desafios

presentes nesse tema são o diagnóstico rápido e correto sobre a causa do aborto assim como o tratamento efetivo do processo de perda gestacional pois estes desafios representam duas etapas distintas que tem que ser manejadas adequadamente (Acosta and Knight 2013).

Grande parte dos tratamentos utilizados atualmente na prática médica trata apenas a causa do aborto enquanto que o final da cascata é caracterizado basicamente por inflamação + trombofilia frequentemente, independe do fator desencadeador (Falcon, Cotechini et al. 2012). Desta forma uma abordagem com fins anti-inflamatórios e anti-trombóticos poderia representar um ganho para as terapias atuais, por tratar o processo abortivo de maneira ampla (Luna, Nunes et al. 2015). Para esse tipo de terapia é necessário a elucidação dos mecanismos dessas classes de fármacos durante a gestação e durante a perda gestacional.

A ampla distribuição da enzima fosfodiesterases tipo-5 e a disponibilidade de inibidores seletivos para essa enzima facilitou o desenvolvimento de novos estudos para o tratamento patologias como hipertensão pulmonar, cardiopatias e restrição de crescimento fetal entre outras (Lin, Lin et al. 2006, Panda, Das et al. 2014). Por outro lado, níveis circulantes de fator de crescimento placentário podem estar diminuídos em mulheres com pré-eclâmpsia, ao passo que o receptor para VEGF 1 pode estar aumentado nessa mesma população. Regulação entre fatores de crescimento da família do fator de crescimento vascular endotelial, assim como seus receptores, podem estar envolvidos na fisiopatologia dessa complicação gestacional (Autiero, Waltenberger et al. 2003).

A partir do entendimento dos fatores que influenciam direta ou indiretamente no processo gestacional e por consequência no abortivo, podem-se estudar alvos terapêuticos envolvendo essas moléculas ou vias intracelulares. É muito importante que surjam novas terapias para tratar o aborto recorrente seja este de causa identificada ou idiopática pelo fato de atualmente não existir relato de um esquema de tratamento eficaz para essa patologia gestacional tão relevante, que afeta milhares de mulheres mundialmente todos os anos.

2. JUSTIFICATIVA

Três em cada 10 mulheres que engravidam têm abortos espontâneos por causas variadas, incluindo más-formações, abortos recorrentes, pré-eclâmpsia e trombofilia materna. Atualmente o tratamento de grande parte das perdas gestacionais é realizado de forma paliativa com análogos da heparina que diminuem o risco de um evento trombótico ou com terapias hormonais, contudo ainda existem grandes limitações na terapêutica atual.

Por sua ação anti-inflamatória e vasodilatadora, o Sildenafil poderia atenuar os efeitos dos eventos inflamatórios e possivelmente trombóticos durante o aborto. Uma associação com Heparina poderia ser uma oportunidade terapêutica para casos de aborto no futuro.

A pré-eclâmpsia é uma síndrome hipertensiva gestacional que, atualmente é a primeira causa de morte materna no Brasil. É necessária a investigação do papel do fator de crescimento placentário no desenvolvimento da vascularização fetal, visto que esta molécula é encontrada diminuída em mulheres grávidas com pré-eclâmpsia e essa dimuniução pode está associada a patologias no feto, principalmente relacionadas ao sistema cardiovascular.

3. OBJETIVOS

3.1. Objetivo Geral

Caracterizar o efeito e o mecanismo de ação isolado do Sildenafil ou em tratamento conjunto com Heparina sobre a evolução clínica, incidência de AR, manutenção da integridade placentária em modelo de aborto induzido através da injeção de LPS em camundongos fêmeas grávidas. Assim como, avaliar a influência dos fatores de crescimento PGF e VEGF durante o desenvolvimento cerebral fetal em um modelo animal de PE (*knockout PGF-/-).*

3.2. Objetivos Específicos por capítulo

Capítulo 1

- Avaliar a evolução clínica e morte fetal pelo modelo experimental de AR, após tratamento dos animais com Sildenafil/Heparina ou em tratamento conjunto Sildenafil+Heparina;
- Avaliar por histopatologia e ultraestrutura a integridade das células da placenta;
- Avaliar por morfometria os espaços sanguíneos da região do labirinto da placenta;
- Avaliar por imunofluorescência a expressão da citocina IL1-β em placenta;
- Avaliar por imunohistoquímica a expressão da citocina TNF-α e do fator de transcrição NFκB em placenta;
- Avaliar por western blotting os níveis de TNF-α, IL1- β e NFκB total em placenta.

Capítulo 2

- Avaliar por histopatologia diferentes áreas do tecido placentário;
- Avaliar por morfometria a área das células trofoblásticas gigantes e a presença/incidência de vasos com características hemorrágicas;
- Avaliar por ultraestrutura a integridade das células em diferentes áreas do tecido placentário;
- Avaliar por imunofluorescência a expressão da molécula P-Selectina em placenta.

Capítulo 3

- Avaliar por imunofluorescência a expressão dos fatores de crescimento PGF e VEGF em cérebro de fetos de camundongos BLACK6;

- Avaliar por PCR quantitativo (RT-PCR) a transcrição para genes de PGF, VEGF , VEGFR1 e VEGFR2 em cérebro de fetos de camundongos BLACK6;

- Avaliar o fluxo cerebral pela oclusão das carótidas comuns laterais em camundongos adultos BLACK-6 e *knockout* para PGF-/-;

- Avaliar a vascularização durante o desenvolvimento fetal no rombencéfalo de fetos de camundongos BLACK-6 e *knockout* para PGF-/- pela coloração de lectina.

Capítulo 4

- Avaliar a saúde materna e fetal através de exame de anatomia grossa dos sítios de implantação. Medidas da circunferência abdominal, comprimento e peso dos fetos. Também avaliar a medida da largura, comprimento e peso das placentas;

- Avaliar por imunohistoquímica a expressão de eNOS e PGF em placenta;

- Avaliar por tromboelastografia o sangue de mulheres voluntárias saudáveis para ensaio *in-vitro* da interação do Sildenafil, Heparina ou combinação das drogas durante a coagulação;

- Avaliar por contagem total de células brancas, hemácias e contagem de plaquetas no sangue de camundongos submetidos ao modelo de AR e tratados com Sildenafil/Heparina ou em tratamento conjunto Sildenafil+Heparina;

- Avaliar por análise Multiplex o perfil de citocinas e fatores de crescimento no líquido amniótico de camundongos fêmeas grávidas submetidos ao modelo de AR e aos tratamentos;

- Avaliar por tromboelastografia o sangue de camundongos submetidos ao modelo de AR, tratados com Sildenafil/Heparina ou em tratamento conjunto Sildenafil+Heparina;

4. REVISÃO BIBLIOGÁFICA

4.1 Gestação saudável e desenvolvimento fetal

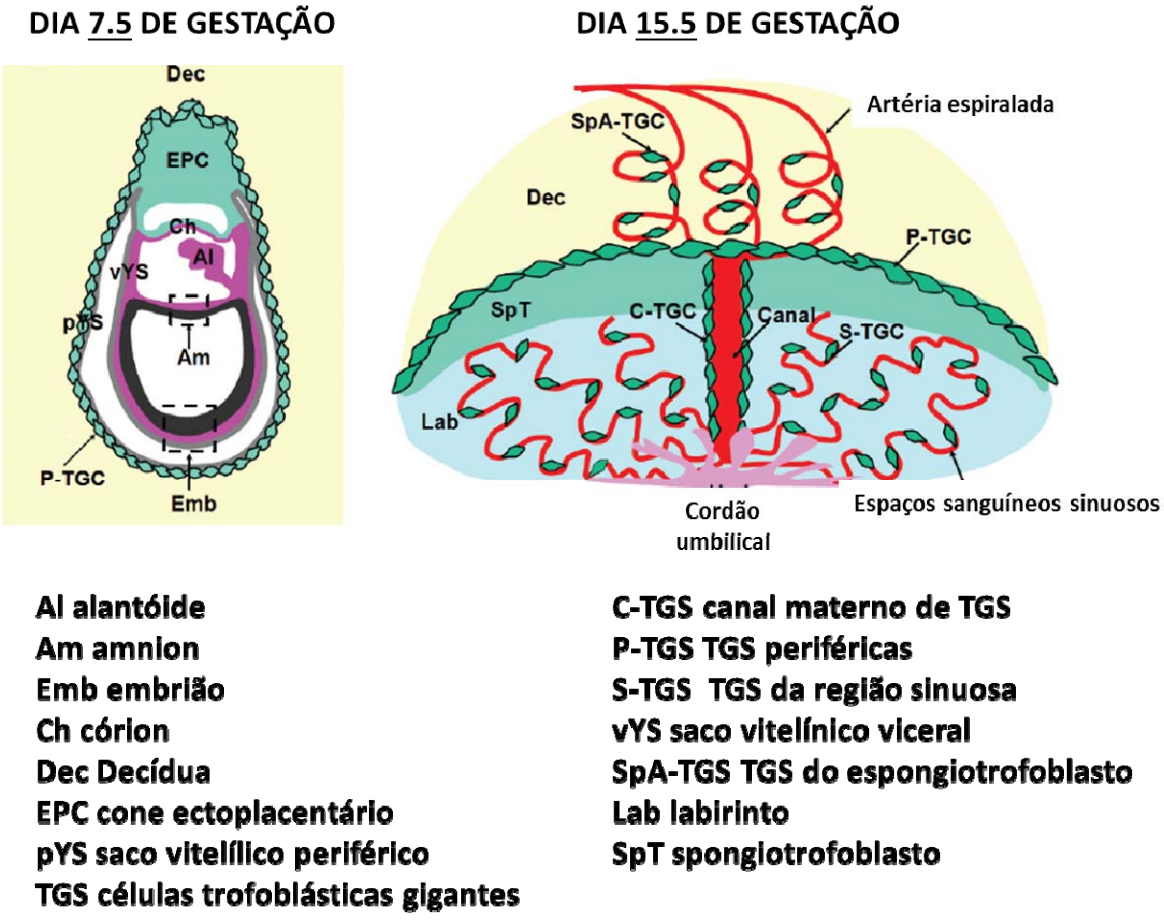
A Implantação embrionária acontece principalmente por invasão de células do sincitiotrofobasto na parede uterina (Mowbray, Jalali et al. 1997). Numerosos fatores são responsáveis pelo sucesso do evento de invasão e pela implantação completa do blastocisto seguido da diferenciação celular e desenvolvimento da gestação. A supressão momentânea do sistema imune materno durante a gestação ainda não está completamente esclarecida, acredita-se que este evento seja um dos principais fatores responsáveis pelos abortos espontâneos nos primeiros meses de gravidez (Arck, Ferrick et al. 1999, Christiansen 2013). Células *Natural Killers* uterinas (uNK) tem sido investigadas intensamente, por possuírem um papel importante na receptividade uterina ao embrião e tendem a diminuir a sua quantidade a medida que a gestação progride (Tayade, Hilchie et al. 2007). Desta forma fica demonstrado que vários outros fatores também tem função fundamental desde a fecundação até o parto natural saudável não apenas as células uNK (Costa 2015). O desenvolvimento embrionário, seguido do fetal é basicamente dividido em segmentação, gastrulação e organogênese. Todos esses eventos são de suma importância para o nascimento viável de uma criança (Zeng, Baldwin et al. 2004). Fatores ambientais, genéticos ou fisiológicos podem influenciar em uma ou mais fases da gestação resultando em déficit no desenvolvimento, o que pode estar relacionado com sofrimento fetal (Borte, Wang et al. 2011, Cooke 2014). O desenvolvimento fetal saudável é caracterizado pela autoregulação imune a nível celular, molecular e genético de forma constante garantindo o amadurecimento placentário correto em cada fase da gestação. O resultado do correto balanço

endócrino, celular e molecular é a garantia da proteção do feto até o momento do parto (Challis, Newnham et al. 2013).

Após o início do desenvolvimento embrionário inicia-se a placentação, caracterizada principalmente pela formação do arcabouço vascular para a troca sanguínea entre mãe e feto. A placenta é um órgão materno-fetal responsável pelo armazenamento de substancias como ferro, cálcio e aminoácidos, adicionalmente pela transferência de vários fármacos (Cooke 2014). A placenta representa a maior fonte de progesterona durante a gravidez, desta forma, danos ao tecido placentário podem acarretar em disfunção hormonal e complicações gestacionais (Costa 2015). A placentação humana implanta-se na região média da decídua frequentemente, esse órgão transitório se estabelece na quarta semana de gestação e tem característica córion vilosa, a mesma é dividida em cotilédones que são unidades funcionais independentes (Borekci, Aksoy et al. 2006).

Por sua vez a placentação dos murinos é classificada como hemotricorial, labiríntica e tem por característica principal ser zonária, onde cada região é responsável por respostas individuais e possuem tipos celulares distintos (Hu and Cross 2010) (Figura 1). Modelos animais desenvolvidos usando camundongos para investigação da gestação tem sido bastante aplicados pois estes animais possuem características gestacionais semelhantes aos humanos, mesmo mantendo diferenças evolutivas bem distintas (Deb, Chaturvedi et al. 2004, Suenaga, Kitahara et al. 2014). Para investigação da ação de fármacos durante a gestação e a influência dos mesmos no desenvolvimento fetal, modelos murinos têm sido bastante úteis (Stanley, Andersson et al. 2012, Zhao, Chen et al. 2013).

Figura 1 - Representação do processo de placentação em murinos com detalhe para os tipos celulares envolvidos.



Ref: Modificado de Dong hu e James C. Cross, 2010.

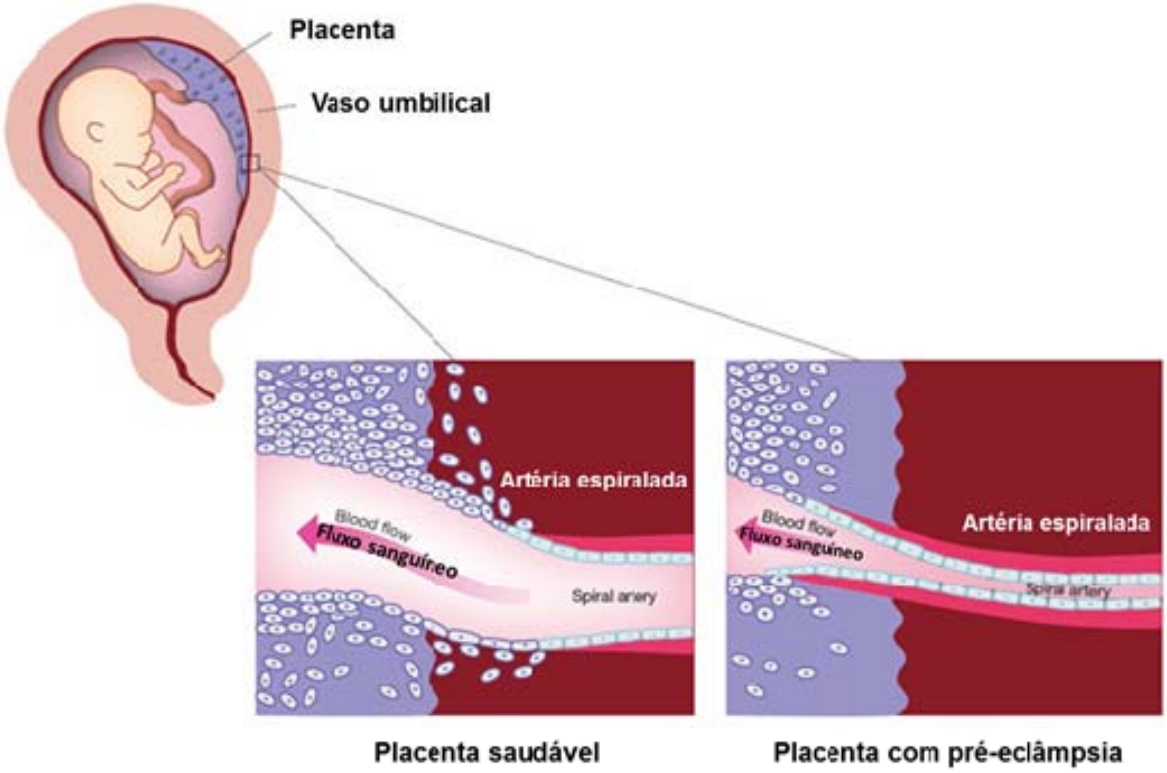
4.2 Patologias associadas à perda gestacional

Diversas patologias, síndromes ou sintomas que se desenvolvem durante a gestação são muito comuns e podem afetar até 90% das mulheres grávidas (Negi, Pande et al. 2014). Entretanto algumas desses fatores podem afetar não só a saúde materna, mas também a saúde do feto, resultando em injúria e até morte fetal (Sanson, Friederich et al. 1996, Zhao, Chen et al. 2013). Recente publicação em 2013 demostrou que complicações gestacionais causaram mundialmente aproximadamente 293.000 mortes. Os fatores mais comuns relacionados à morte dessas mulheres estavam hemorragia, complicações do aborto, pressão arterial alta e sepse durante a gravidez (Mortality and Causes of Death 2015). Estresse e fatores psiconeurológicos também podem ser preponderantes para o desenvolvimento ou manutenção de uma patologia durante a gestação (Rice, Jones et al. 2007). Existem inúmeros fatores que podem conectar as complicações gestacionais fatais supracitadas umas as outras, entre eles estão principalmente inflamação e coagulopatias. Mesmo quando o aborto se inicia por causas nutricionais, genéticas, acidentes mecânicos entre outras causas a inflamação (Rogers and Velten 2011) se torna necessária para sinalizar a morte fetal para os outros sistemas fisiológicos ou para provocar a morte fetal. Inflamação na maioria das vezes vem acompanhada de trombofilia ou hemorragia (Ku, Arkel et al. 2003, Kwak-Kim, Yang et al. 2009). Ainda não está clara a causalidade entre fatores moleculares e as complicações gestacionais, principalmente como esses eventos estão ligados à mortalidade fetal.

A hipótese de Barker, proposta pelo epidemiologista David Barker em 1990 descreve que crianças nascidas com restrição de crescimento fetal (RCF) e nascidas de parto prematuro tendem a apresentar uma maior incidência de doenças cardiovasculares na vida adulta (Barker 2007, Barker, Osmond et al. 2009). Outros estudos demonstram que o status de saúde da mãe influencia diretamente no futuro status da criança nascida em condições não saudáveis como a presença de obesidade, tabagismo e consumo de álcool durante a gestação

(Dietert 2014, Leese 2014, Smith and Ryckman 2015). Pré-eclâmpsia (PE) é uma das mais frequentes complicações gestacionais, caracterizada por pressão arterial elevada, proteinúria, deficiência de fatores de crescimento placentário (PGF) entre outros sintomas. Atualmente ainda não existe cura para PE e o parto é a única forma relativamente segura de parar os danos causados pela doença. Mulheres que tem PE em uma gravidez tendem a ter novamente em outas gestações. A resistência ao fluxo sanguíneo provocada pela má formação das artérias espiraladas pode provocar diminuição da nutrição e respiração do feto, por esta razão PE está frequentemente associada à RCF (Figura 2) (Cotechini, Komisarenko et al. 2014, Sharp, Heazell et al. 2014). Por outro lado, trombofilias maternas (TM) são caracterizadas por um aumento da tendência em formar trombos durante a gestação o que pode acarretar, por exemplo: o descolamento da placenta entre outras complicações, levando inclusive ao aborto (Rey, Kahn et al. 2003). O aborto recorrente (AR) por sua vez pode ter causas previamente identificadas ou ser idiopático, desta forma se tornando um desafio para a medicina, pois os tratamentos são realizados sem um protocolo estabelecido e o índice de sucesso dos tratamentos atuais continua muito baixo (Daya, Gunby et al. 1999, Di Nisio, Peters et al. 2005, Whitley and Ural 2014). Abordagens terapêuticas utilizando tratamentos profiláticos, após o diagnóstico de AR devem ser consideradas para prevenir uma nova perda gestacional.

Figura 2 - Imagem representativa da vascularização uterina durante gestação saudável e com pré-eclâmpsia.



Ref: Modificado de Karumanchi e Levine, 2006.

4.3 Envolvimento da inflamação na gravidez e no processo de injúria fetal

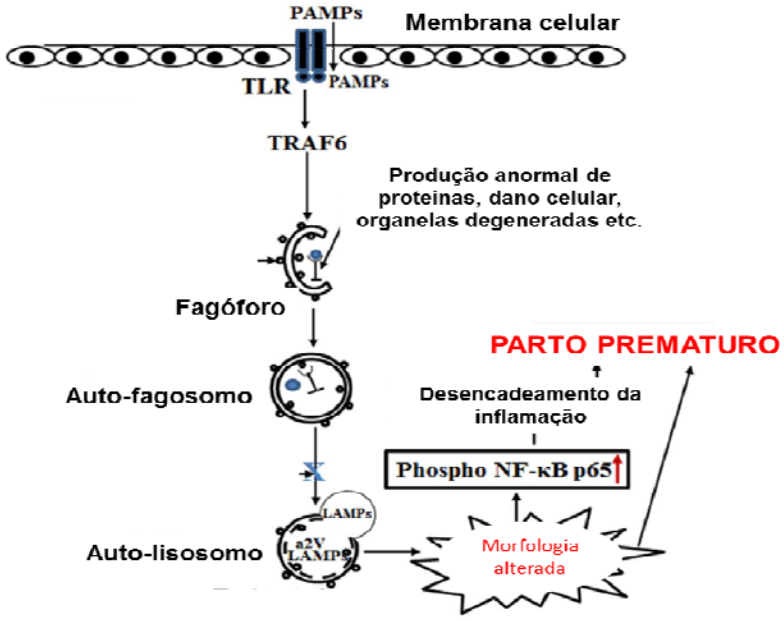
Citocinas pró-inflamatórias, anti-inflamatórias, moléculas de adesão assim como fatores de crescimento podem estar em desequilíbrio para o desencadeamento do processo abortivo (Christiansen 2013, Eskicioglu, Lacin et al. 2014, Ratsep, Carmeliet et al. 2014). Em sua maioria, independentemente da causa, a expulsão do feto se caracteriza como um evento mediado por inflamação principalmente pela citocina TNF-α (Renaud, Cotechini et al. 2011, Luna, Nunes et al. 2015). Facilitação de apoptose de células trofoblásticas e descolamento placentário podem ocorrer em decorrência ou por consequência de um déficit vascular durante implantação uterina (Zeng, Baldwin et al. 2004, Kusinski, Baker et al. 2009, Challis,

Newnham et al. 2013). Inflamação pode desencadear evento trombótico com formação de coágulos em regiões placentárias importantes de troca sanguínea, principalmente em casos de TM (Falcon, Cotechini et al. 2012). Mulheres que já tiveram experiência de uma perda gestacional por causa idiopática apresentam comumente desregulação na sinalização celular imune (Krieg, Fan et al. 2012). Várias vias de sinalização estão envolvidas na fisiopatologia da PE e do AR, entretanto ainda são limitadas as informações relacionadas a esse tema. Não existindo, atualmente tratamentos eficazes para essas patologias que juntas podem atingir até 30% das mulheres grávidas no mundo. Várias vias de sinalização têm sido estudadas para o desenvolvimento de novas terapias contra o aborto ou para o entendimento da fisiopatologia desse evento tão complexo, vias como a via janus kinase (JAK-STAT), a do fator nuclear-κB (NF-κB) e a da proteína ativada por mitógeno (MAPK) entre outras (Simsek, Gul et al. 2013, Borg, Yong et al. 2015, Hashino, Tachibana et al. 2015).

O fator nuclear-κB regula direta ou indiretamente a transcrição gênica de mediadores da resposta inflamatória como das citocinas TNF-α e IL-1β que por sua vez, influenciam no crescimento, diferenciação e ativação de células imunes como os macrófagos (Ku, Arkel et al. 2003, Lim, Barker et al. 2013, Luna, Nunes et al. 2015). Tem sido descrita a influência do NF-κB durante a perda gestacional, pois a alta taxa de fosforilação desse fator pode ter um papel importante na fisiopatologia tanto de AR, TM como da PE (Huang, Su et al. 2014, Koch, Frommhold et al. 2014). A atividade no NF- κB durante o parto é bastante relevante pois o mesmo está relacionado com o controle entre maturação fetal e a atividade uterina para liberação do mesmo (Lindstrom and Bennett 2005). Mais recentemente tem sido descrita que desregulação dessa via de sinalização é preponderante em casos de partos prematuros (Challis, Newnham et al. 2013). O fator nuclear-κB pode está ativado na fase de resposta a atutofagosomos e a liberação de lisossomos por células imunes circulantes no tecido

gravídico (Figura 3). Ainda não está estabelecida a relação entre causa e efeito durante a atuação do NF-κB na gestação, tendo em vista que o mesmo é ativado em momentos de injúria e da mesma forma, as citocinas produzidas por essa via podem realizar um *feedback* positivo para a fosforilação do mesmo. Mais estudos acerca desse tema são importantes para o entendimento da interação entre a inflamação e a gestação.

Figura 3 – Diagrama de como a inflamação e autofagossomos influenciam na fisiopatologia do parto prematuro.

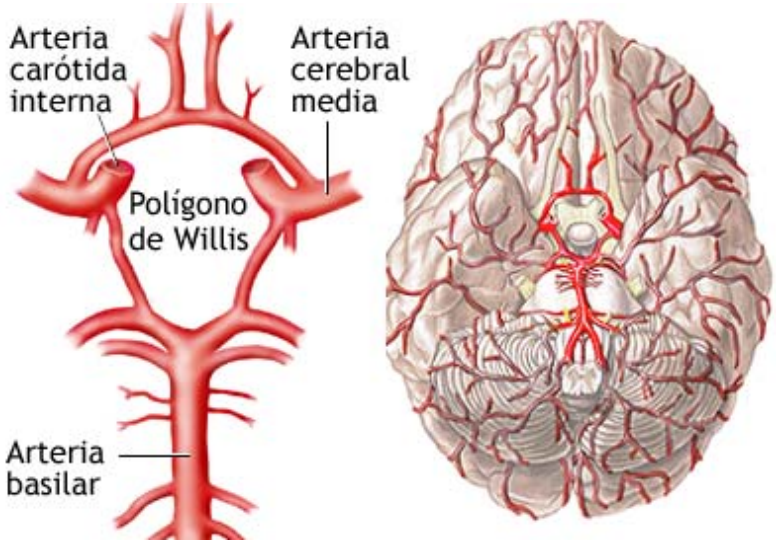


Ref: Modificado de Varkha Agrawal at al, 2015.

4.4 Papel dos fatores de crescimento e outras moléculas no desenvolvimento da vascularização fetal

Fatores de crescimento placentário (PGF) ou endotelial (VEGF) são essenciais para o desenvolvimento e manutenção da vascularização placentária. Esses fatores também são importantes para angiogênese no sítio de implantação para que haja invasão embrionária no miométrio. Em mulheres com PE podem ser encontrados, baixos níveis de PGF circulante, esses dados estão muitas vezes relacionados com insuficiência placentária clinicamente relevante (Powe, Levine et al. 2011, Goel and Rana 2013, Cotechini, Komisarenko et al. 2014). A molécula de PGF sinaliza através do receptor 1 para VEGF (Flt-1), com isso a distribuição desse receptor na fisiopatologia da PE tem sido também investigada (Makrydimas, Sotiriadis et al. 2008, Chappell, Mouillesseaux et al. 2013). Em alguns casos específicos pacientes com PE apresentam níveis de Flt-1 circulantes mais elevados, quando comparados com pacientes com uma gestação sem complicações. Inibidores de Flt-1 estão sendo investigados como terapia alternativa para PE. Mesmo com grande quantidade de bibliografia sobre o tema, a influência da via PGF-Flt-1 na PE e no desenvolvimento vascular fetal ainda não foi descrita claramente. Níveis maternos e fetais adequados dessas substâncias são fundamentais para uma adequada angiogênese em todas as fases do desenvolvimento. Com a presença de uma adequada formação vascular existe por consequência o risco diminuído de doenças cardiovasculares como infarto, esquemia e acidente vascular cerebral (AVC) na vida adulta (Herrera-Garcia and Contag 2014, Lu, Mao et al. 2014). Tem sido descrito que crianças nascidas de mães que desenvolveram PE durante a gestação têm menos vasos comunicantes na região cerebral. Adicionalmente animais geneticamente deficientes em PGF apresentaram má-formação no polígono de Willis que é a estrutura vascular central do sistema nervoso central (SNC).

Figura 4 – Desenho anatómico demostrando a vascularização cerebral, em detalhe o polígono de Willis e suas conexões. Por ADAM *heath care company*.



Ref: Por ADAM *heath care company*.

Moléculas de adesão da classe das Selectinas têm sido descritas por terem um papel importante durante a implantação embrionária (Eskicioglu, Lacin et al. 2014). Os níveis de S-Selectina (S-Sel) estão relacionados com o aumento da receptividade uterina ao embrião. Os níveis de P-Selectin (P-Sel) podem ser utilizados como marcador inflamatório em algumas patologias pelo fato da mesma está associada com a adesão de macrófagos ao endotélio de tecidos durante injúria ou patologia. O balanço dessa proteína tem sido estudado e os dados ainda são controversos. Por isso, o papel de P-Sel durante a gestação e no aborto permanece não esclarecido totalmente (Fu, Wu et al. 2009). Em placenta, a P-Sel é expressa constitutivamente, desempenhando um papel importante na manutenção da arquitetura tecidual (Burrows, King et al. 1994, Dye, Jablenska et al. 2001). Bibliografia sobre o papel

da P-Sel em casos de dano placentário e a relevância clínica desses achados ainda é limitada. Estudo recente demostrou uma ligação entre diminuição de P-Selectina em tecido placentário com a degeneração de células trofoblásticas e endoteliais acompanhada de deficiência na adesão célula-célula.

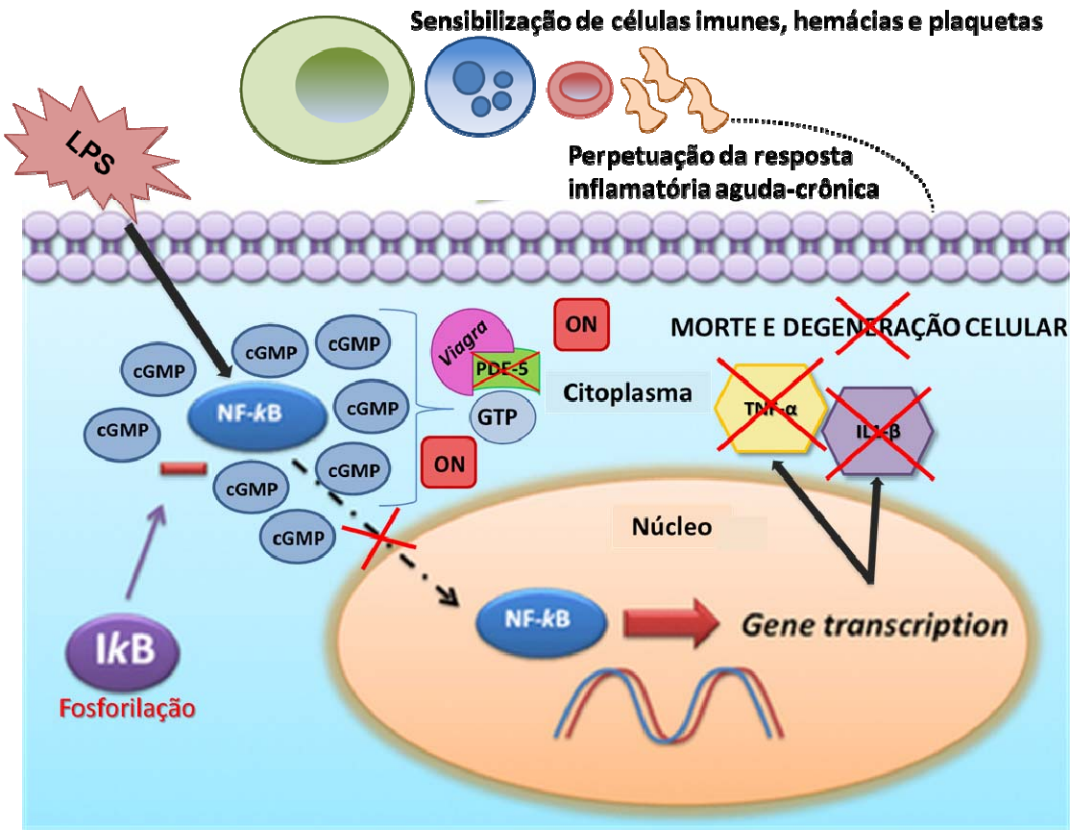
Outro relevante mediador químico durante a gravidez é o óxido nítrico (NO) que relaxa o músculo liso vascular através da ativação da enzima guanilato ciclase (GC), produzindo níveis elevados de monofosfato cíclico de guanosina (cGMP). Estão presentes em tecidos gravídicos as enzimas, sintase de óxido nítrico induzível (iNOS) e a sintase de óxido nítrico endotelial (eNOS) (Khan, Kusakabe et al. 2012, Toda, Toda et al. 2013). Doadores de ON têm sido utilizados como terapias em modelos animais de perda gestacional incluindo de PE. O aumento dos níveis de NO é fisiologicamente importante para mediação da angiogênese principalmente nas primeiras semanas da gestação humana e primeiras horas da gestação de camundongos. Além de atuar com um sinalizador entre células residentes assim como em células imunes circulantes, o NO tem outras atividades nos tecidos gravídicos, como vasodilatação de artérias espiraladas e relaxamento do musculo liso uterino. A produção de NO pela eNOS tem sido descrita por ter características anti-inflamatórias, geralmente pela regulação da sinalização pela proteína quinase ativada por AMP (AMPK), em diferentes tecidos (Donato, Morgan et al. 2015, Nunes, Raposo et al. 2015). Por sua vez tem sido descrito que NO produzido pela enzima iNOS pode ter características pró-inflamatórias. O entendimento da complexa modulação na via NO-cGMP pode ajudar no entendimento da resposta vascular em placenta em casos de perdas gestacionais. Após o embasamento científico necessário a via NO-cGMP pode se tornar promissora para tratar patologias gestacionais onde o aborto é uma característica comum entre elas.

4.5 Oportunidades terapêuticas para tratar o aborto

O Citrato de Sildenafil (Sil) é um inibidor potente e seletivo da PDE5 que por sua vez está presente em vários tecidos, tais como vascular, uterino e placentário (Lin, Lin et al. 2006, Kouvelas, Goulas et al. 2009). Vista a ampla distribuição da PDE-5 e o papel dessa enzima em funções fisiológicas importantes, o Sil pode ser eficaz no tratamento de muitas patologias e injúrias. No processo de remodelamento de pequenos vasos durante a implantação embrionária, o GMPc é um mediador importante do efeito do NO endógeno, que é fortemente expresso no trofoblasto invasivo. (Bolnick, Kilburn et al. 2015). Recentemente, vários são os modelos de doenças com caráter inflamatório, que avaliaram o Sildenafil obtendo sucesso terapêutico (Galie, Ghofrani et al. 2005, Raposo, Nunes et al. 2013, Zhang, Guo et al. 2013). A administração crônica de Sil reduziu os níveis de marcadores inflamatórios e vasculares tais como proteína-C reativa, interleucina-6, molécula de adesão intercelular (ICAM) e molécula de adesão vascular (VCAM) (Price, Gingell et al. 1998). Modelos animais para RCF são utilizados para demonstrar a eficácia desse fármaco em aumentar o fluxo de nutrientes para o feto (Dastjerdi, Hosseini et al. 2012, Dilworth, Andersson et al. 2013). Clinicamente, Sil tem sido reportado como um fator benéfico em aumentar receptividade uterina tratamento de pré-eclâmpsia e partos prematuros experimentalmente (Jerzak, Kniotek et al. 2008, El-Far, El-Motwally Ael et al. 2009, Stanley, Andersson et al. 2012). Por ambas características vasodilatadora e anti-inflamatória, o tratamento profilático com Sil demostrou ser eficaz em proteger do os fetos de sofrimento e morte em gestações expostas a altas doses de Lipopolissacaridesos (LPS) (Luna, Nunes et al. 2015). Sildenafil pode hipoteticamente

atuar como um medicamento multialvo em casos de complicações gestacionais, pois pode ser apresentar-se como vasodilatador mantendo o feto nutrido e respirando mesmo com a obstrução das artérias espiraladas, assim como, diminuir o perfil inflamatório do complexo abortivo; desta forma manter o feto vivo e saudável até o termo (Patnaik, Haddad et al. 2007, Middeldorp 2013). Mais estudos, incluindo estudos clínicos bem desenhados são necessários para o efetivo tratamento de pacientes com AR, TM e PE com Sildenafil.

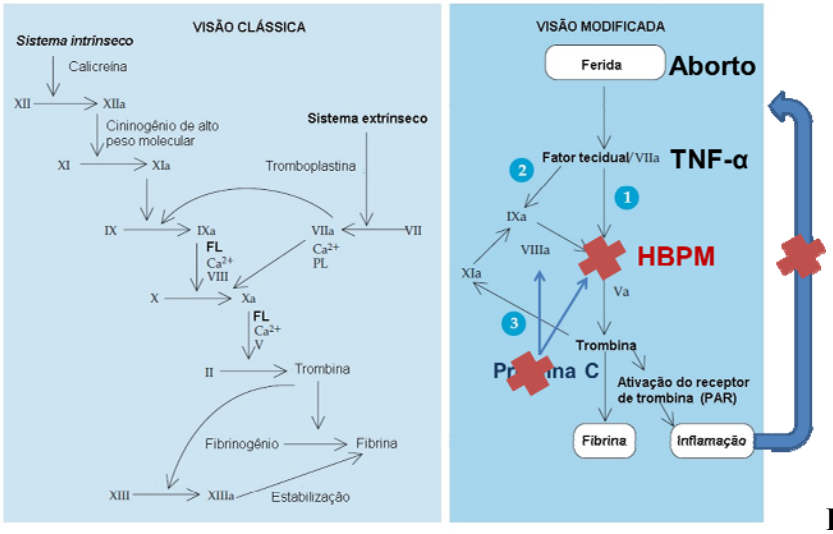
Figura 5 - Esquema do possível mecanismo de ação simplificado do Sildenafil como anti-inflamatório, especialmente inibindo a resposta via NFκB induzida por LPS. Por Rayana Luna, não publicado.



Ref: Por Rayana Luna 2014, não publicado.

Heparina (Hep) e seus análogos fazem parte de uma classe de drogas antitrombóticas, pois influenciam de variadas formas na cascata de coagulação (Lee and Kong 2015). Mais recentemente as Heparinas de baixo peso molecular (HBPM) como a Dalteparina e a Enoxeparina têm sido descritas pela inibição do Fator Xa, inibindo dessa forma a geração de trombina pela pró-trombina (Di Nisio, Peters et al. 2005, Lee and Kong 2015). Por sua vez, trombina não formada será um fator inibitório para a formação de fibrina pelo fibrinogênio. Tratamentos com Hep podem adicionalmente inibir a ativação do sistema complemento, que é a principal resposta imune do sistema humoral. O mecanismo de ação da Hep pode atenuar a ativação do fator XII, que se ativado liberaria altos níveis de bradicinina, gerando choque hemodinâmico por inflamação (Patnaik, Haddad et al. 2007). Desta forma, a Hep pode ser relacionada à uma atividade anti-inflamatória secundária moderada. Tem sido descrito também que Hep pode inibir diretamente a inflamação resultando em diminuição dos níveis de TNF-α (Spratte, Schonborn et al. 2015). HBPM tem sido utilizada sozinha ou em combinação com outras drogas para tratamento de complicações gestacionais por vários anos (de Jong, Goddijn et al. 2013, Gomaa, Elkholy et al. 2014, Whitley and Ural 2014). Tratamentos têm sido reportados utilizando HBPM em pacientes com TM, AR e PE, mesmo que a eficácia desse tipo de tratamento ainda seja considerada controversa (Roberge, Demers et al. 2015, Schleussner, Kamin et al. 2015). Alternativas para o tratamento de TM, AR e PE devem ser avaliadas para o desenvolvimento inclusive de um tratamento conjunto com HBPM, visto que a mesma apresenta eficácia limitada.

Figura 6 - Representação das vias de coagulação e como essas podem influenciar no processo abortivo.



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6. APÊNDICES

CAPÍTULO 1

Sildenafil (viagra(®)) blocks inflammatory injury in lps-induced mouse abortion: a potential prophylactic treatment against acute pregnancy loss?

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Sildenafil (Viagra®) blocks inflammatory injury in LPS-induced mouse abortion: A potential prophylactic treatment against acute pregnancy loss?

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Abstract

Introduction: Recurrent pregnancy losses (RPL) are common women's health issues. Inflammatory and thrombotic events have been associated with RPL including excessive production of cytokines, in particular TNF-a. However, mechanisms behind gestational losses are not yet fully understood. Sildenafil inhibits phosphodiesterase Type-5 (PDE5). This drug increases intracellular cyclic guanosine mono- phosphate, having vasodilatory and, more recently described, anti-inflammatory properties. PDE5 is present in murine and human uterus and placenta. Sildenafil is already used clinically for treatment of human fetal growth restriction (FGR). Our objective was to determine if Sildenafil alone or in combination with Heparin had protective effects in pregnant Swiss albino challenged to abort by lipopoly- saccharide (LPS).

Methods: Treatments (Sildenafil (50 mg/kg/day), Heparin (500 IU/Kg/day) or Sildenafil þ Heparin at the same doses) were initiated the morning of copulation plug detection (gestational day (gd0)). On the 15th day of pregnancy, an intraperitoneal injection of LPS (100 mg/kg) was administered. Untreated, pregnant mice challenged by LPS served as controls. Results: Assessments at 48 h after LPS revealed that Sildenafil þ Heparin prevented fetal loss. Early assessments at 2 h after LPS indicated that the pretreatments prevented induction of inflammatory cytokine production (TNF-a, IL-1b/NF-kb) and preserved placental histopathology.Discussion: Combined Sildenafil þ Heparin therapy was superior to either treatment alone in most an- alyses. The known safety of Sildenafil and Heparin in human pregnancy suggests that usage of these combined agents may be of value for treatment of patients with impending pregnancy loss or prophylactically in women with a history of recurrent miscarriages.

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1. Introduction

It is estimated that 20e30% of women who conceive experience one or more miscarriages [1e3]. One to five per cent of all women suffer recurrent pregnancy loss (RPL), defined as 3 or more consecutive losses [4]. However, the mechanisms responsible for fetal loss are not fully understood. Hematological, immunological, hormonal and genetic anomalies have been addressed as underlying causes of RPL [5]. Human endometrial microarray an- alyses between RPL and elective terminations in women with previous live offspring identified many significantly dysregulated genes in RPL patients that had immune function or were cell- signaling associated [4]. Patients with idiopathic RPL are a thera- peutic challenge and they are more prone to adverse obstetric and neonatal outcomes including a high risk for fetal growth restriction (FGR) [6].

Miscarriages are often associated with thrombosis [7,8] impli- cating the coagulation cascade as an additional element in female infertility [9]. Thrombosis may be a sequel to reduced utero- placental perfusion [10,11] and low-molecular weight Heparin(LMWH) has been a therapeutic treatment of choice [12,13]. How- ever use of LMWH therapy is controversial and, in some cases anti- miscarriage effect is not seen [14e16]. LMWH therapy is not currently recommended for RPL. Sildenafil Citrate (Viagra®), a vasodilator, is also described as an anti- inflammatory agent [17e19]. In pregnant women, treatment with Sildenafil may improve blood flow to the placenta and fetus and is currently being investigated as a treatment in fetal growth restriction (FGR) [20e22]. In pregnant mice, Sildenafil enhances fetal growth, even in the absence of abnormal placental circulation [23].

Sildenafil has an excellent tolerability profile and, although it was originally approved for therapeutic use in cases of erectile dysfunction, is now used to treat multiple human diseases such as:, pulmonary hypertension, Raynaud's syndrome besides of erect dysfunction [24e28]. This drug induces the accumulation of cyclic guanosine monophosphate through selective inhibition of the enzyme phosphodiesterase-5 (PDE5) [24,29,30]. PDE5 is expressed in myometrium, endometrium, decidua and placental tissues of woman and female mice [31e33]. Given its anti-inflammatory, vasodilatory and safety properties, we postulated that Sildenafil might have prophylactic benefit against acute pregnancy loss. LPS- induced abortion model in mice was selected for proof of principle studies.

Human RPL and LPS-induced mouse abortions have been asso- ciated with pro-inflammatory cytokines and particularly, tumor necrosis factor alpha (TNF-a) [9,10,34e36]. The nuclear transcrip- tion factor kB (NF-kB) pathway regulates the inflammatory response including the gestational inflammatory complex [37]. In addition to acute inflammation, rodent LPS exposure induces thrombophilia [10,36], making the LPS-induced abortion model of dual relevance to human RPL.

2. Methods

2.1. Animals

The study was approved by the Aggeu Magalh~aes Research (FIOCRUZ) Center Institutional Animal Care and Use Committee (IACUC) and complies with the guidelines of the Research Center (FIOCRUZ-PE) Ethics Committee for Animal Experimentation (Brazil; Permit Number: P-408-68 licensed on 08/08). 48 female and 20 male mice, each weighing 30 g were used to obtain 40 Swiss albino pregnancies for study. Pregnancy was achieved by placing proestrous females with stud males. Copulation was confirmed the morning after mating by the vaginal plug (considered day 0.0 of pregnancy). Two studies were conducted that differed only in the timing for outcome analyses. Preliminary studies were used to validate the effectiveness of the dosage chosen. The first study analyzed fetal loss 48 h after LPS. The second study recovered tis- sues for analyses at 2 h after LPS injection when all implantation sites retained a viable appearance.

A total of 40 mated females were divided into 5 groups of 8 pregnancies each as follows:

i. Untreated pregnancy control: received neither prophylactic drug (water only for drinking) or LPS (sterile saline placebo treatment).
ii. LPS treatment only-abortion control: received neither pro- phylactic drug (water only for drinking), however were injected (intra- peritoneal) with 0.5 ml LPS (*Escherichia coli*

serotype SigmaeAldrich 0111-B4 100 mg/kg in sterile saline) on the 15th day of pregnancy. Euthanasia was 48 or 2 h later.

iii. Sildenafil prophylaxis: 50 mg/kg of Sildenafil (Viagra®, Pfizer) were administered through the drinking water from the 1st to 15th day of pregnancy. Mice were weighed and their water consumption was measured daily to accurately increase Sildenafil concentrations in the water to deliver a constant dosage of Sildenafil on each day of pregnancy. LPS was administered on gd15 Euthanasia was 48 or 2 h after LPS.

iv. Heparin prophylaxis: LMWH (Fragmin, Pfizer) was adminis- tered at a dose of 500 IU/kg subcutaneously per day to weighed mice in a volume of 0.2 ml from gd1 to gd15 day of pregnancy. LPS was administered as above on gd1. Eutha- nasia was 48 or 2 h after LPS.

v. Sildenafil þ Heparin prophylaxis: received both drugs as described above from gd1-gd15 and also received intra- peritoneal LPS on gd15.

To assess fetal death at 48 h after saline placebo or LPS, gross pathological examination was used with 4 pregnancies studied/ treatment group. Assessments were based on the replacement of bright red-pink coloration of the conceptus by pallid coloration, absence of or decreased movements of fetuses relative to control or absence of a beating fetal heart. For tissue collections at 2 h after saline or LPS, pregnant females were euthanized by anesthesia using ketamine (100 mg/kg) and xylazine (10 mg/kg) using 4 pregnancies/group. Implantation sites in all treatment groups (usually about 13/uterus) appeared similar and healthy at this early time after abortion induction. Placentas were removed and pro- cessed by one of the following methods. Due to the large litter sizes obtained, all of the techniques described below could be conducted on tissues from multiple implantation sites from each female.

2.2. Histopathology

Placental tissues were fixed in 4% buffered PFA (PFA) for 24 h, processed and paraffin embedded pre-cutted in half/half or placed into 15% sucrose followed by 30% sucrose, embedded in OCT-Tissue- Tek compound and frozen in n-hexane with liquid nitrogen for cryostat sectioning. Microtome sections (4e5 mm paraffin or 7e8 mm, cryostat) were mounted onto glass slides and stained with hematoxylin-eosin or fluorescent antibodies and assessed using an inverted photomicroscope (Observer Z1, Zeiss Micro Imaging GmbH) at a magnification of 400x or an inverted fluorescence photomicroscope (Zeiss Micro Imaging GmbH). Quantitative ana- lyses of the vascular spaces were undertaken in the placental labyrinthine region using 8 images per group from 4 differentpregnancies. Measurements used Image J (NIH, Bethesda, MD, USA).

2.3. Transmission electron microscopy

Placental fragments were fixed in Karnovsky's solution, post- fixed in 1% osmium tetroxide, dehydrated in an acetone series and embedded in SPIN-PON resin. Semi-thin sections (0.5 mm) were placed on slides and stained with toluidine blue for the morpho- metric analysis. Ultrathin sections (70 nm) were placed on 300- mesh nickel grids, counterstained with 5% uranyl acetate and lead citrate and examined using a FEI Morgani 268D transmission electron microscope.

2.4. Immunohistochemistry

Immunohistochemistry using paraffin-embedded sections used a diaminobenzidine (DAB) staining protocol, while cryostat sec- tions were examined using a fluorescence protocol. Deparaffinized sections were incubated in 1% bovine serum albumin (BSA for blocking) buffer. For antigen retrieval, a citrate buffer was used (pH 6.0) in a humid chamber for 30 min. Sections were then incubated with the anti-mouse polyclonal primary antibody anti-TNF-a 1 mg/ ml (ab34674-Abcam), anti- NF-kB 1 mg/ml (ab31418-Abcam) and

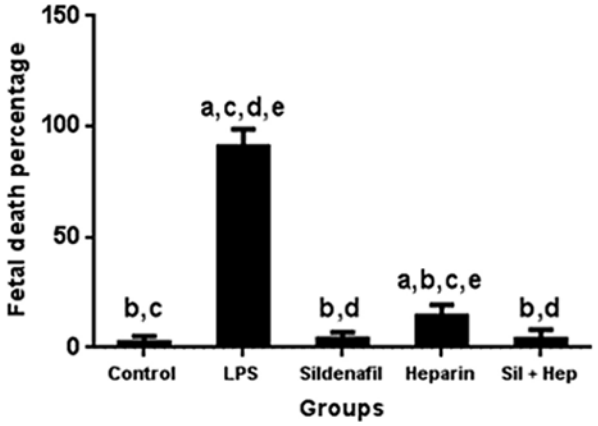

Fig. 1. Percentages of fetal death 48 h after LPS exposure for the following groups: Control, LPS, Sildenafil, Heparin, Sil þ Hep. Data expressed in ±S.D. mean of n ¼ 4 mice in each group. Data expressed as mean ± S.D. of n ¼ 4 mice in each group; ND: not detected. The letter on the top of the columns represents the groups that have had significant differences with that cited group (a - control; b - LPS; c - Sildenafil; d - Heparin and e - Sildenafil þ Heparin) ($p < 0.05$) was found.

biotin-conjugated secondary antibody using an HRP-kit (K0690- DakoCytomation), with DAB used as the chromogen. The speci- mens were then weakly counter-stained with hematoxylin. Sec- tions were examined from multiple implantation sites in each of the 20 pregnancies studied at 2 h after LPS. Cryostat sections were incubated for 1 h with BSA blocking solution. The sections were incubated with antibody IL-1b 1 mg/ml (ab9722-Abcam), and incubated with polyclonal Cy3-conjugated secondary antibodies (705-165-147-Jackson) against rabbit immunoglobulin 0.5 mg/ml (F6257-Sigma-Aldrich) and examined. For each antibody, five stained areas for each protein investigated were measured for pixel density using the GIMP 2.6.11 software program.

2.5. *Western blotting*

Previously dissected placental fragments were homogenized in an extraction cocktail (10 mM EDTA; 2 mM phenylmethane sulfonyl-fluoride, 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM NaVO4, 10 mg of aprotinin/mL, and 100 mM Tris, pH 7.4). Proteins (40 ng) were separated on 10% acrylamide electrophoresis gel and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with anti-mouse polyclonal rabbit antibody, TNF-a 0.1 mg/ml (500-P64-Peprotech), IL-1b 0.1 mg/ml (ab9722-Abcam) anti-NFKB 0.1 mg/ml (ab31418-Abcam) or b-actin 0.1 mg/ml (A2228-SigmaAldrich), followed by peroxidase-conjugated anti-rabbit 0.08 mg/ml (A9169-SigmaAldrich). The bands were visualized by chemiluminescence and the blots were developed on X-ray film. The pixel density of each band was determined using the Image J 1.38 software program and compared between groups. Experiments were performed in triplicate. Immunoblotting for b-actin was performed as a protein control.

2.6. *Statistical analyses*

GraphPad Prism (San Diego, CA, USA) was used for statistical analyses of immunoblotting densitometry data, immunohisto- chemistry and immunofluorescence specific pixel density analyses

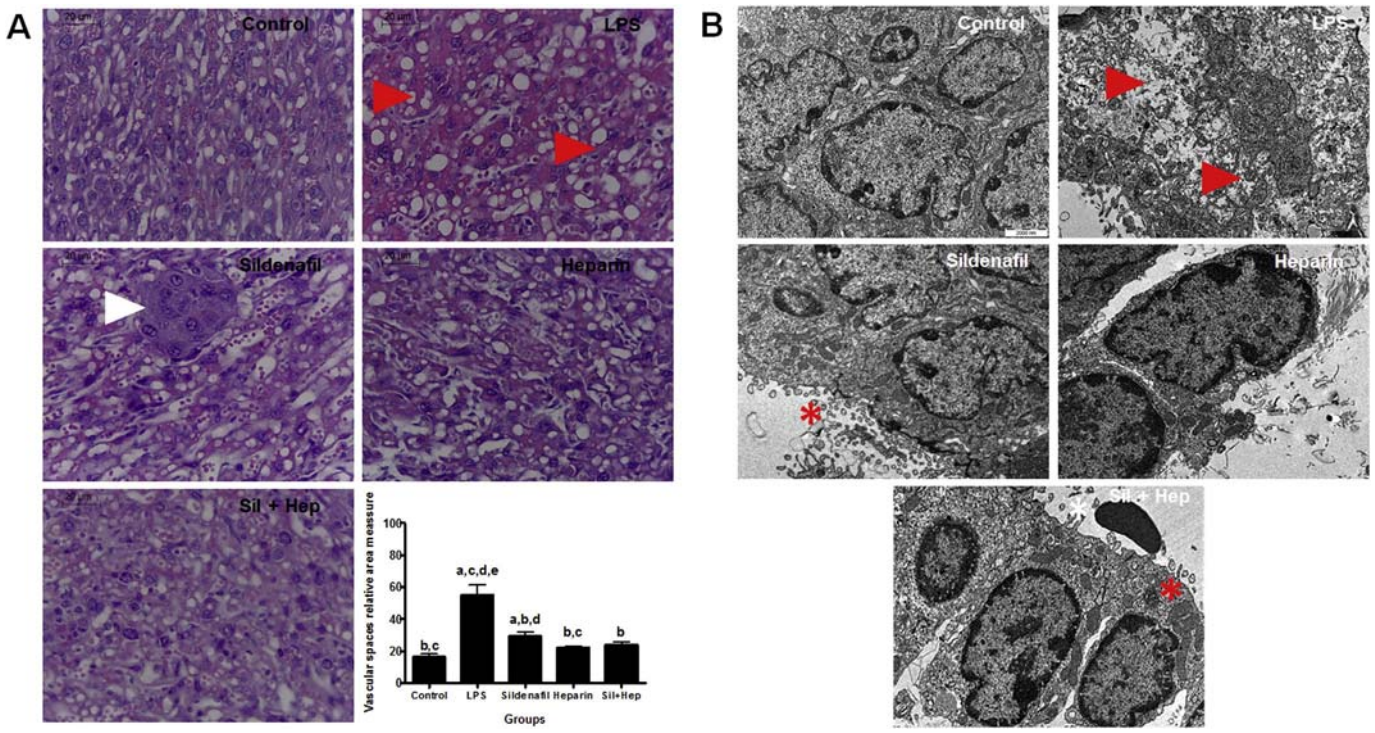

Fig. 2. A: Histopathology of the placental labyrinthine region of the following groups: Control, LPS, Sildenafil, Heparin, Sil þ Hep. Congestion and extravascular edema were observed in LPS group (red arrows). Accumulations of trophoblast giant cells were observed in the Sildenafil group (white arrow). Heparin preserved cellular morphology. Sil þ Hep preserved cellular morphology and tissue architecture, and maintained blood cells inside vessels. Bars, 20 mm. The histogram presents the area in the placental labyrinth occupied by vascular space. Data expressed as mean ± S.D. of n ¼ 4 mice in each group; ND: not detected. The letter on the top of the columns represents the groups that have had significant differences with that cited group (a - control; b - LPS; c - Sildenafil; d - Heparin and e - Sildenafil þ Heparin) ($p < 0.05$) was found. B: Electron transmission microscopy revealing labyrinthine cells in the following groups: Control, LPS, Sildenafil, Heparin, Sil þ Hep. Control shows normal tissue architecture while the LPS group shows widespread severe cell degeneration (red arrows). Pre-treatment with Heparin protected cellular ultrastructure, but some cytoplasmic degeneration was observed. In the Sildenafil and Sil þ Hep group (E). Cells of the labyrinth were protected from LPS damage and microvilli were sustained (red asterisk). Bar, 2000 nm.

and for evaluation of fetal death. Two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test were performed. Data were also subjected to non-parametric test ManneWhitney testing. Statistical significance was set as $p < 0.05$.

3. Results

3.1. Assessment of fetal viability 48 h after LPS

In control females, the spontaneous fetal death rate at gd17 was low at 2.25%. In females receiving LPS only (without prophylaxis), the rate of fetal demise approached 100% in each of the 4 pregnancies. The abortifacient actions of LPS were significantly reduced in the pregnancies treated prophylactically with Sildenafil, Heparin and Sildenafil þ Heparin. Fetal losses were 4%, 14.5% and 3.75% respectively. Sildenafil and Sildenafil þ Heparin achieved preg- nancy success rates that were similar to the unchallenged Control group (non-significant difference) (Fig. 1).

3.2. Assessment of acute tissue damage 2 h after LPS

3.2.1. Morphology

Initial histological scanning overviews revealed that the gd15.0 placentas of mice 2 h after receiving LPS only differed from con- trols in the labyrinth region of mid-sagittal sections. Other regions of the implantation sites had not yet become noticeably compro- mised. Thus, detailed analyses were focused to the placental lab- yrinth. Exposure to LPS with no prophylaxis induced edema, congestion and degeneration in labyrinthine trophoblast cells. All prophylactic treatments attenuated these findings. The Sildenafil group retained cellular architecture similar to the non-challenged control and had blood spaces that appeared to be normal in size. In the Heparin group, cell structure was well-preserved but the labyrinthine blood spaces appeared reduced in size. In contrast, prophylaxis with Sildenafil þ Heparin preserved labyrinth tro- phoblasts and vascular spaces (Fig. 2A). Quantitative analyses of the total labyrinthine vascular space (ie. maternal and conceptus vessels) revealed that LPS treatment greatly dilated vessels of this key placental exchange area compared to untreated controls (Fig. 2A) All treatments prevented ($p > 0.05$) this acute major vascular disturbance. Vessels in the labyrinth of the Sildenafil treated group were slightly dilated when compared to groups treated with Heparin and Sildenafil þ Heparin. This may be due to the vasodilator effect of Sildenafil. Thus, all of the treatments were effective in protecting labyrinthine vessels which should stabilize fetal and maternal hemodynamic properties following LPS administration (Fig. 2A).

Ultrastructurally, unchallenged control placental labyrinthine cells were oval with a nucleus containing prominent nucleoli and they had prominent, numerous microvilli. Exposure to LPS without prophylaxis induced striking degeneration of these cells, particu- larly reduction of trophoblast microvilli. In contrast, prophylaxis with Sildenafil or Sildenafil þ Heparin resulted in well-preserved cellular organelles, including distinct microvilli after LPS chal- lenge. Prophylaxis with Heparin was only less protective against LPS challenge with some mild cytoplasmic damage (Fig. 2B).

3.2.2. Inflammatory responses

Tissue sections from the non-challenged control group had a basal expression of the pro-inflammatory cytokine TNF-a. The LPS

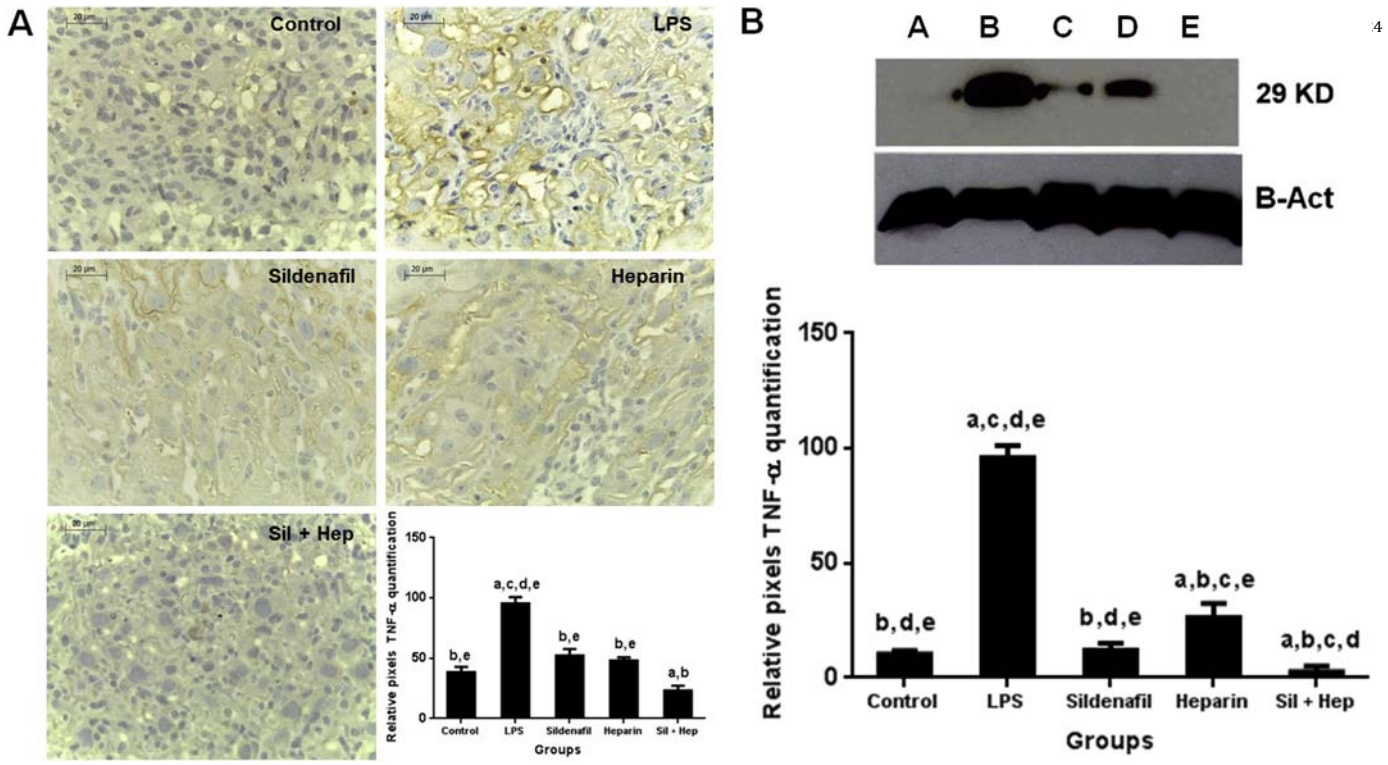

Fig. 3. A: Immunohistochemical localization of TNF-a in the following groups: Control, LPS, Sildenafil, Heparin, Sil þ Hep. The Control group exhibited basal expression and LPS induced increased TNF-a staining. Bar, 20 mm. Quantitative densitometry analysis of TNF-a immunohistochemistry (GIMP2 analyzed). Data are expressed as mean ± S.D. from n ¼ 5 mice for each group. B: Western blotting for TNF-a. Pre-treatments decreased TNF-a expression especially combined Sildenafil and Heparin pre-treatment. Groups are Control, LPS, Sildenafil, Heparin, Sil þ Hep. The largest decrease was found in the Sil þ Hep group (Image J analyzed). Representative blots of lysates obtained from a pool of 8 placentas per group. Data expressed as mean ± S.D. of n ¼ 4 mice in each group; ND: not detected. The letter on the top of the columns represents the groups that have had significant differences with that cited group (a - control; b - LPS; c - Sildenafil; d - Heparin and e - Sildenafil þ Heparin) ($p < 0.05$) was found.

group had increased TNF-a immunostaining. In comparison to the LPS group, the Sildenafil and Heparin groups exhibited less TNF-a labeling, indicating that these treatments were effective in reducing inflammation. Superior reduction in TNF-a was obtained by Sildenafil þ Heparin treatment compared with single agent treatments (Fig. 3A). These findings were confirmed by Western blotting analyses (Fig. 3B). Immunofluorescence analyses revealed a significant increase in IL-1b labeling in the LPS versus the non- challenged control group. Treatment with Sildenafil or Heparin separately or the combined treatment significantly reduced IL-1b labeling in comparison to the LPS group (Fig. 4A). These results were confirmed by Western blotting analyses (Fig. 4B).

Immunostaining for NF-kB specifically localized this transcrip- tion factor. In the unchallenged control group, NF-kB was detected mainly in the cytoplasm of labyrinthine trophoblast cells, a pattern typical of non-activated cells (Fig. 5A). In pregnancies receiving LPS only, intense nuclear labeling was seen in trophoblast cells indi- cating nuclear translocation of phosphorylated, activated NF-kB. Additionally, weaker NF-kB immunostaining was seen over the trophoblast cytoplasm (Fig. 5B). In the Sildenafil and Sildenafil þ Heparin groups, no nuclear NF-kB reactivity was detected. In these groups, a pattern of light cytoplasmic staining was present that was similar to the labeling pattern observed in the non-challenged control group (Fig. 5A). The Heparin only treatment group exhibited slightly less nuclear staining than the LPS only treatment group and the cytoplasm of these cells had a similar labeling pattern to that of the unchallenged control group (Fig. 5A). These data suggest that Sildenafil is superior to Heparin in inhib- iting NF-kB nuclear translocation. Western blotting analyses pro- vided similar information (Fig. 5B).

4. Discussion

Current research goals include the discovery of new drugs that can reduce gestational complications such as premature birth and fetal death [38e43]. The present findings demonstrated that Sil- denafil is efficient in reducing the fetal mortality rate in an LPS- induced murine abortion model and has treatment value in com- bination with Heparin. As reported by Renaud et al., a single in- jection of LPS in pregnant rats (100 mg/kg) induces pregnancy harm that nearly always results in fetal death within 72 h [10]. Aberrant inflammation, particularly elevated TNF-a and implantation site thrombosis are both induced prior to fetal death [44]. The same dose of LPS triggers pregnancy loss and our data suggest that this is also through aberrant inflammation that includes elevation in TNF- a plus other cytokines and in promotion of coagulation that can be reduced by heparin administration [36]. These same pathological mechanisms are reported to accompany spontaneous pregnancy losses in women [45,46]. The present study found fetal loss in mice could be quantified earlier at 48 h after LPS injection and that prophylactic treatments blocked LPS-induced conceptus demise. Treatment with Heparin significantly reduced the percentage of fetal death rate to 14.25%. More significantly, treatment with Sil- denafil or Sildenafil þ Heparin completely blocked LPS-mediated fetal demise demonstrating the greater efficacy of these treat- ments in comparison to Heparin alone.

We implemented a unique study design in the mouse which provides a basis for future prophylactic studies in women who have had previous recurrent miscarriages and may benefit from a treatment begun before or during the first weeks of pregnancy. The decrease of peripheral natural killer cells was described in women

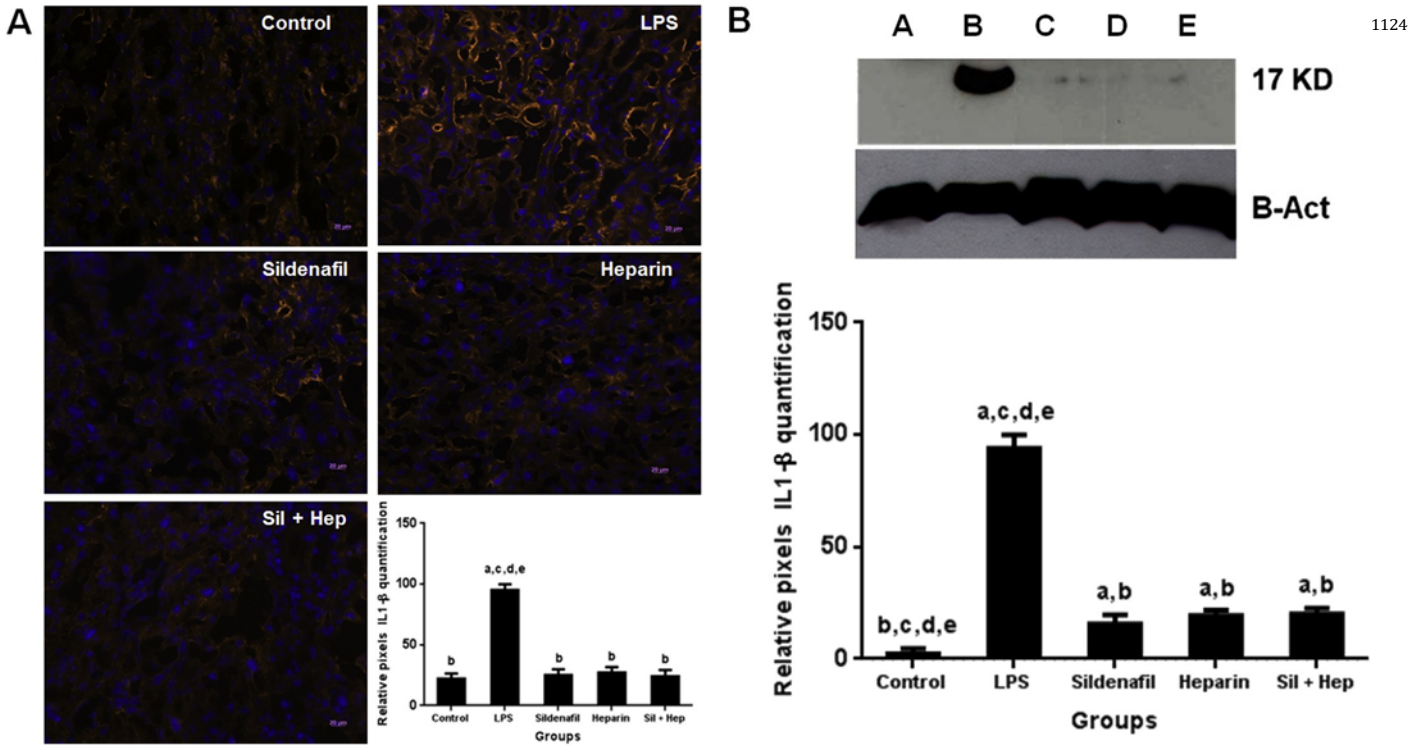

Fig. 4. A: Immunofluorescence for IL1-b reactivity of the following groups: Control, LPS, Sildenafil, Heparin, Sil þ Hep. Detectable IL1-b increased in the LPS group but was not elevated in groups receiving prophylactic treatments. Bar, 20 mm. Quantitative densitometry analysis of IL1-b immunofluorescence (GIMP2 analyzed). Data are expressed as mean ± S.D. from n ¼ 5 mice for each group. B: Western blotting for IL1-b. Decreased the expression for IL1-b was observed in all the treatments versus the LPS challenged group. (Image J analyzed). Representative blots of lysates that were obtained from a pool of 8 placentas per group, 3 replicates in each group. b-actin was used for standardization. Data expressed as mean ± S.D. of n ¼ 4 mice in each group; ND: not detected. The letter on the top of the columns represents the groups that have had significant differences with that cited group (a - control; b - LPS; c - Sildenafil; d - Heparin and e - Sildenafil þ Heparin) ($p < 0.05$) was found.

treated with oral Sildenafil during pregnancy [47]. The literature contains a case report which suggests that intra-vaginal application of Sildenafil (25 mg during 24 days) in women with histories of previous spontaneous pregnancy losses who presented with first trimester (wk 6e11) with threatened abortion (vaginal bleeding) may have value in prevention of miscarriage [48]. This report is limited because the pregnancies are only reported as "ongoing" and gestational outcomes are not reported; only plasma and leukocyte alterations induced at 13 and 24 days after initiation of treatment. While this study assumed a continuous provocative challenge against the continuation of the pregnancies, our study addressed a single provocative episode. Both studies report linkage of TNF-a with impending or ongoing fetal demise.

In our model, histopathological analysis demonstrated that LPS rapidly (2 h) induced tissue and cellular alterations, especially in the labyrinth region, the interface important in exchange of blood nutrients and wastes between mother to the fetus [49e51]. These data confirm findings described in a previous study [38], which reports high levels of tissue inflammation 2 h following exposure to LPS. Treatment with Sildenafil and Heparin attenuated tissue degeneration, with the best results achieved using the two drug combination. The groups treated with Sildenafil exhibited some groups of giant trophoblast cells inside of the labyrinth region. This finding has been reported as harmful, since these cells replace other areas of the placenta in the presence of pathological processes such as neoplasia [52e55]. In the present study, however, neoplastic features were not apparent and the trophoblasts were concentrated in regions close to damaged tissue, forming what seemed to be a protective barrier against further injury. This cell type produces and stores a number of hormones and cytokines in some areas of the placenta and is therefore crucial to the maintenance of a healthy pregnancy [52,56,57].

LPS causes placental degeneration due to acute inflammation [10,58]. The ultrastructural analysis demonstrated cell degenera- tion induced by LPS, especially in the labyrinthine region. All treated groups showed improvements in comparison to the LPS group. In the Sildenafil group, cells with preserved membranes and nuclear chromatin were found. Treatment with Heparin did not completely protect from LPS-induced cellular damage. Treatment with Sildenafil þ Heparin gave a labyrinth with well-preserved cells morphologically similar to those in the unchallenged control group. The control group data replicate data described in previous studies involving untreated placental tissue [59,60]. Studies involving LPS report ultrastructural damage to spiral arteries three hours following exposure to the endotoxin [10]. In one study, Sildenafil was used for the treatment of uterine growth restriction and was found to lead to better fetal development in humans [21], however, no ultrastructural data are reported in humans.

Inflammation [61] is described as an important factor in recur- ring miscarriages of many species in naturally or artificially conceived pregnancies [7,62e64] with TNF-a having a pivotal role [36]. While the mechanism by which TNF-a mediates fetal death remains unclear, it may involve luteal hormone insufficiency or placental damage resulting in vascular insufficiency with reduced

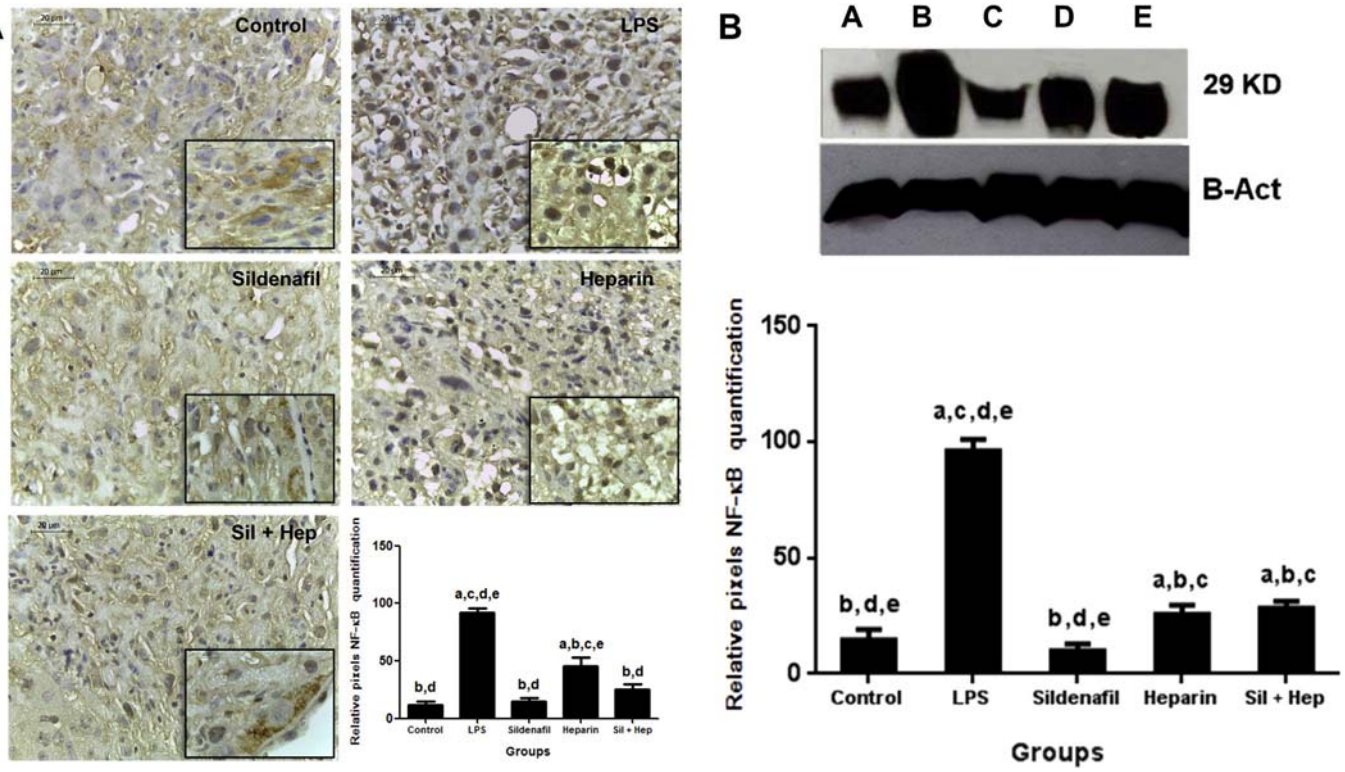

Fig. 5. A: Immunohistochemistry for NF-kB of the following groups: Control, LPS, Sildenafil, Heparin, Sil þ Hep. NF-kB expression increased in the LPS group and was mainly seen in the nucleus in comparison to the unchallenged Control group that displayed cytoplasmic staining. Treatment with Sildenafil and Sildenafil with Heparin maintained the pattern of the untreated Control group. Bar, 20 mm. Highlight: magnification immunohistochemistry for specific localization of NF-kB of the following groups: Control, LPS, Sildenafil, Heparin, Sil þ Hep. Staining was cytoplasmic in the unchallenged Control group whereas in the LPS group, reactivity was located mostly over the nucleus NF-kB staining remained predominantly cytoplasmic in groups receiving Sildenafil. Bar, 10 mm. Quantitative nuclear densitometry analysis of NF-kB immunohistochemistry (GIMP2 analyzed). Data are expressed as mean ± S.D. from n ¼ 5 mice for each group. B: Western blotting for NF-kb. Pre-treatments decreased NF-kb expression. Groups are Control, LPS, Sildenafil, Heparin, Sil þ Hep. The largest decrease was found in the Sildenafil group. (Image J analyzed) Representative blot of lysates obtained from a pool of 8 placentas per group, 3 replicates for each group. b-actin was used for normalization. Data expressed as mean ± S.D. of n ¼ 4 mice in each group; ND: not detected. The letter on the top of the columns represents the groups that have had significant differences with that cited group (a - control; b - LPS; c - Sildenafil; d - Heparin and e - Sildenafil þ Heparin) ($p < 0.05$) was found.

uteroplacental flow [65]. Others demonstrated involvement of TNF- a in pregnancy loss three hours after exposure to LPS [10,66,67]. Our data suggest lethal damage is more acute. Entanercept, a TNF-a inhibitor, maintains rat gestation and fetal growth rates after LPS challenge [36]. The phosphodiesterase-4 inhibitor Rolipram is re- ported to decrease pro-inflammatory cytokines, such as TNF-a, up to four hours following exposure to LPS [68]. Prophylactic treat- ment with Sildenafil in combination with this agent might also be beneficial for women experiencing RPL and be safe for the preg- nancy. The response in the Sildenafil þ Heparin group decreased TNF-a expression below levels in the Control group. A possible explanation for this is that Sildenafil [17] and Heparin [69] have anti-inflammatory effects using different pathways and synergis- tically reduce TNF-a below physiological levels. Future studies will be needed to see if lower than normal levels of TNF-a have any negative impact on pregnancy or post-partum maternal or offspring health.

Quantification of IL-1b, had a different pattern. Combined treatment with Sildenafil and Heparin did not reduce the immu- nostaining for IL-1b below that achieved by either drug alone. Others report that nuclear factor kB (NF-kB) has an important role in gene regulation at the onset of the inflammatory response by regulating mediators such as TNF-a and IL-1b [64,70]. A possible broad role for Sildenafil in blocking the inflammatory cascade mediated by NF-kB has been described. This hypothesis is sup- ported in the current study by the immunohistochemical localiza- tion of NF-kB. In the LPS group, NF-kB staining was mainly nuclear whereas staining in the non-challenged and treatment groups was cytoplasmic. This confirms that treatment blocked this important inflammatory pathway and predicts the prevention of tissue damage and implantation site viability 48 h after LPS challenge. Identification of the involvement of the NF-kB pathway is of key importance for the development of novel treatments for miscarriage.

This is the first description of the protective role of Sildenafil (Viagra®) alone and in a combined treatment with Heparin after LPS-challenge in mouse pregnancies. The action of Sildenafil (Via- gra®) in inhibition of NF-kB transcription may be its critical role in reducing inflammatory mediators such as TNF-a and IL-1b. Heparin has anticoagulant and anti-inflammatory features, as it inhibits the complement system [69,71,72] and is therefore able to protect placental tissue. While a dominant therapeutic effect was attrib- uted to Sildenafil, a synergism was apparent between Sildenafil and Heparin, and the combined administration of these two drugs proved the most effective approach to prevention of placental injury. Thus, Sildenafil appears to be a safe and effective prophy- lactic therapy for women who experience RPL. It may also have value in treatment of impending acute fetal loss but new studies will be required to determine if the duration of these treatments can be shortened while retaining efficacy. Additional studies are also needed to address earlier gestational time points more typical of RPL and to gain a better understanding of the relationship be- tween the NF-kB pathway of inflammation and fetal loss.

5. Conclusion

The labyrinthine exchange region of the placenta was identified as acutely vulnerable to LPS-induced murine pregnancy failure. The placenta could be protected from LPS-induced injury by drugs blocking inflammatory and thrombotic pathways. Treatment with Sildenafil and, especially its combined use with Heparin, main- tained integrity of labyrinthine trophoblast cells against acute LPS- induced inflammation and sustained fetal viability. Protection by Sildenafil involved the blockade of LPS-induced NF-kB signaling and inflammatory cytokine elevation (TNF-a and IL-1b). Sildenafileither alone or in combination with Heparin, may be a valuable prophylactic pre-conception or in early pregnancy for RPL patients.

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CAPÍTULO 2

Effects of sildenafil citrate and heparin treatments on placental cell morphology in a murine model of pregnancy loss

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Effects of Sildenafil Citrate and Heparin Treatments on Placental Cell Morphology in a Murine Model of Pregnancy Loss

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Key Words
Dalteparin · Inflammation · Recurrent miscarriage · Sildenafil

Abstract

Lipopolysaccharide (LPS) injections during pregnancy are well established as models for pregnancy complications, including fetal growth restriction (FGR), thrombophilia, preterm labor and abortion. Indeed, inflammation, as induced by LPS injection has been described as a pivotal factor in cases of miscarriage related to placental tissue damage. The phosphodiesterase-5 inhibitor sildenafil (Viagra®) is currently used to treat FGR cases in women, while low-molecular weight heparin (Fragmin®) is a standard treatment for recurrent miscarriage (RM). However, the pathways and cellular dynamics involved in RM are not completely understood. The aim of this study was to evaluate the protective effect of sildenafil and dalteparin in a mouse model of LPS-induced abortion. Histopathology, ultrastructural analysis and immunofluorescence for P-selectin were studied in two different placental cell types: trophoblast cells and labyrinth endothelial cells. Treatment with sildenafil either alone or in combination with heparin showed the best response against LPS-induced injury during pregnancy. In conclusion, our results support the use of these drugs as future therapeutic agents that may protect the placenta against inflammatory injury in RM events. Analyses of the ultrastructure and placental immunophysiology are important to understand the mechanism underlying RM. These findings may spark future studies and aid in the development of new therapies in cases of RM.

Abbreviations used in this paper

FGR	fetal growth restriction
LPS	lipopolysaccharide
LMWH	low-molecular weight heparin
PDE-5	phosphodiesterase-5
P-Sel	P-selectin
RM	recurrent miscarriage
Sil+Hep group	sildenafil with heparin
TEM	transmission electron microscopy

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Introduction

Sildenafil (Viagra®) is a selective phosphodiesterase-5 (PDE-5) enzyme inhibitor [Abbott et al., 2004], which causes intracellular accumulation of cyclic guanosine monophosphate since PDE-5 is responsible for maintaining normal levels of this important second messenger [Raposo et al., 2013; Zhang et al., 2013]. Anti-inflamma- tory activity is also attributed to other PDE inhibitors that may cause nucleotide cyclic monophosphate accumula- tion in the cytoplasm, blocking inflammatory pathways [Roumeguere et al., 2010; Raposo et al., 2013; Garcia et al., 2014]. Sildenafil has an excellent tolerability profile and has been safely used to treat several diseases, such as erec- tile dysfunction [Mulhall et al., 2013], pulmonary hypertension [Bogdan et al., 2012] and Raynaud's syndrome [Schwartz et al., 2012]. More recently, sildenafil has been tested for the treatment of patients with female infertility [Jerzak et al., 2008], and improvements were achieved in the treatment of fetal growth restriction (FGR) [Dastjerdi et al., 2012; Dilworth et al., 2013]. FGR is an important perinatal condition [DuBois et al., 2014] that is remedied by improved blood flow during pregnancy as a result of the vasodilator effects of sildenafil [Wareing et al., 2005]. Dalteparin is a low-molecular-weight heparin (LMWH) with anticoagulation effects. It enhances the in- hibition of factor Xa and blocks the conversion of throm- bin from antithrombin [O'Brien et al., 2014]. LMWH is effective in preventing clot formation [Middeldorp, 2013] and has been used to treat recurrent miscarriage (RM) with [Mutlu et al., 2015] or without [Di Nisio et al., 2005] thrombotic involvement. The miscarriage process begins with major inflammation processes followed by activa- tion of the coagulation cascade [Falcon et al., 2012; de Jong et al., 2013]; therefore, heparin may be effective in preventing miscarriage. There are reports on associated therapies involving heparin with aspirin [de Jong et al., 2014] and with prednisolone to treat RM. It was found that treatment with LMWH can decrease the levels of in- flammation in animal models [Kwak-Kim et al., 2009; Li et al., 2015; Mulloy et al., 2016]. The effects of LMWH alone, however, are not consistent although LMWH is used prevalently as an obstetric therapy for RM around the world [de Jong et al., 2013].

The pathways involved in the pathogenesis of RM have not been elucidated [Xu et al., 2013]. Additional studies in reproductive biology are necessary to improve treat- ment of RM in pregnancy loss, which affects 3 in 10 wom- en of reproductive age [Scroggins et al., 2000]. Miscar- riages have many causes, including maternal immune in- competence [Amarante-Paffaro et al., 2011; Christiansen, 2013], hormonal insufficiency [Aisemberg et al., 2013] and preeclampsia [Block-Abraham et al., 2014]. Altera- tions in the placental circulation [Cotechi-ni et al., 2014] as well as placental cellular morphology [Monfared, 2014] lead to fetal distress and, consequently, fetal death. Often times, high levels of P-selectin (P-Sel) are related to inflammation patterns, perhaps because the involvement of this molecule has different roles in various organs and systems [Magro et al., 2004; Pusch et al., 2015]. Endothe- lial dysfunction is associated with increased P-Sel expres- sion, but the presence of this protein is important to nu- merous physiological processes, particularly during preg- nancy [Uszynski et al., 2008]. Low levels of constitutive proteins in the placental tissue, for example adhesion molecules and growth factors such as P-Sel [Burrows et al., 1994] and placental growth factor [Ratsep et al., 2014], can also contribute to pregnancy complications. More studies are needed to clarify the mechanisms of RM, in- cluding the use of different techniques to evaluate placen- tal alterations in cases of pregnancy loss.

Previous findings from our group showed that silden- afil can block inflammation [Luna et al., 2015] through the nuclear factor-κB pathway. Combinatorial treatment with sildenafil and heparin strongly decrease the protein levels of tumor necrosis factor-α in the placenta [Luna et al., 2015]. It is well known that two of the main mecha- nisms involved in miscarriage are inflammation and thrombosis [Kwak-Kim et al., 2009; Falcon et al., 2012]. Placental tissue has been studied as an important target organ for new therapeutic approaches in cases of RM, since interactions between the mother and the fetus occur through the placenta [Silasi et al., 2010; Boyd, 2013]. The aim of this study was to investigate the changes in the pla- cental tissue during an LPS-induced abortion. In addi- tion, combination treatment with sildenafil and heparin (Sil+Hep) was found to protect the placenta in an RM- like model.

Materials and Methods

Ethical Approval

The Animal Care and Use Committee of the Aggeu Magalhães Research (FIOCRUZ) Center authorized this study. The protocol complies with the guidelines of the Research Center (FIOCRUZ- PE) Ethics Committee for Animal Experimentation; 24 female and 10 male mice (each weighing on average 30 g) were used to obtain 20 Swiss albino pregnancies for the study. Pregnancy was achieved by placing pro-estrous females with stud males. Copulation was confirmed the morning after mating by the vaginal plug (consid- ered gestational day 0). This study recovered tissues for analyses 2 h after LPS injection when all implantation sites retained a viable appearance. For tissue collections 2 h after saline or LPS injection, pregnant females were euthanized by anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg) using 4 pregnancies/group. Placentas were removed and processed using one of the following methods.

Animals

The methods are summarized in a diagram (fig. 1). Swiss albino pregnant mice aged 60 days and weighing 30 g were divided into 5 groups:

Control: Mice did not receive the prophylactic drug (water only for drinking) or LPS (saline placebo treatment). The animals were euthanized on the 15th day of pregnancy.

LPS: Mice did not receive the prophylactic drug (water only for drinking) but were injected with 0.5 ml LPS i.p. (*Escherichia coli* serotype 0111-B4, 100 µg/kg in sterile saline; Sigma-Aldrich) on the 15th day of pregnancy and euthanized 2 h after injection.

Sildenafil: 50 mg/kg of sildenafil (Viagra®; Pfizer) was admin- istered in their drinking water from days 0 to 15 of pregnancy to mice. Mice were weighed and their water consumption was mea- sured daily to alter sildenafil concentrations in the water to ensure that mice received a constant dosage of sildenafil on each day of pregnancy. LPS was administered on day 15 of gestation and eu- thanasia was 2 h after LPS injection.

Heparin: LMWH (Fragmin®; Pfizer) was administered at a dose of 500 IU/kg s.c. per day to previously weighed mice in 0.2 ml total volume solution from days 0 to 15 day of pregnancy. LPS was administered on day 15 of gestation and euthanasia was performed 2 h after LPS injection.

Sil+Hep: Mice received both drugs as described above from days 0 to 15. The animals also received intraperitoneal LPS injec- tion on day 15 of gestation and were euthanized 2 h later.

Histopathology

Placental tissues were fixed in 4% buffered paraformaldehyde for 24 h. The samples were dehydrated in an ethanol series, cleared in xylene and embedded in paraffin (Merck, Rio de Janeiro, Brazil). Sections (5 µm) were cut on an RM 2035 microtome (Reichert S; Leica). Placental tissues were mounted onto glass slides, stained with hematoxylin and eosin and assessed using an inverted photo- microscope (Observer Z1; Zeiss Micro Imaging GmbH) at a mag- nification of ×400.

Morphometric Analysis

Quantitative analyses of vessels with a hemorrhagic character- istic were performed through selective quantification between ves- sels containing high amounts of red blood cells and showing signs of cellular disruption. Five different fields of view in a total of 50 vessels were analyzed per group in the labyrinth area. In the spon- giotrophoblast area of the placenta, all the giant trophoblast cells were measured separately in 5 different fields, and a total of 50 cells were analyzed per group. For both analyses, the average number of vessels or cells per picture was normalized by percentage. Mea- surements were performed using ImageJ (NIH, Bethesda, Md., USA) on 5 images per group from 4 different pregnancies.

Transmission Electron Microscopy

Small fragments of placenta were fixed in Karnovsky's solution, postfixed in 1% osmium tetroxide, dehydrated in an acetone series and embedded in SPIN-PON resin (Embed 812-Electron Micros- copy Science, Washington, Pa., USA) as previously published [Wanderley et al., 2013]. Semithin sections (0.5 µm) were placed on slides and stained with toluidine blue for morphometric analysis; different areas were chosen for ultrathin sectioning. Ultrathin sec- tions (70 nm) were placed on 300-mesh nickel grids, counter- stained with 5% uranyl acetate and lead citrate, and examined using a transmission electron microscope (TEM; FEI Morgani 268D).

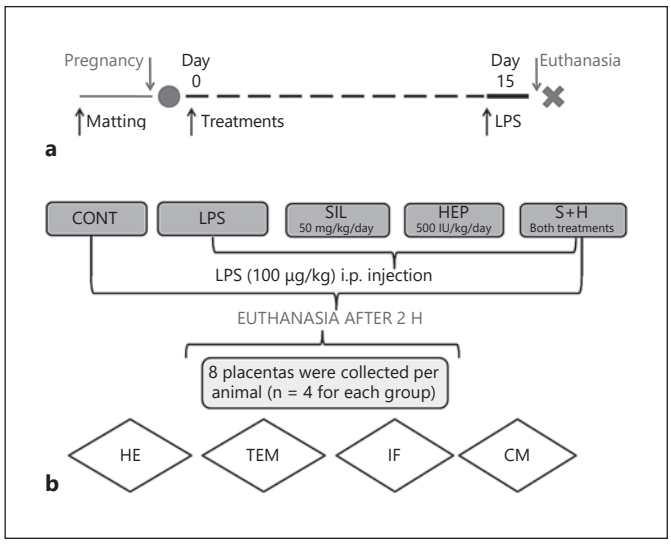


Fig. 1. a Treatment timelines for experimental animal groups. Ar- rows indicate the time point when each step (mating, pregnancy, treatments, LPS injection and euthanasia) was initiated. **b** Meth- odology of the animal protocol. CONT = Control group; LPS = LPS group; SIL = sildenafil group; HEP = heparin group; S+H = sildenafil with heparin group. Sildenafil was given to mice orally in the drinking water, while heparin was given by subcutaneous injection once every day from days 1 to 15 of pregnancy. Placentas were collected separately for each of the following techniques: HE, TEM, Immunofluorescence (IF) and cellular morphometric analy- sis (CM).

Immunofluorescence

Placental tissues were fixed in 4% buffered paraformaldehyde for 24 h, placed into 15% sucrose followed by 30% sucrose, embed- ded in OCT-Tissue-Tek compound (Sakura Finetek, Torrance, Calif., USA) and frozen in n-hexane with liquid nitrogen for cryo- stat sectioning. Cryostat sections (8 µm) were incubated in 1% bo- vine serum albumin for blocking and then incubated with anti- mouse polyclonal primary antibody for P-Sel (1 µg/ml; LS-B3578- Lifespan Bio Science). Sections were incubated with polyclonal Cy3-conjugated secondary antibodies (705-165-147; Jackson ImmunoResearch) against rabbit immunoglobulin (0.5 µg/ml; F6257; Sigma-Aldrich) and multiple implantation sites were examined for each of the 20 pregnancies studied.

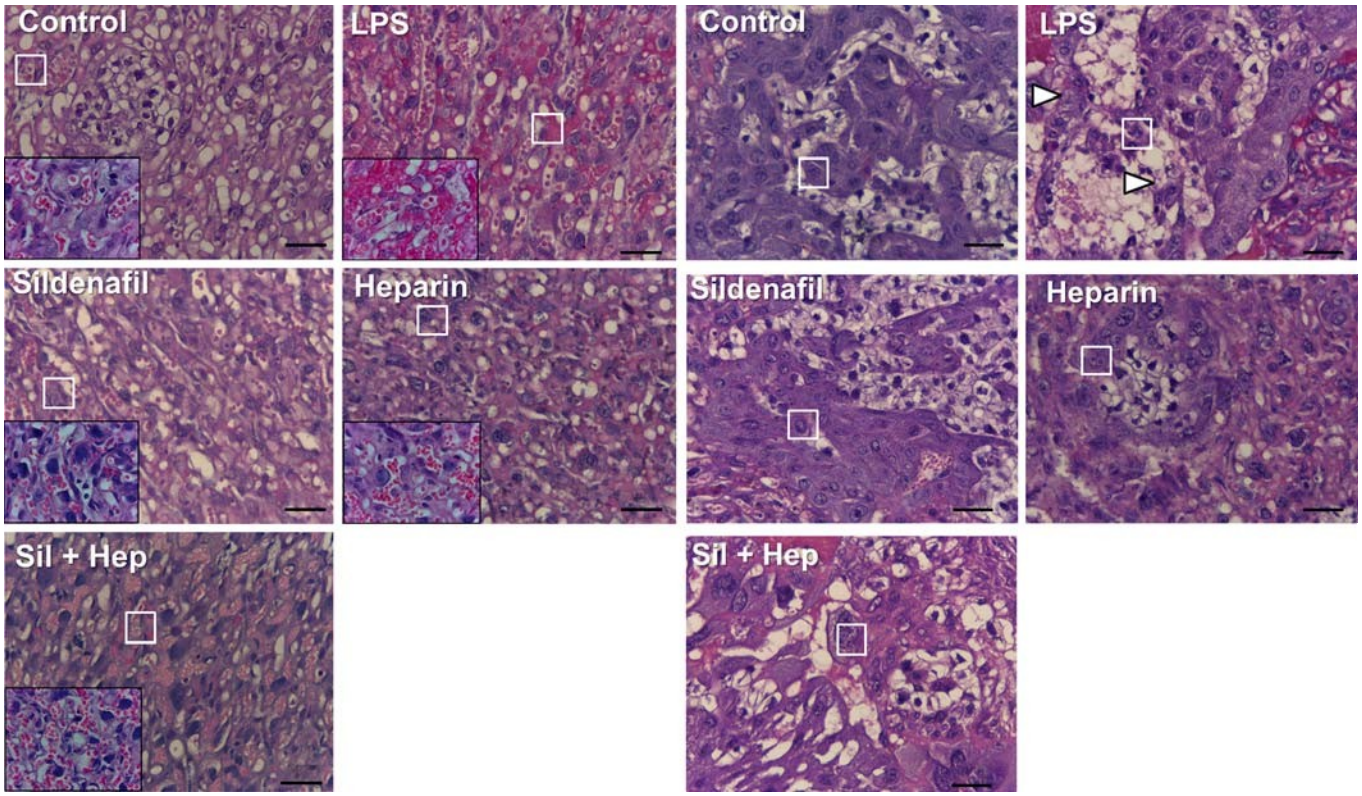
Statistical Analysis

Immunofluorescence images were assessed for statistical sig- nificance. All the statistical analyses were performed using Graph- Pad Prism 6.05 (San Diego, Calif., USA). For P-Sel, pixel density was measured in 5 stained areas using a software program (GIMP 2.6.11). Different placental (spongiotrophoblast and labyrinth) re- gions were measured separately. For each picture, morphometric analysis was performed, and the relative specific pixel density was analyzed using one-way ANOVA followed by Dunnett's and/or Tukey's post hoc test. $p < 0.05$ was regarded as statistically signifi- cant.

Results

Labyrinth Region

Histopathological analysis of placental tissues 2 h after LPS exposure showed placental labyrinth damage. The labyrinth region contained endothelial cells with signs of edema as well as hemorrhagic foci in the vascular spaces and vessels. Some cells in the labyrinth area also presented signs of distress such as vacuolization. The number of vessels thatcontained signs of hemorrhage were increased in the LPS group (fig. 2b). Treatments were effective in maintaining vessel histo-architecture and significantly decreased hemorrhagic foci (fig. 2a, b). In the Sil+Hep group, healthy red blood cells were seen inside vessels, which demonstrates that blood flow was not altered, since no blood clots were observed (fig. 2a). Overall, treatment



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with sildenafil, heparin or the combination of both drugs protected vessels from the onset of hemorrhagic-like events in the placenta caused by LPS exposure (fig. 2a: inset).

Ultrastructure analysis of the LPS group showed a de- generative pattern among the endothelial cells including electrodense nuclei, which also showed light hyperplasia. The LPS injury also caused detachment of endothelial cells from the basal lamina (fig. 3a). Groups treated with sildenafil preserved their cell structure and were more similar to the control group. The heparin group also did not show the signs of cell degeneration seen in the LPS group (fig. 3a). The most protective responses were ob- served in the groups that received sildenafil alone and Sil+Hep. The concurrent action of both drugs protected the placental tissue against LPS-induced injury in the en- dothelial cells in the labyrinth area (fig. 3a).

Assessment of immunofluorescent staining shows the control group has a constitutive expression of the adhe- sion molecule P-Sel in the labyrinth region of the placen- ta. The LPS-challenged group showed significantly de- creased P-Sel expression ($p < 0.05$) in the placental laby- rinth (fig. 4a, b). Treatments with sildenafil either alone or in combination with heparin were beneficial for main- taining higher levels of P-Sel, similar to what was found in the control group (fig. 4a). Pretreatment with heparin also had a protective effect with regard to P-Sel expres- sion, but sildenafil showed a superior response against the injury caused by LPS in the mouse model of pregnancy loss (fig. 4b). Quantitative fluorescence pixel analysis confirmed the levels of P-Sel expression in the vessels from the placental labyrinth region (fig. 4b).

Trophoblast Cells

Two hours after LPS injection in pregnant mice, histo- pathological analysis of giant trophoblast cells showed nuclear degeneration, which is an early sign of arresting cells. The spongiotrophoblast cells were also injured by LPS treatment resulting in damage to giant trophoblastic cells (fig. 2c). Additionally, in the LPS group, cellular ar- chitecture showed hemorrhagic signs even in the absence of a change in the overall size of the cells (fig. 2d). All pro- phylactic treatments reduced the effect of LPS injury and protected the giant trophoblast cells in mouse placenta. Although some degeneration was observed in the heparin group, the damage was not as strong as in the LPS group (fig. 2c). In the sildenafil group and in the Sil+Hep group, cells were significantly bigger, which demonstrates some cytoplasmic accumulation or high cellular activity (fig. 2d). The treatment groups had differences in the maintenance of the spongiotrophoblast placental layer histostructure (fig. 2c); data were confirmed by morpho- metric analysis of the giant trophoblast cells (fig. 2d).

Ultrastructural analysis confirms the results seen in histopathologicalanalysis. The LPS-treatedgroupshowed constricted nuclei with dimorphism and high amounts of heterochromatin (electrodense; fig. 3b). Following com- bination treatment, the giant trophoblast cells had ex- tended cytoplasm, which was observed as a dilatation of the rough endoplasmic reticulum cistern in the cytoplasm, which may be related to intense protein synthesis (fig. 3b). Cells from the heparin group had some vacuoles in the cytoplasm and mild changes in the nuclear architecture, and the cells were protected against LPS damage. In the majority of cells, regularly shaped nuclei and healthy mi-

Fig. 2. a Histopathological analysis of the labyrinth layer of placen- tal tissues 2 h after LPS injection in the study groups. The vessels of the labyrinth region evidenced degeneration, including edema and hemorrhagic foci. All treatments showed improvement com- pared to the LPS group. While heparin appeared to protect the placental tissue, the sildenafil group and the Sil+Hep group were the best at preserving placental vessels. Scale bars: 20 μm (×200). Detailed images of the vessels in the labyrinth area of the placenta were obtained using a ×1,000 objective lens (insets). White squares indicate the region of the labyrinth examined further by TEM to visualize the ultrastructure of the endothelial cells. **b** Quantifica- tion of hemorrhagic characteristics in the labyrinth confirms that all treatments were beneficial in preventing deposits, while hemor- rhage was increased in the LPS group. **c** Histopathological analysis of the spongiotrophoblast layer of the placenta 2 h after LPS injec- tion in the study groups. The giant trophoblast cells show signs of damage including anucleated cells and cellular dimorphism (ar- rowheads). The heparin group showed partial protection against LPS damage in this cellular type. The sildenafil group and the Sil+Hep group had placental architecture similar to the control group, but the cells were larger in the sildenafil and especially in the Sil+Hep group compared to the other groups (control, LPS and heparin). Scale bars: 20 μm. White squares show the region of the spongiotrophoblast examined further by TEM to visualize the ul- trastructure of the giant trophoblast cells. **d** Quantification of the area of each individual giant trophoblast cell shows differences be- tween the cells from the control, sildenafil and Sil+Hep groups but not compared to the LPS group. A slight increase was also observed in the heparin group. **b**, **d** Data are means ± SD. $n = 4$ mice in each group. The letters on the top of the columns represent the groups that had significant differences from the cited group: control (a); LPS (b); sildenafil (c), and heparin (d) and Sil+Hep (e). $p < 0.05$ was found to be significant.

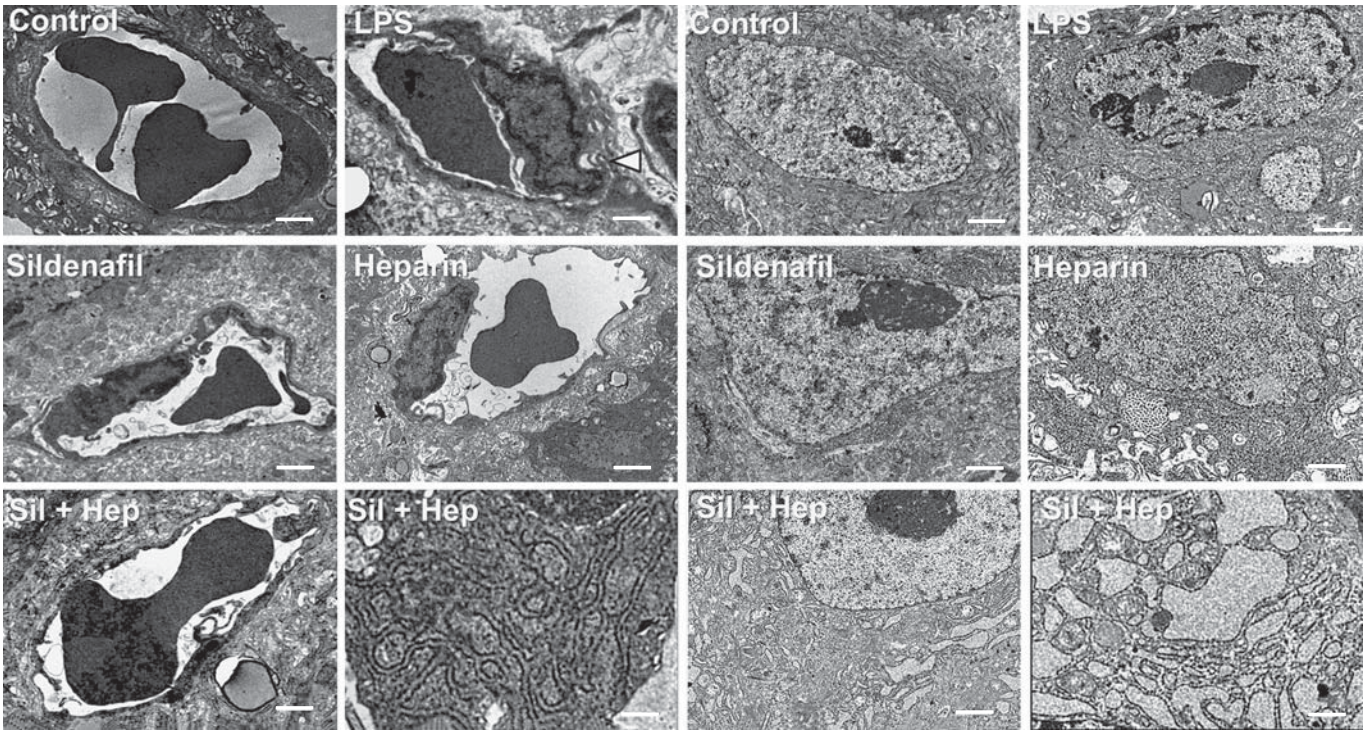

Fig. 3. a TEM analysis of endothelial cells in the study groups. LPS exposure causes endothelial cell degeneration and initiation of de- tachment between endothelial cells and the basal lamina (arrow- head). Sildenafil and heparin groups showed improvements in the structure of vessels and endothelial cells compared to the LPS group. Combined sildenafil and heparin treatment also provided protection to placental endothelial cells. Scale bars: 2,000 μm. Inset from the Sil+Hep group was demonstrated in an additional pic- ture. Scale bar: 1,000 μm. **b** TEM analysis of giant trophoblast cells in the study groups. Giant trophoblast cell ultrastructure in the LPS group showed signs of intense distress. The nuclear membrane presented an irregular shape and areas of invagination were visible. Placental tissue in the heparin group was protected from LPS damage. Treatment with sildenafil alone showed characteris- tics similar to the control group with conserved membranes, prominent nucleoli and no signs of cellular death. The combined treatment revealed endoplasmic reticulum hypertrophy due to an enlargement of cisterns; however, the overall nuclear and cellular anatomy was similar to the control group. Scale bars: 5,000 μm. Inset from the Sil+Hep group was demonstrated in an additional picture. Scale bar: 2,000 μm.

cochondria were present in the control, sildenafil and Sil+Hep groups, being in contrast to the stressed mito- chondria observed in the LPS group (fig. 3b).

P-Sel expression was analyzed in the spongiotropho- blast area, where giant trophoblastic cells are predomi- nant. The LPS-treated group had decreased expression of P-Sel (fig. 4c). Placentas from the control group as well as the sildenafil and Sil+Hep groups expressed similar levels of P-Sel in the cells from the spongiotrophoblast area, which includes the giant trophoblast cells (fig. 4d). Treat- ment with sildenafil either alone or in combination with heparin preserved the constitutive levels of P-Sel in this specific area of mouse placenta after LPS exposure. Re- sults were confirmed by relative densitometry analysis in all groups (fig. 4d). Although the heparin group showed only a small increase in P-Sel expression in the same re- gion, the expression was statistically different from the control group (fig. 4d).

Discussion

Inflammation is one of the most important factors in cases of RM [Krieg et al., 2012; Hua et al., 2013; Luna et al., 2015]. Sildenafil prevents apoptosis of human first- trimester trophoblast cells exposed to oxidative stress [Bolnick et al., 2015]. In this study, sildenafil-mediat- ed survival was reversed by either cyclic guanosine monophosphate or nitric oxide antagonists; in fact, the agonists, for the same molecules were cytoprotective

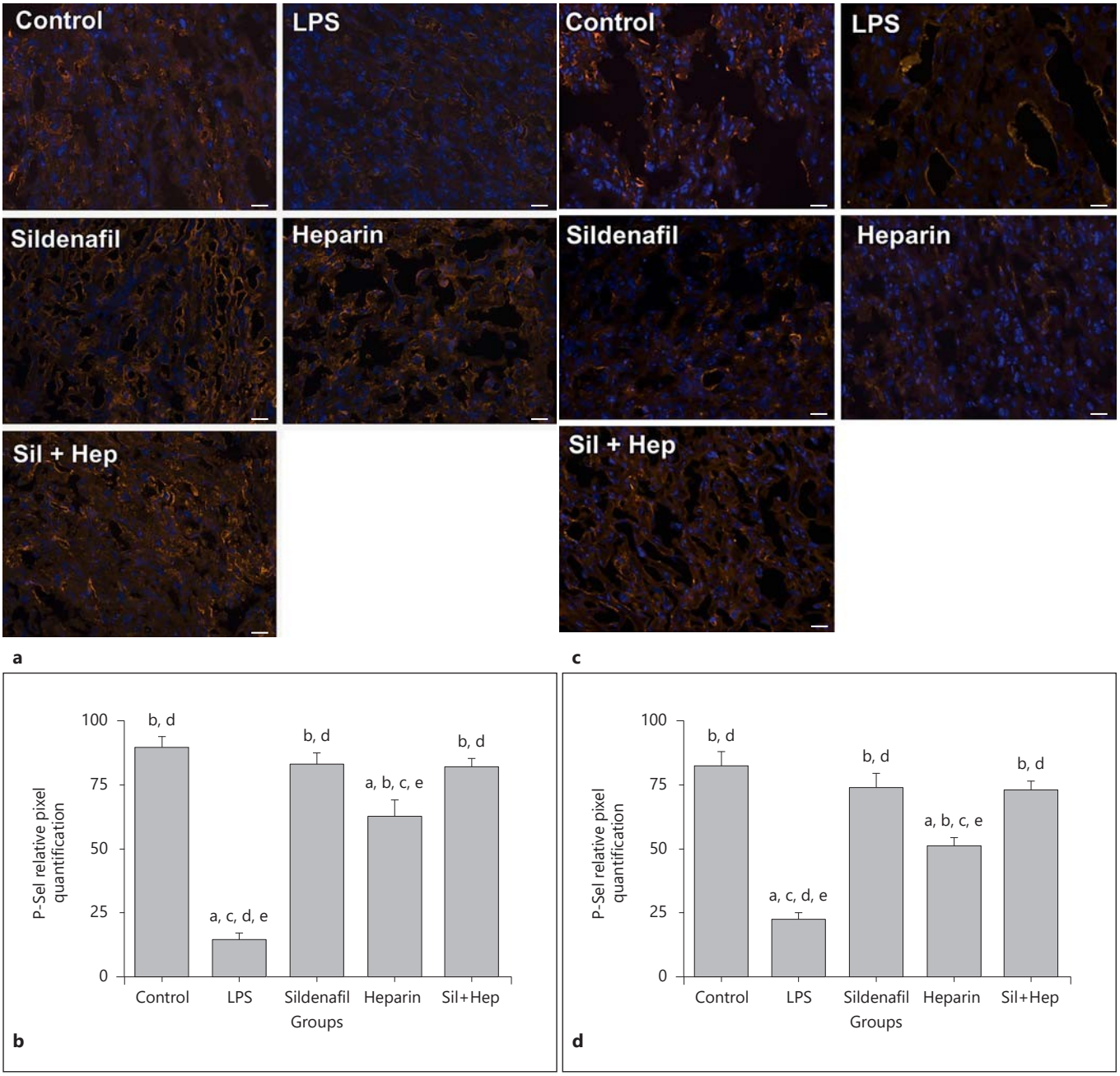

Fig. 4. a Immunofluorescent staining for P-Sel in the labyrinth area containing giant trophoblast cells. Constitutive expression of P-Sel was observed in the control group. The LPS group had decreased P-Sel expression. Treatment with sildenafil reestablished the basal pattern of P-Sel expression while the heparin group was signifi- cantly different from the control group. **b** Graph showing confir- mation of results by densitometry analysis of P-Sel from immuno- fluorescent photographs. **c** Immunofluorescent staining for P-Sel in the spongiotrophoblast area containing giant trophoblast cells. Constitutive expression of P-Sel was observed in the control group. The LPS group showed decreased expression of P-Sel compared to controls. Treatment with sildenafil reestablished the basal pattern of P-Sel expression while the heparin group presented a significant difference compared to the control group. **d** Graph showing con- firmation of results by densitometry analysis of P-Sel in immuno- fluorescent images. **b**, **d** Data are means ± SD. n = 4 mice in each group. The letters on the top of the columns represent the groups that had significant differences from each cited group: control (a); LPS (b); sildenafil (c), and heparin (d) and Sil+Hep (e). $p < 0.05$ was found to be significant. **a**, **c** Scale bars: 20 μm.

[Bolnick et al., 2015]. Other PDE-5 inhibitors are de- scribed to have anti-inflammatory properties [Bogdan et al., 2012; Raposo et al., 2013; Garcia et al., 2014]. Our results demonstrate the influence of inflammation and the consequent alterations in placental tissue morpholo- gy. The labyrinth area is described as a crucial border at the maternal-fetal interface. Inflammation induces cellu- lar degeneration and can consequently damage tissues with specific physiological changes following placental failures as described previously [Cotechini et al., 2014].

The ultrastructural changes followed by the protein as- sessment of P-Sel supported the findings of the histological analysis. Treatment with the PDE-5 inhibitor sildenafil and/or the LMWH dalteparin changed the cellular dynam- ics and protected vessels in the labyrinth area from the damage caused by LPS injury. A recent clinical trial using LMWH to treat unexplained RM did not show any evi- dence of efficacy from this therapeutic approach [Pasquier et al., 2015]. Coagulopathies have a big impact on miscar- riage events and maternal thrombophilia is a common complication in cases of FGR, RM and placental abruption [Patnaik et al., 2007; Mutlu et al., 2015]. Blood flow ex- change between mother and fetus is extensive in the laby- rinth area of the mouse placenta [Suenaga et al., 2014], and healthy vascularization provides the growing fetus with sufficient nutrition [Dye et al., 2001]. The results of this study show that LPS injection leads to fetal distress and dysregulation of placental blood flow followed by hemor- rhage. Interestingly, treatment with sildenafil, heparin or Sil+Hep counteracts these effects and normalizes blood flow, blood clots or focal hemorrhage was not observed.

Giant trophoblasts are present primarily in the spon- giotrophoblast layer of rodent placenta [Monfared, 2014] and show a high production and accumulation of a vari- ety of molecules such as hormones, cytokines and para- crine factors [Hu and Cross, 2010]. The spongiotropho- blast area was injured by LPS exposure. Consequently, either the cytoplasm or the nuclear structure of giant tro- phoblastic cells was damaged. Sildenafil blocks the in- flammatory pathway, thus preventing degeneration in important areas of the placenta, including the spongio- trophoblast area. In our study, the histology and ultra- structure of the spongiotrophoblast area were preserved 2 h after LPS exposure in both sildenafil-treated groups. Inflammation and coagulopathy are closely linked and key regulators of fetal death [Middeldorp, 2013]. Also, anti-inflammatory pathways may influence giant cell physiology [Hashino et al., 2015]. Indeed, an ablation of sinusoidal giant trophoblast cells resulted in FGR fol- lowed by fetus lethality, thus illustrating the importance of this cell type [Outhwaite et al., 2015]. Mouse tropho- blast cells showed physiologic pattern in the groups treat- ed with sildenafil. The ability of sildenafil to increase pro- tein synthesis in secretory cells was described previously [Saraiva et al., 2009; Gomes et al., 2014]. The enlargement of rough endoplasmic reticulum observed in the silde- nafil-treated groups is likely related to increased glyco- protein synthesis by the giant trophoblast cells. Despite the larger appearance of the cells in our study, no signs of cellular death were observed.

Adhesion molecules such as P-Sel play a role in em- bryo implantation and placentation, and can also be a marker of healthy placentas, even though the presence of selectins in the placenta are not clearly understood throughout gestation [Dye et al., 2001; Zenclussen et al., 2001]. Paracrine signaling and cell-to-cell communica- tion have been noted in the placenta of rodents and hu- mans [Dos Santos et al., 2015]. Both treatments are also successful in maintaining the ultrastructural integrity of endothelial cells and in regulating the expression of P-Sel in both cell types analyzed from the labyrinth and spon- giotrophoblast areas of the placenta. All prophylactic treatments were significantly beneficial. Sildenafil either alone or in combination with heparin was superior com- pared to heparin monotherapy in our study. More studies are needed, however, to understand the protective effect of both drugs on placental vessels and giant trophoblast cells in a murine model of pregnancy loss caused by aber- rant inflammation.

In this study, we demonstrated how the effects of LPS- induced pregnancy loss can affect the ultrastructure in different areas of placental tissue. Our results show how the placenta reacts to early LPS exposure, with or without the protective effect of pre-treatment using sildenafil and/ or heparin. The development of alternative techniques as well as the consolidation of animal models is important for understanding cellular dynamic processes due to the in vivo effects of the drugs, especially during pregnancy. The variety of cell types in placental tissue and how they interact with each other in healthy or compromised preg- nancies is still unclear. These results may help to improve current RM treatment regimens in the future. This is the first description of the effects of sildenafil and heparin treatments on the morphology of placental tissue using different parameters, including histopathology, ultra- structure and P-Sel expression, in a murine model of RM. These findings corroborate previous studies that de- scribed the effect of inflammation and coagulation in in- duced abortion in animal models of miscarriage [Krieg et al., 2012; Cotechini et al., 2014]. Our results reinforce the

role of sildenafil (Viagra®) treatment – either alone or in combination with LMWH (Fagrimin®) – to treat cases of placental damage. Due to the protective effect of these drugs, they may be useful as a new therapeutic approach in cases of RM mainly related to placental failure.

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Disclosure Statement

The authors have no conflict of interest.

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CAPÍTULO 3

Placental growth factor (PGF) deficiency is associated with impaired cerebral vascular development in mice

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molecular human reproduction ORIGINAL RESEARCH

Placental growth factor deficiency is associated with impaired cerebral vascular development in mice

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study hypothesis: Placental growth factor (PGF) is expressed in the developing mouse brain and contributes to vascularization and vessel patterning.

study finding: PGF is dynamically expressed in fetal mouse brain, particularly forebrain, and is essential for normal cerebrovascular development.

what is known already: PGF rises in maternal plasma over normal human and mouse pregnancy but is low in many women with the acute onset hypertensive syndrome, pre-eclampsia (PE). Little is known about the expression of PGF in the fetus during PE. *Pgf* $^{2/2}$ mice appear normal but recently cerebral vascular defects were documented in adult *Pgf* $^{2/2}$ mice.

study design, samples/materials, methods: Here, temporal–spatial expression of PGF is mapped in normal fetal mouse brains and cerebral vasculature development is compared between normal and congenic *Pgf* $^{2/2}$ fetuses to assess the actions of PGF during cere- brovascular development. *Pgf*/PGF, *Vegfa*/VEGF, *Vegf* receptor (*Vegfr*)1 and *Vegfr2* expression were examined in the brains of embryonic day (E)12.5, 14.5, 16.5 and 18.5 C57BL/6 (B6) mice using quantitative PCR and immunohistochemistry. The cerebral vasculature was compared between *Pgf* $^{2/2}$ and B6 embryonic and adult brains using whole mount techniques. Vulnerability to cerebral ischemia was investigated using a left common carotid ligation assay.

main results and the role of chance: *Pgf*/PGF and *Vegfr1* are highly expressed in E12.5-14.5 forebrain relative to VEGF and *Vegfr2. Vegfa*/VEGF is relatively more abundant in hindbrain (HB). PGF and VEGF expression were similar in midbrain. Delayed HB vascularization was seen at E10.5 and 11.5 in *Pgf* $^{2/2}$ brains. At E14.5, *Pgf* $^{2/2}$ circle of Willis showed unilateral hypoplasia and fewer collateral vessels, defects that persisted post-natally. Functionally, adult *Pgf* $^{2/2}$ mice experienced cerebral ischemia after left common carotid arterial occlusion while B6 mice did not.

limitations, reasons for caution: Since *Pgf* $^{2/2}$ mice were used, consequences of complete absence of maternal and fetal PGF were defined. Therefore, the effects of maternal versus fetal PGF deficiency on cerebrovascular development cannot be separated. However, as PGF was strongly expressed in the developing brain at all timepoints, we suggest that local PGF has a more important role than distant maternal or placental sources. Full PGF loss is not expected in PE pregnancies, predicting that the effects of PGF deficiency identified in this model will be more severe than any effects in PE-offspring.

wider implications of the findings: These studies provoke the question of whether PGF expression is decreased and cerebral vascular maldevelopment occurs in fetuses who experience a preeclamptic gestation. These individuals have already been reported to have elevated risk for stroke and cognitive impairments.large scale data.

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Keywords: cerebral vessels / circle of Willis / fetal development / PGF / pre-eclampsia / stroke

Introduction

Women who experience pre-eclampsia (PE), an acute hypertensive syn- drome of pregnancy, are at increased cardiovascular disease risk later in life (Powers et al., 2012). Fewer studies address effects of PE on offspring cardiovascular health. One long-term cohort study reported greater risk for stroke but notcoronary heartdisease in offspring of preeclamptic preg- nancies aged 59–69 (Kajantie et al., 2009). Elevated stroke sensitivity was postulated to result from reduced fetal brain growth or from a 'brain- sparing response' that redirected blood to the brain, permanently altering fetal cerebral vessels (Kajantie et al., 2009). Genetic, epigenetic, and envir- onmental factors that alter placental function are also proposed to de- crease cardiovascular fitness in offspring of preeclamptic pregnancies (Davis et al., 2012; Herrera-Garcia and Contag, 2014). Suboptimal placen- tal production of angiogenic factors such as placental growth factor (PGF, previously PLGF) and/or increased release of anti-angiogenic proteins such as sFLT1 characterize PE (Powers et al., 2012; Goel and Rana, 2013). While PGF deficiency precedes clinical signs of PE in many women, significant outcomes due to fetal PGF deficiency have not been investigated (Carmeliet et al., 2001). However, PGF/Pgf expression is reported in human and mouse oocytes and in embryos from zygote to blastocyst stages (Gene Expression Omnibus database: GDS3958, GDS812 and GDS813) (Zeng et al., 2004; Xie et al., 2010). Thus, dysregu- lation of the angiogenic pathways that contribute to PE may begin prior to implantation and before placental lineage commitment in the blastocyst. If so, dysregulated angiogenesis would be predicted in both placental and inner cell mass-derived fetal tissues. Genetic or epigenetic factors affecting gene expression in the blastocyst lineages may also explain the higher risk of pre-eclampsia in subsequent pregnancies of women who have had pre-eclampsia (Herna´ndez-D´ıaz et al., 2009).

Like vascular endothelial growth factor-A (VEGF), PGF binds to membrane-bound and soluble forms of the FLT1 receptor (VEGFR1). 2004; Errico et al., 2004), maternal plasma PGF is unlikely to cross the pla- centa although it may induce signaling via placentally expressed VEGFR1s or neuropilins (NRP) (Baston-Buest et al., 2011). Data on fetal PGF pro- duction are minimal. One study reported week 7 – 9 human amniotic fluid had a PGF concentration approximately half that of matched maternal serum (Makrydimas et al., 2008). Over midgestation, PGF and VEGF levels increase in amniotic fluid with VEGF at a 6-fold greater concentration than PGF (Kalampokas et al., 2012; Papapostolou et al., 2012). At term, PGF is generally undetectable in amniotic fluid or cord blood from fetuses whose mothers have quantifiable plasma PGF (Staff et al., 2005).

We postulated that deficiency in fetal PGF would disturb normal cere- brovascular development and initiated comparisons in fetal mice suffi- cient or genetically-ablated for PGF. Cerebral arterial casts prepared from Pgf 2/2 mice exhibited elongated shape, vascular disorganization and frequently an incomplete Circle of Willis (cW) (Ratsep et al., 2015a). We therefore undertook

mouse fetal time course studies of re- gional brain vascular development to assess when and where PGF contributes to cerebrovascular development.

Materials and Methods

Animals

Male and female B6 mice were purchased from Charles River Canada (St. Constant QU). B6-Pgf 2/2 mice were bred at Queen's University from foundation pairs provided by VIB vzw B-9052 Zwijnaarde, Belgium (Carmeliet et al., 2001). Mice were housed in micro-isolator cages enriched with igloos and nesting materials maintained on an environmental rack and
had free access to water and food. Males and pregnant or nursing females were housed individually while non-pregnant females were housed with lit- termates up to 4 per cage according to our facility's standard procedures. A 12 hour light-12 hour dark cycle was used with an ambient temperature of 238C. Mice were monitored daily for health and sentinels were assessed
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regularly to ensure the room that was bioBUBBLE HEPA air filtered

However, only VEGF and not PGF binds to the Kinase insert domain
receptor (KDR/VEGFR2) (Autiero et al., 2003; Cao, 2009). Both VEGF receptors engage multiple co-receptors and participate in complex signal- ing pathways. Humans express four isoforms of PGF while mice only have one, which corresponds to the human PGF-2 (Dewerchin and Carmeliet,
2012). Pgf 2/2 mice are viable and develop apparently normally, resulting
in a consensus that PGF is of limited importance developmentally or in maintenance of homeostasis but acts under disturbed states such as preg- nancy, ischemic heart repair and tumor vessel induction (Dewerchin and Carmeliet, 2014). PGF is made not only by placenta but by other cell types such as endothelium and hepatocytes (Steenkiste et al., 2011) and it can be induced in adult neurons, Schwann cells and astrocytes (Beck et al., 2002; Hayashi et al., 2003; Freitas-Andrade et al., 2008; Chaballe et al., 2011). Because PGF is a large glycoprotein (Christinger et al.,remained free of pathogens. All animal handling was conducted in a laminar flow hood. Females were paired overnight with males. Copulation plug detection was designated embryonic day (E) 0.5. Pregnant females were euthanized by cervical dislocation between E10.5 – E18.5, and the fetuses were removed and dissected. For some experiments, neonatal mice (both sexes) at post-natal day (P) 7 or adult non-pregnant female and male mice were euthanized using an injected overdose of sodium pentobar- bital or inhaled isoflurane followed by decapitation.

Ethical approval

All procedures were approved by the Queen's University Animal Care Com- mittee (reference number: Croy-2013-006-01) and were consistent with the Canadian Council on Animal Care national standards for ethical animal care and use.

Quantitative real-time PCR for *Pgf, Vegfa, Vegfr1* and *Vegfr2* expression

For quantitative real-time PCR (RT– PCR) analyses, B6 fetal brains (E12.5, 14.5, 16.5, and 18.5 with n ¼ 5 fetal brains, each from a different litter at each time point) were dissected using RNAse-free treated forceps and a Heer- brugg microscope (Wild-Leitz, Canada). Three dissected regions, forebrain (FB), midbrain (MB) and hindbrain (HB), were flash frozen in liquid nitrogen. Isolated brain tissues were homogenized and the RNA was purified using RNA binding columns (Norgen Biotek Corp, Thorold, ON, Canada) as per manufacturer's instructions. RNA concentration and purity were determined using a Nanodrop 2000C UV-Vis spectrophotometer (Thermo Scientific, Wil- mington, DE, USA) and the extracted RNAwas stored at 2808C. The mRNA was reverse transcribed and amplified using the RT^2 First Strand cDNA synthe- sis kit (Qiagen, Mississauga, ON, Canada). Products were stored at 2208C. Primer assays for genes of interest (*Pgf*— PPMO3669C), (*Vegf*— PPMO03041F),

(*Vegfr1/Flt1*—PPMO 3066F), (*Vegfr2/Kdr*—PPMO3057A)

obtained from Qiagen (Qiagen, Mississauga, ON, Canada). Plate-based Light- Cycler 480 reactions (Roche Diagnostics, Laval, QC, Canada) were performed using RT^2 SYBR Green Q-PCR master mix with final reaction volumes of 25 ml. All samples were run in triplicate including Actb as the control. Cycling condi- tions were denaturation: 958C; 15 min, amplification: 45 cycles; 958C for 15 s; 558C for 30 s; 728C for 30 s, melting curve: 70– 958C at a rate of 0.18C/s. Melt curve analyses were performed using LightCycler Software for each gene to verify the specificity of individual PCR products. Relative quantification of each gene was performed using DDCt method and *Actb*.

PGF and VEGF immunolocalization

Heads were collected from B6 fetuses at E12.5, 14.5, 16.5 and 18.5 (n ¼ 3 fetal heads from 3 different litters at each time point), fixed in 4% (w/v) par- aformaldehyde (PFA) (12 h) and paraffin-embedded. Sections (5 mm) were deparaffinized, rehydrated, blocked in 3% bovine serum albumin and incu- bated with the anti-mouse polyclonal primary antibodies (0.25 mg/ml anti-PGF (Abcam—ab0542, Toronto, ON, Canada) and 1 mg/ml anti-VEGF (Abcam—ab46162)) for 2 h at room temperature (228C). Goat anti-rabbit secondary antibodies conjugated to Alexa Fluor 488 (Invitrogen, Ottawa, ON, Canada—A11008) were used for both PGF and VEGF staining in sep- arate sections. At this point, the PE-conjugated anti-CD31 (BD Pharmingen, Mississauga ON—MEC13.3) was added and sections were incubated for 1.5 h at room temperature. Nuclear staining was completed with 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies—D1306, Burling- ton, ON, Canada). Slides were mounted using Prolong gold anti-fade (Life technologies, Burlington, ON, Canada—9071) and examined using a Zeiss M1 Imager microscope. Multiple fetuses from three different pregnant mice were used per time point.

HB vascularization whole mount immunofluorescent staining

B6 and *Pgf* $^{2/2}$ E10.5 and 11.5 fetuses were fixed in 4% PFA for 2 h. Fetuses from 3 litters were used for each time point and genotype. At E10.5, 21 HBs from 32 B6 fetuses and 18 HBs from 38 *Pgf* $^{2/2}$ fetuses were successfully pre- pared. Dissected HBs were analyzed whole (n ¼ 10 B6 and 9 *Pgf* $^{2/2}$) or in cross-sections (n ¼ 11 B6 and 9 *Pgf* $^{2/2}$). At E11.5, 26 HBs from 27 B6 fetuses and 29 HBs from 33 *Pgf* $^{2/2}$ fetuses were successfully prepared. 17 B6 and 21 *Pgf* $^{2/2}$ HBs were imaged whole while 9 B6 and 8 *Pgf* $^{2/2}$ HBs were analyzed as cross-sections. HBs were isolated and the vasculature stained using the protocol of Fantin *et al.* (2013). Briefly, isolated HBs were blocked using 0.1% Triton-X (BioShop, Burlington, ON, Canada—TRX506) and 10% normal goat serum (NGS) (Sigma, Oakville, ON, Canada—G9023) in PBS. After washing, HBs were incubated overnight with 40 mg/ml Tetramethylrhodamine (TRITC)-conjugated isolectin B4 (IB4; Sigma, Oakville, ON, Canada—L5264), an endothelial cell marker. The ventricular plexus was imaged using a Zeiss M1 Imager fluorescence microscope (Zeiss; Toronto, ON, Canada) equipped with an AxioCam and Axiovision 4.8 software. Cross sections of the HB were imaged with a Quorum Wave FX Spinning Disc confocal microscope (Quorum; Guelph, ON, Canada). Three dimensional confocal images were reconstructed with MetaMorph software (Molecular Devices; Sunnyvale, California, USA). Analysis was performed using ImageJ by a single blinded reviewer. HBs were processed in batches and measurements from each HB were not linked to fetal size or sex.

Circle of Willis whole mount immunofluorescent staining

Formation and connectivity of cW were evaluated in B6 and Pgf $^{2/2}$ E14.5 and P7 mice. Fetal studies used all pups from three litters for each genotype. Neonatal studies used at least three pups from each of three litters per geno- type. Males and females were studied at both stages. Fetuses were fixed in 4% PFA overnight at 48C before dissection. P7 mice were anesthetized using pentobarbital 50 mg/kg intraperitoneally and perfused via the left ventricle with 1 ml 4% PFA after which the heads were immersion-fixed in 4% PFA overnight. Skulls were removed for visualization of the anterior cW. After blocking in PBS with 0.1% Triton-X and 10% NGS, brains were incubated with a 10 mg/ml IB4 solution. After washing, the anterior cW was visualized using a Zeiss M1 Imager microscope. The posterior cerebral and communi- cating arteries were not analyzed due to technical difficulties in dissecting posterior brain. Image analysis was performed using ImageJ. Fetal and neonatal body and brain dimensions as well as cW vessel diameters, connectivity and vessel numbers were quantified by a single blinded reviewer.

Ink perfusion and circle of Willis imaging

Mice (n ¼ 4 male and 3 female B6 and n ¼ 3 male and 4 female Pgf $^{2/2}$) were anesthetized intraperitoneally with 50 mg/kg sodium pentobarbital. Mice were perfused with 2 ml PBS through the left ventricle followed by 2 ml Pelikan black ink (Wallack's Art Supplies, Kingston, ON). The brains were isolated and images of the cW were obtained with a Zeiss Lumar.V12 stereo microscope (Carl Zeiss, Oberkochen, Germany) with Motic Images Plus 2.0 (Motic, Hong Kong, China). Measurements of cW dimensions, vessel length, angles and diameters as well as number of vessels were com- pleted using ImageJ by a blinded reviewer.

Left common carotid artery ligation ischemic infarct assay

Adult B6 and Pgf $^{2/2}$ mice were anesthetized with isoflurane (Pharmaceut- ical Partners of Canada, Richmond Hill, ON, Canada) in 20%:80% O_2:N_2 and maintained at 36 + 0.58C using a rectal probe and heating pad. Cerebral blood flow was measured with laser Doppler flowmetry (Perimed, Periflux System 5010, North Royalton, OH, USA). The left common carotid artery (LCCA) was occluded using a 6–0 silk suture for 30 min then the anesthetized mice were decapitated (n ¼ 14 B6 and 15 Pgf $^{2/2}$ male and female mice). In a subset of the mice, (n ¼ 6 B6 and 9 Pgf $^{2/2}$) cerebral blood flow was measured before LCCA ligation and throughout the experiment. The percent of initial blood flow remaining after LCCA ligation was calculated and compared. Brains were rapidly excised under a drip of ice-cold phosphate-buffered saline (PBS), cut into 1 mm thick coronal sections using an adult mouse brain matrix (Zivic Instruments, #BSMAS001-1, Pitts- burgh, PA, USA) and stained with 0.5% 2,3,5-triphenyltetrazolium chloride in PBS (TTC; Sigma Aldrich, #T8877, Mississauga, ON, Canada). Images of these tissues were captured using a Zeiss Lumar.V12 stereo microscope (Carl Zeiss, Oberkochen, Germany) with Motic Images Plus 2.0 (Motic, Hong Kong, China). The presence of an ischemic tissue was determined by a blinded reviewer and the size was measured using ImageJ. Size of the ischemic area was corrected for edema using the ratio of the size of the unaffected to the affected hemispheres.

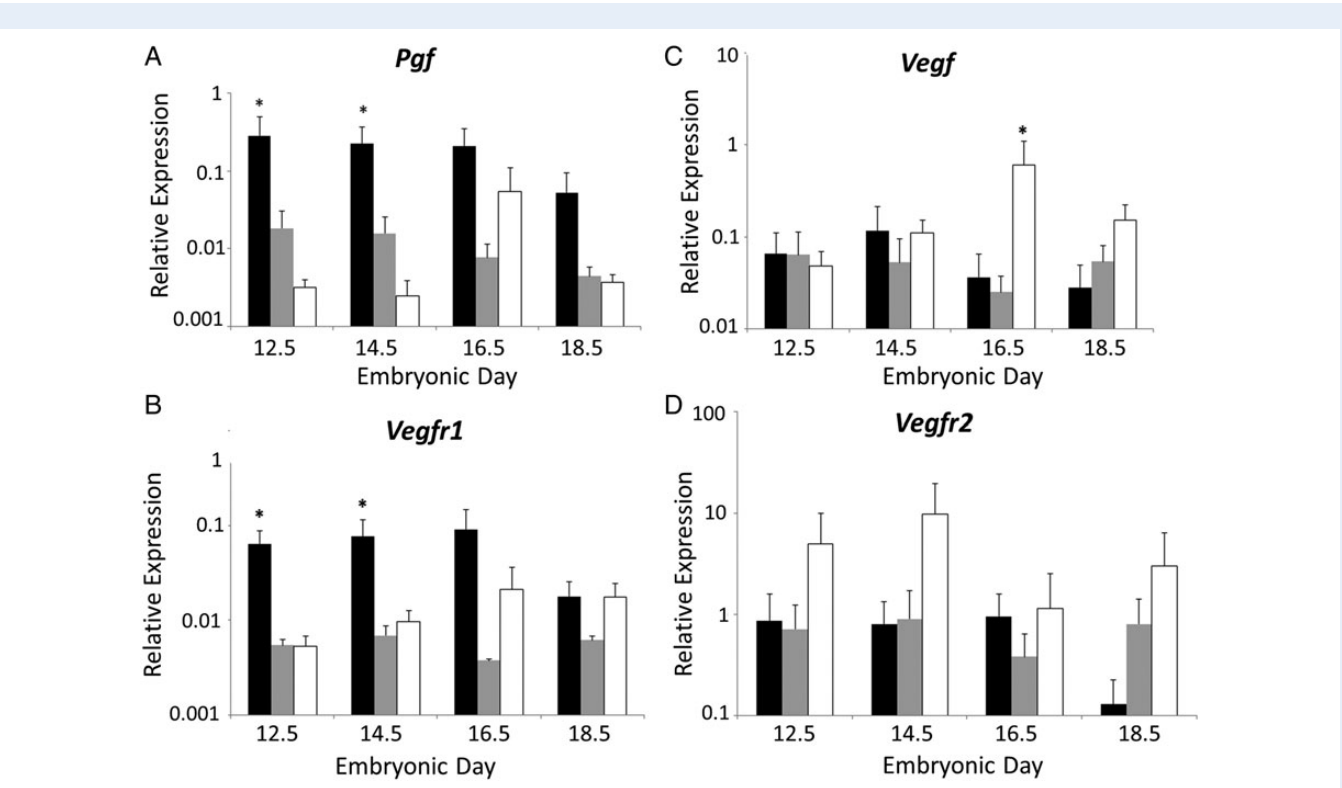

Figure 1 Relative gene expression in B6 fetal brains. Quantitative PCR data are shown for relative expression of mRNA for *Pgf* (A), *Vegfr1* (B), *Vegf* (C) and *Vegfr2* (D) by fetal brain regions. The brains were dissected into forebrain (FB) (black bars), midbrain (MB) (gray bars) and hindbrain (HB) (white bars) at embryonic day (E) 12.5, 14.5, 16.5 and 18.5. Results were considered significant when P, 0.05 (*) or P, 0.02 (**) for comparisons between the brain areas and P, 0.05 ($^{\#}$) or P, 0.02 ($^{\#\#}$) for comparisons between the gestational times. b-actin was used as the reference gene.

Statistical analysis

The gene expression data obtained by qPCR was analyzed with an ANOVA for repeated measures using the PROC GLM procedure of the Statistical Analysis System (SAS 9.0; Toronto, Ontario, Canada). Effects of brain region, embryonic day and interactions were included in the model. Correla- tions between genes were assessed using the PROC CORR procedure of SAS. Two-way analysis of variance (ANOVA) and Bonferroni-corrected post-tests were used to test the statistical significance of differences in HB vascularization and cW development with one mouse or one fetus as a unit of analysis (Graphpad Prism; San Diego, California, USA). An unpaired t-test was used to analyze the difference between genotypes in cerebral blood flow after LCCA ligation.

Results

Pgf/Vegf and Vegfr1/Vegfr2 expression time courses in B6 fetal brain

Relative abundance of transcripts for Pgf, Vegf, Vegfr1 and Vegfr2 was measured in fetal FB, MB and HB homogenates at mid to late gestation (Fig. 1). Brain regions and gestational times were compared independently. Relative Pgf expression was significantly higher in FB than MB or HB at E12.5 (P ¼ 0.001) and at E14.5 (P ¼ 0.04). At E16.5, relative Pgf expression was significantly greater when FB was compared with MB (P , 0.05) but not with HB. By E18.5, no statistically significant differ- ences were present between relative *Pgf* expression in FB, MB and HB. Relative *Pgf* expression in MB did not change over gestation. Relative *Vegfr1* expression was statistically significantly higher in FB at E12.5 (P ¼ 0.011) and E14.5 (P ¼ 0.011) compared with either MB or HB. At E16.5, FB had greater relative *Vegfr1* expression than MB but not HB. Relative *Vegfr1* expression in MB and HB did not change throughout the gestational study interval

and was not significantly different when compared with other brain areas. Relative expression of *Vegf* was also stable across gestation, except for a peak in expression in E16.5 HB. This was the only time point at which relative *Vegf* expression was signifi- cantly higher in HB than in FB or MB (P ¼ 0.001). There were no signifi- cant changes in relative expression of *Vegfr2* at any of the analyzed gestational times or between the FB, MB and HB. These data suggest dynamically regulated, anatomically specific importance for PGF in midgestational development of forebrain vessels.

Immunolocalization of PGF and VEGF in B6 fetal brain

To confirm that the pathways that promote angiogenesis in developing brain include signaling via PGF protein, immunofluorescent staining was undertaken for PGF/CD31/DAPI and VEGF/CD31/DAPI on transverse sections of fetal B6 brains. At E12.5 (Fig. 2A), PGF expression was predominant in FB, most notably in the diencephalon. Expression was seen in both nervous and vascular tissue. In MB, PGF and VEGF appeared to be expressed at similar levels by fluorescence quenching time measurements in the maxillary component of the first branchial arch and trigeminal ganglion. In HB, VEGF was more highly expressed than PGF in the roof but not in vessels of the marginal layer. In the latter images, the anti-CD31 fluorescent staining was overwhelmed by high endothelial cell expression of PGF and VEGF.

At E14.5 (Fig. 2B), PGF and VEGF expression appeared unchanged in the superior dural venous sinus and lateral ventricle/corpus striatum of the FB. Equivalent expression of PGF and VEGF was also observed in the diencephalon (hypothalamus) and trigeminal ganglion. In HB areas, VEGF localization was predominant in the transverse dural venous sinus and especially in cells of fourth ventricle.

At E16.5 (Fig. 3A), FB had greater expression of PGF than VEGF. This difference was stronger in the wall of telencephalon than in the lateral ventricles. In E16.5 MB, VEGF expression was high in the transverse dural venous sinus while PGF expression was strong in the mesencephalic vesicle. In the selected HB areas, primitive cerebellum and its vessels showed the highest VEGF expression.

At E18.5 (Fig. 3B), expression of PGF and VEGF was localized to vessels of the white matter and superior horn of lateral ventricle as well as to vessels in the hippocampus and transverse dural venous sinus. Patterns of PGF and VEGF expression were similar in FB and MB. In contrast, in HB, the posterior part of the cerebellum had greater VEGF localization while the posterior semi-circular canal exhibited comparable levels of PGF and VEGF staining.

HB vascularization in E10.5 and 11.5 Pgf$^{2/2}$ and B6 mice

Mouse fetal HB vascularization is usually assessed at E10.5. However, we observed that *Pgf* $^{2/2}$ mice were smaller than B6 at E10.5 and therefore made comparisons at E10.5 and E11.5 (Fig. 4A and B). At both times, *Pgf* $^{2/2}$ fetuses were growth-restricted compared with B6 with signifi- cantly shorter head lengths (P ¼ 0.006) at E10.5 and significantly less width (P ¼ 0.001) at E11.5 (Supplementary Fig. S1). The majority of *Pgf* $^{2/2}$ fetuses had no developmental delays as assessed by Theiler staging (data not shown) (Kaufman, 1992). Two E11.5 *Pgf* $^{2/2}$ fetuses at lower Theiler stages were excluded from analysis. Vessels in the E10.5 *Pgf* $^{2/2}$ HB ventricular plexus were significantly thinner than in B6 (P ¼ 0.0008, Fig. 4C) and *Pgf* $^{2/2}$ HB vessel junctions per area were significantly greater (P , 0.001, Fig. 4D). E10.5 *Pgf* $^{2/2}$ HB cross- sections revealed narrower sprout diameters (P ¼ 0.022, Fig. 4E) than B6, further suggesting that HB vascularization is impaired at E10.5 by PGF deficiency. By E11.5, differences in CNS development were more apparent and HB thickness was significantly lower (P ¼ 0.0002, Fig. 4F) in *Pgf* $^{2/2}$ fetuses despite normalization of the HB vasculature with

respect to vessel diameter, number of junctions and sprout diameter. Thus, the transient delay in vascular development linked with PGF defi- ciency was sufficient to impact CNS structural development.

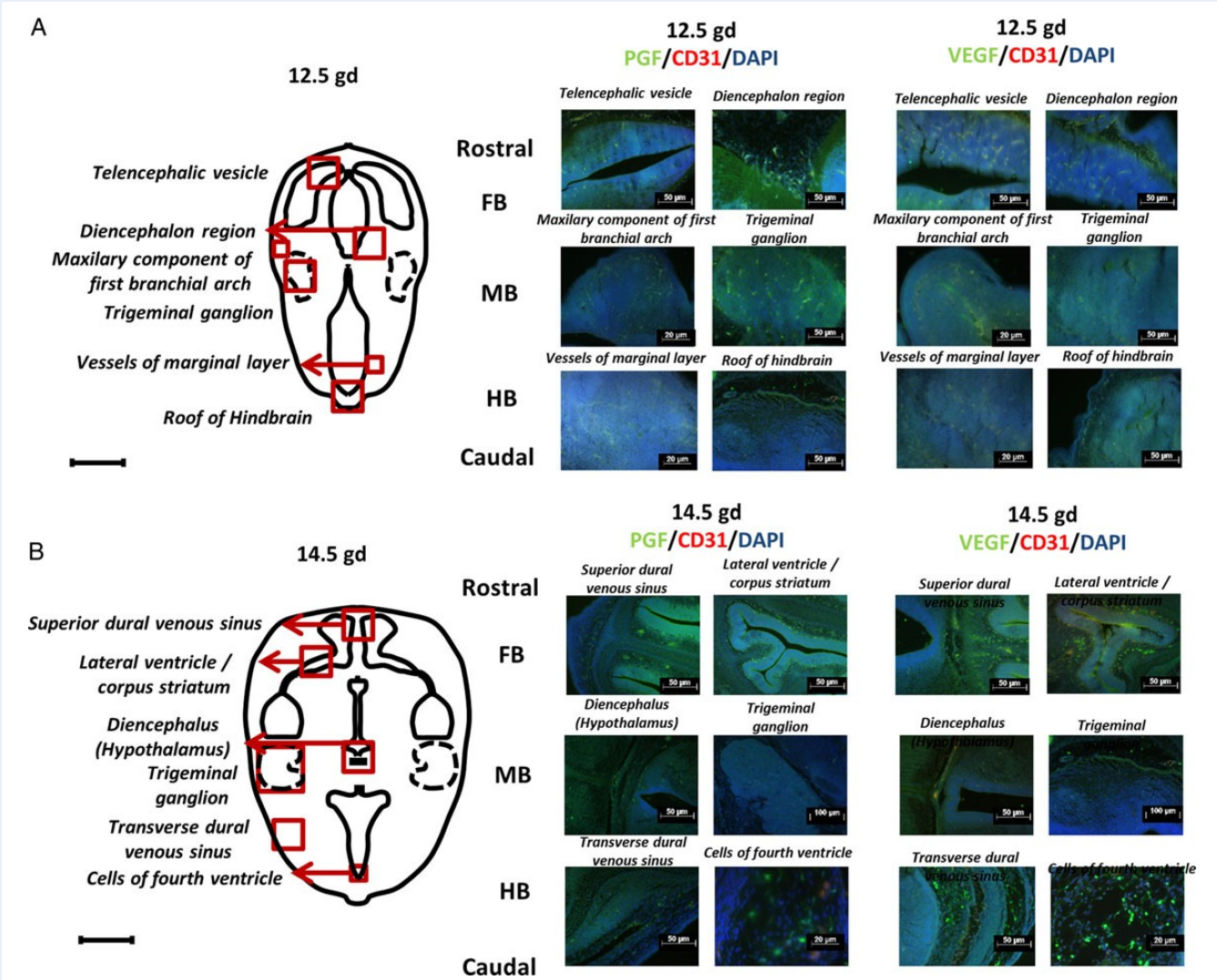

Figure 2 Immunolocalization of placental growth factor (PGF) and vascular endothelial growth factor (VEGF) in embryonic day (E) 12.5 and E14.5 B6 fetal brains. Images depict immunofluorescent staining of PGF (green), VEGF (green) and CD31 (red) and nuclear staining by 4′,6-diamidino-2-phenylindole (DAPI) (blue) in fetal brain at E12.5 (A) and E14.5 (B). Two areas from each region (Fore-, mid- and hind-brain; FB, MB and HB) were chosen. The schematic drawings locate the following structures at E12.5 (telencephalic vesicle, diencephalon region, maxillary component of first branchial arch, trigeminal ganglion, vessels of marginal layer, roof of HB) and at E14.5 (superior dural venous sinus, lateral ventricle/corpus striatum, diencephalon (hypothalamus), trigeminal ganglion, transverse dural venous sinus, cells of fourth ventricle).

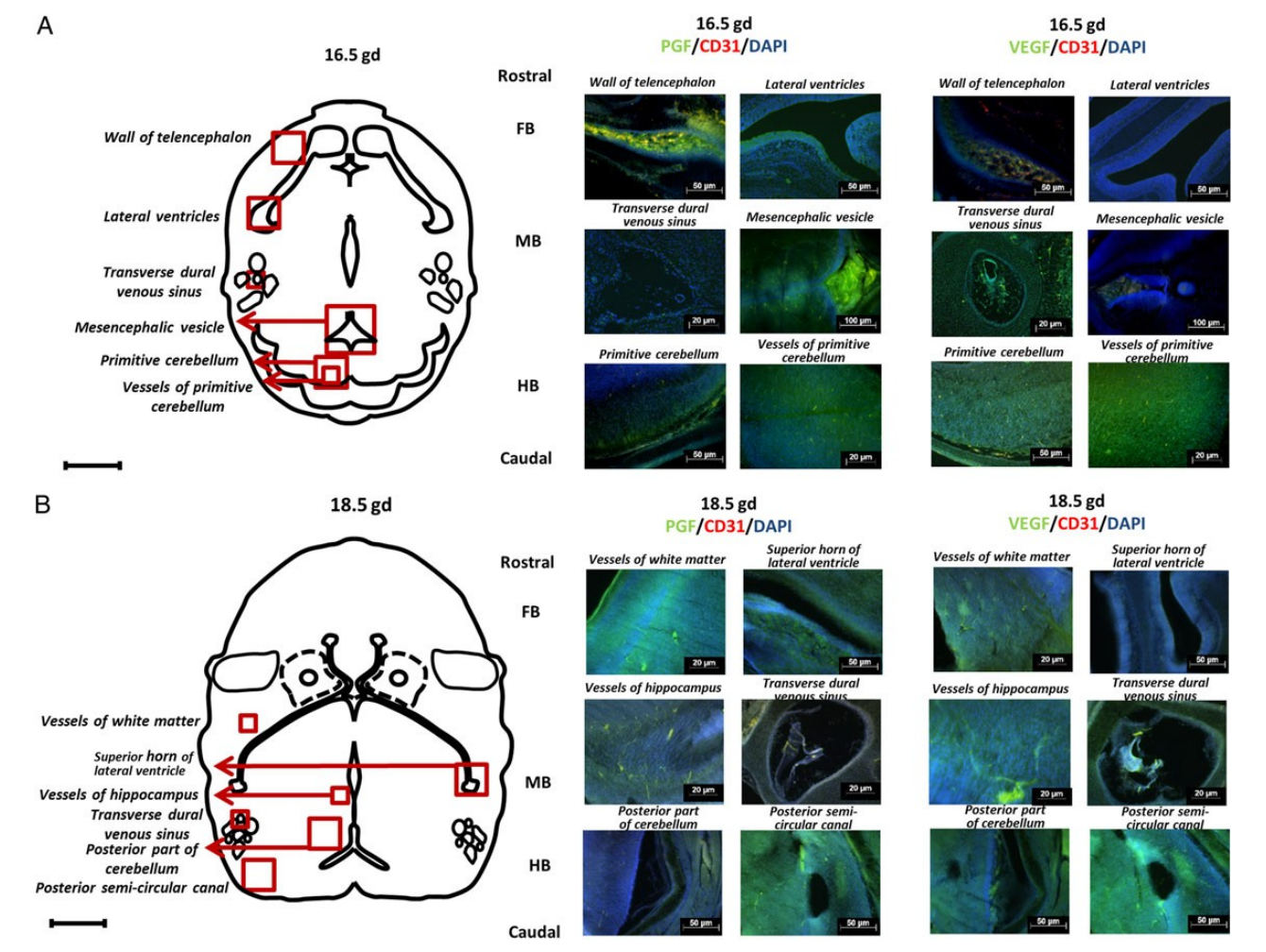

Figure 3 Immunolocalization of placental growth factor (PGF) and vascular endothelial growth factor (VEGF) in embryonic day (E) 16.5 and 18.5 B6 fetal brains. Images depict immunofluorescence staining of PGF (green), VEGF (green) and CD31 (red) and nuclear staining by 4′,6-diamidino-2-phenylindole DAPI (blue) in fetal brain at E16.5 (A) and E18.5 (B). Two areas from each region (Fore-, mid- and hind-brain; FB, MB and HB) were chosen. The schematic drawings show the location of the wall of telencephalon, lateral ventricles, transverse dural venous sinus, mesencephalic vesicle, primitive cerebellum, and vessels of primitive cerebellum at E16.5 as well as the vessels of white mater, superior horn of lateral ventricle, vessels of hippocampus, transverse dural venous sinus, posterior part of cerebellum, and posterior semi-circular canal at E18.5.

Cicle of Willis in E14.5, P7 and adult Pgf $^{2/2}$ and B6 mice

The anteriorcWwas visualized and imaged in E14.5 and P7 Pgf $^{2/2}$ and B6 brains (Fig. 5A and B). Although body weights were similar for both geno- types, P7 Pgf $^{2/2}$ brains were smaller than B6 (P , 0.001, Supplementary Fig. S1). Analyses of arterial diameters and the number of vessels revealed defects in the Pgf $^{2/2}$ cW. In normal B6 brains, the anteriorcerebral arter- ies (ACA) were different in size with one ACA on average 80% the width of the other. However, in Pgf $^{2/2}$ E14.5 and P7 brains, this difference in

HB vascularization in E10.5 and 11.5 Pgf $^{2/2}$ and B6 mice

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excluded from analysis. Vessels in the E10.5 *Pgf* $^{2/2}$ HB ventricular plexus were significantly thinner than in B6 (*P* ¼ 0.0008, Fig. 4C) and *Pgf* $^{2/2}$ HB vessel junctions per area were significantly greater (*P* , 0.001, Fig. 4D). E10.5 *Pgf* $^{2/2}$ HB cross- sections revealed narrower sprout diameters (*P* ¼ 0.022, Fig. 4E) than B6, further suggesting that HB vascularization is impaired at E10.5 by PGF deficiency. By E11.5, differences in CNS development were more apparent and HB thickness was significantly lower (*P* ¼ 0.0002, Fig. 4F) in *Pgf* $^{2/2}$ fetuses despite normalization of the HB vasculature with respect to vessel diameter, number of junctions and sprout diameter. Thus, the transient delay in vascular development linked with PGF defi- ciency was sufficient to impact CNS structural development.

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The anterior communicating artery (AComA) was absent in a significant proportion of E14.5 Pgf 2/2 brains; 45.5% had no AComAs, 50% had one AComA and only 4.5% had more than one AComA. In B6, 58.3% and 41.7% of fetuses had one or more than one AComA respectively. This reduction in the presence of AComAs is reflected in a significantly decreased average number in Pgf 2/2 mice (P ¼ 0.0005; Fig. 5E). Similarly, in adulthood, the average number of communicating vessels was significantly less in Pgf 2/2 mice, particularly in the female mice (P , 0.05, Fig. 6B). The numberof collateral vessels presentin the anteriorcWalso differed in Pgf 2/2 mice. At E14.5, 50% of B6 fetuses had an extra vessel, 25% had more than one extra vessel and only 25% did not have an extra vessel while in Pgf 2/2 fetuses, 86.4% had no extra vessels, 13.6% had one extra vessel and none had more than one extra vessel. The average number of collat- eral vessels was significantly less in Pgf 2/2 mice as a result (P , 0.001;Fig. 5F). However, there was no statistically significant difference in the number of collateral vessels in the adult *Pgf* $^{2/2}$ circle of Willis (Fig. 6C). Overall, while the anterior cW is still complete in *Pgf* $^{2/2}$ mice, its reduced number of vessels suggests that *Pgf* $^{2/2}$ mice may be vulnerable to cerebrovascular insults due to collateral vessel deficiencies.

Left common carotid artery ligation (LCCA)

LCCA was conducted on male and female B6 and *Pgf* $^{2/2}$ mice to assess susceptibility to ischemia. During the surgeries, it was noted that some *Pgf* $^{2/2}$ animals had abnormal vascular anatomy including absence, duplication or hypoplasia of the internal carotid artery (ICA). In B6 mice, no infarct was identified after 30 min ligation (Fig. 7A). However, in two *Pgf* $^{2/2}$ females (out of 15 *Pgf* $^{2/2}$ mice total), infarcts were identified with volumes of 45.91 and 46.77 mm^3 respectively (Fig. 7B). The percentage of cerebral blood flow remaining after LCCA ligation was also significantly less in *Pgf* $^{2/2}$ mice (*P* ¼ 0.0102, Fig. 7D). Therefore, increased *Pgf* $^{2/2}$ susceptibility to an ischemic episode may be associated with structural arterial variations, particularly in the ICA.

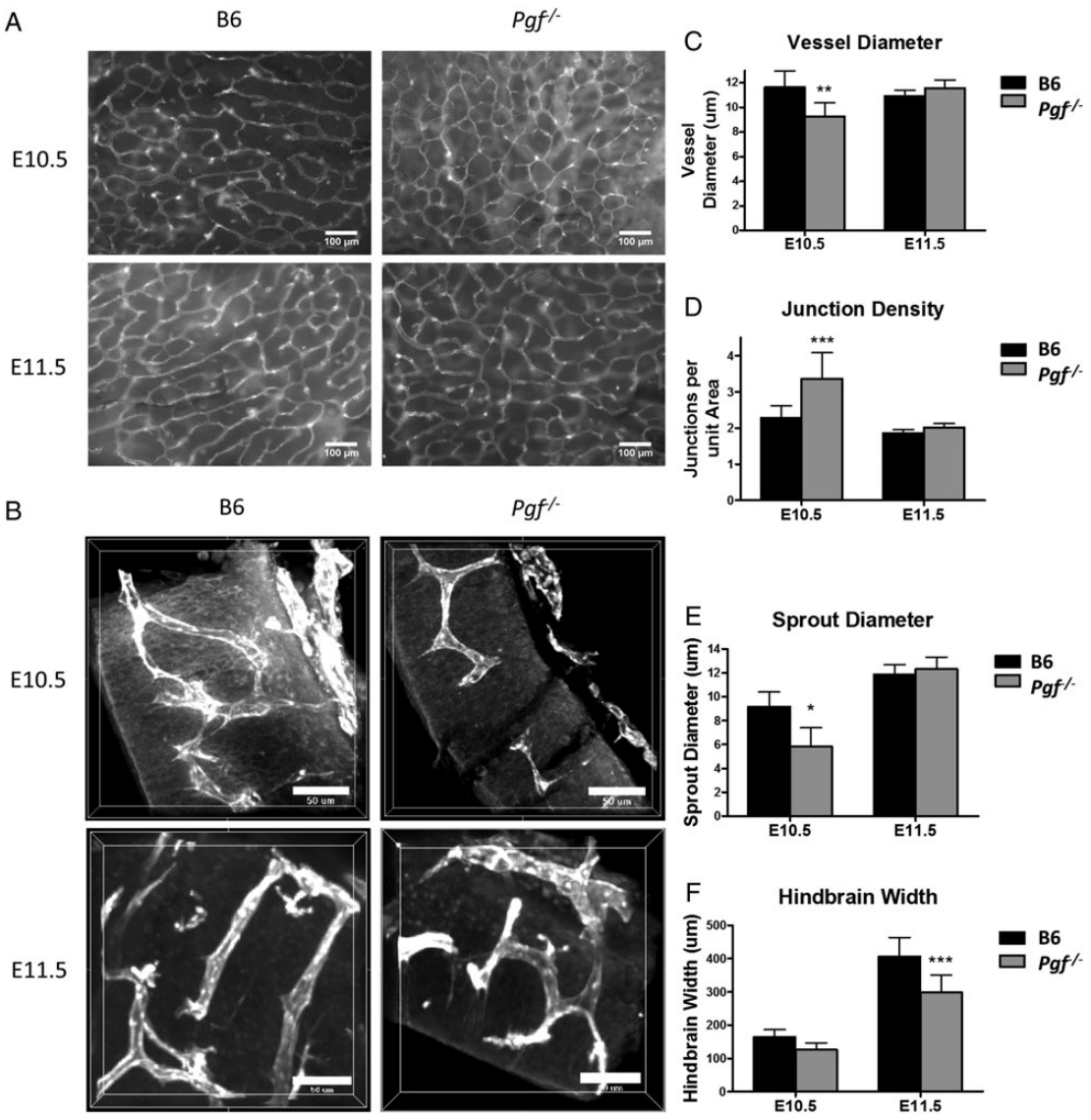

Figure 4 Whole mount vascular staining of hindbrain (HB) at embryonic day (E) 10.5 and 11.5. HB vascularization was compared between B6 and $Pgf^{2/2}$ fetuses at E10.5 and 11.5 by examining the isolectin B4 (IB4) stained (white) ventricular plexus (A) and sprouting in transverse sections of the hindbrain (B). In the ventricular plexus, vessel diameter was thinner in the $Pgf^{2/2}$ HB vasculature at E10.5 (C). Conversely, the number of junctions per area was significantly greater in the E10.5 $Pgf^{2/2}$ HB vascular plexus (D). Confocal imaging of the HB cross-sections revealed smaller sprout diameter (E) and reduced HB thickness (F) in the $Pgf^{2/2}$ HBs. Means with 95% confidence intervals are shown with P , 0.05, P , 0.01, and P , 0.001 represented by *, **, and *** respectively.

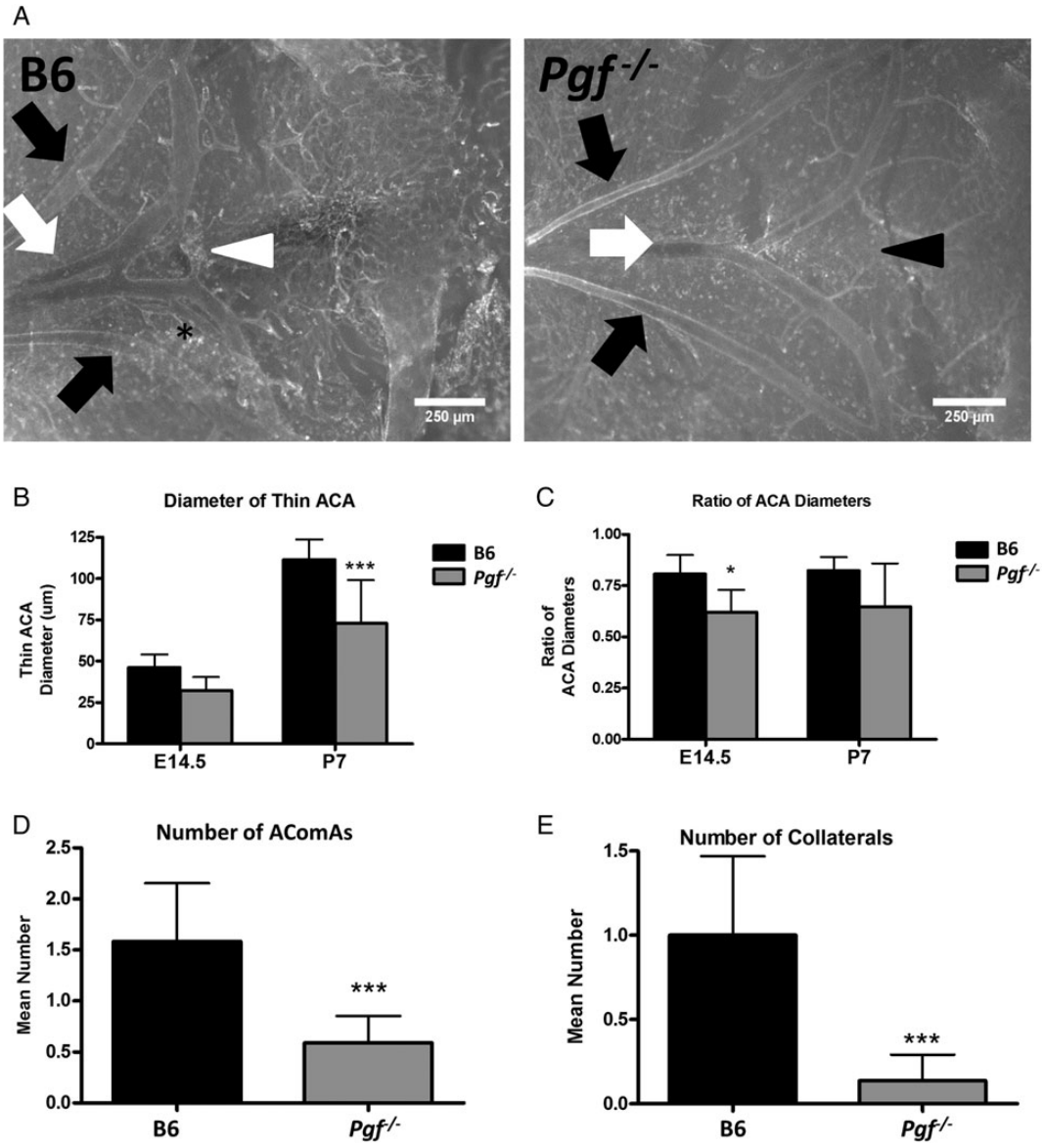

Figure 5 Whole mount vascular staining of circle of Willis (cW) at embryonic day (E) 14.5 and post-natal day (P) 7. Whole mount immunofluorescence with isolectin B4 (IB4; white) provided visualization of the anterior cerebral arteries (ACAs: white arrows), olfactory arteries (black arrows) and the anterior communicating artery (AComA; white arrowhead) in B6 and $Pgf^{2/2}$ brains at E14.5 (A) and P7 (not shown). In many B6 brains, an extra vessel along the ACA was present (asterisk) while in some $Pgf^{2/2}$ brains, the AComA was absent (black arrowhead). Significant changes in the $Pgf^{2/2}$ cW included a narrower diameter of one ACA (B) but not both ACAs, resulting in a decreased ratio of the ACA diameters (C). The number of vessels present in the anterior cW of $Pgf^{2/2}$ mice was fewer than in B6 mice. The mean number of AComAs in the $Pgf^{2/2}$ cW at E14.5 was fewer than in B6 (D). Similarly, at E14.5, the mean number of collateral vessels in the $Pgf^{2/2}$ cW was reduced (E). Means with 95% confidence intervals are shown with $P < 0.05$, $P < 0.01$, and $P < 0.001$ represented by *, **, and *** respectively.

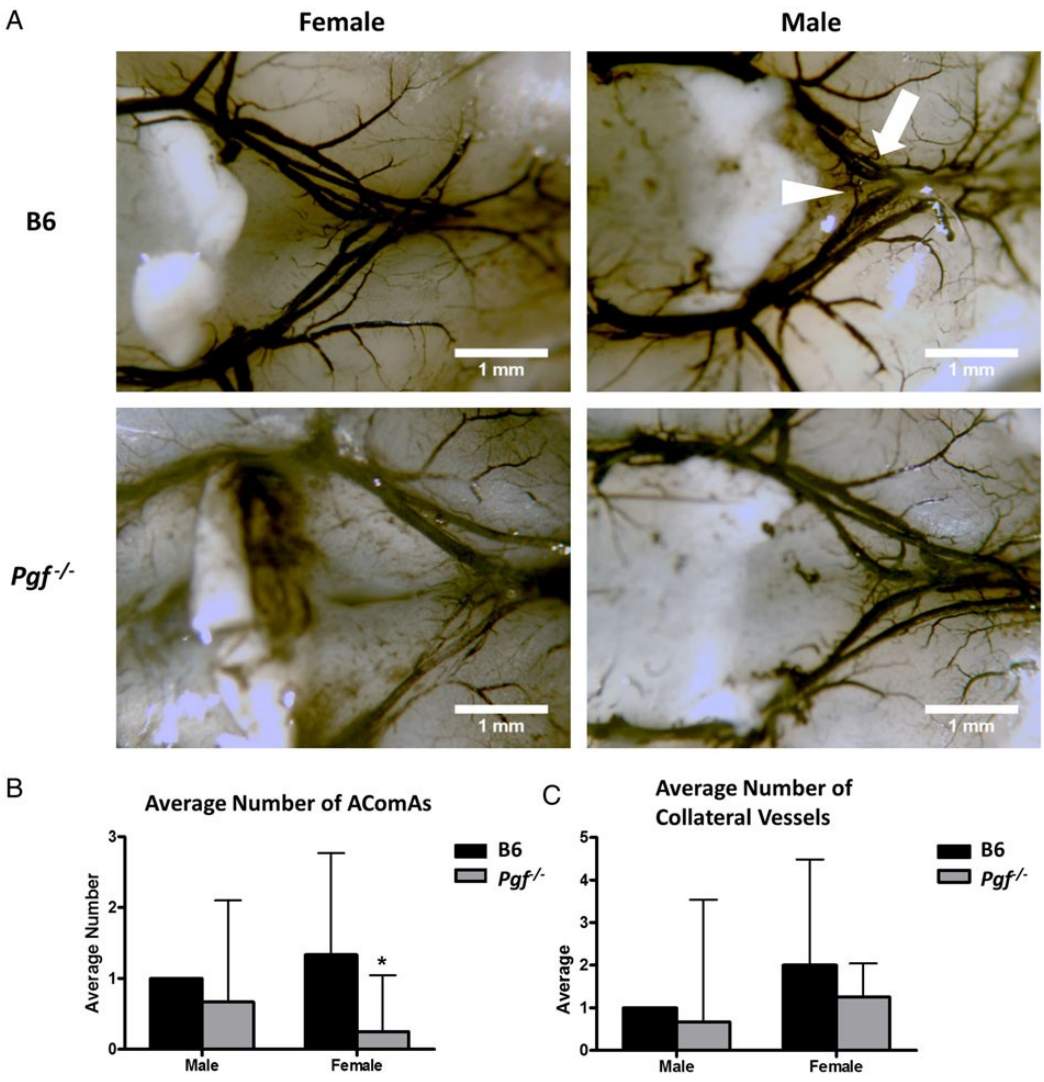

Figure 6 Ink perfusion and imaging of the adult circle of Willis. The circle of Willis was imaged in adult male and female B6 and *Pgf*$^{2/2}$ mice (A). The average number of anterior communicating arteries (AComAs) (arrowhead) was decreased in female *Pgf* $^{2/2}$ mice (B). There was no significant difference in the number of anterior collateral vessels on the anterior cerebral arteries (arrow) in either male or female *Pgf* $^{2/2}$ mice (C). Means with 95% confidence intervals are shown with *P* , 0.05 represented by *.

Discussion

PGF deficiency in mice deviates cerebrovascular development and impacts vessels of varying sizes. HB vascularization was delayed in *Pgf* $^{2/2}$ mice and this was accompanied by a narrower HB, a difference that persisted following normalization of the vascular bed at E11.5. Based on relative expression analyses, it appears that VEGF is more important for HB vascularization than PGF. VEGF compensation, driven by hypoxia, may explain the normalization of HB vessels at E11.5. However, reports of increased systolic arterial pressure in *Pgf* $^{2/2}$ mice (Steenkiste *et al.*, 2011; Aasa *et al.*, 2015) suggest that vascular differences persist into adulthood.

PGF deficiency permanently affected the developing cW. Murine internal carotid, cerebral and basilar arteries form prior to E11.5 from the transient aortic arches (Hiruma and Nakajima, 2002). The anterior communicating artery (AComA) completes the cW between E11.5–15.5 (Yang *et al.*, 2013). Although the anterior cW was complete in *Pgf* $^{2/2}$ mice, defects Predictive of decreased blood flow were identified. Incomplete cW development was previously reported in mice deficient in vascular smooth muscle cell Notch signaling (Proweller *et al.*, 2007; Yang *et al.*, 2013). In these animals, AComA narrowing was found despite normal abundance of VEGFA protein (Yang *et al.*, 2013). ACA unilateral hypoplasia with fewer communicating and collateral arteries was observed in *Pgf* $^{2/2}$ mice. These changes may have limited impact on brain health under normal conditions but would increase sensitivity to ischemia when blood flow is obstructed. This hypothesis is supported by the infarcts observed in two *Pgf* $^{2/2}$ females after LCCA occlusion and probably explains why radiotelemetry experiments were previously unsuccessful in 80% of adult *Pgf* $^{2/2}$ males and females (Ratsep *et al.*, 2015a). In our experiments, LCCA occlusion was maintained for onlY 30 min, a relatively mild insult. Although only two animals developed infarcts, the number may have been higher with a longer protocol. In fact, significantly greater decreases in cerebral blood flow were identified in the *Pgf* $^{2/2}$ mice after LCCA occlusion compared with B6 mice even without appearance of an infarct. Although both animals experiencing infarcts were female and AComA differences were identified in adult female *Pgf* $^{2/2}$ brains, too few ischemic events occurred to conclude there is a sex difference. However, sexually dimorphic gene expression has been reported in preimplanta- tion mouse embryos suggesting that developing offspring will respond differently to stresses (Lowe *et al.*, 2015). In preeclamptic pregnancies, the inflammatory, angiogenic and apoptotic responses differ by fetal sex (Muralimanoharan *et al.*, 2013). Further, sexually dimorphic effects on placental and brain function after *in utero* stress or alcohol exposure have been reported (Lan *et al.*, 2015). The brain has been identified as sexually dimorphic both structurally (Goldstein *et al.*, 2001; Gong *et al.*, 2011; Yan *et al.*, 2011) and vascularly (Tontisirin *et al.*, 2007; Krause *et al.*, 2011). Therefore, we suspect that PGF deficiency and *in utero* experience of PE will impact male and female brains differently. Although PGF is known to be important for collaterogenesis in wound healing and ischemia (Dewerchin and Carmeliet, 2012), it is not yet pos- sible to assign the cerebrovascular phenotype we observed directly to a PGF deficiency, rather it reflects disturbances within angiogenic path- ways. Notch signaling is intricately entwined with PGF and VEGF signaling with effects upstream and downstream of *Vegfr1*. Delta-like ligand 4/NOTCH signaling increases expression of *Vegfr1* while decreasing expression of *Pgf* in endothelial cells (Harrington *et al.*, 2008). Conversely, inhibition of the NOTCH pathway rescues branching defects in *Vegfr1*$^{2/2}$ endothelial cells (Chappell *et al.*, 2013). In humans, the cW is formed between days 40 and 55 of pregnancy (Van Overbeeke *et al.*, 1991; Degani, 2009). The human cW is a highly variable structure, particularly in its posterior components which are in- complete in 22% of normal individuals (Ryan *et al.*, 2013). Defects in the cerebral vasculature following gestational PGF deficiency could explain the higher incidence of stroke in offspring of preeclamptic pregnancies. Although PE occurs acutely in women after week 20 of gestation, low PGF levels in maternal plasma are present by the first trimester in many women who progress to PE (Vatten *et al.*, 2007; Romero *et al.*, 2008). Thus, the timeframes for suboptimal placental PGF production and cW development overlap. In our knockout model, PGF expression is absent in all tissues, including the placenta and the brain. While the pla- centa has not been excluded as the source of PGF that regulates cerebral vascular development and other studies implicate placental hormones and

neurotransmitters in offspring brain development (Bonnin *et al.*, 2011; Penn *et al.*, 2014), we have now documented localized and dynamic expression of PGF in developing brain tissue. This study is the first to describe PGF levels in the developing mouse brain. Previous reports address PGF expression in human brain tumors (Donnini *et al.*, 1999) and after ischemic injury in cerebral vessels, neurons and astrocytes in mice and rats (Beck *et al.*, 2002; Du *et al.*, 2010). In adult human brain, PGF is expressed in neurons but not glial cells (Xu *et al.*, 2012a). PGF was increased in human cerebrospinal fluid after seizures (Xu *et al.*, 2012b) suggesting PGF has an important neuro- protective, homeostatic role. Neuroprotection could be due to improved neoangiogenesis, (Gaa'l *et al.*, 2013) to direct effects on neural cells or to combined effects. Since PGF affects both neuronal and vascular structures, our study cannot determine the temporality of deficits in collateral vessels and brain growth in *Pgf* $^{2/2}$ mice. Impaired brain development may result from vascular deficiencies caused by the lack of PGF. Conversely, impaired brain development caused directly by the lack of PGF may lead to impaired vascularization. PGF is known to act directly on endothelial cells, but also has roles in mural cell and macrophage recruitment (Dewerchin and Car- meliet, 2012). Deficient mural cell recruitment may also impairarteriogen- esis (Gaa'l *et al.*, 2013). Similarly, macrophages have important roles in the formation of anastomoses and are important for collateral vessel forma- tion (Pipp *et al.*, 2003; Duelsner *et al.*, 2012). Therefore, absence of PGF during development may lead to reduced angiogenesis and arteriogenesis through several different pathways. Developmental analysis of the different brain regions is important to understand disorders that present in childhood or adult life. In this study, we chose regions that mature into significant structures including the cerebral cortex, thalamus, cerebellum, and venous plexus. PGF and VEGF and their receptors were expressed by both cerebral tissue and by vessels in the FB, MB and HB at all gestational time points although the strength of expression varied between brain regions and developmental stages. Based on expression strength, PGF should have the greatest influ- ence on the FB, the primordium of brain regions associated post-natally with executive and cognitive functions, while VEGF was more predom- nant in the HB and would have greater influence on areas related to the future fourth ventricle and cerebellum. Smaller head circumference at birth (Kajantie *et al.*, 2009), cognitive impairments, and mood disor- ders at advanced ages are reported in offspring of preeclamptic pregnan- cies compared with controls matched for current and gestational-ages (Tuovinen *et al.*, 2010, 2012, 2013). Recent pilot studies of 10 children age 7 – 10 years, who experienced PE gestations compared with sex, gestation-length and age-matched controls, demonstrated cerebral vas- cular and anatomic differences that were accompanied by PGF deficiency in maternal term plasma (Ratsep *et al.*, 2015b). This literature and the findings reported here regarding anomalous cerebral vascularization in *Pgf* $^{2/2}$ mice and its impact on early brain tissue structure suggest that

cerebral angiograms and further anatomic magnetic resonance imaging scans of offspring of preeclamptic pregnancies may provide a new dimen- sion in understanding lifelong cerebral consequences for offspring from PE gestations.

Conclusion

We characterized the expression of PGF in the developing mouse brain from mid to late gestation, identified multiple cerebral vascular defects induced by PGF deficiency and provided evidence that disturbed cerebral vascularization can precede an alteration in CNS structure and blood flow. While PGF is already described as a therapeutic target in neurologic diseases, our results indicate that normal levels of PGF during develop- ment have critical importance. Further, fetal PGF deficiency would increase susceptibility to stroke due to differentiation of relatively narrow cerebral vessels and failure to develop a normal frequency of collateral vessels.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors' roles

R.L.L., V.R.K., A.J. and B.A.C. designed the studies. R.L.L. performed the PCR and immunofluorescence analysis of PGF expression in developing mouse brain. K.K. and M.B. assisted with the PCR experiments and their analyses. V.R.K. performed the whole mount comparisons of HB vascu- lature and the circle of Willis. N.P. performed the artery ligation surgeries for the ischemia assays with assistance from M.T.R. P.C. provided the *Pgf* $^{2/2}$ mice. R.L.L., V.R.K. and B.A.C. wrote the manuscript.

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Conflict of interest

None declared.

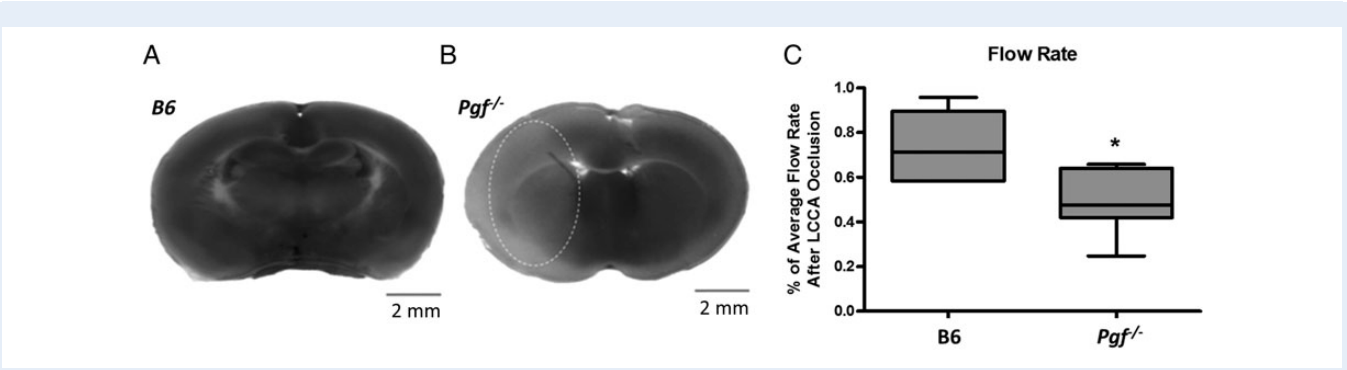

Figure 7 Infarct size and blood flow after left common carotid artery (LCCA) occlusion. Staining revealed no apparent infarct in B6 brain slices (A) but infarcted tissue (pale area encircled) in 2 female *Pgf* $^{2/2}$ mice (B). The percentage of cerebral blood flow remaining after LCCA was significantly decreased in the *Pgf* $^{2/2}$ mice (C). * represents P , 0.05.

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CAPÍTULO 4

Therapeutic outcomes of the Phosphodiesterase-5 inhibitor treatments during pregnancy in improving maternal-fetal health after induced abortion

Luna, R. L., artigo em elaboração para sumissão à Fertility Sterility

(Qualis: A1)

PROMISSING THERAPEUTIC OUTCOMES OF PHOSPHODIESTERASE-5 INHIBITOR TREATMENTS DURING PREGNANCY IN IMPROVING MATERNAL-FETAL HEALTH AFTER INDUCED ABORTION

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ABSTRACT

This study investigated the effect of Sildenafil (Sil) associated with Low Molecular Weight Heparin (LMWH) (Hep) on coagulation process during pregnancy loss and how it reflects in the health of the fetus. Pregnancy complications indeed results in fetal demise. The essential mechanisms of coagulation involved in Recurrent Pregnancy Loss (RPL) as well as the effect of Sildenafil during pregnancy need to be clarified. RPL mouse model was induced by lipopolysaccharides (LPS) injection (100µg/Kg) at gestational day (gd) 15.0, control group was injected with sterile saline. Mice were pre-treated with Sil (50mg/kg) and/or with Hep (500IU/kg) starting at 0.0gd to 15.0gd. Thromboelastography (TEG) and Cell Blood Counting (CBC) analysis were performed. Placentas were analysed by Immunohistochemistry (IHC) for endothelial nitric oxide synthases (eNOS) and placental growth factor (PGF) and the amniotic fluids by multiplex. Fetuses were clinically analysed from each dam. Healthy women's blood samples were treated ex-vivo with Sil (200ng/ml) and LMWH (0.2 IU/ml), TEG was also performed immediately after treatment. The LPS group presented lower levels of platelets counting, in all the CBC analysis the Sil+Hep group showed similar results to the control group. Combined treatment rescued pregnancies and improved fetal heath and addicionally Sil alone increased the number of implantations sites and fetal size. Sil+Hep group showed high level staining for eNOS and similar PGF expression as was shown in the Control group. Sil alone did not change the coagulation pattern in normal women's blood samples, also did not exacerbate the LMWH hypocoagulation effects. Sildenafil represents a safe therapeutic approach to RPL, including as an adjuvant to LMWH treatment to the maintenance of fetal health even after pregnancy injury.

KEY-WORDS: Miscarriage, Phospodiesterase-5 inhibitor, Low molecular weight heparin, Lipopolysaccharides, Amniotic fluid.

INTRODUCTION

Sildenafil (Viagra®, Pfizer) induces Cyclic Guanosine Monophosphate (cGMP) accumulation through selective phosphodieterase-5 (PDE5) inhibition (Boolell, Allen et al. 1996). Food and drug administration (FDA) approves the therapeutic use of this molecule in cases of erectile dysfunction, pulmonary hypertension and Raynauld's syndrome (Badesch, Hill et al. 2007). Sildenafil has an excellent tolerability and safety profile (Boolell, Allen et al. 1996, Abbott, Comby et al. 2004). Other diseases regulated by NO-cGMP pathway and can also beneficiate with PDE-5 inhibition (Fries, Shariat et al. 2005, Zhang, Guo et al. 2013, Gomes, Carvalho Mda et al. 2014). Besides vessels and lung, PDE- 5 has also been known to exert physiological effects on other cells such as platelets and retinal cells (Schwarz, Kapur et al. 2007). The presence of PDE-5 on the platelet surface and light influence in the coagulation cascade has been previously shown; however, the relationship of enzyme inhibition remains to be known. Studies have been published describing the effects of Sil in cases of FGR (Dastjerdi, Hosseini et al. 2012, Panda, Das et al. 2014). The promotion of vasodilation in murine uterine arteries, as well as increased fetal weight during challenged-pregnancy have been demonstrated (Dilworth, Andersson et al. 2013). A recent publication by our group showed that a pre-treatment with Sildenafil could reduce fetal death induced by high doses of Lipopolysaccharide injection (Luna, Nunes et al. 2015). The association of Sildenafil and Heparin (Hep) was demonstrated to treat different stages of abortion however exists a lack of information about the influence of Sil alone or in combination with Hep in the fetal side of pregnancy and how the fine balance between a demising and a health fetus occur.

Low molecular weight heparin (Fragmin©, Pfizer) is a Xa inhibitor from the common coagulation cascade has been indicated to treat acute deep venous thrombosis as well as preventing clotting and other coagulopathies (Company 2014). Treatments involving this type of Heparin cause a block of thrombin formation, which ultimately hinders clot formation due to a lack of fibrinogen (Oberkersch, Attorresi et al. 2010, Noci, Milanini et al. 2011, Shi, Bai et al. 2013). The use of anti-coagulation therapies are common in women that are diagnosed with Thrombophilia (de Jong, Goddijn et al. 2013). Indeed, different types of Heparin have been considered as a safe therapeutic approach for pregnant women (Patnaik, Haddad et al. 2007, Shi, Bai et al. 2013, Grandone, Villani et al. 2015). LMWH and other anti-coagulation drugs have been used to treat complicated pregnancies (Nelson-Piercy, Letsky et al. 1997, Grandone, Villani et al. 2015), regardless of whether the pregnancy is natural or conceived from IVF cycles (Noci, Milanini et al. 2011). Treatments using heparin also have been appear as beneficial in several non-thrombophilia cases, however, the outcomes are ambiguous (Kaandorp, Di Nisio et al. 2009, Noci, Milanini et al. 2011, Rodger 2015, Schleussner, Kamin et al. 2015), in addiction the therapy may be associated with higher bleeding risk (Bain, Wilson et al. 2014). Preeclampsia is another pregnancy complication which has previously been treated with LMWH alone or in combination with other drugs (Roberge, Demers et al. 2015). The interaction between LMWH and Complement pathway activation was previously demonstrated for preventing either complement dependent or other inflammatory mechanisms responsible Pregnancy Loss (PL) (Oberkersch, Attorresi et al. 2010). Subcutaneous injections of LMWH and the lack of conclusions regarding the efficacy in cases

of RPL open a new therapeutic opportunity for alternative treatments that may rather support or substitute LMWH administration process.

Fetal death has been associated with many coagulation disorder such as factor V Leiden and antiphospholipid syndrome among others that cause thrombogenic effects on placental vasculature (Grandone, Margaglione et al. 1997, de Jong, Goddijn et al. 2013). There is also an increased risk of pregnancy loss in women with a deficiency of antithrombin, protein C, and protein S (Sanson, Friederich et al. 1996). Pathological analysis indicates extensive placental infarction and thrombosis in the placenta (Laskin, Chuma et al. 1994) in cases of RPL (Dawood, Farquharson et al. 2003, Rey, Kahn et al. 2003). Decidual inflammation has been associated with fibrin deposition and thromboembolism in cases of spontaneous recurrent miscarriage (RM) (Krieg, Fan et al. 2012). Pregnancy complications such as preeclampsia (PE), Fetal growth restriction (FGR) and coagulopathy diseases in general may have an influence either during the implantation and/or fetus development stages, and therefore are related to RPL (de Vries, Dekker et al. 1997, Powers, Roberts et al. 2012). The presence of a thrombotic event during pregnancy often times is associated with fetal distress which may be followed by fetal death (Coulam, Branch et al. 1999, Middeldorp 2013, Mutlu, Mutlu et al. 2014). The maintenance of fetal health even after injury, cellular stress, inflammation and coagulation process still a challenge. From our knowledge based in an up to date bibliography analyze, does not exist a standard high effective treatment for cases of pregnancy loss which can protect placental tissue as well as protect the fetus against miscarriage event.

ADD HERE BACKGROUND AND REFERENCES ABOUT THE CYTOKINES FOUND SIGNIFICANTLY CHANGED IN THE MULTIPLEX ASSAY

The main objective of this study was to evaluate the influence of Sildenafil in the coagulation cascade during pregnancy loss as well as the action of Sil preventing fetal demise improving fetal heath by balancing molecule factors. The influence of both inhibitions of PDE-5 (Sildenafil) and the Xa Factor (Dalteparin) were evaluated in an *in-vivo* and *in-vitro* experiments, in both animal and humans samples respectively. Therefore we aim to compare the results from Sil to Hep or a combinatorial treatment with Sil+Hep. We hypothesized that Sildenafil may be a safe potential treatment for cases of PL related to fetus distress, whether alone or in combination with Heparin.

MATERAIALS AND METHODS

Animal studies protocol

Outbred virgin Albino Swiss aged 60 days and weighing approximately 30g were used in this study. Mice were mated in a 2:1 female to male ratio overnight in the pro-oestrus phases. Copulation was confirmed the morning after mating by the vaginal plug and considered the 0.0 gestation day (0.0gd). The mice were handled at the animal care facility at Queen's university following all guidelines and ethical protocols of Queen's University Animal Care (number: 2014-1503). 25 pregnant mice were divided in a 1:1 ratio for the following groups:

Control: received neither prophylactic drug (water only for drinking) nor LPS (saline placebo treatment).

LPS abortion-model: intra-peritoneal (i.p.) injection with 0.5 ml LPS (*Escherichia coli* serotype Sigma-Aldrich 0111-B4 100µg/Kg in sterile saline) on 15.0gd.

Sildenafil treatment: 50 mg/Kg of Sildenafil (Viagra®, Pfizer) was administered through the drinking water from 0.0gd to 15.0gd. Mice were weighed and their water consumption was measured daily to accurately increase Sildenafil concentrations in the water to deliver a constant dosage of Sildenafil on each day of pregnancy. LPS was administered on 15.0gd.

Heparin treatment: LMWH (Fragmin, Pfizer) was administered at a dose of 500 IU/kg subcutaneously per day to weighed mice in a volume of 0.2 ml from GD1 to15.0gd, LPS was administered on 15.0gd.

Sildenafil + Heparin treatments: received both drugs from gd 0-15 as described above. LPS was administered on 15.0gd.

All the dams were euthanized 2 hours after LPS injection. Mice were anesthetised using pentobarbital (40mg/kg) and 1ml of blood was collected by cardiac puncture. Implantation sites in all treatment groups (usually approximately 12/dam) appeared similar in general at this early time after abortion induction however the mother abdominal arteries were separately analysed by gross anatomy. 1 ml of amniotic fluid was collected from each pregnancy. For Maternal-fetal health measurements fetuses were measured by height (mm), abdominal circumference (mm) and weighted (mg) among each pre-divided studied group. Placentas were removed, measured by length and width (mm), weighed and processed to the followed techniques.

Human studies protocol

Healthy non-pregnant women with no pre-existing coagulopathies or pre-existing thrombotic family history were recruited for this study. The volunteers most have not taken any anti-coagulant therapy. The Queen's University Faculty of Health Sciences Research Ethics Board approved this study (number: 6007631) and all the 6 volunteers signed a written consent agreeing with the rules of this study. The treated human blood samples were immediately submitted to thromboelastography (TEG) analysis after the treatments or saline. The bloods from the volunteers in a maximum volume of the 50ml were divided in the followed groups:

Control: 10µl of sterile saline was add to the blood.

Molecular Sildenafil: 10µl of pre-diluted solution containing Sildenafil citrate (200mg/ml) was add to the blood

Molecular Heparin: 10µl of pre-diluted solution containing Low Molecular Weight Heparin (0.2IU/ml) was add to the blood

Sildenafil + Heparin: 5µl of each of the Sildenafil and Heparin were added at the same time to the blood. The same doses were kept from the previous groups.

Thromboelastography

Thromboelastography was performed using the TEG 5000, Haemoscope; Braintree, MA, USA machine according to the manufacturer's instructions. Pre-citrated whole blood (340 µL) obtained via cardiac puncture from the animals or via brachial venipuncture from the humans volunteers.

Samples were recalcified with 20 µL 0.2 M $CaCl_2$ and placed into the TEG cup. Major TEG parameters were analyzed including (i) R time; time to formation of initial fibrin threads, (ii) MA; maximum width of the trace reflecting strength of the clot (iii) LY30; percent lysis at 30 min after MA and (iv) CI; clotting index; an overall evaluation of coagulation. Quality control was maintained per manufacturer's instructions.

Complete blood cell counts

Whole blood samples obtained via cardiac puncture from the animals were subjected to automated blood picture analyses using Cell Counter Analyzer (Scil Vet ABC, animal blood counter, SCILVET). Total Red blood cells (RBCs), total white blood cells (WBCs) with differential counting (lymphocytes, granulocytes and monocytes), and platelets counts were analyzed.

Multiplex cytokine array

Amniotic fluids collected from each dam from multiple gestational sites were used. A cytokine/ chemokine/ growth factor biomarkers were measured simultaneously using a Discovery Assay® (Mouse Cytokine Array/Chemokine Array 32-Plex (Eve Technologies Corp, Calgary, AB, Canada). The multiplex assay was performed using the Bio-Plex™ 200 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and a Milliplex Mouse Cytokine / Chemokine kit (Millipore, St. Charles, MO, USA) according to their protocol. The consisted of G-CSF, GM-CSF, IFNγ, IL-1α, IL-6, IL-10, CXCL10, CXCL1, CXCL3, MCP-1, CCL3, CCL4, CCR5, TNFα and VEGF. Cytokine proteins were quantified

through the detection of conjugate which is in direct proportion to the amount of target analyte. Quantification was completed with a standard curve, and values were recorded in pg/mL. The assay sensitivities of these markers range from 0.1 – 33.3 pg/mL. Protein concentrations were recorded and analysed, The average values of each molecule were compared between the five groups.

Immunohistochemistry

Paraffin-embedded sections of placenta were stained for eNOS and PGF using an anti-mouse polyclonal primary antibody anti- eNOS 0.5µg/ml (Abcam - AB66127) and PGF 0.5µg/ml (Abcam - ABC375) Biotin-conjugated secondary antibody using an HRP-kit (K0690-DakoCytomation) with DAB was used as the chromogen. The specimens were weakly counter-stained with hematoxylin. Sections were examined from multiple implantation sites in each of the 25 pregnancies studied at 2h after LPS injection. For each antibody, five stained areas for each protein investigated were measured for pixel density using the GIMP 2.6.11 (GNU Image Manipulation Program, UNIX platforms) software program.

Statistical analysis

GraphPad Prism (San Diego, CA, USA) was used for statistical analyses of immunohistochemistry specific pixel density analyses, for all the evaluation of fetal and placental heath and the average values from the Multiplex analyses also were compared using the one-way analysis of variance (ANOVA) followed

by Bonferroni's post hoc test. Data were also subjected to non-parametric test Mann–Whitney testing. Statistical significance was set as $p < 0.05$ or $p<0,01$.

RESULTS

Effect of Heparin and Sildenafil treatment on global hemostasis in mice (in vivo)

There was a significant reduction in R time and a significant increase in LY30 in the LPS group indicating that in the animals from this group may happened a quicker beginning of clot formation as well as a longer coagulation process when compared to the Control. Indeed mice treated with Sildenafil presented decrease of the R time and the MA parameter, no changes were observed in the LY30% or CI (Figure 3C). Heparin group have shown a hypocoagulable pattern recognized by a higher R time and LY30% and a smaller CI which represents a later coagulation process. A significant reduction in MA and CI was found in the sildenafil group only, a significant increase in R time and a reduction in CI in heparin only group, when compared to the control. Even though there was a significant increase in R time and MA in the sildenafil and heparin group, the result from the combined treatment showed closer similar parameters compared to the control group (Figure 3C).

Effect of heparin and sildenafil treatment on global homeostasis in humans *(in vitro)*

There was no significant difference in the TEG parameters of the control and sildenafil only group. Heparin only and sildenafil and heparin combined groups showed hypocoagulable patterns as shown by a significant increase in R time and reduction in MA, LY30 and CI (Figure 3B).

Variation in blood cells parameters in different treated groups

We thoroughly examined the blood picture of the mice in all the treatment groups and control. The LPS group mice did not show a higher WBCs counts, compared to all the control group. The mice under the heparin alone and sildenafil alone treatments had less WBCs than the control and lesser than the LPS group. There was also a significant reduction in platelet counts in LPS treated group. However, there was no significant difference in the platelet count between the treated groups and control. Reed blood cells (RBC) were significantly was reduced in the Hep group, compared to control, LPS and also Sildenafil treated groups.

Gross anatomy evaluation from pregnancy and pregnancy loss and Maternal-Fetal measurements

Amniotic fluid cytokine profile

Placental expression of eNOS and PGF

DISCUSSION

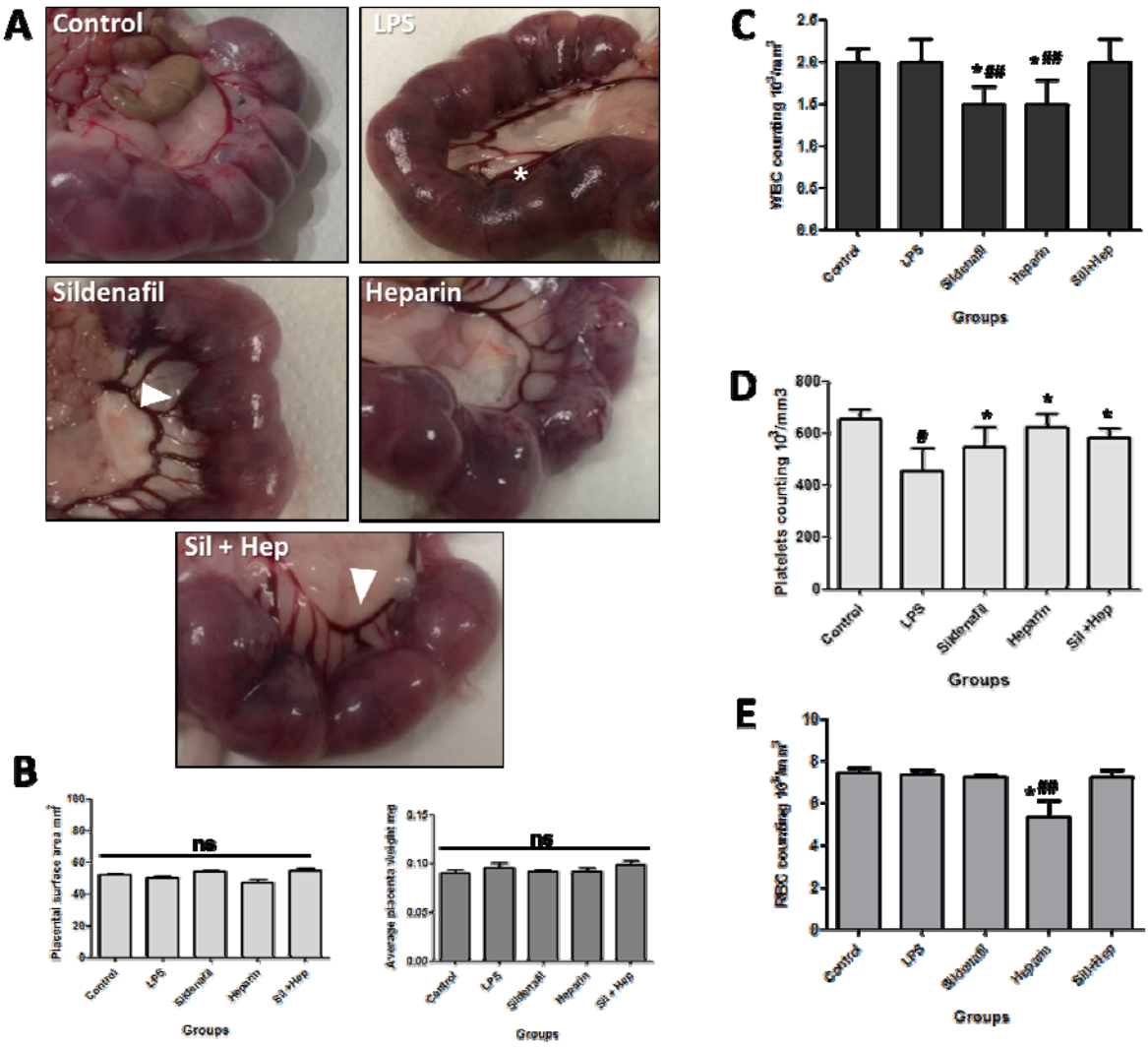


Figure 1: A: Gross anatomy of gd 15.0. Control group showed normal pink colour and arteries conserved with a normal caliber. LPS group showed dark

colour gestational sacks including darker areas (white asterisk), the arteries showed beginning of thrombus development. In Sil group, the gestational sacks showed a characteristic pink colour, lighter than LPS group, and presented a vasodilatory effect in the abdominal arteries. Hep group presented pink-like normal colour gestational sacks, did not show thrombus in the arteries. Sil + Hep presented lighter pink colour similar to the control group, however did not show thrombus in the arteries and parts of arteries presented as vasodilated. **B:** No difference in the placental weight was found between the groups and no difference in the placental area was found between the groups. **C:** Mouse blood analysis. White blood cells counting did not differ from the LPS group to the Control group**.** The Hep and Sil groups presented a significant decrease compared to the Control and LPS groups. **D:** Platelets counting showed a decrease in the LPS group, while no difference was found in the treated groups. **E:** Red blood cells counting showed that only the treatment with Hep significantly decreased the total RBC number, compared to the Control and LPS groups. Data expressed as ± S.D of mean. *p<0,05, **p<0,01 compared to the LPS group, $^{\#}$p<0,05, $^{\#\#}$p<0,01 compared to the control group.

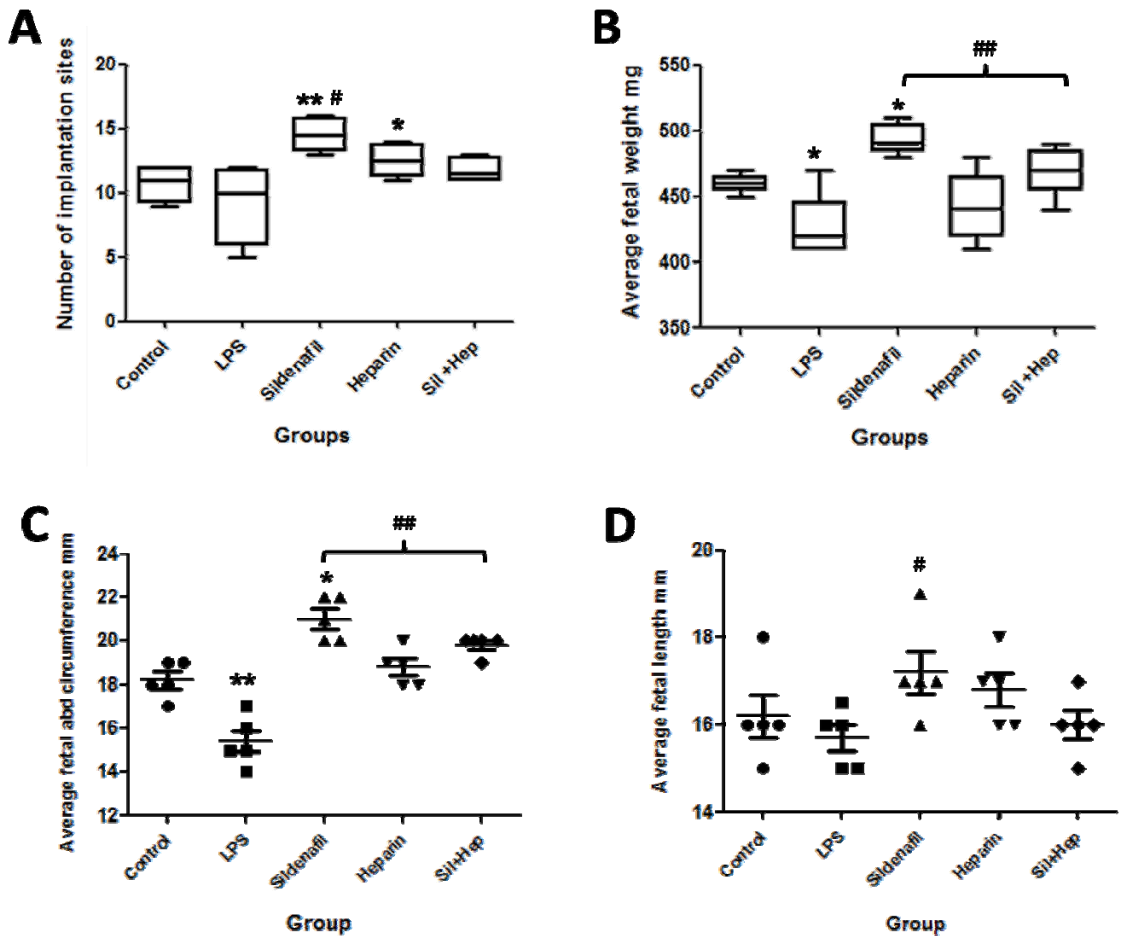
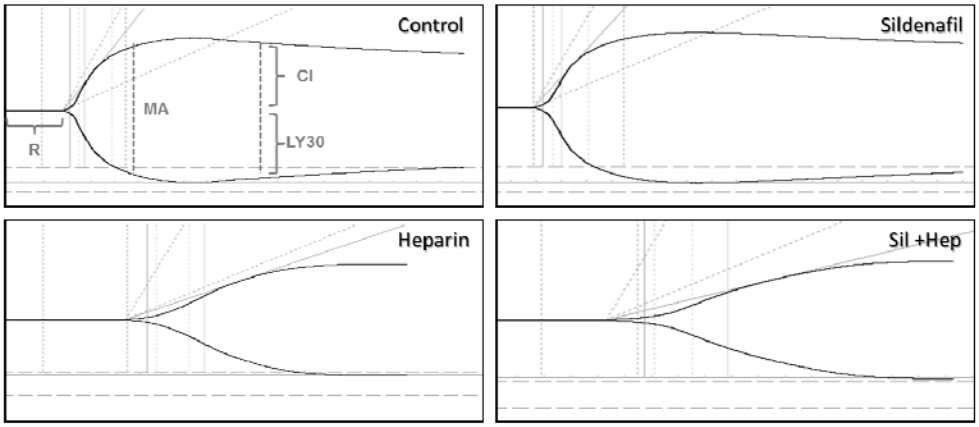


Figure 2: **A:** Results from the animal model of RPL. Mean of number of implantation sites divided by female and by group. Statistical difference was found between control and Sil+Hep group. **B:** Average fetal weight divided by group. All treated groups showed an increased fetal weight compared to the LPS group, sildenafil group was the only that presented significant higher fetal weight compared to the control group. **C:** Average fetal circumference represents the nutrition status of the fetus. The LPS group showed a significant reduction in this measurement. All the treatments showed higher average than LPS group, only the Sildenafil group presented a significant increase compared to the Control group. **D:** Average fetal length represents the development status

of the fetus. The LPS group showed a decreased length. Only the Sildenafil group presented a significant increase compared to the Control group. Data expressed as ± S.D of mean. *p<0,05, **p<0,01 compared to the LPS group, $^{\#}$p<0,05, $^{\#\#}$p<0,01 compared to the control group.

A

Human protocol - *In vitro* study



B

n = 6	R (min)	MA (mm)	LY30 (%)	CI
Control	10.47	62.84	1.74	1.53
Sildenafil	9.59	61.71	1.35	1.37
Heparin	36.10$^{\#\#}$	45.87$^{\#\#}$	00.00$^{\#\#}$	-6.55$^{\#\#}$
Sil + Hep	41.72$^{\#\#}$	39.06$^{\#\#}$	00.00$^{\#\#}$	-8.78$^{\#\#}$

C

Animal protocol - *In vivo* study

n = 4	R (min)	MA (mm)	LY30 (%)	CI
Control	7.80	60.00	0.10	2.10
LPS	5.53$^{\#}$	58.01	4.80$^{\#\#}$	2.66
Sildenafil	5.75	40.03*	0.07**	1.44*
Heparin	12.33**	54.35	0.43**	00.00**
Sil + Hep	8.03*	64.05*	00.00**	2.90

Figure 3: A: Human blood analysis. Representative TEG traces for all studied groups. The angle of the trace and the pattern shows that the Sildenafil group presented a more similar pattern to the control group. The Heparin pre-treated blood showed a hypocoagulopathy-like trace. **B:** Thrombelastography parameters within the table containing R: reaction time, MA: maximum amplitude LY30% (percent lysis at 30 min after MA) and CI: (coagulation index). There was no significant difference found in the Sildenafil group. Only Heparin and Sil+Hep combined groups had significantly higher R and MA and significantly lower LY30 and CI. **C:** Mouse blood analysis representative TEG parameters for all the studied groups. LPS group showed evidence of hypercoagulability while heparin group showed hypocoagulability patterns. Sildenfil and heparin combined group showed more closely similar parameters compared to the control group. Data expressed as ± S.D of mean. *p<0,05, **p<0,01 compared to the LPS group, $^{\#}$p<0,05, $^{\#\#}$p<0,01 compared to the control group.

Figure 4: MULTIPLEX RESULTS

Figure 5: **A**: Immunohistochemical localization of eNOS in placental tissue. The Control group exhibited basal expression of this enzyme, and LPS did not induce an increase of eNOS staining as well as the treatments with Sildenafil and Heparin alone. The Sil+Hep group expressed a higher expression and consecutively staining for eNOS Bar, 20 μm. **B**: Immunohistochemical localization of PGF in the placental tissue. The trophoblast cells stained to PGF appeared in the Control group, LPS group showed a reduction of expression. The Sildenafil group showed comparable numbers of stained cells as in the Control group. Heparin Group and the Sil+Hep were superior compared to the LPS group, however still numerically lower than Control. 20 μm. Graphs show the quantitative densitometry analysis of eNOS immunohistochemistry (GIMP2 analyzed) which was performed to confirm the qualitative results and PGF counting cells results. **C:** The groups were coded as: A: Control, B: LPS, C: Sildenafil, D: Heparin, E: Sil+Hep. Data expressed as ± S.D of mean. *p<0,05, **p<0,01 compared to the LPS group, $^{\#}$p<0,05, $^{\#\#}$p<0,01 compared to the control group.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as affecting the impartiality of the research reported.

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7. CONCLUSÕES GERAIS

No presente estudo demostramos a eficácia do tratameto com Sildenafil, sozinho ou em associação com Heparina de baixo peso melecular, na manutenção da saúde fetal assim como na proteção placentária contra injúria causada pelo processo abortivo. Sildenafil atuou como anti-inflamatório inibindo a expressão de citocinas importantes como o TNF-α e IL-1β através da via do NFκB. Paralelamente o fator de crescimento PGF foi visto como essencial para o desenvolvimento vascular fetal, principalmente em tecido cerebral. Medicamentos que influenciem na síntese de PGF podem representar uma opção terapêutica para PE.

O uso de modelos animais para a investigação pré-clínica de patologias diversas é relevante. O benefício apresentado no presente estudo pelo tratamento com Sildenafil pôde ser potencializado pela associação desse fármaco com a Dalteparina, visto que ambos os mecanismos de ação dessas substâncias seriam sinérgicos e principalmente paralelos, utilizando inclusive vias de sinalização diferentes. Nossos resultados suportam, depois de estudos clínicos bem desenhados, o uso do Sildenafil como terapia profilática para tratamento de aborto e injuria fetal associado à pré-eclâmpsia ou não.

8. OUTRAS ATIVIDADES

8.1 Doutorado sanduiche – CANANDÁ

- Bolsa: aprovada pelo CNPq em 2013 para 12 meses.
- Perído de vigência: 01/02/2014 a 01/02/2015
- Orientadora do exterior: Dr Anne Croy
- Instutuição: Queens University – Kingston

B. Anne Croy, DVM, PhD
Professor and Tier 1
Canada Research Chair in Reproduction
Development and Sexual Function

DEPARTMENT OF BIOMEDICAL AND
MOLECULAR SCIENCES

June 2, 2015

Evaluation of CNPq□sponsored, PhD Visitorship of Rayana Luna Leal

To Whom It May Concern at CNPq:

Due to my ongoing difficulties with the evaluation website, I am reporting on the 1 year visit of this scholar to my laboratory by letter.

Rayana Leal is an outstanding, highly motivated and technically skilled PhD candidate. Rayana joined my laboratory on February 1, 2014, supported by CNPq. Her visit was completed on January 21, 2015 when she returned to her home laboratory at Universidade Federal de Pernambuco (UFPE).

My laboratory studies pregnancy in mice. During her time here, Rayana was a fully active participant in our research. She completed all the required training programs of our institution in biosafety, hazardous materials handling and animal care. She conducted research that supported not only her work but collaborative studies with other graduate students. The techniques that she employed were mouse breeding and handling, fetal dissections in mice, immunohistochemistry, electron microscopy and molecular biology, including realtime PCR quantification. Rayana also audited a graduate course on Pregnancy and attended the 60hr Human Placenta Summer Workshop. The latter is a wetlab course for 16 research investigators who along with the instructors come from around the world. She developed her professional network and a broader appreciation for the human applications of her research.

On a weekly basis, Rayana attended and presented at a reproductive immunology journal club and at weekly laboratory research meetings. I worked with her to revise a manuscript on her Brazilian research that is under review as a revised submission in the journal “Placenta”. This revised format was also presented at several regional and local research meetings. These

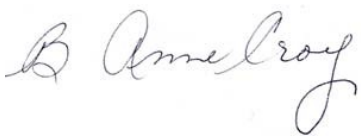
included the Southern Ontario Reproductive Biology, J. A. Low Obstetric Research and Faculty of Health Sciences Research Days. In collaboration with Drs. Maha Othman and Rob Siemans, we were able to support extension of Rayana's Brazil-based work to a new set of pregnant mice receiving endotoxin plus her drug of interest. This extension used equipment to measure blood clot formation that was available here by not in Rayana's home laboratory. The data generated resulted in an abstract to an international thrombosis meeting and a manuscript ready for submission.

The main project Rayana worked on in my laboratory related to deficiency of the molecule placenta growth factor (PGF). This molecule is often deficient in women who progress in pregnancy to the complication of preeclampsia. Children from such pregnancies have mild cognitive impairments and we are seeking the structural and molecular basis for this. Rayana conducted RNA and protein-based, time course studies on mouse fetal brains across the 2nd half of pregnancy. She taught herself fetal brain structure and development from mouse atlases and worked very independently. She wrote the first draft of a manuscript in English that describes this work and is the first author of the paper that includes authorship with other students in my laboratory. This manuscript is also in revision for the journal Cardiovascular Research.

Rayana also participated in the social activities of my laboratory and I tried to ensure that she experienced special features of Canada such as ice skating and seeing Maple Syrup being made.

Rayana is very organized and excelled in the amount of work she completed and in the thoroughness of her work. She is committed to research and reasons logically and well. I anticipate she will have a successful, long term career in research. It was a pleasure to work with her during her year in Canada. Thank you for your support that enabled me to work with this talented research trainee.

Sincerely



Anne Croy, DVM, PhD, Professor,
Director, Queen's University
Group for Research in The
Reproductive and Developmental
Origins of Health, Disability and
Disease,
Fellow, Canadian Academy of Health Sciences

8.2 cursos palestras e certificações

- *Pregnancy course*: participação como ouvinte na disciplina oferecido a Pós-graduação em Ciências Biomédicas na Queens Univesity, (60h).

- *Placenta Summer Workshop:* participação como aluna do curso teórico-prático sobre placenta humana na Queens University, Kingston, ON, Canada (60h).

- *Animal handling*: curso de manipulação de animais de laboratório, sendo dada a certificação internacional pela Queens University animal facility ao final do curso (20h).

- Organização de Congresso internacional da Sociedade Americana da imunologia da Reprodução (ASRI) em Kingston, ON, Canadá.

- Palestrante convidada: FAFIRE no III Encontro de Biologia da FAFIRE, Recife, PE, Brasil: Título: Viagra para infertilidade feminina?

- Colaboradora: projeto de pesquisa do estudante visitante Kasra Khalaj da Queens University, no laboratório de Ultraestrutura, Recife, PE, Brasil.

- Aceite: Curso de Princípios e Prática da Pesquisa Clínica da Escola de Sasúde Publica da Faculdade de medicina de Harvard, Boston, MA, Estados Unidos (9 meses *on-line based course*).

9. ANEXOS

9.1 Artigos em coautoria durante o período do doutorado

Brain Res. 2015 Nov 19;1627:119-33. doi: 10.1016/j.brainres.2015.09.008. Epub 2015 Sep 25.

Involvement of AMPK, IKβα-NFκB and eNOS in the sildenafil anti-inflammatory mechanism in a demyelination model.

Nunes AK[1], Rapôso C[2], Rocha SW[3], Barbosa KP[4], Luna RL[4], da Cruz-Höfling MA[5], Peixoto CA[4].

Author information

Abstract

Sildenafil (Viagra®) has recently been found to have a neuroprotective effect, which occurs through the inhibition of inflammation and demyelination in the cerebellum. However, the mechanism of action of sildenafil remains unknown. AMPK, the regulatory protein of the lipid and glucose metabolism, plays a protective role by activating the eNOS enzyme. The production of a nanomolar concentration of NO by eNOS has an anti-inflammatory effect through the cGMP signaling pathway and plays an important role in the regulation of the nuclear transcription factor (NFkB), preventing the expression of inflammatory genes. The present study investigated whether AMPK-eNOS-NO-cGMP-IKβα-NFkB is involved in the mechanism of action of sildenafil in a cuprizone-demyelination model. Neuroinflammation and demyelination induced by cuprizone in rodents have been widely used as a model of MS. In the present study, five male C57BL/6 mice (7-10 weeks old) were used. Over a four week period, the groups received: cuprizone (CPZ) 0.2% mixed in feed; CPZ in the diet, combined with the administration of sildenafil (Viagra®, Pfizer, 25mg/kg) orally in drinking water, starting concurrently (sild-T0) or 15 days (sild-T15) after the start of CPZ. Control animals received pure food and water. The cerebella of the mice were dissected and processed for immunohistochemistry, immunofluorescence (frozen), western blotting and dosage of cytokines (Elisa). CPZ induced an increase in the expression of GFAP, IL-1β TNF-α, total NFkB and inactive AMPK, and prompt microglia activation. CPZ also induced a reduction of IKβα. The administration of sildenafil reduced the expression of the pro-inflammatory cytokines IL-1β and TNF-α and increased the expression of the anti-inflammatory cytokine IL-10. In addition, the administration of sildenafil reduced expression of GFAP, NFkB, inactive AMPK and iNOS, and increased IKβα. Interestingly, sildenafil also reduced levels of NGF. In general, the sild-T0 group was more effective than sild-T15 in improving clinical status and promoting the control of neuroinflammation. The present study offers evidence that sildenafil has anti-inflammatory and neuroprotective effects, which are probably achieved through modulation of AMPK-IKβα-NFκB signaling. In addition, eNOS may play a role in the sildenafil neuroprotective mechanism, contributing to the activation of AMPK. However, other pathways such as MAPK-NFkB and the downstream proteins AMPK (AMPK-SIRT1-NFκB) should also be further investigated. An understanding of these mechanisms of action is critical for the clinical use of sildenafil to control neuroinflammation in neurodegenerative diseases such as MS.

KEYWORDS: AMPK; Cuprizone; Demyelination; NFkB; Neuroinflammation; Sildenafil

PMID: 26404052 [PubMed - in process]

Publication Types

Biol Reprod. 2015 Feb;92(2):44. doi: 10.1095/biolreprod.114.124677. Epub 2014 Dec 23.

Placental growth factor influences maternal cardiovascular adaptation to pregnancy in mice.

Aasa KL[1], Zavan B[2], Luna RL[3], Wong PG[4], Ventura NM[4], Tse MY[4], Carmeliet P[5], Adams MA[4], Pang SC[4], Croy BA[4].

Author information

Abstract

In healthy human pregnancies, placental growth factor (PGF) concentrations rise in maternal plasma during early gestation, peak over Weeks 26-30, then decline. Because PGF in nongravid subjects participates in protection against and recovery from cardiac pathologies, we asked if PGF contributes to pregnancy-induced maternal cardiovascular adaptations. Cardiovascular function and structure were evaluated in virgin, pregnant, and postpartum C56BL/6-Pgf(-) (/) (-) (Pgf(-) (/) (-)) and C57BL/6-Pgf(+/+) (B6) mice using plethysmography, ultrasound, quantitative PCR, and cardiac and renal histology. Pgf(-/-) females had higher systolic blood pressure in early and late pregnancy but an extended, abnormal midpregnancy interval of depressed systolic pressure. Pgf(-/-) cardiac output was lower than gestation day (gd)-matched B6 after midpregnancy. While Pgf(-) (/) (-) left ventricular mass was greater than B6, only B6 showed the expected gestational gain in left ventricular mass. Expression of vasoactive genes in the left ventricle differed at gd8 with elevated Nos expression in Pgf(-) (/) (-) but not at gd14. By gd16, Pgf(-) (/) (-) kidneys were hypertrophic and had glomerular pathology. This study documents for the first time that PGF is associated with the systemic maternal cardiovascular adaptations to pregnancy.

KEYWORDS: cardiac remodeling; cardiovascular risk; fetal growth; placenta; ultrasound

PMID: 25537372 [PubMed - indexed for MEDLINE] PMCID: PMC4490891 **Free PMC Article**

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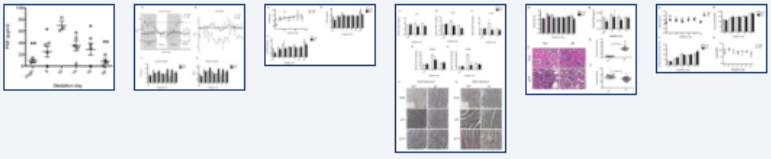

Publication Types, MeSH Terms, Substances, Grant Support

Toxicol Appl Pharmacol. 2014 Oct 1;280(1):159-68. doi: 10.1016/j.taap.2014.05.015. Epub 2014 Jun 8.

Effect of the combination of metformin hydrochloride and melatonin on oxidative stress before and during pregnancy, and biochemical and histopathological analysis of the livers of rats after treatment for polycystic ovary syndrome.

Lemos AJ[1], Peixoto CA[2], Teixeira AA[3], Luna RL[2], Rocha SW[2], Santos HM[3], Silva AK[2], Nunes AK[2], Wanderley-Teixeira V[4].

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Abstract

The aim of the present study was to analyze the effect of a combination of metformin hydrochloride and melatonin on oxidative stress together with a biochemical and histopathological analysis of the livers of Wistar rats induced with PCOS. The results indicated that a combination of the drugs was more effective in the reduction of plasmatic levels of liver enzyme alanine aminotransferase, nitric oxide and total glutathione, and decreased the inflammatory response and histopathological damage, producing results that were significantly similar to animals from the control group. A mixture of the drugs produced more effective results against liver toxicity caused by PCOS, encouraging the normalization of biochemical parameters. During pregnancy, there was reduced oxidative stress compared to monotherapeutic use of these drugs. Interestingly, the combination of the drugs caused a physiological reaction similar to responses identified in healthy rats without induction of the PCOS control group. However, the clinical and physiological effectiveness of the combination should be further explored, especially with respect to the possible side effects on offspring.

KEYWORDS: Liver; Melatonin; Metformin hydrochloride; Polycystic ovary syndrome; Pregnancy; Rats

PMID: 24918699 [PubMed - indexed for MEDLINE]

Brain Res Bull. 2014 May;104:60-73. doi: 10.1016/j.brainresbull.2014.04.002. Epub 2014 Apr 13.

Role of iNOS-NO-cGMP signaling in modulation of inflammatory and myelination processes.

Rapôso C[1], Luna RL[2], Nunes AK[3], Thomé R[4], Peixoto CA[5].

Author information

Abstract

Nitric oxide (NO) is the main activator of the soluble guanylate cyclase (sGC)-guanosine 3'5' cyclic monophosphate (cGMP) pathway. The Open/close is regulated by phosphodiesterases (PDEs), which break down cGMP. It has been reported that levels of NO in the central nervous system (CNS) can greatly increase during demyelination and/or neuroinflammation. Controversially, in demyelination models, mice without iNOS may develop more severe cases of disease. Furthermore, cGMP accumulation caused by PDE inhibitors has an anti-inflammatory/neuroprotective effect in MS-models. The role of the NO-cGMP pathway in the nervous tissue is, therefore, complex and not fully understood. The aim of the present study was to contribute to existing knowledge of the role of this pathway in the CNS. Wild type (WT - C57BL/6) and iNOS(-/-) animals were treated with sildenafil (25mg/kg) for 8 weeks. Control animals were not treated. VCAM and ICAM (adhesion proteins), GFAP and Iba-1 (astrocyte and microglia markers, respectively), PKG (cGMP-dependent protein kinase), sGC, eNOS (constitutive endothelial NO sinthase) and GSTpi (a marker of mature oligodendrocytes) were evaluated in the cerebellum using immunohistochemistry or western blotting. Myelin was assessed by luxol fast blue staining and electron transmission microscopy. Treatment with sildenafil reduced ICAM and VCAM levels (anti-inflammatory effect) and increased GFAP and Iba-1 expression (clearance phenotype) in WT animals. The expression of VCAM, ICAM, GFAP, PKG and sGC was lower in iNOS(-/-) mice than in WT control animals. The treatment of iNOS(-/-) animals with sildenafil resulted in an increase of all proteins (pro-inflammatory effect). There was overexpression of eNOS in untreated iNOS(-/-) mice. The myelin structure of iNOS(-/-) animals was damaged in comparison with WT control. Sildenafil increased GSTpi and resulted in an improved myelin structure in iNOS(-/-) mice. In conclusion, NO-cGMP signaling plays a role in the regulation of inflammation and myelination processes. The accumulation of cGMP produced opposite effects in WT and iNOS(-/-) mice. This can be explained by the overexpression of eNOS in iNOS(-/-) mice, unbalancing cGMP signaling, or cGMP has a dual role in inflammation. Drugs that modulate the NO-sGC-cGMP pathway may be clinically beneficial in the treatment of neuroinflammatory/demyelinating disorders, but further studies of the regulation of this pathway are required.

KEYWORDS: Adhesion molecules; Astrocytes; Cerebellum; Microglia; Oligodendocytes; Sildenafil

PMID: 24727400 [PubMed - indexed for MEDLINE]

Mediators Inflamm. 2013;2013:321460. doi: 10.1155/2013/321460. Epub 2013 Jul 22.

Sildenafil (Viagra) protective effects on neuroinflammation: the role of iNOS/NO system in an inflammatory demyelination model.

Raposo C[1], Nunes AK, Luna RL, Araújo SM, da Cruz-Höfling MA, Peixoto CA.

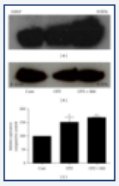
Author information

[1]Departamento de Histologia e Embriologia, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Rua Monteiro Lobato 255, 13083-862 Campinas, SP, Brazil. cataraposa@gmail.com

Abstract

We recently demonstrated that sildenafil reduces the expression of cytokines, COX-2, and GFAP in a demyelinating model induced in wild-type (WT) mice. Herein, the understandings of the neuroprotective effect of sildenafil and the mediation of iNOS/NO system on inflammatory demyelination induced by cuprizone were investigated. The cerebella of iNOS(-/-) mice were examined after four weeks of treatment with cuprizone alone or combined with sildenafil. Cuprizone increased GFAP, Iba-1, TNF- α , COX-2, IL-1 β , and IFN- γ expression, decreased expression of glutathione S-transferase pi (GSTpi), and damaged myelin in iNOS(-/-) mice. Sildenafil reduced Iba-1, IFN- γ , and IL-1 β levels but had no effect on the expression of GFAP, TNF- α , and COX-2 compared to the cuprizone group. Sildenafil elevated GSTpi levels and improved the myelin structure/ultrastructure. iNOS(-/-) mice suffered from severe inflammation following treatment with cuprizone, while WT mice had milder inflammation, as found in the previous study. It is possible that inflammatory regulation through iNOS-feedback is absent in iNOS(-/-) mice, making them more susceptible to inflammation. Sildenafil has at least a partial anti-inflammatory effect through iNOS inhibition, as its effect on iNOS(-/-) mice was limited. Further studies are required to explain the underlying mechanism of the sildenafil effects.

PMID: 23970812 [PubMed - Indexed for MEDLINE] PMCID: PMC3736464 Free PMC Article



9.1 Certificados das aprovações em comissões de ética

Protocol Detail Report

Printed By: Kaur, Harmanpreet
2/16/2016 10:22:50 AM

Report Comments

Protocol Information

Version # 1

Reference Number	1503		
Protocol Number	2014-1503		
Protocol Type:	Original		
Principal Investigator:	Othman, Maha	**Approval Date:**	9/19/2014
Submittal Date:	9/12/2014	**Effective Date:**	9/19/2014
Author:	Kaur, Harmanpreet	**Renewal Date:**	9/19/2015
Status:	Approved	**Next Review Date:**	9/19/2015
Inactive Date:	10/29/2014	**Expiration Date:**	9/19/2018

Administrative Data 1

Reference Number 1.1

The Reference Number is system generated.
1503

Principal Investigator 1.2

Select the Principal Investigator.

Othman, Maha	othman@queensu.ca	

Department 1.3

Indicate the PI's home department within the University.

Biomedical and Molecular Sciences

Created By 1.4

This field is automatically populated by the person who initially logs in to create the protocol request. This person will automatically have rights to access, edit, renew and amend this protocol.

Othman, Maha	othman@queensu.ca	

Author 1.5

Select the name of an alternate associate who will be considered one of the core team and therefore will be able to create, access, and edit any protocols they have been named against. This person will automatically be authorized to order animals. An appropriate designate example is a lab manager or senior technician.

Kaur, Harmanpreet	hk846@queensu.ca	

Queen's University

QUEEN'S UNIVERSITY HEALTH SCIENCES & AFFILIATED TEACHING HOSPITALS RESEARCH ETHICS BOARD-DELEGATED REVIEW
December 17, 2012

Dr. Maha Ahmed Ali Othman
Department of Biomedical and Molecular Sciences
Queen's University

Dear Dr. Othman
Study Title: DBMS-019-12 Platelet-type Von Willebrand disease: Novel studies in the PT-VWD mouse model
File # 6007631

I am writing to acknowledge receipt of your recent ethics submission. We have examined the protocol and the information/consent form for your project (as stated above) and consider it to be ethically acceptable. This approval is valid for one year from the date of the Chair's signature below. This approval will be reported to the Research Ethics Board. Please attend carefully to the following listing of ethics requirements you must fulfill over the course of your study:

Reporting of Amendments: If there are any changes to your study (e.g. consent, protocol, study procedures, etc.), you must submit an amendment to the Research Ethics Board for approval. Please use event form: HSREB Multi-Use Amendment/Full Board Renewal Form associated with your post review file **# 6007631** in your Researcher Portal (https://eservices.queensu.ca/romeo_researcher/)

Reporting of Serious Adverse Events: Any unexpected serious adverse event occurring locally must be reported within 2 working days or earlier if required by the study sponsor. All other serious adverse events must be reported within 15 days after becoming aware of the information. Serious Adverse Event forms are located with your post-review file **6007631** in your Researcher Portal (https://eservices.queensu.ca/romeo_researcher/)

Reporting of Complaints: Any complaints made by participants or persons acting on behalf of participants must be reported to the Research Ethics Board within 7 days of becoming aware of the complaint. Note: All documents supplied to participants must have the contact information for the Research Ethics Board.

Annual Renewal: Prior to the expiration of your approval (which is one year from the date of the Chair's signature below), you will be reminded to submit your renewal form along with any new changes or amendments you wish to make to your study. If there have been no major changes to your protocol, your approval may be renewed for another year.

Yours sincerely,

Albert L. Clark

Chair, Research Ethics Board
December 17, 2012

FIOCRUZ
Fundação Oswaldo Cruz
Centro de Pesquisas Aggeu Magalhães

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificado de Aprovação

Certificamos que o projeto intitulado: **Avaliação dos efeitos do sildenafil sobre a placenta e incidência de células natural killer uterinas em modelo de aborto induzido por lipopolissacarideos em camundongos,** protocolado sob nº 71/2014 pelo (a) pesquisador (a) Christina Alves Peixoto. Está de acordo com a Lei 11.794/2008 e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS do Centro de Pesquisas Aggeu Magalhães/Fundação Oswaldo Cruz (CEUA/CPqAM) em 27/05/2015. Na presente versão, este projeto está licenciado e tem validade até maio de 2017.

Quantitativo de Animais Aprovados	
Espécie	Nº de Animais
Camundongos Mus músculos Albino Swiss macho	240
Camundongos Mus músculos Albino Swiss fêmea	480
Total	720

We certify that project entitled **Avaliação dos efeitos do sildenafil sobre a placenta e incidência de células natural killer uterinas em modelo de aborto induzido por lipopolissacarideos em camundongos.** Protocol nº 71/2014, coordinated by Christina Alves Peixoto. Is according to the ethical principles in animal research adopted by the Brazilian law 11.794/2008 and so was approved by the Ethical Committee for Animal Research of the Centro de Pesquisas Aggeu Magalhães/Fundação Oswaldo Cruz on May, 27, 2015. In present verson this project is licensed and valid until May, 2017.

Recife (PE, Brazil) november, 05, 2015.

Drª Sheilla Andrade de Oliveira
Coordenadora CEUA/CPqAM

Drª Sheilla Andrade de Oliveira
Coordenadora da Comissão de Ética no Uso de Animais – CEUA
Mat. SIAPE 1554975
e-mail: sheilla@cpqam.fiocruz.br
CPqAM/Fiocruz

Av. Professor Moraes Rego, s/n - Cidade Universitária – Campus da UFPE
Recife - PE - CEP: 50.670-420
Telefone: (81) 2101-2500/2101-2600 Fax: (81) 3453-1911
www.cpqam.fiocruz.br

9.2 Participação e apresentações em congressos

- Apresentação Oral no J.A. Low Research Day Program, Kingston ON, Canadá.

J.A. Low Research Day Program

8:00 am *Poster and Oral Presentation Set-up (Coffee and Continental Breakfast)*

8:40 am Opening Remarks (Robert Reid, Research Director)

Morning Session Chair: Dr. Graeme Smith

8:45 am **(O1) Malia Murphy (PhD. Candidate),** Graeme N. Smith,
Pre-eclampsia is associated with early postpartum endothelial dysfunction and elevated cardiovascular risk.

9:00 am **(O2) Carolina C. Venditti (Ph.D. Candidate),** Richard Casselman, Iain Young, S. Ananth Karumanchi, Graeme N. Smith
Maternal carbon monoxide exposure reduces hypertension and proteinuria in a mouse model of preeclampsia.

9:15 am **(O3) Matthew T. Rätsep (Ph.D. Candidate),** Bruno Zavan, Nicki Peterson, Leandra Tolusso, Vanessa Kay, Nicole Ventura, Stephen C. Pang, Albert Jin, Michael A. Adams, B. Anne Croy
The Role of Placental Growth Factor in Regulating Fetal Brain Vascular.

9:30 am **(O4) Rayana Luna (Ph.D. Candidate),** Anne Croy, Christina Peixoto
Impact of Sildenafil (Viagra®) on fetal survival and inflammatory responses in LPS-challenged pregnant mice.

9:45 am **(O5) Soo Hyun Ahn (Ph.D. Candidate),** Andrew K. Edwards, Diane S. Nakamura, Conrad Reifel, Bruce A. Lessey, Chandrakant Tayade
Expression of Interleukin 17A in plasma and endometriotic lesions from women with endometriosis and post laparoscopic removal of endometriotic lesions.

10:00 am **(O6) Mallikarjun Bidarimath (Ph.D. Candidate),** Andrew K. Edwards, Jocelyn M. Wessels, Kasra Khalaj, Rami T Kridli, Chandrakant Tayade
Distinct microRNA and their putative target mRNA expression in endometrial lymphocytes, endometrium and trophoblast during spontaneous porcine fetal loss.

10:15 am *Health Break & Poster Viewing (30 minutes)*

10:45 am **(O7) Jeff Emack (Meds 2015),** Marie-Andree Harvey
Study Proposal: Does a Subsequent Delivery Following an Obstetric Anal Sphincter Injury Affect the Rate of Anal Incontinence and Negatively Impact Quality of Life?

11:00 am **(O8) Michael Chaikof (Meds 2015); Ashley Waddington, Robert L. Reid**
Identifying and Improving Knowledge Gaps about Emergency Contraception in Front-Line Health Care Providers: A Pilot Study.

- Apresentação oral no Congresso da SORB 2015, Kingston, ON, Canadá.

Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, ON; [2]Department of Obstetrics and Gynecology, Hadassah-Hebrew University Medical Center, Mt. Scopus, Jerusalem, Israel; [3]Departments of Physiology and [4]Obstetrics and Gynecology, Faculty of Medicine, University of Toronto, Toronto, ON; [5]Maternal and Fetal Health Research Centre, St. Mary's Hospital, The University of Manchester, Manchester, UK; [6]Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON.

1:45-2:00 PM **The Anti-inflammatory Effect of Sildenafil (Viagra®) on Fetal Survival and Placental Protection in LPS-challenged Pregnant Mice**
Rayana Luna[1,2], A. Croy[1], C. Peixoto[2], [1]Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON; [2]Ultrastructure Laboratory – Aggeu Magalhães Research Center – FIOCRUZ, Brazil.

ORAL SESSION IV: *Administrative Building Boardroom*
Chair: Dr. Laura Graham, University of Guelph

1:00-1:15 PM **A Study of the Relationships among Luteal Ultrasonographic Anatomy, Blood Flow and Circulating Concentrations of Progestins in Asian Elephants (Elephas maximus)**
Stephan Botha[1,2], C. Niemuller[3], E. Delitala[1], K. Harper[1], R. Rebus[1], B. Richardson[1], A. Shoji[1], P. M. Bartlewski[1], [1]Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, ON; [2]African Lion Safari and Game Farm Ltd., Flamborough, ON; [3]Kingfisher International Inc., Stouffville, ON.

1:15-1:30 PM **Successful Cryopreservation of Asian Elephant Semen Using Simple, Inexpensive Techniques**
Danielle Arnold[1], C. Gray[2], T. L. Roth[3] and L. H. Graham[1], [1]Department of Animal and Poultry Science, University of Guelph, Guelph, ON; [2]African Lion Safari, Cambridge, ON; [3]Center for Research in Endangered Wildlife, Cincinnati Zoo, Cincinnati, Ohio, USA.

1:30-1:45 PM **Hair Reproductive Hormones as a Technique to Assess Reproduction in Canada Lynx (*Lynx canadensis*) by Enzyme Immunoassay**
Christine V. Terwissen[1], D. L. Murray[1], G. F. Mastromonaco[2], [1]Department of Biology, Trent University, Peterborough, ON; [2]Reproductive Physiology, Toronto Zoo, Toronto, ON.

1:45-2:00 PM **Six New Cases of Chromosome Rearrangements in the Canadian and Vietnamese Pig Populations**
T. Anh Quach, T. Revay, M. Macedo, W. A. King, Department of Biomedical Sciences, University of Guelph, Guelph, ON.

CONCURRENT SESSIONS B:

ORAL SESSION V: *Administrative Building Atrium*
Chair: Dr. Jonathan LaMarre, University of Guelph

2:00-2:15 PM **Maternal High Fat Nutrition Increases Primordial Follicle Number in Neonates and Follicle Atresia in adult Offspring**

- Apresentação em pôster no HRST 2014, Kingston, ON, Canadá

38 ULTRASTRUCTURAL ANALYSIS OF PLACENTAL TISSUE AFTER SILDENAFIL AND HEPARIN TREATMENT IN A MODEL OF MOUSE ABORTION INDUCED BY LPS. Rayana Luna[1,2], Anne Croy[1], Christina Peixoto[2]. [1]Department of Biomedical and Molecular Sciences, Queen's University [2]Ultrastructure Laboratory, Aggeu Magalhaes Research Center, FIOCRUZ, Brazil.

Miscarriage is a thrombotic and inflammatory process. Mechanisms behind the gestational complications leading to fetal loss are not yet fully understood. Sildenafil (Viagra®) is a vasodilator that is used to treat fetal growth restriction (FGR). This drug is a phosphodiesterase type 5 (PDE5) inhibitor that increases the intracellular level of cGMP. The objective of the present study is to investigate the ultrastructural effects of Sildenafil alone or in combination with heparin in a model of mouse abortion induced by lipopolysaccharides (LPS). On gestation day (gd)0, Sildenafil (50 mg/Kg p.o.), heparin (500 IU/Kgs.c.) or Sildenafil (50 mg/Kgp.o.) + heparin (500 IU/Kgs.c.) were administered. On gd15, LPS (100 µg/Kg i.p) or saline was administered. Histopathology and transmission electronic microscopy analysis was performed. Sildenafil and combined heparin therapy was superior to Sildenafil treatment alone in protecting placental ultrastructure against LPS injury. Analysis of the placenta following exposure to LPS contributes to the understanding of the mechanisms involved in placental damage during miscarriage. Treatment with Sildenafil, and particularly Sildenafil in combination with heparin, maintained the integrity of placental

- Apresentação em pôster da ASRI 2015, Kingston, ON, Canadá

- Apresentação em pôster do aluno de estágio Murillo Tenório na FESBE 2015, São Paulo, SP, Brasil.

REUNIÃO ANUAL

FeSBE 2015 FEDERAÇÃO DE SOCIEDADES DE BIOLOGIA EXPERIMENTAL

Certificamos que

o trabalho 12.026 intitulado SILDENAFIL (VIAGRA®) IMPROVES FETAL HEALTH IN MURINE MODEL OF PREGNANCY LOSS: AN ANTI-INFLAMMATORY AND ANTI-THROMBOTIC APPROACH. Luna RL, Costa MTT, Nunes AKS, Vasconcelos AGO, Oliveira WH, Araujo SMR, Rocha SW, Othman M, Croy BA, Peixoto CA - Ultrastructure Laboratory - Aggeu Magalhães Research Center - FIOCRUZ, Brazil - UFPE Department of Biomedical and Molecular Sciences - Queens, foi apresentado sob a forma de painel na

XXX Reunião Anual da Federação de Sociedades de Biologia Experimental - FeSBE, realizada na Faculdade de Medicina da Universidade de São Paulo em São Paulo - SP, de 09 a 12 de setembro de 2015.

Comissão Organizadora

CNPq MEDICINA FAPESP FAPERJ

- Prêmio Travel Award do SLIMP 2015, Mar del Plata, Argentina