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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

AVALIAÇÃO DOS EFEITOS DO SILDENAFIL SOBRE A PLACENTA EM UM MODELO DE PERDA GESTACIONAL INDUZIDA POR LIPOPOLISSACARÍDEOS EM CAMUNDONGOS

RAYANA LEAL DE ALMEIDA LUNA

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Ciências Biológicas como parte dos requisitos para a obtenção do título de Mestre em Ciências Biológicas, na área de Biotecnologia pela Universidade Federal de Pernambuco.

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(Orientadora)

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“O primeiro passo para a vitória é a vontade de vencer”

(Mahatma Gandhi)

RESUMO

Infertilidade que é um importante problema de saúde pública mundial e é caracterizada pela incapacidade em não poder gerar filhos e está relacionada com vários eventos, como é o caso de eventos de perdas gestacionais recorrentes. Dentro desse âmbito das infertilidades o aborto se apresenta como um fator recorrente e preponderante, o mesmo pode ser por cauã genética, insug=ficiencia hormonal ou morbidades que acomentem a mãe. O aborto além de outras características se apresenta como um processo inflamatório e trombótico que envolve principalmente alta expressão da citocina pró-inflamatória TNF-α., porém os mecanismos relacionados com as complicações gestacionais que levam às perdas fetais ainda não estão completamente entendidos. Estudos anteriores já identificaram a presença da enzima Fosfodiesterase tipo-5 (PDE5) no útero, placenta e tecidos da decídua, por sua vez o Sildenafil (Viagra®) inibe a PDE5 aumentando os níveis de GMPc no meio intracelular . Esse fármaco desempenha um importante papel como vasodilatador, e propriedades anti-inflamatorias tem sido recentemente descobertas, o Sildenafi possui um ótimo nível de segurança e alto perfil de tolerabilidade e não possui atividade teratogênica também. O presente estudo investigou a ação do Sildenafil em um modelo de perda gestacional induzida por Lipopolisacarideos (LPS) em camundongos Albino Swiss, fêmeas, grávidas. Seguindo metodologia já descrita, a aplicação intra-peritoneal do LPS (100µg/Kg) realizada no 15° de gestação e todos os tratamentos foram realizados desde o primeiro dia de gestação. Foi avaliada a ação do Sildenalfil (50mg/Kg/dia) e tratamento associado com Heparina (500UI/Kg/dia). Os resultados demonstraram o efeito benéfico do Sildenafil e em tratamento coadjuvante com Heparina após injúria induzida por LPS, promovendo a manutenção da estrutura celular e molecular da placenta. Assim, após estudos aprofundados bem dirigidos este fármaco poderá representar uma opção terapêutica para perdas gestacionais, principalmente aquelas que possuem um envolvimento inflamatório e trombótico.

Palavras chave: Sildenafil, Inflamação, Placenta, Gravidez.

ABSTRACT

Infertility is a worldwide public health problem characterized by the inability to procreate and is often related to recurring miscarriages. Miscarriage is a thrombotic, inflammatory process involving the excessive production of the cytokine TNF-α. However, the mechanisms related to the gestational complications that lead to fetal loss are not yet fully understood. Type 5 phosphodiesterase (PDE5) is found in the uterus, placenta and decidua. Sildenafil (Viagra®) is a vasodilator that inhibits PDE5 by increasing the level of intracellular cyclic guanosine monophosphate and has recently been discovered to exhibit anti-inflammatory properties. The aim of the present study was to investigate the action of sildenafil in a miscarriage model induced by lipopolysaccharides (LPS) in pregnant female Swiss albino mice. Treatments were initiated on the first day of pregnancy with sildenalfil (50 mg/Kg/day), heparin (500 UI/Kg/day) or sildenalfil + heparin. A group without treatment served as the control. On the 15th day of pregnancy, an intra-peritoneal injection of LPS (100 µg/Kg) was administered (except in the control group). Treatment with sildenafil and sildenalfil + heparin led to the maintenance of the cellular and molecular structure of the placenta following LPS-induced injury. Further studies are needed to confirm the protective effect of sildenalfil from miscarriage, especially in cases with thrombotic, inflammatory involvement.

Keywords: Sildenafil. Inflammation. Placenta. Pregnancy.

LISTA DE IMAGENS E FIGURAS

LISTA DE ABREVIATURAS

AMPc Adenosina Monofosfato Cíclico.

bHCG - Gonadotrofina Coriônica Humana Beta.

eNOS - Óxido nítrico sintase endotelial.

GMPc - Guanosina Monofosfato Cíclico.

FDA – Food and Drugs administration.

Hep – Heparina.

IL – Interleucina.

IL-1β - Interleucina 1β.

iNOS - Óxido nítrico sintase induzível.

LB – Labirinto.

LPS – Lipopolissacarídeo.

MET- Microscopia eletrônica de transmissão.

NF-ĸB - Fator nuclear KB.

NO - Óxido nítrico.

NOS - Óxido nítrico sintase.

PDES- Fosfodiesterases

PDE4 - Enzima fosfodiesterase Tipo 4.

PDE5 - Enzima fosfodiesterase Tipo 5.

PKA - Proteína quinase dependente de AMPc.

PKG - Proteína quinase dependente de GMPc.

RCIU - Restrição de crescimento intra-uterino.

Sil - Sildenafil.

SP – Sponjiotrofoblásto.

TC - Células trofoblásticas.

TM – Trombofilia Materna

TNF-α - Fator de necrose tumoral-α.

SUMÁRIO

1. INTRODUÇÃO

O uso de inibidores da Fosfodiesterase-5 (PDE5) como o Sildenafil é aprovado pela Foods and Drug Administration (FDA) para o tratamento de disfunção erétil e hipertensão pulmonar, porém novas abordagens têm sido propostas para a sua utilização, como mais recentemente para o tratamento de pacientes acometidos com a síndrome cardiovascular de Renold. A ampla distribuição PDE5 por e a disponibilidade de inibidores seletivos para essa enzima facilitou o desenvolvimento de novos estudos para o tratamento de insuficiência cardíaca, depressão e inflamação. Tem sido reportado a ação anti-inflamatória do Sildenafil em modelos de patologias como: Diabetes tipo II, Esclerose múltipla, doenças hepáticas entre outras.

Dez a cada cem mulheres que tentam engravidar tem problemas relacionados à infertilidade e 20 a 30% das mulheres que engravidam tem um ou mais abortos espontâneos. Levando em consideração que as Trombofilias ocupam um importante lugar na incidência dos abortos recorrentes e que esses eventos são um importante problema de saúde pública mundial, é nessessário maior investimento em pesquisas sobre esse tipo de patologia e os mecanismos celulares envolvidos nesse processo. Atualmente esse tratamento é feito com análogos da heparina que influenciam na cascata de coagulação e diminuem o risco trombose, porém mesmo com os esforços médicos ainda há uma grande parcela de mulheres que não podem gerar filhos, dada a limitação da terapêutica atual.

Os processos trobóticos e inflamatórios estão intimamente relacionados durante a gestação e aparecem ligados a casos de abortos recorrentes. Ainda não existe um tratamento eficaz para esses eventos de perdas gestacionais. Portanto, há necessidade de novas propostas farmacológicas que auxiliem na manutenção da gravidez; visto que a PDE5 pode ser encontrada no útero, anexos embrionários e outros tecidos gravídicos. Por sua ação anti-inflamatória e vasodilatora, o Sildenafil pode atenuar os efeitos de um evento trombótico durante a gestação e possivelmente até evitá-lo. Para isso é necessário a elucidação dos mecanismos de ação deste fármaco no durante a gestação. O Sildenafil já está sendo ultilizado atualmete para o tratamento de Restrição de crescimento uterino (RCIU) e poderia ser aplicado a outras patologia que acontecem ndurante o processo gestácional, podendo assim se tornar um futoro potencial terapeutico para esses casos de perdas fetais recorrentes.

2. OBJETIVOS

2.1. Objetivo Geral

Caracterizar o efeito do Sildenafil sobre a morte fetal e manutenção da integridade placentária em modelo de perda gestacional, induzido através da exposição ao LPS em camundongos fêmeas grávidas.

2.2. Objetivos Específicos

- Avaliar a evolução clínica do modelo experimental através da quantificação de morte fetal por aborto;
- Avaliar por Histopatologia a integridade do tecido placentário;
- Avaliar a Ultraestrutura das células: do labirinto, endoteliais placentárias e trofoblásticas gigantes através de Microscopia Eletrônica de Transmissão.
- Avaliar por Imunofluorescência a expressão da proteína de adesão: P-Selectina e da citocina pró-inflamatória IL1-β;
- Avaliar por Imunohistoquímica a expressão da citocina pró-inflamatória TNF-α e das sintases de Óxido Nítrico (eNOS) e (iNOS);
- Quantificar por Western Blotting a expressão das citocinas pró-inflamatórias IL1-β e TNF-α;

3. REVISÃO DA LITERATURA

3.1. Estrutura e fisiologia da placenta

A Placenta é o órgão materno-fetal onde acontecem as trocas de nutrientes e gases e é constituída principalmente de duas faces: uma porção fetal e outra materna. O sangue materno chega ao espaço interviloso vindo das artérias espiraladas do endométrio, que por sua vez é drenado pelas veias endometriais. As numerosas vilosidades coriônicas são banhadas continuamente por sangue materno que possui os materiais nutritivos necessários para o desenvolvimento fetal, assim como, produtos da excreção do feto. A placenta possui três principais funções: metabolismo, transporte de gases e nutrientes e secreção endócrina através da síntese de hormônios como progesterona e principalmente a gonadotropina coriônica humana (hCG). Essas atividades abrangentes são essenciais para a manutenção da gravidez e promoção do desenvolvimento do feto [1]. A placenta tem sua origem fetal originária do saco coriônico e a parte materna derivada do endométrio. O processo de placentação envolve complexas alterações no metabolismo materno, expressão de citocinas, hormônios e uma fina regulação gênica mediada principalmente pelos genes Homeobox.

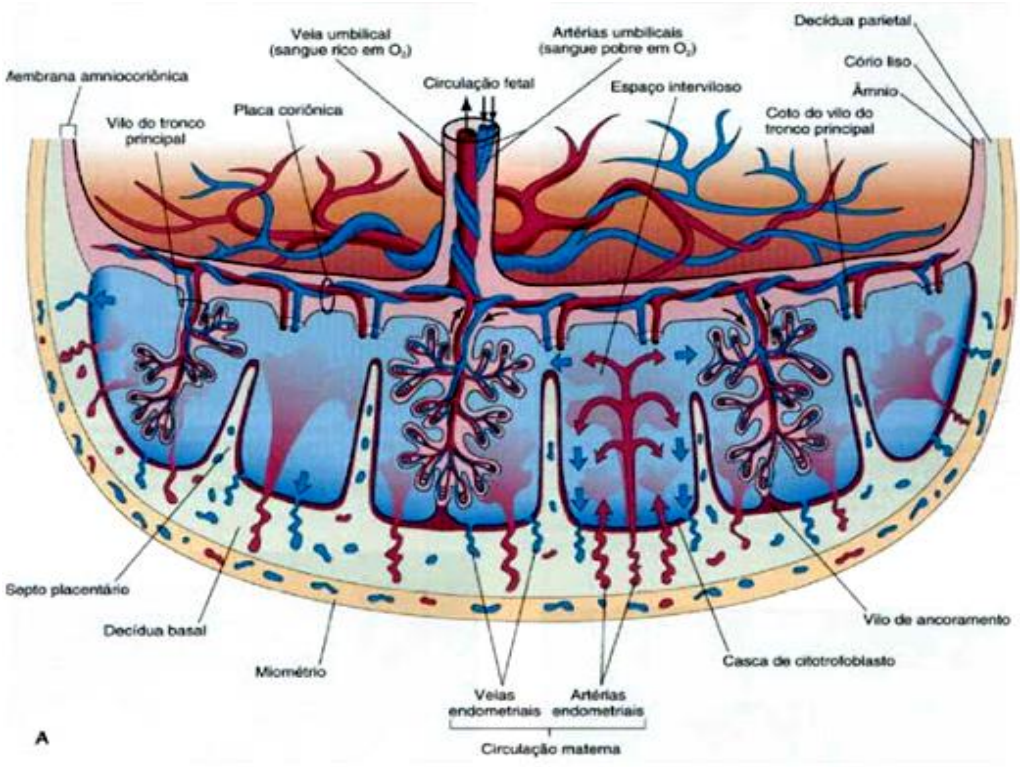

Figura 1: (CALLEN PW. 4ed.)

A evolução das espécies de acordo com sua placentação se desenvolveu a partir da placenta Difusa, Zonal, Cotiledonária e Discal, onde essa última ocorre em Humanos, primatas e roedores nos quais os vasos sanguíneos maternos dilatados desembocam em lacunas [2], localizadas em sua maior parte na região do Labirinto. A formação do labirinto está associada com a remodelação tecidual e diferenciação celular, que irão formar os envelopes perivasculares bilaminares distintos de sinciciotrofoblastos os quais ficarão em contato direto com os as estruturas denominadas vasos fetais [3] [4]. O labirinto placentário é a principal área de intercâmbio materno-fetal e consiste de uma malha de sangue materno delimitada por células trofoblásticas. Ele contém células do trofoblasto, células de origem mesodérmica e do estroma e vasos sangüíneos, que se ramificam para produzir uma grande área de superfície para trocas gasosas e de nutrientes [5]. Na gravidez, nutrientes são transportados ao feto via circulação placentária, sua integridade é essencial para manter o desenvolvimento fetal. Em modelos de perda gestacional têm se observado que uma das regiões que mais sofre com a injúria é a região do labirinto, podendo se tornar alvo para novos estudos que envolvem o processo abortivo [6]. Outras regiões com tipos celulares diferenciados também já foram bastante descritas [7]. Nos roedores, particularmente no camundongo fêmea e na rata, a placenta definitiva é discóide e se estabelece por volta do décimo dia de gestação. Ela possui anatomicamente e fisiologicamente três regiões distintas: o labirinto placentário, que é a maior porção do disco placentário; o espongiotrofoblasto, também chamado de zona juncional ou trofoespôngio, que é formado da parede original do blastocisto e permanece unicelular; e o sinciotrofoblasto, também chamado de camada de células gigantes trofoblásticas, que é formado a partir da penetração das células trofoblásticas no endométrio [8]. O espongiotrofoblasto, camada média, é um compartimento constituído por pelo menos dois subtipos celulares [9]. As células O espongiotrofoblasto atua como um compartimento glandular endócrino, para manter a secreção de progesterona pelo corpo lúteo, além de outras funções. O espongiotrofoblasto e, secundariamente, o sinciotrofoblasto, produzem hormônios luteotróficos e lactogênicos durante a gestação [10]. O sinciotrofoblasto, localizado mais externamente na periferia da unidade fetoplacentária, é constituído por células gigantes trofoblásticas que possuem núcleos volumosos ou mais de um núcleo, formando um sincício que é resultado da fusão das células citotrofoblásticas . As células trofoblásticas gigantes são as primeiras a mediar o processo de implantação e invasão uterina pelo concepto. Mais tarde, elas produzem diversos hormônios e citocinas que regulam o fluxo de sangue materno para o local da implantação [11].

3.2. Mediadores químicos na gravidez

As adaptações anatómicas, fisiológicas e bioquímicas à gravidez são profundas, muitas delas iniciam-se quase logo após a fecundação e prolongam-se por toda a gestação. Durante a a gravidez, o útero sofre uma série de alterações morfofuncionais cujo objetivo é acomodar o concepto em crescimento. Essa ampla reorganização tecidual depende do equilíbrio entre proliferação e morte celular, que desempenha papel crucial para a implantação do embrião e manutenção da gestação [12]. A gravidez é um processo fisiológico caracterizado pela instalação de um enxerto semialogênico existindo complexas alterações no metabolismo materno sendo a maior parte delas ocorridas em resposta a estímulos fisiológicos produzidos pelo feto. Quando há desequilíbrio dos mediadores químicos envolvidos nesse processo, é desencadeado o aborto, que além de outras características se apresenta como um processo inflamatório possivelmente mediado por TNF-α. As moléculas de adesão como a P-Selectinas, tem sido descrita como cruciais para a implantação no embrião no epitélio uterino bem como para a manutenção e desenvolvimento do embrião, essas também parecem estar envolvidas com a migração elucocitária, evento muito importante para a manutenção da gstação saudável [13] [14]. Outro importante mediador químico da gravidez é o óxido nítrico (NO), que relaxa o músculo liso vascular através da ativação da enzima guanilato ciclase, produzindo níveis elevados de cGMP [15]. Várias isoformas de NO sintase já foram identificadas no útero, principalmente a óxido nítrico sintase induzível (iNOS) e a óxido nítrico sintase endotelial (eNOS) [16] Sabe-se que o NO produzido pea iNOS tem características pró-inflamatórias enquanto que o produzido pela eNOS atua como anti-inflamatório provavelmente ativando a via que envolve a proteína quinase depedente de AMP (AMPK). Nesse contexto o citrato de Sildenafil (Viagra), pode ser benéfico possivelmente por aumentar os efeitos vasodilatadores do NO, devido à inibição da degradação do cGMP [17]. Entretanto, os mecanismos pelos quais o Sildenafil atua de forma benéfica na prevenção do aborto por causa inflmatória e trombótica ainda necessitam de esclarecimento.

3.3 Infertilidade e aborto

A Infertilidade pode atingir homens e mulheres e de forma geral se caracteriza por inabilidade de gerar gametas viáveis e consequantemente não pode gerar filhos [18, 19] .

Fatores ambientais sociais e fisiológicos estão intimamente ligados com os diversos tipos de infertilidade. Amplas são as causas e sintomas das infertilidades femininas, além disso, morbidades como a obesidade, doenças metabólicas, pressão alta e idade influenciam diretamente na manutenção de uma gestação. Durante as fases da gravidez que vão da fecundação, passam pela impalntação do blatocisto e pacentação e ainda o desenvolvimento embrionário e fetal podem haver complicações [7]. A intima reação materna com o concepto ainda não está bem entedida, pois envolve muitos fatores que se diferenciam entre as gestações [3]. Quando existem alterações anormais de quaquer natureza durante o processo gestacional, como insuficiencia hormonal, mutações genéticas graves no embrião, doenças pré existentes, incompatibilidade genética materna/paterna, trobofilia, choques mecâncicos e outros é desencadeado o processo que vai culminar na expulsão e morte do feto. Quando há desequilíbrio dos mediadores químicos envolvidos na gestação saudável, é desencadeado o aborto, que além de outras características se apresenta como um processo inflamatório possivelmente mediado por TNF-α [6]. Os mecanismos precisos pelos quais o TNF-α promove a morte fetal ainda não são claros, mas podem envolver insuficiência hormonal lútea, facilitação de aponecrose do trofoblasto levando à ruptura da placenta ou alternativamente, produzindo insuficiência vascular uterina-placentária e, consequentemente um deficit funcional no fluxo sanguíneo para o feto [20, 21]. Vários estudos têm demonstrado que o fator nuclear-Kβ (NF-Kβ) desempenha um papel importante na regulação dos genes responsáveis pela geração de mediadores da resposta inflamatória, tais como TNF-α e IL-1β, desempenhando um papel regulatório no crescimento, diferenciação e ativação de células imunes (SCHINS et al., 2000). Três das patologias mais comuns na gestação de humanos, representadas por retardo do crescimento intra-uterino, aborto espontâneo e pré-eclâmpsia, parecem estar associadas com alterações no desenvolvimento vascular da placenta. Já existem estudos experimentais realizados em animais e grupos de mulheres gravidas que vizam tratamentio da Pré-ecampsia [22] e do retardo de crescimento uterino [23] com o Sildenafil. Esse estudo se propós a estudar a ação do Sildenafil em um modelo animal de aborto espontâneo, visto que ambas as formas de infertilidades citadas estão interligadas.

3.4 Fisiopatolgia das Trombofilias

O termo trombofilia é utilizado para descrever a tendência aumentada para ter fenômenos tromboembólicos, seja esta de origem hereditária ou adquirida [24]. a entrega da

maior parte das substâncias essenciais ao crescimento e metabolismo do feto e placenta, assim como a remoção dos resíduos metabólicos, depende de uma perfusão adequada do espaço interviloso placentar [1]. A perfusão placentar depende diretamente do fluxo sanguíneo uterino total. Mulheres com (TM) podem apresentar perdas fetais por trombose e posterior infarto placentário, pois o sucesso gestacional depende de uma adequada circulação útero-placentária, e anormalidades nessa rede vascular estão relacionadas com várias patologias, como: aborto, óbito fetal, partos prematuros e pré-eclampsia, sendo as Trombofilias um fator determinante e prejudicial nesses eventos [25]. Déficits funcionais na rede de perfusão útero-placentária podem estar relacionados com a conversão insuficiente da musculatura elástica das arteríolas espiraladas pelo trofoblasto invasivo [26]. O risco de tromboembolismo venoso (TEV) é cinco vezes maior em mulheres grávidas do que em mulheres não grávidas. Coagulopatias devido a Trombofilias hereditárias são indicadas como causas hematológicas de perdas fetais recorrentes [27], no entanto, estes achados ainda não estão completamente estabelecidos na literatura [24]. O tratamento para TM atualmente é feito com Heparina de baixo peso molecular, porém ainda não é totalmente eficaz. Outros fatores como a inflamação estão interligados no processo abortivo por causa trombótica [20] [6], assim como abortos por causas inflamatórias podem ter uma via final envolvendo a formação de trombos principalmente nas artérias espiradas impedindo a passagem do fluxo sanguíneo para o feto [28]. Atualmente o tratamento para Trombofiia Materna é realizado com Heparina de baixo peso molecular que diminui o risco de um evento trombótico por atuar inibindo o fator Xa e interagindo com a antitrombina III, evitando assim a ativação da cascata de coagulação, agregamento plaquetário e consequentemente o trombo. Porém esse tratamento ainda não é totalmente eficaz e ainda existem muitas mulheres que apresentam perdas fetais recorrentes por causa trombótica.

3.5 Inibidor de fosfodiesterase tipo 5–PDE5 (Sildenafil)

As PDEs de mamíferos formam uma grande família e estão subdivididas de acordo com a ordem de descoberta, seqüência de aminoácidos e características catalíticas e regulatórias. Estas enzimas hidrolisam os nucleotídeos cíclicos, AMPc e GMPc, às suas formas inativas 5´monofosfatos [17, 29, 30]. O Sildenafil é um inibidor potente e seletivo da PDE5 que por sua vez está presente em vários tecidos tais como o vascular, o muscular liso, uterino e placentário [31], [32]. Este fármaco foi resultado de um programa que iniciou em

1985, na Sede de Investigação Européia Pfizer em Sandwich, UK, com finalidade de desenvolver um inibidor de PDE5 para estimular a via NO-GMPc para o tratamento de angina pectoris em pacientes com doença arterial coronária. Muitas são as patologias que podem ser beneficiadas e tratadas com o Sildenafil, sendo atualmente utilizado no tratamento da hipertensão pulmonar [33]. A PDE5 pode ser encontrada no miométrio, endométrio, tecidos da decídua e até mesmo nos tecidos placentários de ratas [34]. O uso do Sildenafil diminui a contratilidade uterina durante o parto, sugerindo que este possa ser usado inclusive para casos de parto prematuro [35]. Este fármaco também tem sido identificado recentemente como um vasodilatador *in vitro* em pequenas artérias do endométrio [23]. O GMPc é fortemente expresso no trofoblasto invasivo, onde o mesmo medeia o efeito do NO endógeno [36]. Estudos recentes mostram Sildenafil apresenta-se como anti-inflamatório em patologias diversas [15] [33] [29]. Recentemente o esse farmaco tem sido utilizado parao tratamento da RIU-restrição de crescimento intra uterino, por aumentar o calibre dos vasos que levam os nutrientes para o feto [23]. A ligação do GMPc ao sítio alostérico da PDE5 ativa a fosforilação da enzima pela PKG, resultando em uma maior afinidade deste nucleotídeo cíclico ao domínio R e aumento de sua taxa de hidrólise pelo sítio catalítico. Este efeito está envolvido na regulação por feedback negativo dos níveis celulares de GMPc. Sua administração promove um efeito de feedback positivo, representado pela inibição da degradação, com acúmulo do GMPc, através da interação do Sildenafil com o domínio C [37]. O Sildenafil por sua ação vasodilatadora e anti-inflamatória pode ser benéfico para tratar alguns tipos de infertilidade feminina, principalmente aquelas em que os fatores relacionados a inflamação e fluxo sanguíneo são cruciais.

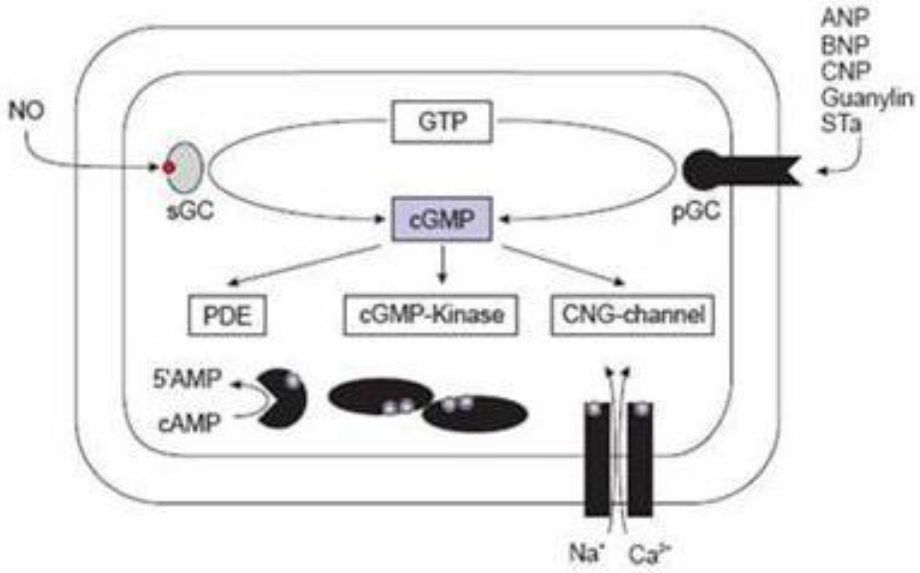

Figura 2: (HOFMAN, 2010)

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Sildenafil (Viagra®) maintains placental integrity and prevents fetal death in loss pregnancy model induced by lipopolysaccharides

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Abstract

Infertility is an important problem of worldwide public health, but the mechanisms related to gestational complications are not completely understood yet. Among other characteristics, abortion represents an inflammatory and thrombosis process that mainly involves high expression TNF-α. Sildenafil plays an important role as vasodilator, and anti-inflammatory properties have recently been found. This study investigated the action of Sildenafil in a pregnancy loss model induced by Lipopolysaccharide in female pregnant mice. The action of Sildenafil (50mg/kg/day) in a single treatment and in an association treatment with Heparin (500/UI/kg/day) was assessed. The results demonstrated the benefic effect of Sildenafil in the

single treatment and in the coadjuvant treatment with Heparin after injury induced by LPS, promoting the maintenance of the cellular and molecular structure of the placenta. Thus, it represents a therapeutic option for pregnancy loss and with inflammatory and thrombotic involvement.

Keywords: Sildenafil; inflammation; placenta; pregnancy.

Introduction

Sildenafil induces cGMP accumulation through selective phosphodiesterase-5 (PDE5) inhibition [15]. The PDE5enzyme can be found in myometrium, endometrium, decidua and placental tissues in female mice [34]. Such drug was approved for therapeutic use in erectile dysfunction and is also currently used for the treatment of pulmonary hypertension and it has most recently been used for Raynaud-s syndrome [17, 38, 39]. Despite its excellent tolerability profile, these are the only diseases currently treated with Sildenafil. Studies have shown that selective PDE5 inhibitors such as Sildenafil and Vardenafil (Levitra; Bayer) raise cGMP/cAMP. PDE4 inhibitor Rolipran decreased the inflammation in the uterus and placenta after 4 hours of LPS exposure, providing protective effects [40]. Experimentally, these drugs have been used for many pathologies involving female infertility [23].

It is estimated that 20–30% of women who become pregnant experience one or more spontaneous pregnancy loss, that is, the loss of a pregnancy without outside intervention before 20 weeks of gestation [41]. The mechanisms responsible for these pregnancy losses are not well understood. The imbalance of chemical mediators involved in pregnancy can trigger the abortion process, possibly being mediated by cytokine Pro-inflammatory TNF-α [6]. Besides, S-selectins and P-selectins have been described as crucial for embryo implantation

into the uterine epithelium and for the maintenance and development of embryos [13]. Another important chemical mediator in the pregnancy process is nitric oxide (NO), which can relax smooth and vascular musculature through activation of the guanylate cyclase, producing high levels of cGMP. Many isoforms of NO synthase were identified in uterus, particularly the inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) [16].

An important element involved in female infertility is related to the coagulation cascade, once abortions have a thrombotic pathway [20]. Reduced uteroplacental perfusion is a possible contributing factor to pregnancy loss and pregnancy-related morbidities [6, 25]. Inherited and acquired Thrombophilias have emerged as one of the leading causes of pregnancy loss and pregnancy-related complications. It appears inflammation and thrombosis are intimately linked [14]. Women with Maternal Thrombophilia (MT) may experience pregnancy losses as a result of placental infarction due to thrombosis, because the gestational success depends on a proper uteroplacental circulation [21, 26]. Sporadic clot formation could interrupt uterus placental blood flow, inevitably leading to placental insufficiency and intrauterine fetal demise [6]. Currently, Heparin (low weight) is used as a treatment for maternal Thrombophilia, however, it is not completely efficient [24].

Considering the anti-inflammatory and vasodilator properties of Sildenafil (Viagra) as a result of its effect on the inhibition of PDE5, the purpose of the present study was to investigate the action of this drug in a model of pregnancy loss by LPS - on a single treatment and in a coadjuvant treatment with Heparin -, thus determining: 1) Quantification of fetal death; 2) Placental injury (histology and ultrastructure); 3) induced nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS) (immunohistochemistry); 4) P-Selectin molecule adhesion (immunofluorescence) 5) expression of TNF-α (immunohistochemistry and western bloting) 6) IL-1β (immunofluorescence and western blot).

Materials and methods

Experimental procedures

Eight female Swiss Albino mice at the age of 60 days, weighing 30g, were used per group. The control group did not receive any treatments, nor LPS induction. Pregnancy loss group received only LPS intraperitonial injection. The Sildenafil group was treated from the 1st to the 15th day of pregnancy with a 50mg/Kg dosage of Sildenafil Citrate administrated through the water (Viagra, Pfizer Inc., New York, NY, USA) and received the LPS injection on the 15th day. The Heparin group was treated with low weight molecular Heparin (Fragmin, Pfizer Inc., New York, NY, USA) 500UI/kg per day from the 1st to the 15th day of pregnancy and also received LPS the on 15th day of pregnancy. The Sild + Hep group received both of treatments and the LPS inducement. The model of pregnancy loss by LPS was reproduced according to Renauld *et al.* 2011 [6]. Mice were examined for health status, acclimated to the laboratory environment at 25°C and 12-h light/dark photoperiod, and were then housed in metal cages. Pregnancy was achieved by housing proestrus phase females with males. After 48 h, copulation was confirmed by vaginal plug. On the 15^{th} day of pregnancy, 2 h after the LPS injection, females were euthanized by anesthesia with ketamin and xilasin. The placentas were removed and processed through all the available techniques. Fetal death analysis was performed 24h after LPS induction. The fetal signals were assessed: absence of movements, cardiac pulsation, and coloration abnormal coloration.

Histopathology

Placenta fragments were fixed in 10% formalin for 24h, processed and embedded in paraffin. Sections of 4-5 mm were cut and mounted on glass slides. The slices were stained with hematoxylin-eosin and assessed through an inverted microscopy (Observer Z1, Zeiss MicroImagingGmbH) equipped with a camera, and through a 4.7.4 image analysis program (AxionCamMRmZeiss) at a magnification of 400x. The groups were compared to each other.

Immunohistochemistry

The placentas were immediately removed and -fixed in the same fixative overnight. Samples were dehydrated in an ethanol series (Isofar Chemical Co., RJ, Brazil), cleared in xylene and embedded in paraffin (Merck, catalog number: 1071642504). Sections of 5 µm thickness were cut using an RM 2035 microtome (Reichert S, Leica), re-hydrated, washed in 0.05 M PBS, and incubated in a buffer with 1% bovine serum albumin (BSA, fraction V) (Miles, Naperville, IL, USA) for 1 h. Endogenous peroxidase was blocked and antigen retrieval was performed, pre-treating the sections using a 20 mM citrate buffer, pH 6.0, at 100°C, for 30 min. All groups were incubated with the rabbit polyclonal primary antibodies anti-TNF-α (ab34674 Abcam Cambridge, MA, USA), anti-eNOS (ab66721 Abcam Cambridge, MA, USA) and anti-iNOS (ab3523, Abcam Cambridge, MA, USA) at a dilution of 1:100 overnight at 4°C. After washing, sections were overlaid for 1 h with a biotin-conjugated secondary antibody using a HRP-kit (DakoCytomation, CA, USA, Biotinylated Link Universal HRP; catalog number: K0690) and visualized with diaminobenzidine (DAB) as the chromogen. The slices were then weakly counter-stained with Harris' hematoxylin and mounted in entellan (Merck, catalog number: 1079610100). Stained areas were measured

using the GIMP 2.6.11 image analysis software (GNU Image Manipulation Program software, CNET Networks, Inc. Australia).

Immunofluorescence

Placentas were dissected and immersed in 15% sucrose overnight, followed by 30% sucrose for a second night (36h total). Specimens were then embedded in OCT-Tissue-Tek compound (Sakura Finetek, Torrance, CA, USA) and frozen in n-hexane (Dinâmica, São Paulo, SP, Brazil) with liquid nitrogen. Cryo-sections of 8 μm were permeabilized with 0.3% Triton X-100 and incubated for 1h with blocking solution (3% BSA plus 0.2% Tween 20 in Tris buffered saline). Subsequently, the sections were incubated with antibodies for P-Selectin (LS-B3578, Lifespan Bio science Inc. USA) or IL-1β (ab9722, Abcam Cambridge, MA, USA) at a dilution of 1:100. Primary antibodies were incubated overnight, and then incubated with polyclonal Cy3-conjugated secondary antibodies (Jackson, catalog number 705-165-147) against rabbit immunoglobulin (Sigma–Aldrich, catalog number F6257, 1:200) for 1 h. The slices were washed and mounted in fluorescent Prolong Gold Antifade medium (Life Technologies, catalog number: P36930) for observation under an inverted fluorescence microscope (Zeiss MicroImaging GmbH) equipped with a camera (Zeiss AxioCam MRM). Stained areas were measured using the GIMP 2.6.11 software (GNU Image Manipulation Program software, CNET Networks, Inc. Australia).

Western blotting

The placentas of each group was homogenized in an extraction cocktail (10 mM EDTA, Amresco, Solon, USA; 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 – Sigma–Aldrich). The samples were mixed and homogenized to form a pool from each group. Homogenates were centrifuged and frozen until they were used for immunoblotting. The proteins (40 mg of total protein were used for all gels) were separated on 10% electrophoresis gel under reduced conditions and were electrophoretically transferred to nitrocellulose membranes (BioRad, catalog number 162-0115). After blocking, the membranes were incubated for 4h with rabbit polyclonal antibody, TNF-α (1:1000 dilution, catalog number: 500-P64, Peprotech, NJ, USA), or IL-1β (1:1000 dilution, ab9722, Abcam Cambridge, MA, USA). After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit (1:8000 dilution, catalog number: A9169), anti-mouse (1:1,000 dilution, catalog number: A0168), or anti-goat secondary antibody (1:100,000 dilution, catalog number: A5420) - all from Sigma–Aldrich. An enhanced chemiluminescence reagent (Super Signal, Pierce, catalog number: 34080) was used to make the labeled protein bands visible and the blots were developed on X-ray film (Fuji Medical, Kodak, catalog number: Z358487-50EA). For quantification, the pixel density of each band was determined using the Image J 1.38 software (available at http://rsbweb.nih.gov/ij/download.html; developed by Wayne Rasband, NIH, Bethesda, MD, USA). For each protein investigated, the results were confirmed in three sets of experiments. Immunoblotting for β-actin was performed as a control for the above mentioned protein blots. After protein blot visualization with enhanced chemiluminescence, protein antibodies were stripped from the membranes, which were reprobed with monoclonal anti-β-actin antibody (1:1000 dilution, Sigma–Aldrich, catalog number A2228), and protein densitometry was then performed. The ratio of each protein/β-actin studied was calculated and compared between groups.

Transmission electron microscopy

Small fragments of placenta were immersed in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate (Sigma–Aldrich) buffer, pH 7.2, and were then post-fixed in the same fixative overnight. After that, the placenta fragments were washed twice in the same buffer and post-fixed in a solution containing 1% osmium tetroxide (Sigma–Aldrich), 2 mM calcium chloride and 0.8% potassium ferricyanide (Sigma–Aldrich) in 0.1 M sodium cacodylate buffer, pH 7.2, dehydrated in acetone series and embedded in SPIN-PON resin (Embed 812 – Electron Microscopy Science – EMS, Washington, PA, USA) [42]. Resin polymerization was performed at 60°C for 3 days. Semithin sections (0.5 µm thickness) were placed in glass slides, stained with toluidine blue, and used for the morphometric analysis of placental regions. Ultrathin sections (70 nm thickness) were placed in 300-mesh nickel grids, counterstained with 5% uranyl acetate (EMS) and lead citrate (Sigma–Aldrich), and examined using a FEI Morgani 268D electron transmission microscope.

Statistic analysis

The densitometry values of the immunoreactive bands (immunoblotting), immunohistochemistry, immunofluorescence and Fetal Death were analyzed using the GraphPad Prism software package (San Diego, CA, USA). One-way analysis of variance (ANOVA), followed by Dunnett's and/or Tukey's post-test, were used to compare the groups. The results were expressed in ± S.E means, when appropriated. A p-values < 0.05 indicated statistical significance.

Results

For fetal death, the implementation of the model via LPS resulted in almost 100% of deaths 48h after the LPS injection. All pregnant females had significant fetus losses, which did not occur to the control group. Among the groups treated, the one that had the higher percentage of fetal mortality was the one treated with Heparin, which is still significantly lower than the LPS group's, thus indicating its benefic therapeutical effect. Treatment with Sildenafil and coadjuvant treatment with Sildenafil and Heparin significantly decreased the number of females that had pregnancy losses, as well as it decreased the total percentage of fetal deaths. The latter result was similar to that found in the control group, which is close to zero (Figure 1).

During the histological analysis it was possible to find different areas of the placenta tissue: the labyrinth (LB) region, which is important for nutrient exchange for having the blood spaces which are also known as fetal vessels; the Espongiotroflobasto (SP) region: a basal region where there are, among other cells, trophoblast cells (TC), with special emphasis to the giant trophoblast cells, which are important for their secretor functions and for storing hormones such as b-hCG and progesterone, which are essential for maintaining the thickness of the endometrium during pregnancy. Well conserved structural features were observed in the placental tissue of the control group. In the LPS group, all cell types described lost their architectural pattern, and the most affected region by the tissue injury was the labyrinth. Therefore, the pregnancy loss model probably harmed the maternal fetal blood flow. In the group treated only with Sildenafil, both labyrinth cells and trophoblast cells were well preserved. Besides, it was also possible to find trophoblast cells neogenesis, as well as their increased presence in the spongiotrophoblast region, and also unexpectedly in the labyrinth region, delimitating the injury tissue areas. The Heparin group also had well preserved cells in the labyrinth and trophoblast areas. However, their architectural pattern in the tissue was disorganized, indicating possible sequelae induced by LPS. Finally, the Sild + Hep group was

the one that better presented protection against the damage caused by the exposure to LPS. Cells were well-preserved and well-defined. However, an increase in trophoblast cells was found, as described in the Sildenafil group. Furthermore, the Sild + Hep group presented a greater number of blood cells in the labyrinth region, indicating a possible increase in the blood flow (Figure 2).

In Figure 3, we conducted an evaluation of the labyrinth region (40x) of all groups, once it was the area that had most alterations in previous analysis. The exposure to LPS caused edema, congestion and degeneration in the labyrinth cells. All treatments resulted in the improvement of such conditions. In the Sildenafil group, vascularization is normalized. However, it was possible to find the presence of trophoblast cells and giant trophoblast cells which were abnormally located mainly in the labyrinth region. In the Heparine group, the labyrinth's cell structure was well preserved but the blood spaces were reduced, which may decrease the overall maternal fetal circulation. In the group that had combined treatment with Sildenafil and Heparin, the spaces in which fetal blood circulates had normal contour and size, as well as normal cells in the labyrinth region.

The ultrastructural analysis of the labyrinth cells from the control group found oval cells with nucleus containing well defined nucleoli, while the exposure to LPS induced drastic alterations which were made visible by intense degenerations in the labyrinth cells. On the other hand, the group treated with Sild+Hep had well preserved cells with evident microvilli (Figure 4). The placental vessels of the control group had well preserved endothelial cells and erythrocytes. After the damage caused by LPS, the endothelial cells were contracted and had spaces between them and the basal lamina. In turn, the lumen space was reduced, and there was also the presence of platelet agglomerates. The Sild +Hep group had well preserved endothelial cell ultrastructure, as well as a well preserved intravascular space containing standard features red cells (Figure 5). During the ultrastructural analysis of the giant

trophoblast cells in the control group, it was possible to find regular nuclei with well defined nucleoli, as well as well preserved cytoplasmic cisterns. In the LPS group, these cells had nuclei morphologically irregular, and condensation of the chromatin, which is characteristic of apoptosis process. Also, it was possible to observe the presence of vacuoles and degenerated cisterns in the cytoplasm. In turn, the Sild + Hep group had well preserved giant cells with hypertrophied smooth endoplasmic reticulum, denoting an cellular activation (Figure 6).

The immunohistochemical analysis for endothelial nitric oxide enzyme (eNOS) in the control group revealed a basal expression of this enzyme, which was not significantly altered by the exposure to LPS. The Sildenafil group and the Heparin group had a uniform labeling, which was significant in comparison to the control group. The Sil+Hep group had an overexpression of eNOS, indicating its possible benefic role in reversing the pregnancy loss model induced by exposure to LPS (Figure 7).

The nitric oxide enzyme's inducible synthase (iNOS) is an important inflammatory marker. There was a significant increase in the presence of this enzyme in the LPS group. Treatment with Sildenafil led to a significant increase for iNOS expression. Similarly, Hep group showed high levels of iNOS labeling. Unexpectedly, the Sild+Hep group had a significant reduction in the presence of this nitric oxide isoform (Figure 8).

The P-selectin adhesion molecule is constitutively in the placenta throughout pregnancy. Immunofluorescence for the P-selectin adhesion molecule confirmed the large amount of this molecule in placental tissues during pregnancy. In the control group, the presence of P-selectin was high and homogeneously distributed throughout the tissue. In the LPS group, this presence was significantly reduced. In turn, treatment with Sildenafil significantly increased

the presence of P-selectin when compared to the control group. The same result was found in the treatment with Sild +Hep (Figure 9).

The expression of proinflammatory TNF-α cytokine is involved in several tissue damaging processes and is regarded as a crucial mediator in the abortive process. The LPS group had increased immunostaining for TNF-α, mainly in the labyrinth area. In contrast, the group treated with Sildenafil reduced TNF-α labeling, indicating that the treatment was effective in reducing inflammation. A similar result was obtained with the treatment with Heparin. The Sil + Hep group had the best results, having significant reductions in the staining of TNF-α when compared to single treatments (Figure 10). These results were confirmed by western blotting analysis (Figure 12).

IL1-β is another important proinflammatory cytokine. Immunofluorescence analyses for IL-β revealed that the LPS group had a significant increase in this cytokine's labeling. The treatment with Sildenafil, Heparine, and Sild + Hep significantly reduced the presence of IL1-β, when compared to the LPS group (Figure 11). These results were confirmed by IL1-β presence analysis by western blotting analysis (Figure 12).

Discussion

According to Renaud et al (2011), one single LPS injection in pregnant mice (100 µg/kg) induces a complex pregnancy damage which almost 100% of times results in fetal death after up to 72h after exposure to LPS (9).Using a modified protocol, this study found similar results 48h after the induction of the model. The treatments with Sildenafil and Sild+Hep significantly reduced fetal mortally, also indicating a bigger protective role than in the single

treatment with Heparin. Currently, most research try to discover and describe new drugs that might reduce complications such as premature delivery and fetal death (19). This study demonstrated that Sildenafil is efficient in reducing the mortality rate implemented by a pregnancy loss model induced by LPS.

The histopathological analysis demonstrated that LPS induced a great cell degeneration area, especially in the labyrinth, which is an important region for maternal blood transfusion to the fetus (20). These data confirm previous findings (9), which registered high levels of tissue inflammation 2h after exposure to LPS. Treatments with Sildenafil, Heparin, or Sild.+Hep.had lower tissue degeneration. The latter group had the best results, and it had the higher placental vascularization. The groups treated with Sildenafil had trophoblast cells proliferation and/or neogenesis in the placenta. This has been reported as harmful, once the cells are distributed in other areas of the placenta when there is pathologic process ongoing (21-24). However, in this case, proliferation did not have neoplastic features, and the trophoblast cells were concentrated in regions that were closer to the tissue damage forming what seemed to be a protective barrier against the injury. This cell type produces and storages a number of hormones, such as estrogen, progesterone, and Beta human chorionic gonadotrophin (bHCG) (14, 25). Besides, it synthesizes cytokines in some of the placental areas, thus, being crucial to pregnancy preservation ([10, 43, 44].

It has been reported that LPS causes severe tissue damages due to acute inflammation in the gestational complex (28). Ultrastructural analysis demonstrated cell degeneration induced by LPS, especially in the labyrinth region. Furthermore, placental vessels presented damaged endothelial cells. After treatment with Sild + Hep, the labyrinth region had well preserved cells, as well as placental vessels with endothelial cells morphologically similar to that of the control group. There was also an increase in the number of erythrocytes present within the vessels, indicating an increased blood circulation, which confirms the data obtained in

histopathological analysis. The preservation of placental vessels is crucial to proper fetal development (29). Studies involving LPS show that 3h after exposure to endotoxin, there is ultrastructural damage in spiral arteries (9). Sildenafil has been used for the treatment of uterine growth restriction (RCU) for it increases the fetus' blood flow, thus contributing to a better fetal development (7). Our data confirm Sildenafil's ability to increase placental blood flow. Treatment with Sild + Hep promoted the preservation of trophoblast cells. It also promoted the activation of giant trophoblast cells. New studies are being developed in our laboratory in order to deepen knowledge on giant trophoblast cells, so it is possible to understand their role in infertility and their potential as therapeutic targets.

Nitric oxide synthases are important for various physiological events (30) and gestational development (11). NO synthases are commonly found in the myometrium, placenta and decidual tissues of the gestational complex (11, 31). These enzymes produce the necessary NO for the synthesis of secondary messengers, such as GMPc, thus maintaining gestational development (31). The results obtained in this study demonstrated a role for NO in a model of induced pregnancy loss by LPS. Analyzes for iNOS showed basal presence in the control group and increased presence in the LPS group. However, the presence in the Sil + Hep. group was equal to the one from the control group, confirming the beneficial effects of the joint treatment. The relationship between NO and cellular inflammation is very complex, because when NO is concentrated at high levels it is cytotoxic and pro-inflammatory. Such effects are opposite when NO synthesis occurs at nanomolar concentrations through eNOS and Kinase protein – which depends on GMP (PKG) activation. Therefore, the NO produced by eNOS is considered anti-inflammatory (32, 33), which represents effects that are related to the direct or indirect inhibition of NF-kB (34). The Sild + Hep group had an intense reaction for eNOS. Our data confirm that an increase in the presence of eNOS in this group possibly acted as a balancing factor, reducing tissue damage caused by LPS.

The role of adhesion molecules in pregnancy is not well known. It is possible they are related to uterine leukocyte migration, which is necessary for fetal development (10, 14, 35), as previously found in uterine and placental tissue (36, 37). In this study, basal levels of P-selectin were detected in the placenta of control group animals. However, LPS induction caused significant reduction in the presence of P-selectin. Treatments with Sildenafil, Heparin or Sild + Hep recovered the levels of P-selectin found in the control group. The importance of selectins has been reported for being involved in processes such as: uterine embryo implantation and fetal development (10). The joint treatment with Sildenafil + Heparin once again had the best results, demonstrating the synergy between these two drugs in the general protection of placental tissue.

Inflammation may happen in response to an injury, aggression, or pathological process (34). During pregnancy, inflammation has been described as an important factor in cases of recurrent abortions both in natural or artificial pregnancies (12, 38, 39). Therefore, TNF-α plays a crucial role in mediating the inflammatory response in the gestational complex. The mechanism by which TNF-α mediates fetal death remains unclear. However, it may involve luteal hormonal insufficiency, aponecrose facilitation in trophoblast, or placental damage, resulting in the vascular insufficiency of the uteroplacental flow (19, 40, 41). Previous studies have demonstrated the involvement of TNF-α in the inflammatory process involved in pregnancy loss, showing increased levels 3 hours after exposure to LPS (9). The phosphodiesterase-4 inhibitor (Rolipram) decreased levels of proinflammatory cytokines, such as TNF-α, even 4h after the exposure to LPS (6). Sildenafil may protect the placental tissue inflammation caused by exposure to LPS with single or joint treatment with heparin, confirming the aforementioned findings. This is the first description of the anti-inflammatory role of Sildenafil in a pregnancy loss model induced by LPS in pregnant female mice.

The quantification of another important pro-inflammatory cytokine, IL-1β, had the same presence pattern as those found in the analysis for TNF-α. Treatment with Sildenafil or Sild + Hep significantly reduced immunostaining for IL-1β. Studies have demonstrated that the nuclear -KB factor (NF-Kβ) plays an important role in gene regulation at the beginning of the inflammatory response by regulating mediators such as TNF-α and IL-1β (42, 43). The possible role of Sildenafil in the inflammatory cascade mediated by NF-Kβ has been described (1). In this study, which involves the mechanisms of the abortion process induced by exposure to Lipopolysaccharides, Sildenafil could act by inhibiting the NF-Kβ via and thereby inhibiting the transcription of proinflammatory cytokines. However, more studies are needed in order to deepen our understanding of the relationship between the NF -Kβ via and recurrent fetal losses that have an inflammatory component. Heparin has anticoagulant features, and also anti-inflammatory features, once it inhibits the complement system (17, 44, 45). Therefore, it is able to protect the placental tissue. Hence, the synergism between Sildenafil and Heparin was clear in this study, once after being analyzed using all techniques, this group was the most effective in preventing placental injury.

This study was aimed at assessing the placental tissue after exposure to Lipopolysaccharides and at contributing to the understanding of the mechanisms involved in pregnancy loss, which have an inflammatory and thrombotic role. Treatment with Sildenafil promoted good results, but the joint therapy of Sildenafil and Heparin had greater efficacy, protecting the integrity of placental tissue against the damage caused two hours after the exposure to LPS. This treatment protected against fetal death, reduced the presence of proinflammatory cytokines (TNF and IL-1), recovered constitutive levels of iNOS and P-Selectin, as well as, increased the presence of eNOS, and maintained the integrity of the placental tissue. By deepening the studies involving the action of Sildenafil in abortion cases, this drug may become an option for therapeutic single or combined treatment with Heparin in the future.

Declaration of interest

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

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Figure legends

Figure 1: Quantification of fetal death after 48h of LPS exposure. All the groups had decreases in fetal deaths. Data expressed in ± S.E.M mean from n = 4 mice for each group; ND: Not detected; *P < 0.05 and **P < 0.01 compared to LPS group, $^{\#\#}$P < 0.01 compared Control group.

Figure 2: Histopathology of placenta of the following groups: (A) control, (B) LPS, (C) Sildenafil, (D) Heparin, (E) Sild + Hep. Distinct regions observed: (LB): labyrinth, (SP): spongiotrophoblast containing trophoblast cells (TC). Tissue degeneration in the LPS group is indicated (black arrows). Neogenesis and proliferation of trophoblast cells observed in the Sildenafil group (white arrow). Bars, 20μm.

Figure 3: Histopathology of placental labyrinth region of the following groups: (A) control, (B) LPS, (C) Sildenafil, (D) Heparin, (E) Sild + Hep. In the LPS group, congestion and edema were observed (black arrows). Agglomeration of trophoblast cells (giants) observed in the Sildenafil group (white arrows). Heparin group preserved cellular morphology. Sild + Hep preserved cellular morphology and architecture, and increase blood cells inside vessels. Bars, 20μm.

Figure 4: Electron transmission microscopy revealing labyrinth cells with preserved architecture in Control group (A). In LPS group (B) widespread severe cellular degeneration is showed (white arrows); otherwise, treatment with Sild+ Hep (C) preserved the cells of Labyrinth against the LPS damage. It was possible to observe the presence of microvilli (white asterisk). Bar, 2000 nm.

Figure 5: Electron transmission microscopy. Control group (A) presented well-preserved blood vessels; whereas in the LPS group (B) endothelial cells were damaged and corrugated (white arrow) and the luminal space was reduced. Also several platelets agglomerations were

frequently seen (insert). In the Sild+ Hep group (C) the treatment protected all kinds of cells, the endothelial cells and erythrocytes presented morphological pattern characteristics. En: endothelial cell, Er: erythrocytes. Bar, 2000 nm.

Figure 6: Electron transmission microscopy showing giant trophoblast cells in Control group (A). In LPS group (B) some giants cells presented nuclear condensations (apoptotic bodies) (white arrow) and regions of cytoplasmic degeneration. In the Sild+ Hep group (C) I was possible to observe preserved giant cells with enlarged smooth endoplasmic reticulum (white asterisk), denoting an cellular activation. Bar, 2000 nm.

Figure 7: Immunohistochemical localization for eNOS of the following groups: (A) control, (B) LPS, (C) Sildenafil, (D) Heparin, (E) Sild + Hep. Control group presented a basal expression, and Sildenafil and Heparin groups had a discrete increase detected. Higher expression is observed in Sild + Hep group. Graphic show densitometry analysis of immunohistochemistry photographs for eNOS. Data expressed as ± S.E.M. mean from n = 5 mice for each group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared Control group.

Bar 20 µm.

Figure 8: Immunohistochemical localization for iNOS of the following groups: (A) control, (B) LPS, (C) Sildenafil, (D) Heparin, (E) Sild + Hep. Reduced expression observed in Sild + Hep group. Graphic shows densitometry analysis of immunohistochemistry photographs for iNOS. Data expressed as ± S.E.M mean. from n = 5 mice for each group; **P < 0.01 compared to LPS group, $^{\#\#}P < 0.01$ compared to Control group. Bar, 20 µm

Figure 9: Immunofluorescence for P-Selectin of the following groups: (A) control, (B) LPS, (C) Sildenafil, (D) Heparin, (E) Sild + Hep. Constitutive expression is observed in the control group. The LPS group had a decreased expression for P-Selectin. All the treatments reestablished the basal pattern expression. Graphic shows densitometry analysis of immunofluorescence photographs for P-Selectin. Data expressed as ± S.E.M mean from n = 5 mice for each group; **P < 0.01 compared to LPS group, $^{\#\#}$P < 0.01 compared to Control group. Bar, 20 µm

Figure 10: Immunohistochemical localization for TNF-α of the following groups: (A) control, (B) LPS, (C) Sildenafil, (D) Heparin, (E) Sild + Hep. Control group presented basal level expression and LPS induced increased staining for TNF- α. Treatments decreased TNF-α expression significantly. Data expressed as mean ± S.E.M. from n = 5 mice for each group; **P < 0.01 compared to LPS group, $^{\#\#}$P < 0.01 compared to control group. Bar, 20 µm

Figure 11: Immunofluorescence to IL1-β. (A) control group, (B) LPS group, (C) Sildenafil group, (D) Heparin Group, (E) Sild + Hep group. In the LPS group, the levels of IL1-β were increased and all the treatments decreased IL1-β expression. Graphic shows densitometry analysis of immunofluorescence photographs for IL1-β. Data expressed as mean ± S.E.M. from n = 5 mice for each group; **P < 0.01 compared to LPS group, $^{\#\#}$P < 0.01 compared to control group. Bar, 20 µm

Figure 12: Western Blotting to IL1- β and TNF- α. (A) control group, (B) LPS group, (C) Sildenafil group, (D) Heparin Group, (E) Sild + Hep group. All the treatments decreased the expression of IL1-β and TNF- α. Representative blot of lysates obtained from pool 8 placentas per group; data expressed as mean ± S.E.M. of 3 replications for each group; **P < 0.01 compared to LPS group, $^{\#\#}$P < 0.01 compared to control group. β-actin for standardizing reaction.

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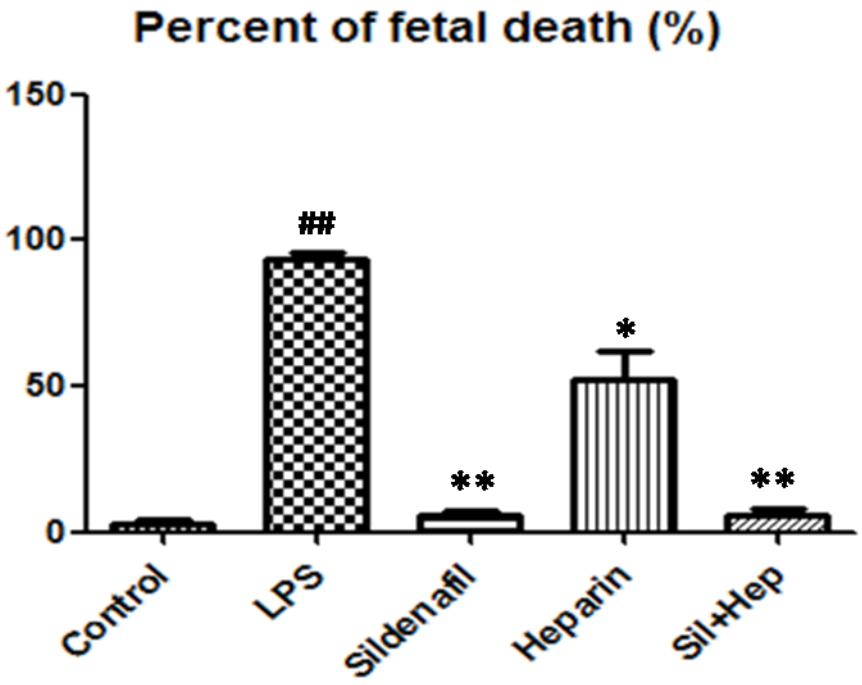
Quantitation of fetal death

Groups	Female 1		Female 2		Female 3		Female 4	
	Life	Death	Life	Death	Life	Death	Life	Death
Control	16	00	20	01	18	01	18	00
LPS	18	16	16	15	19	19	20	18
Sildenafil	20	02	19	01	18	00	16	01
Heparin	15	10	18	13	17	12	19	05
Sil + Hep	17	01	19	02	20	00	18	01


Percent of fetal death (%)
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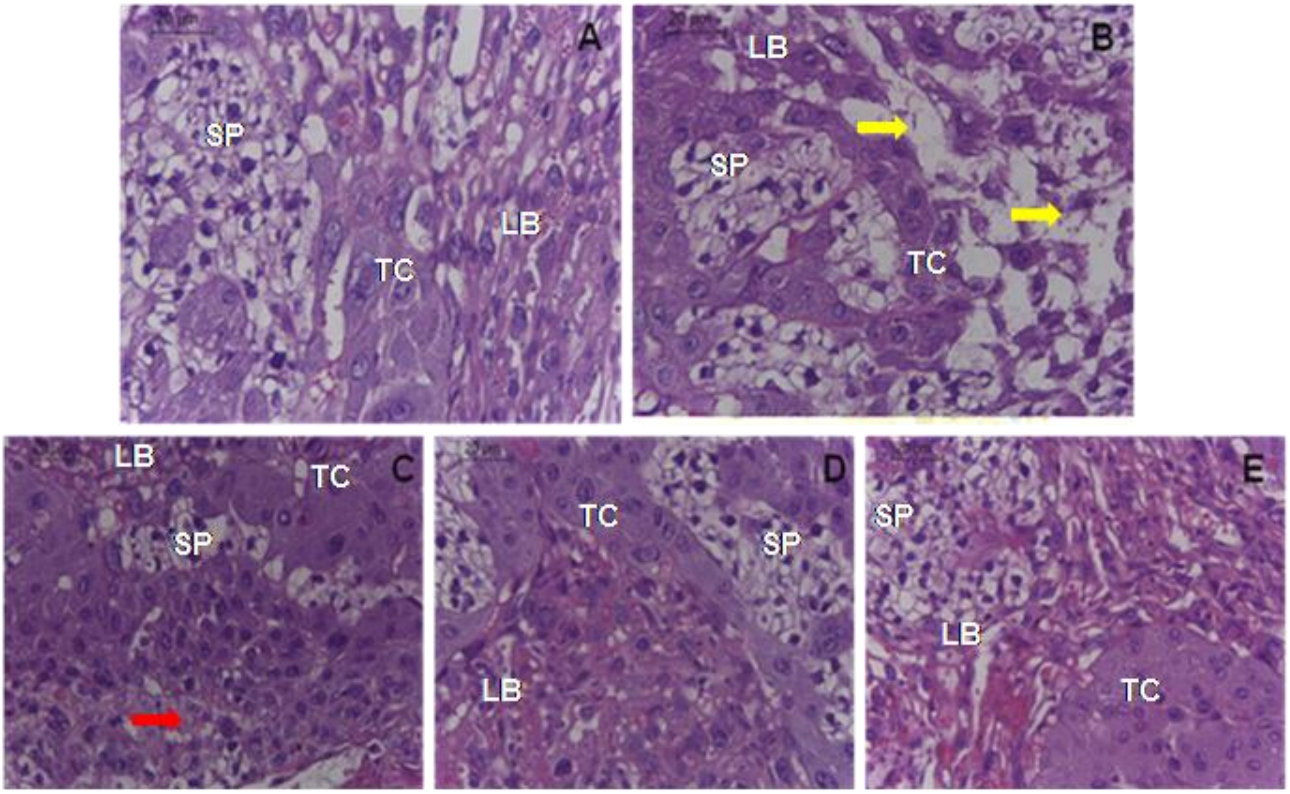
100
50
0
##
*
**
**
Control
LPS
Sildenafil
Heparin
Sil+Hep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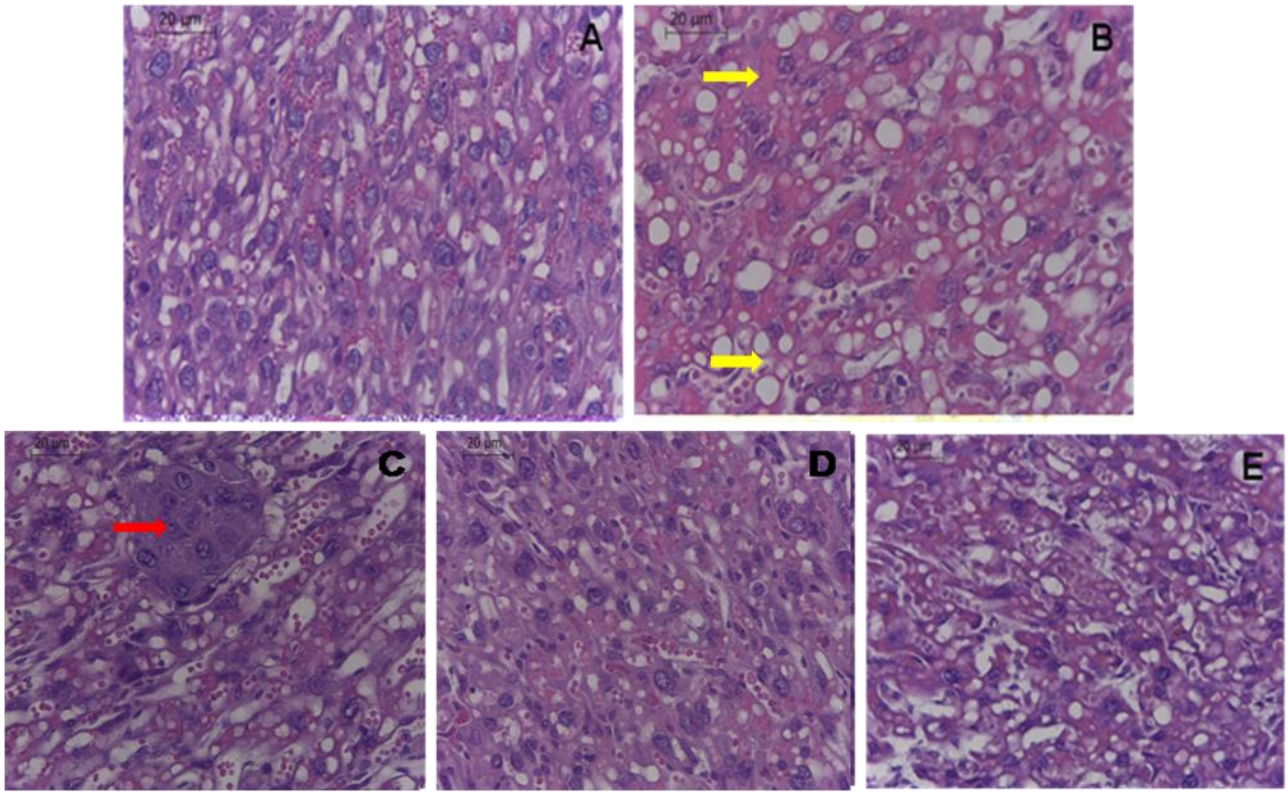
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D
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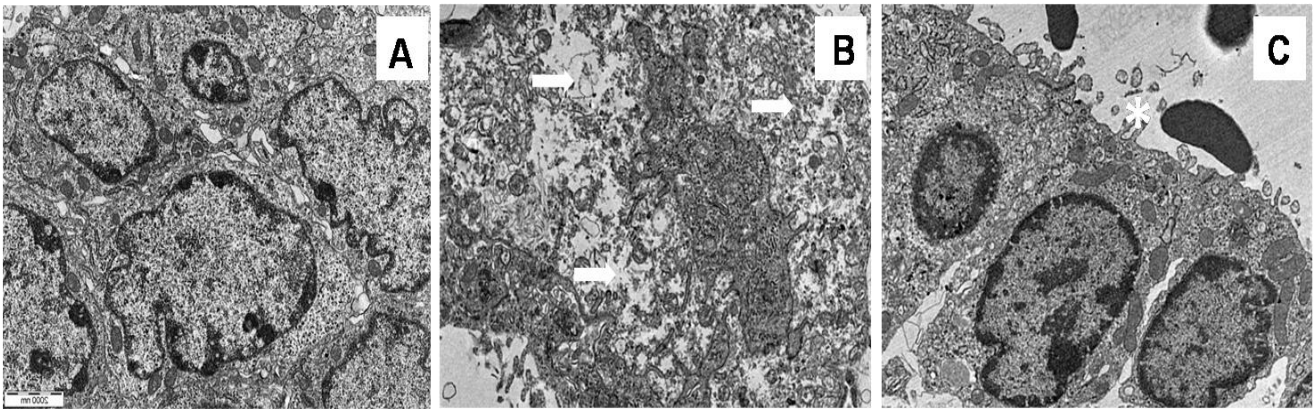
E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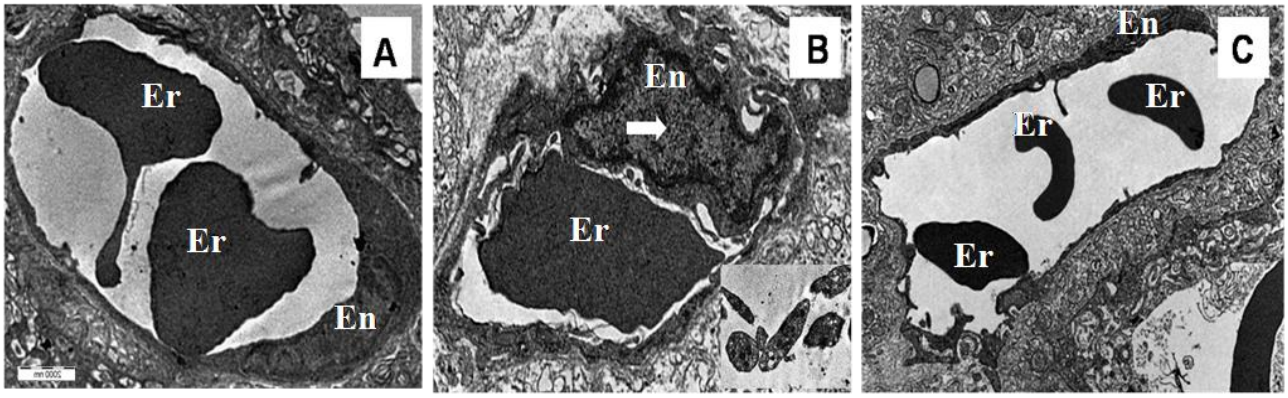
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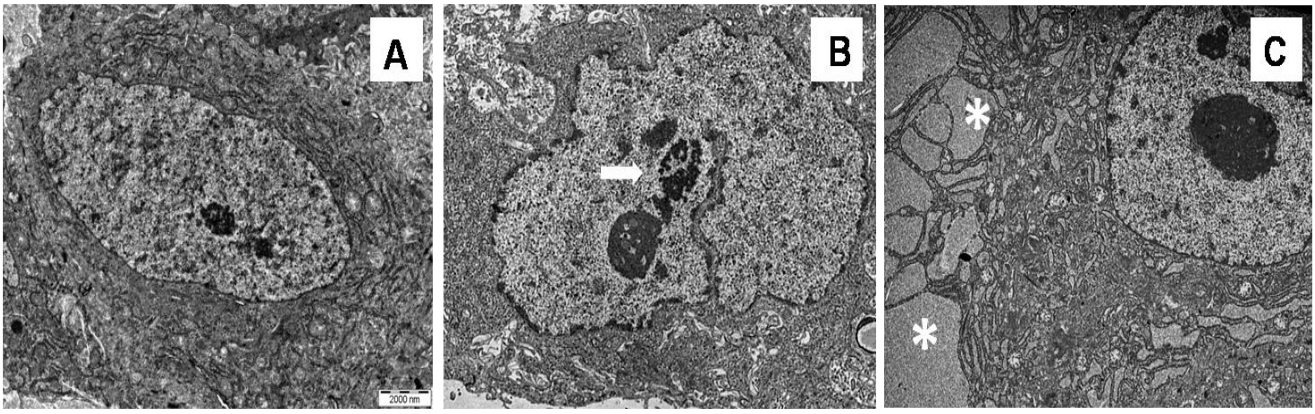
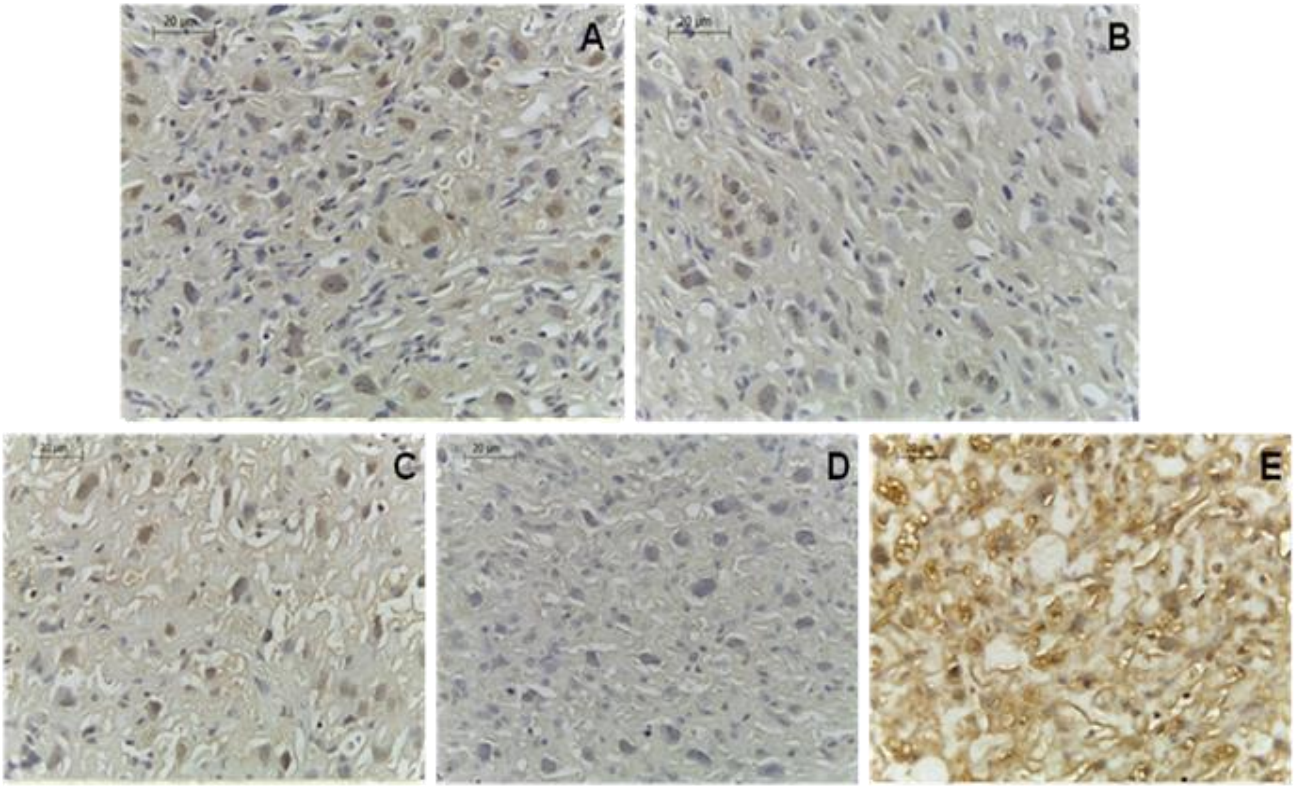

A
2000 nm
B
C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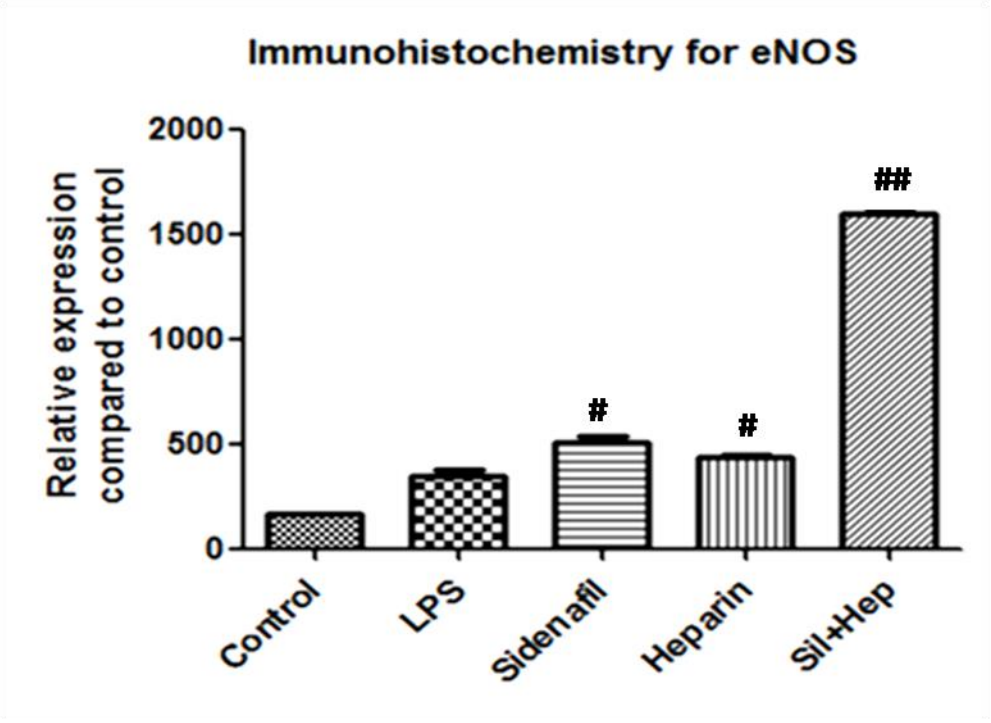
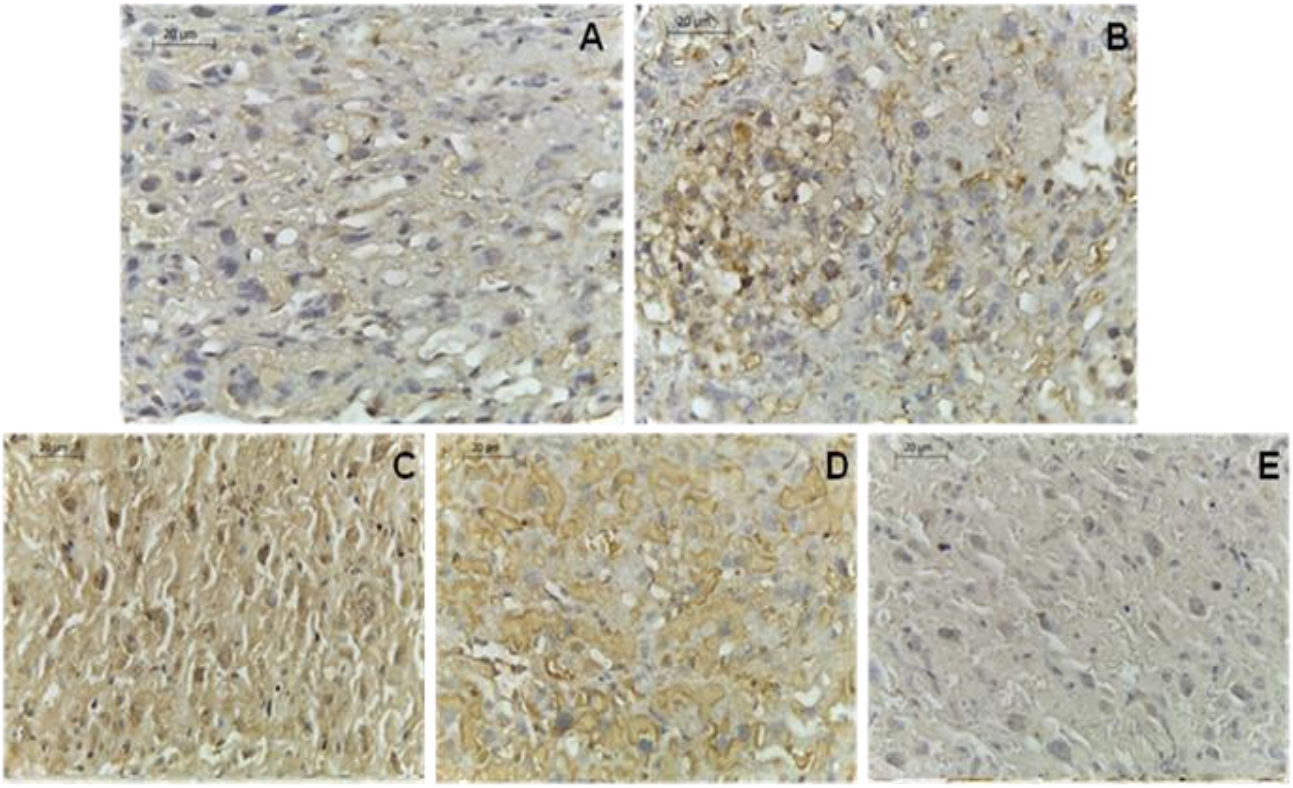

Immunohistochemistry for eNOS
Relative expression compared to control
2000
1500
1000
500
0
#
#
##
Control
LPS
Sidenafil
Heparin
Sil+Hep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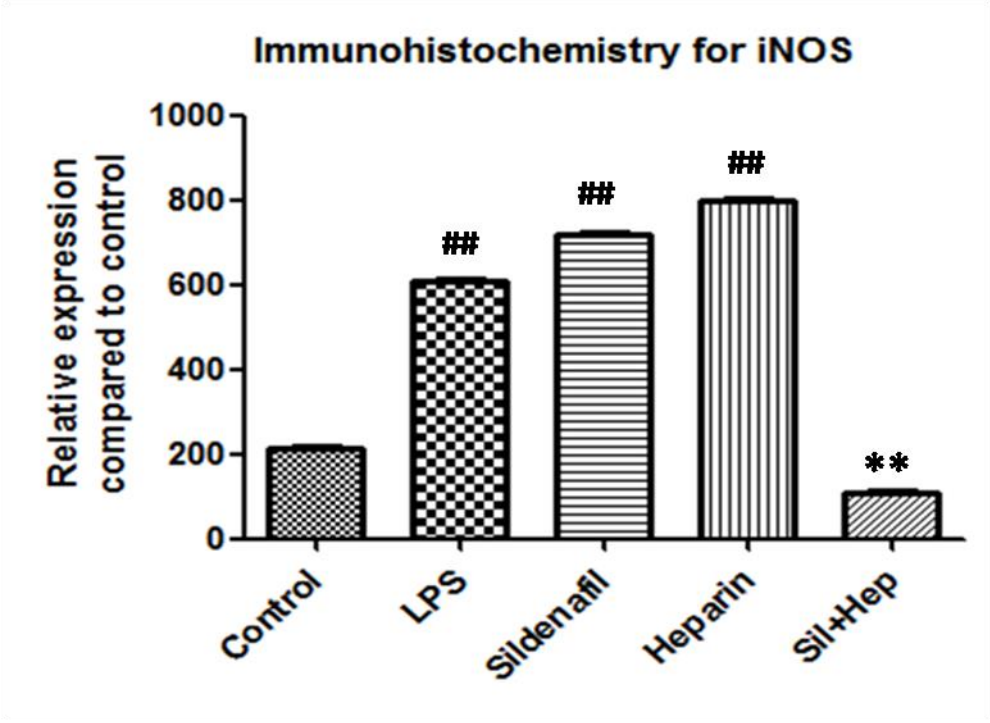
Immunohistochemistry for iNOS
Relative expression compared to control
1000
800
600
400
200
0
##
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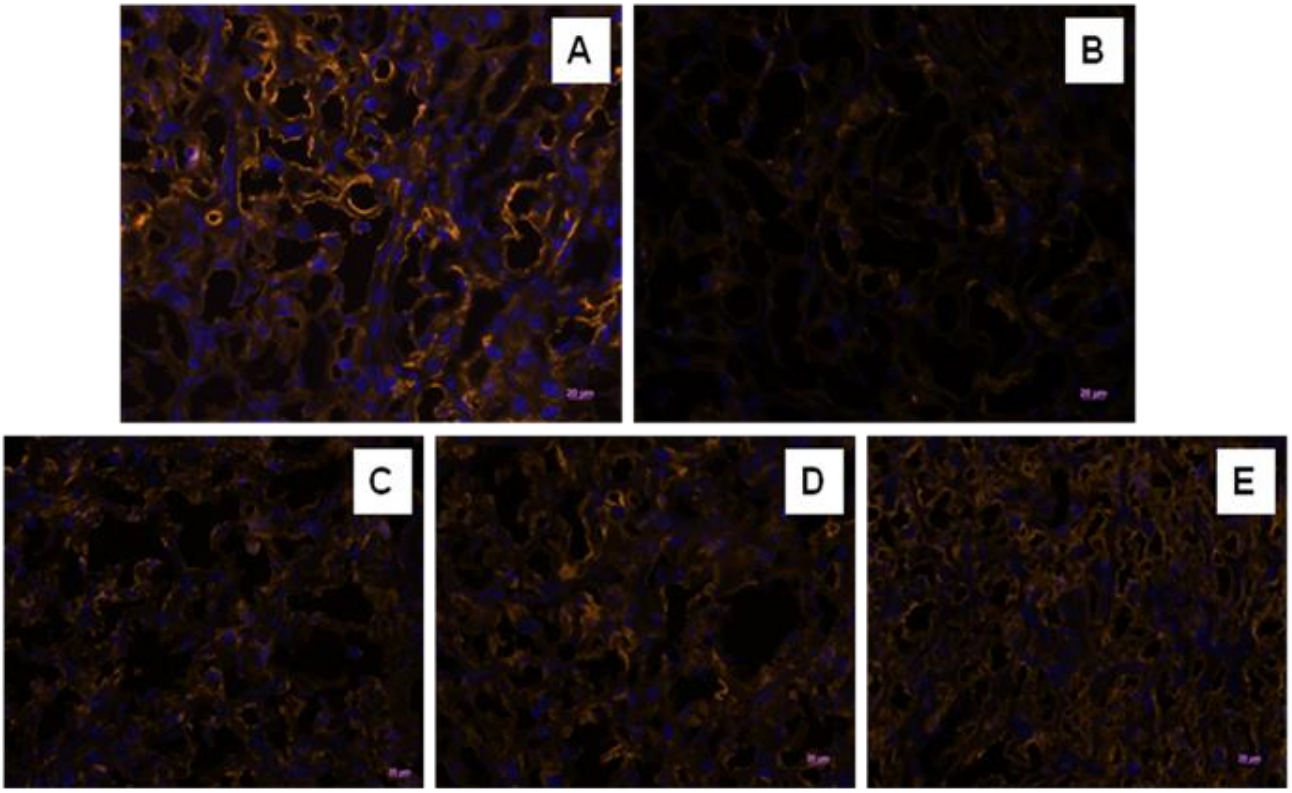
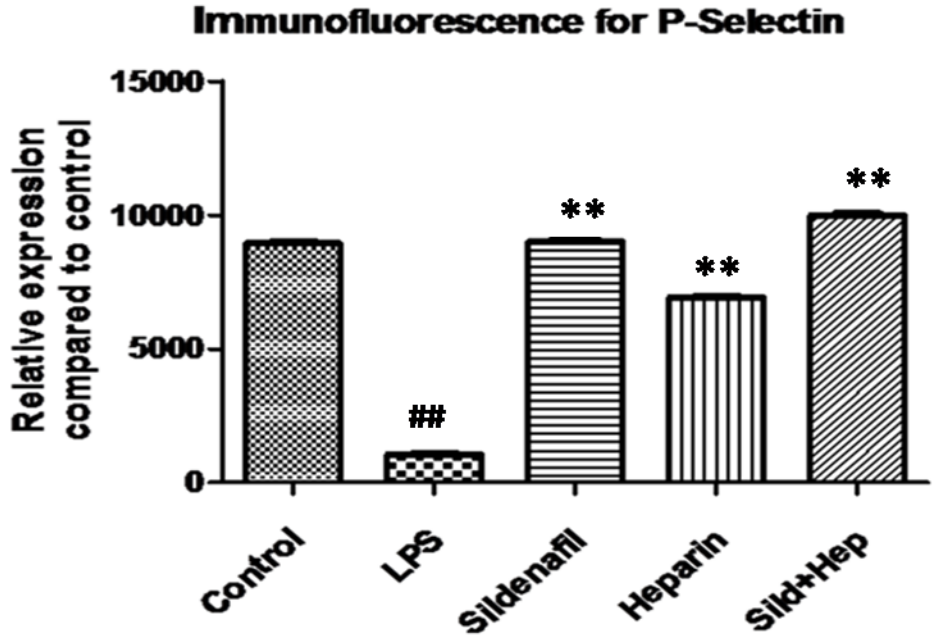
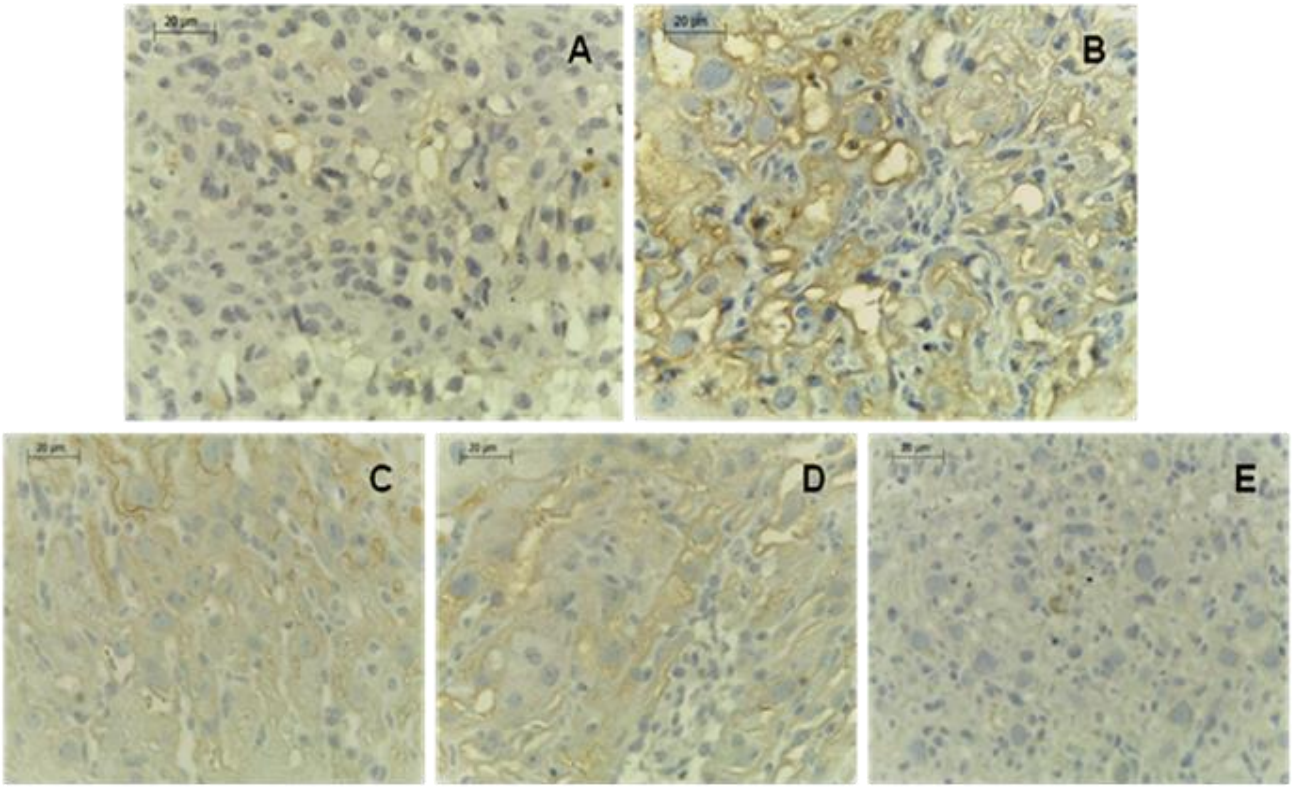
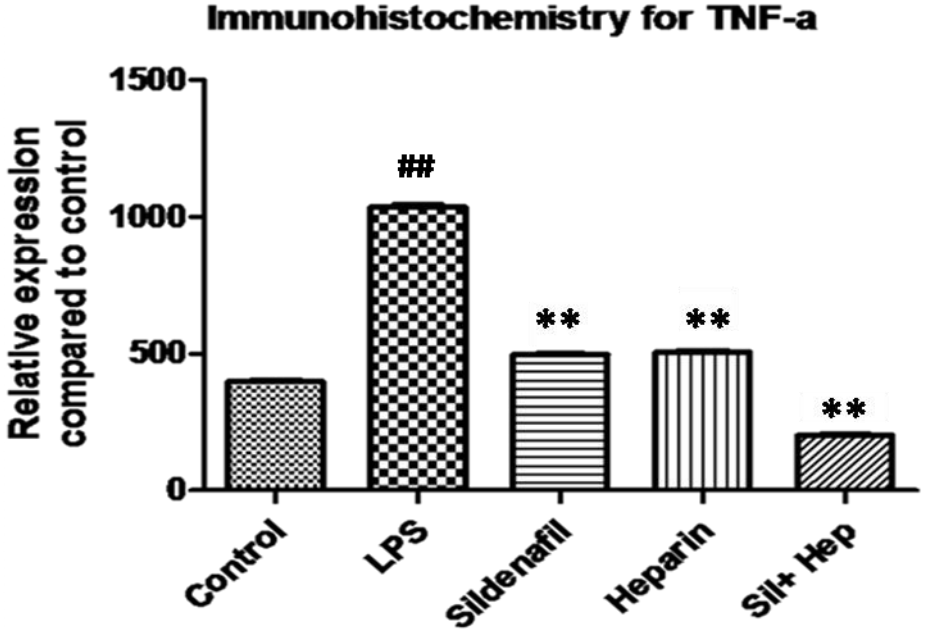
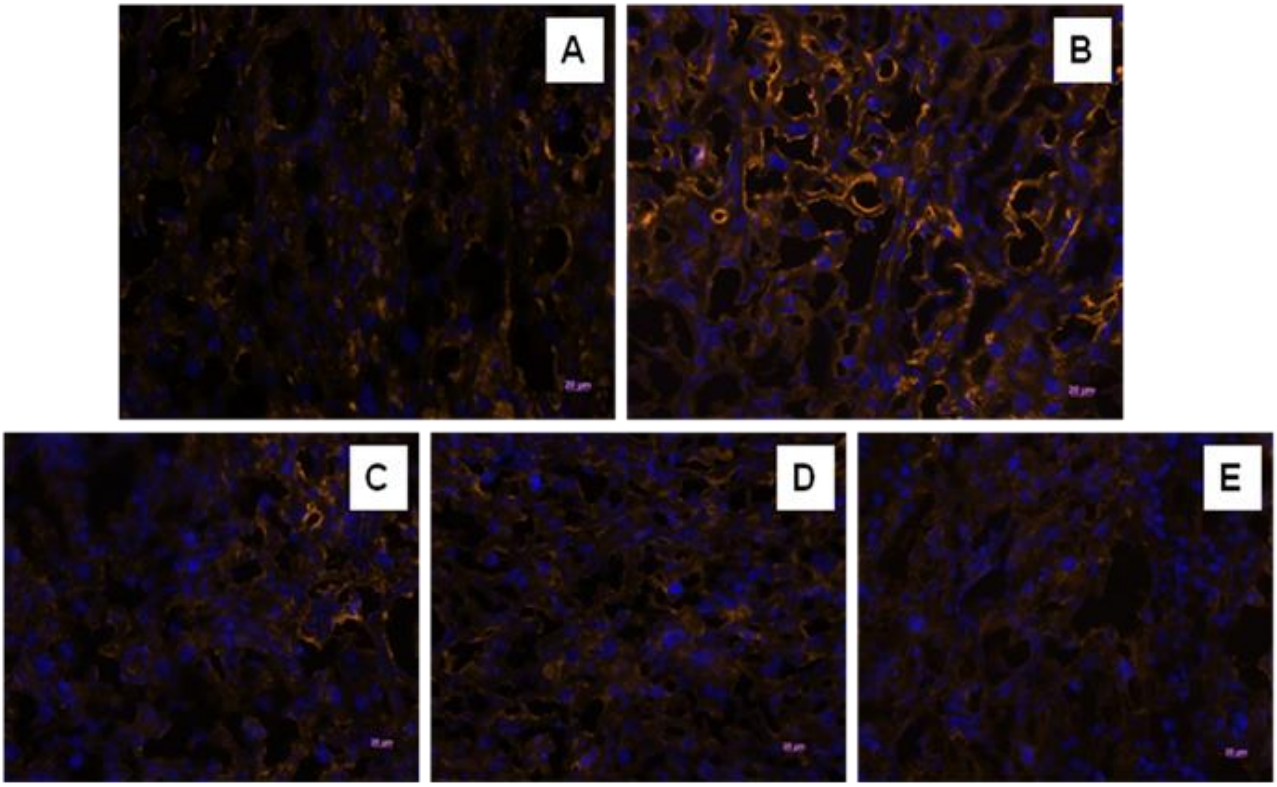
Control
LPS
Sildenafil
Heparin
Sil+Hep

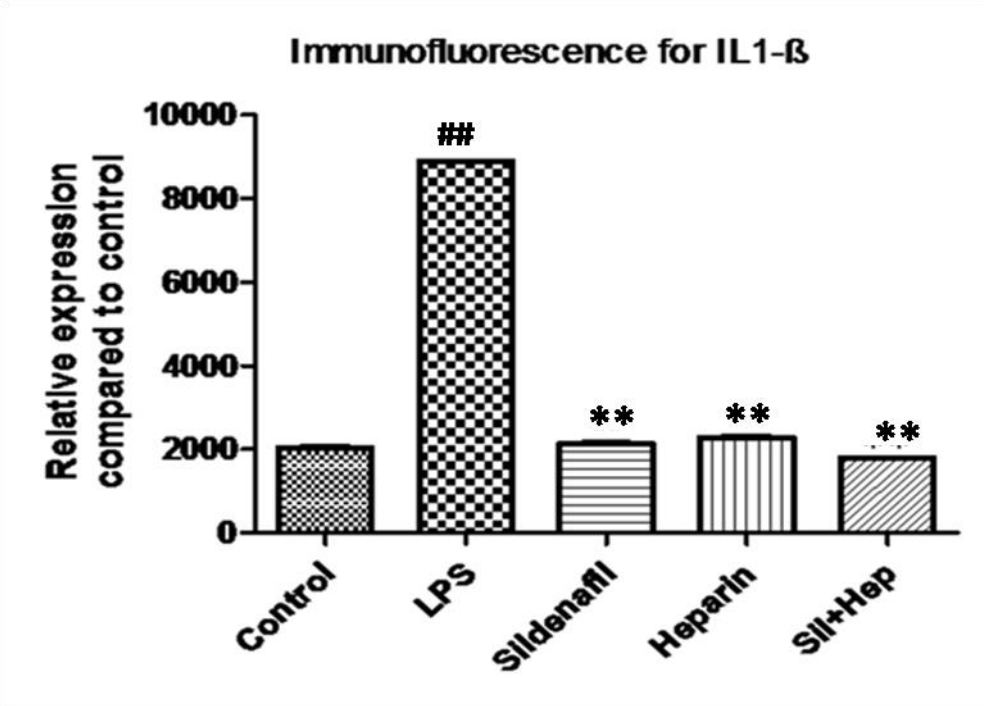

A
B
C
D
E

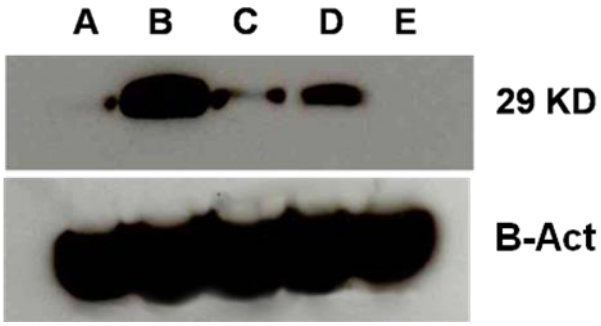

Immunofluorescence for P-Selectin
Relative expression compared to control
15000
10000
5000
0
##
**
**
**
Control
LPS
Sildenafil
Heparin
Sild+Hep

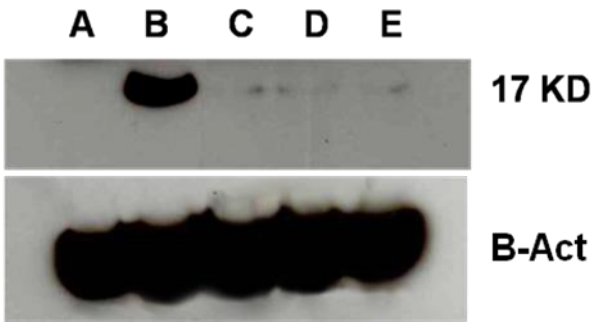

A
B
C
D
E

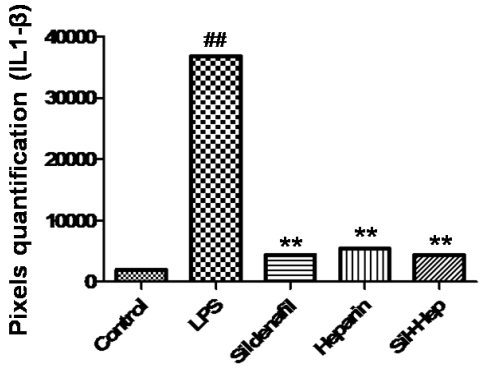

Immunohistochemistry for TNF-a
Relative expression compared to control
1500
1000
500
0
##
**
**
**
Control
LPS
Sildenafil
Heparin
Sil+ Hep

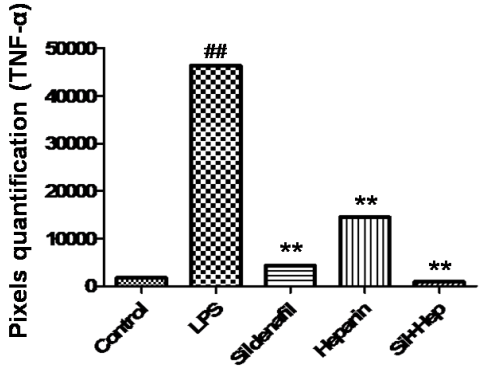

A
B
C
D
E


Immunofluorescence for IL1-ß
Relative expression compared to control
10000
8000
6000
4000
2000
0
##
**
**
**
Control
LPS
Sildenafil
Heparin
Sil+Hep


A B C D E
29 KD
B-Act


A B C D E
17 KD
B-Act


Pixels quantification (IL1-β)
40000
30000
20000
10000
0
##
**
**
**
Control
LPS
Sildenafil
Heparin
Sil+Hep


Pixels quantification (TNF-α)
50000
40000
30000
20000
10000
0
##
**
**
**
Control
LPS
Sildenafil
Heparin
Sil+Hep

5. CONCLUSÕES

O tratamento único com Sildenafil e o tratamento associado Sildenafil–Heparina pode reestabelecer o equilíbrio celular e molecular após a indução do modelo de perda gestacional induzida após 48hs de exposição ao LPS, diminuindo morte fetal a quase zero; alem de manter a integridade tecidual e ultraestrutural da placenta. Uma única injeção de Lipopolissacarideos no 15º dia de gestação resultou em um pico de citocinas inflamatórias. Após 2 horas, os tratamentos diminuíram a inflamação a níveis semelhantes e até menores do que o controle. P-Selectina, molécula constitutiva, importante para a gestação diminuiu sua expressão após indução do modelo, em todos os grupos tratados, os níveis dessa molécula de adesão apresentaram-se aumentados como no grupo controle.

As infertilidades femininas abrangem um grande campos de estudos, porém os mecanismos que envolvem os eventos de abortos recorrentes, ainda não estão totalmente esclarecidos. O Sildenafil (Viagra®) possui um ótimo nível de tolerabilidade e um amplo índice de segurança e vem sendo usado experimentalmente para o tratamento de diversas patologias, inclusive problemas relacionados ao desenvolvimento fetal como na Restrição de Crescimento Uterino; por sua ação vasodilatadora e mais recentemente descrita anti-inflamatória, o Sildenafil em tratamento único ou em tratamento associado com a Heparina pode ser útil para o tratamento de eventos de perdas fetais recorrentes principalmente aquelas que possui um fator inflamatório e trombótico.