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FERNANDA CARDOSO DA SILVA

DISFUNÇÃO ENDOTELIAL PELA INIBIÇÃO DA SÍNTESE DE ÓXIDO NÍTRICO: proposta e caracterização de um modelo celular *in vitro*

Declaramos que os resultados aqui apresentados ainda estão em fase de desenvolvimento e não foram publicados.

PATOS DE MINAS - MG
FEVEREIRO DE 2020

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Patos de Minas – MG

2020

“O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis." José de Alencar

Dedico esse trabalho aos meus pais,
Belchior e Celina, vocês são tudo para
mim! Amo vocês!

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RESUMO

O endotélio é um tipo de tecido epitelial, formado por uma única camada de células, que recobre internamente os vasos sanguíneos e é essencial para a manutenção da homeostase vascular, garantida pela produção de biomoléculas vasoativas. Um desequilíbrio na biodisponibilidade dessas biomoléculas pode desencadear um estado patológico, como a disfunção endotelial (DE), que se caracteriza, principalmente, pela redução na disponibilidade do óxido nítrico (NO) e por um aumento na produção de espécies reativas de oxigênio (EROs). Dada a importância do entendimento da disfunção endotelial para o progresso de patologias como a aterosclerose, por exemplo, faz-se necessário o desenvolvimento de modelos experimentais que simulem tal condição. Assim, esse estudo foi realizado visando desenvolver e caracterizar um modelo celular *in vitro* de DE pela inibição da síntese de NO. Inicialmente, realizou-se o tratamento das células endoteliais derivadas do timo (linhagem tEnd.1) com o inibidor da síntese de NO (L-NAME) nas concentrações 1µM, 10 µM, 100 µM e 1mM por 12, 24, 48, 72, 96 e 120 horas, com e sem retratamento a cada 24 horas. Posteriormente, determinou-se as melhores condições de tratamento para indução de um estado de DE nas células, a partir da avaliação da viabilidade celular e da concentração de nitrito. Os tratamentos com 10 µM e 100 µM de L-NAME, nos tempos de 72 horas sem retratamento e de 96 horas com retratamento foram selecionados. Em seguida, realizou-se a qPCR para os genes $p22^{phox}$, $p47^{phox}$ e eNOS. Os resultados foram significativos para o $p22^{phox}$, em que os níveis relativos de mRNA do gene foram reduzidos em 65% em resposta ao tratamento com L-NAME 100 µM por 96 horas com retratamento. A análise da expressão proteica da eNOS por Western blotting não foi bem-sucedida. Por fim, os resultados de avaliação de peroxidação lipídica pela técnica de Buege e Aust não foram estatisticamente significativos. Em conjunto, os resultados apontam que o tratamento com L-NAME 100 µM por 96 horas com retratamento foi capaz de induzir a DE, diminuindo a disponibilidade de NO, a qual foi verificada tanto pela redução na concentração de nitrito quanto pela redução na expressão de $p22^{phox}$. Entretanto, estudos adicionais, sobretudo visando aprofundar o papel das EROs nas células submetidas ao tratamento com L-NAME, são necessários para o aprimoramento e caracterização do modelo experimental proposto.

Palavras-chave: Óxido nítrico. L-NAME. Estresse oxidativo. $p22^{phox}$. Disfunção endotelial.

ABSTRACT

Vascular endothelium is a type of epithelial tissue, formed by a monolayer of cells, which internally covers blood vessels and is essential for the maintenance of vascular homeostasis, guaranteed by the production of vasoactive biomolecules. An imbalance in the bioavailability of these biomolecules may trigger a pathological state, such as endothelial dysfunction (ED), which is mainly characterized by a reduction in the availability of nitric oxide (NO) and an increase in the production of reactive oxygen species (ROS). Considering the importance of endothelial dysfunction for the progress of pathologies such as atherosclerosis, for example, it is necessary to develop experimental models that simulate such a condition. This study was then carried out to develop and characterize an in vitro cellular model of ED by inhibiting NO synthesis. Initially, the thymus-derived endothelial cells (tEnd.1 line) were treated with the NO synthesis inhibitor (L-NAME) at 1µM, 10 µM, 100 µM and 1mM for 12, 24, 48, 72 , 96 and 120 hours, with and without retreatment every 24 hours. Subsequently, the best treatment conditions for inducing an ED state in the cells were determined based on the evaluation of cell viability and nitrite concentration. Treatments with 10 µM and 100 µM of L-NAME for 72 hours without retreatment and 96 hours with retreatment were selected. Real-time PCR was performed for $p22^{phox}$, $p47^{phox}$ and eNOS genes. Results were significant for $p22^{phox}$, in which the relative levels of gene mRNA were reduced by 65% in response to 100 µM L-NAME for 96 hours with retreatment. Western blotting analysis of eNOS protein expression was not successful. Finally, results of the evaluation of lipid peroxidation using the Buege and Aust technique were not statistically significant. Together, the results show that treatment with 100 µM L-NAME for 96 hours with retreatment was able to induce ED, decreasing the availability of NO, which was verified by the reduction in the concentration of nitrite and by the reduction in the expression of $p22^{phox}$. However, additional studies aiming to determine the role of ROS in cells submitted to treatment with L-NAME are necessary for the improvement and characterization of the proposed experimental model.

Keywords: Nitric oxide. L-NAME. Oxidative stress. $p22^{phox}$. Endothelial dysfunction.

0LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

5-HT: 5-hidroxitriptamina

Ach: Acetilcolina

ADMA: inibidor de eNOS

AT-I: Angiotensina I

AT-II: Angiotensina II

B_2: Receptor beta-2

BK: Bradicinina

cAMP: 3’5’-adenosina-monofosfato-cíclico

CAT: Catalase

CE: Células endoteliais

cGMP: Monofosfato cíclico de guanosina

CMLVs: Células musculares lisas vasculares

COX: Ciclooxigenase

DE: Disfunção endotelial

DILA: Dilatação fluxo-mediada

DPI: Difenilenoiodônio

eNOS: Óxido nítrico sintase endotelial

ECA: Enzima conversora de angiotensina

EDHF: Fator hiperpolarizante derivado do endotélio

EROs: Espécies reativas de oxigênio

ERON: espécie reativa de oxigênio e nitrogênio

ET: Endotelina

ET-1: Endotelina 1

ET_B: Receptor de endotelina

FMN: Mononucleotídeo de flavina

GSH-Px: Glutationa peroxidase

GTP: Trifosfato de guanosina

HAS: Hipertensão arterial sistêmica

iNOS: Óxido nítrico sintase induzível

K^+: Íon potássio

L-arg: L-arginina

L-cit: L-citrulina

L-NAME: Nω-Nitro-L-arginina metil éster

M: Receptor muscarínico

nNOS: Óxido nítrico sintase neuronal

NO: Óxido nítrico

NOS: Óxido nítrico sintase

NOX: NADPH oxidase

$O_2^{\circ-}$: Ânion superóxido

PGH_2: Prostaglandina H_2

PGI_2: Prostaciclinas

SOD: Superóxido dismutases

tEnd.1: Linhagem de células endoteliais derivadas de timo

TP: Receptor para tromboxano-prostanóide

TXA_2: Tromboxano A_2

SUMÁRIO

CAPÍTULO 1

1. INTRODUÇÃO

1.1 Problema

O endotélio vascular é constituído por uma monocamada de células que reveste internamente os vasos sanguíneos e que atua como uma interface dinamicamente mutável (KHADDAJ MALLAT et al., 2017; GIMBRONE JÚNIOR; GARCÍA-CARDEÑA, 2013), sendo essencial para a manutenção da integridade e da homeostase vascular (DAIBER et al., 2019). Nesse sentido, a perda do equilíbrio entre a produção e a liberação de moléculas vasoconstritoras e vasodilatadoras definem um estado denominado disfunção endotelial (DE) (GIMBRONE JÚNIOR; GARCÍA-CARDEÑA, 2013).

A DE é uma condição caracterizada, principalmente, por uma redução na biodisponibilidade de óxido nítrico (NO), devido ao desacoplamento da NO sintase (NOS) (INCALZA et al., 2018). Consequentemente, há o aumento da produção de espécies reativas de oxigênio (EROs) e o desencadeamento de um processo inflamatório associado (DAIBER et al., 2019; INCALZA et al., 2018; RUDIC; SESSA, 1999; VANHOUTTE et al., 2017). A DE pode, então, causar um aumento da vasoconstrição, oxidação e alteração da permeabilidade da membrana plasmática, acúmulo de células do sistema imune (GIMBRONE JÚNIOR; GARCÍA-CARDEÑA, 2013; KONUKOGLU; UZUN, 2016), aumento da formação de agregados plaquetários e proliferação das células musculares lisas vasculares (CMLVs) (YUYUN; NG; NG, 2018).

A DE pode se apresentar como um fator de risco inicial para o desencadeamento de doenças vasculares e metabólicas (DAIBER et al., 2017), sendo um marcador importante em patologias como aterosclerose, hipercolesterolemia, hipertensão arterial sistêmica (HAS), doença arterial periférica e diabetes (FALCONER et al., 2018; HONG et al., 2019). Essas doenças são apontadas como a principal causa de morte no mundo, sendo que, em 2017, foram estimadas 17,8 milhões de morte tendo como causa as doenças cardiovasculares (KAPTOGE et al., 2019).

Portanto, considerando-se que a DE é um evento comum aos distúrbios metabólicos que culminam no desenvolvimento e progresso das patologias que mais matam em todo o mundo,

a compreensão sobre os mecanismos celulares e moleculares envolvidos nessa condição é de extrema importância para a condução dos tratamentos, bem como para a elaboração de novas estratégias terapêuticas.

1.2 Hipóteses

- O tratamento de células endoteliais (CE) com Nω-Nitro-L-arginina metil éster (L-NAME) induz um estado semelhante à DE em CE;
- O tratamento com L-NAME reduz a biodisponibilidade de NO e aumenta a produção de EROs.

1.3 Objetivos

1.3.1 Objetivo Geral

Desenvolver e caracterizar um modelo celular *in vitro* de DE por meio da inibição da síntese de NO.

1.3.2 Objetivos Específicos

- Padronizar as condições de cultivo *in vitro* das CE derivadas de timo (tEnd.1);
- A partir do tratamento com o inibidor da síntese de NO (L-NAME), determinar as melhores condições (concentração e tempo de tratamento) para indução de um estado semelhante à DE;
- Caracterizar o modelo de DE *in vitro* proposto observando as principais alterações na síntese de NO, expressão de NOS e parâmetros de estresse oxidativo.

1.4 Justificativa

Conforme mencionado anteriormente, as doenças cardiovasculares são as doenças não transmissíveis mais frequentes em todo o mundo e estão relacionadas com uma alta taxa de morbidade e mortalidade (KAPTOGE et al., 2019). A DE é um evento comum que precede o estabelecimento de patologias metabólicas e vasculares. Os mecanismos inerentes à DE ainda

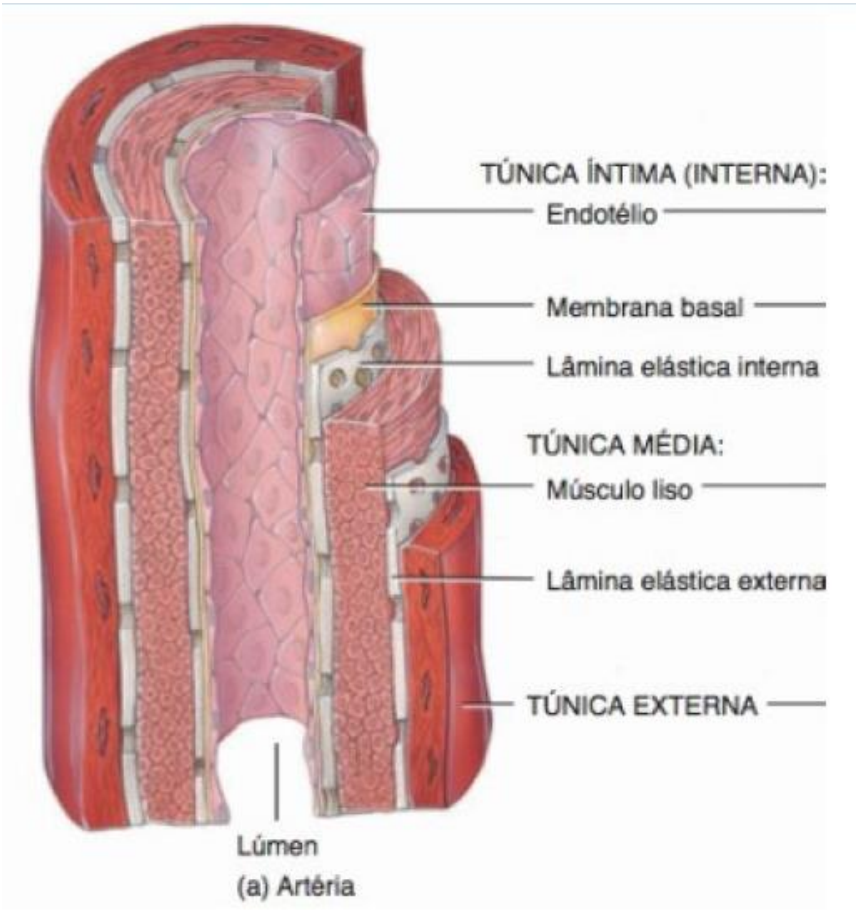
não estão completamente elucidados e os modelos experimentais *in vitro* disponíveis para o estudo dessa condição permanecem escassos. Portanto, o desenvolvimento de um modelo celular de disfunção endotelial poderá permitir o avanço das pesquisas na área, bem como contribuir para a redução de custos e demandas necessárias para a manutenção de modelos animais.

2. REFERENCIAL TEÓRICO

2.1. Funções do endotélio vascular

Os vasos sanguíneos são constituídos pela (i) túnica íntima, formada por uma camada de células endoteliais; (ii) túnica média, composta basicamente por CMLVs; e (iii) túnica adventícia, formada por um tecido fibroso que recobre externamente o vaso (KIERSZENBAUM; TRES, 2016) (Figura 1). O endotélio, objeto de nosso estudo, é constituído por uma monocamada de células, que reveste o interior dos vasos, estando em contato direto tanto com as células musculares lisas vasculares, quanto com as células sanguíneas e o plasma. Essa camada de células não é apenas uma barreira de proteção dos tecidos, mas também apresenta importantes funções, sendo bastante ativa na vasculatura (SENA; PEREIRA; SEIÇA, 2013). Essas células atuam de forma a detectar, integrar e transmitir a sinalização advinda dos tecidos locais e do sangue. Além disso, modulam uma resposta dinâmica liberando os estímulos vasoativos para a circulação (KHADDAJ MALLAT et al., 2017; GIMBRONE JÚNIOR; GARCÍA-CARDEÑA, 2013).

Figura 1- ***Representação das camadas que constituem a parede do vaso sanguíneo: túnica adventícia, túnica média e túnica íntima.***

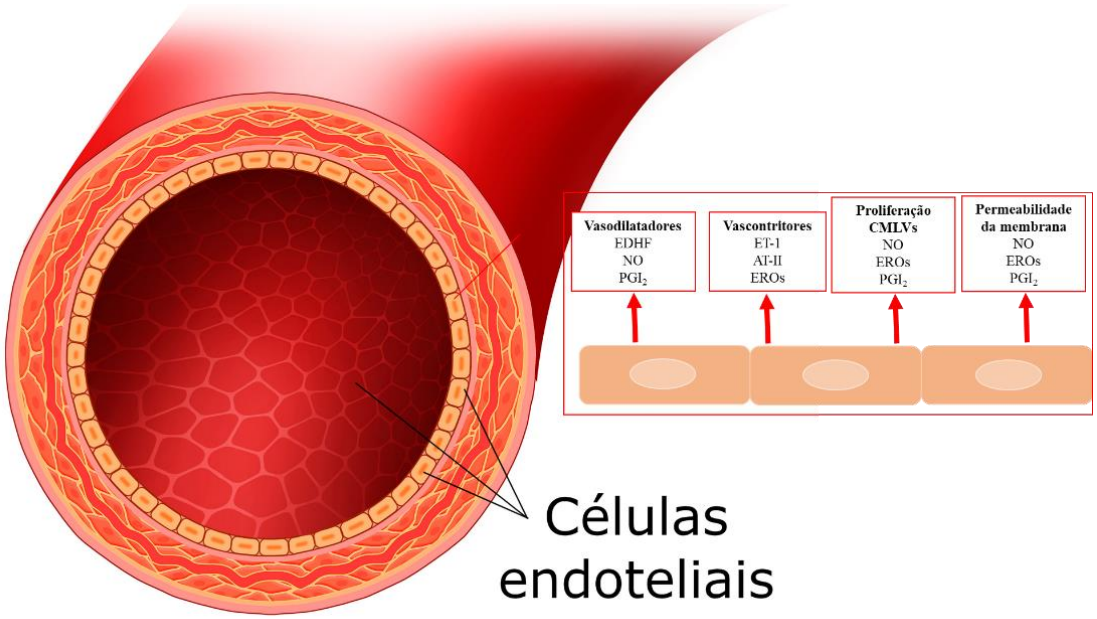


Fonte: (TORTORA, 2013).

O endotélio é essencial para a integridade e manutenção da homeostase vascular (DAIBER et al., 2019) ao regular a angiogênese, tônus vascular, permeabilidade celular e transporte capilar. Ainda, atua na resposta imune, regulando a adesão e infiltração de linfócitos (KHADDAJ MALLAT et al., 2017). Além disso, o endotélio é responsável por produzir biomoléculas capazes de realizar o controle entre a vasodilatação e vasoconstrição, liberação de procoagulantes e anticoagulantes e de protrombóticos e antitrombóticos (FALCONER et al., 2018; JAMWAL; SHARMA, 2018; YUYUN; NG; NG, 2018).

As principais biomoléculas produzidas pelo endotélio são o fator hiperpolarizante derivado do endotélio (EDHF), NO, prostaciclinas (PGI_2), endotelina-1 (ET-1), angiotensina II (AT-II) e EROs (SENA; PEREIRA; SEIÇA, 2013). Essas moléculas realizam importantes funções na vasculatura e o equilíbrio na sua produção é essencial para a manutenção da homeostase vascular. O EDHF, NO e PGI_2 possuem efeito vasodilatador e antiproliferativo; enquanto ET-1, AT-II e EROs possuem efeito vasoconstritor e ativam a proliferação celular (SENA; PEREIRA; SEIÇA, 2013) (Figura 2).

Figura 2 – ***Representação das principais biomoléculas produzidas pelas células endoteliais e suas funções.***



Fonte: Adaptado de SOARES, 2020.

O EDHF é importante no controle do tônus vascular, sendo produzido e liberado pelas CE a partir da ligação de acetilcolina e/ ou bradicinina aos seus receptores. A atuação desse fator através dos receptores presentes na musculatura lisa causa a hiperpolarização e o relaxamento desta, mediado pela ativação dos segundos mensageiros, monofosfato cíclico de guanosina (GMPc) e 3’5’-adenosina-monofosfato-cíclico (AMPc). Esses, por sua vez, desencadeiam a ativação de canais de potássio e a diminuição da concentração de cálcio no interior das células (BATLOUNI, 2001; KHADDAJ MALLAT et al., 2017).

O NO é um importante vasodilatador e será melhor descrito adiante. Já as PGI_2 atuam contribuindo para a vasodilatação e inibindo a agregação plaquetária. Isso se deve ao fato de que tanto as CMLVs quanto as plaquetas possuem os receptores de prostaciclinas. A ligação dessas moléculas aos seus receptores específicos conduz à ativação de adenilato ciclase e proteína quinase e promove o relaxamento da musculatura (ARNAL et al., 1999; BATLOUNI, 2001; KHADDAJ MALLAT et al., 2017). É importante ressaltar que o EDHF e o NO são os responsáveis majoritários pelo relaxamento vascular, com uma pequena contribuição das PGI_2 (BATLOUNI, 2001).

Entre os agentes vasoconstritores destacam-se as endotelinas (ET), as quais são peptídeos vasopressores que atuam diretamente em receptores nas CMLVs, ativando segundos

mensageiros e causando a vasoconstrição. Podem atuar também em receptores presentes nas CE, sendo responsáveis pela manutenção da função endotelial. Em condições fisiológicas, a concentração desses peptídeos é relativamente baixa, porém são os vasoconstritores mais potentes (KHADDAJ MALLAT et al., 2017).

A AT-II é obtida através da conversão da angiotensina I por ação da enzima conversora de angiotensina (ECA) e pode ocorrer em vários tecidos e células, inclusive nas células endoteliais (KRINSKI et al., 2007). Essa molécula desempenha diversas funções na vasculatura, como vasoconstrição, hipertrofia cardíaca, proliferação celular e formação de matriz extracelular (BATLOUNI, 2001).

Finalmente, as EROs apresentam importante função como vasoconstritores diretos e indiretos, podendo diminuir a biodisponibilidade de NO, reduzindo a vasodilatação dependente de NO. A produção e as funções das EROs na vasculatura serão melhor abordadas adiante (BATLOUNI, 2001; KHADDAJ MALLAT et al., 2017).

Diante da importância do endotélio para a manutenção da homeostase vascular, fica evidente a necessidade de se manter o equilíbrio entre a biodisponibilidade de moléculas vasoconstritoras e vasodilatadoras, uma vez que a DE decorre de uma alteração nessa homeostase. Na DE há um desbalanço entre a produção e a degradação de NO e EROs, representando um fator de risco preponderante no desencadeamento de doenças vasculares (GIMBRONE JÚNIOR; GARCÍA-CARDEÑA, 2013).

2.2. Via do óxido nítrico derivado do endotélio

O NO é um radical livre, de caráter inorgânico, que se encontra no estado gasoso em temperatura ambiente (DUSSE; VIEIRA; CARVALHO, 2003). A molécula é constituída por uma ligação covalente do nitrogênio com o oxigênio e apresenta um elétron desemparelhado, isso faz com que essa molécula possua uma alta reatividade (DUSSE; VIEIRA; CARVALHO, 2003). O NO derivado do endotélio é uma importante biomolécula vasomotora, que regula o tônus vascular, inibe a adesão e agregação plaquetária, inibe a inflamação e ativa a angiogênese de pequenos vasos (GHIMIRE et al., 2016).

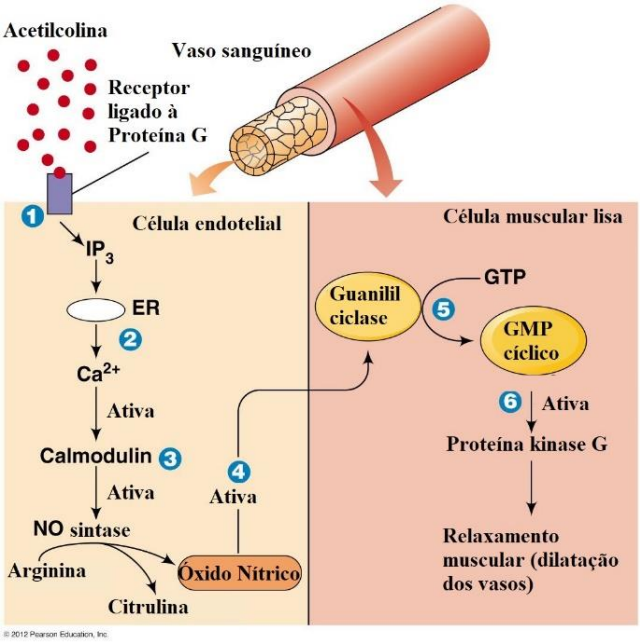
Essa molécula é sintetizada por ação da enzima óxido nítrico sintase (NOS), a qual converte L-arginina em L-citrulina com a redução de oxigênio e concomitante produção de NO (YUYUN; NG; NG, 2018). São reconhecidas três isoformas de NOS nos mamíferos: 1) a óxido nítrico sintase neuronal (nNOS); 2) a óxido nítrico sintase induzível (iNOS); e 3) a óxido nítrico sintase endotelial (eNOS) (HONG et al., 2019). Todas as isoformas das NOS se encontram

ativas na forma de dímeros (YUYUN; NG; NG, 2018) e necessitam de cofatores e coenzimas para a sua ativação como O_2, NADPH, flavinas e biopterinas (FLORA FILHO; ZILBERSTEIN, 2000).

A nNOS é expressa principalmente no sistema nervoso central e periférico, mas também em algumas organelas como as mitocôndrias, complexo de Golgi, retículo endoplasmático e membrana plasmática de alguns tipos celulares (YUYUN; NG; NG, 2018). Essa isoforma é codificada pelo gene *NOS1* (YUYUN; NG; NG, 2018). Já a iNOS, codificada pelo gene *NOS2*, está expressa, principalmente, em macrófagos, regulando a resposta imune; e na musculatura lisa vascular, cuja atividade é modulada por agentes indutores (YUYUN; NG; NG, 2018). Por fim, a eNOS, codificada pelo gene *NOS3*, é expressa principalmente no endotélio vascular, miócitos cardíacos e epitélio renal, sendo responsável pela produção de cerca de 70% do NO presente no plasma sanguíneo (FALCONER et al., 2018; GHIMIRE et al., 2016; SIRAGUSA; FLEMING, 2016) (YUYUN; NG; NG, 2018).

Após sua síntese, o NO pode atuar como vasodilatador direto ou indireto. Para sua atuação direta é necessária sua difusão através da membrana da célula endotelial, atuando nas CMLVs e ativando a enzima guanilato ciclase, que, por sua vez, converte trifosfato de guanosina (GTP) em GMPc. Este conduz à uma cascata de ativação enzimática que culmina na remoção de cálcio das células musculares e, consequentemente, no relaxamento da vasculatura (FALCONER et al., 2018) (Figura 3). Já a vasodilatação indireta ocorre via ação no tronco encefálico, reduzindo o fluxo simpático central e causando a diminuição da liberação de norepinefrina dos terminais nervosos simpáticos (AUGUSTYNIAK et al., 2005).

Figura 3 – *Representação da vasodilatação induzida pelo óxido nítrico produzido pelas células endoteliais*. *IP_3: Inositol trifosfato; Ca^{2+}: Íon cálcio; GTP: Guanosina trifosfato.*



Fonte: Adaptado de PEARSON EDUCATION, 2012.

Além de ser um potente agente vasodilatador, o NO induz a angiogênese, permitindo a síntese de vasos colaterais, e inibe a adesão de leucócitos no endotélio regulando processos inflamatórios (FALCONER et al., 2018). Pode também atuar prevenindo a agregação plaquetária e a ativação de fatores trombogênicos, inibindo a aterogênese (KHADDAJ MALLAT et al., 2017).

Nesse contexto, o NO e drogas doadoras de NO são investigadas quanto ao seu potencial para o tratamento de diversas doenças, como a hipertensão pulmonar e a aterosclerose, devido a sua ação como agente anti-inflamatório, vasodilatador e antitrombótico (EVORA et al., 2012; GHIMIRE et al., 2016; JAMWAL; SHARMA, 2018). Porém, a produção em excesso dessa biomolécula também pode ser prejudicial, visto que, um aumento descomunal na produção de NO pela iNOS, por exemplo, tem sido relacionado com a progressão do câncer de mama (GHIMIRE et al., 2016), um vez que o excesso de NO atua como espécie reativa de oxigênio e nitrogênio (ERON) (QUEIROZ; BATISTA, 1999). Logo, a manutenção da função endotelial e da correta produção de NO é essencial para a manutenção da saúde do indivíduo.

2.3. Espécies reativas de oxigênio (EROs): fontes e funções biológicas

O oxigênio molecular (O_2) é o aceptor final de elétrons na cascata de respiração celular dos seres aeróbicos, devido à sua configuração eletrônica e sua alta eletronegatividade

(RIBEIRO et al., 2005). Ao longo da cascata de respiração são produzidas moléculas intermediárias a partir do O_2, caracterizadas por sua alta reatividade, as quais são denominadas de EROs (INCALZA et al., 2018).

Nos seres aeróbicos, a produção de EROs se dá de forma natural e contínua, sendo o ânion superóxido ($O_2^{\circ-}$), o peróxido de hidrogênio (H_2O_2), o radical hidroxila (OH^-) e o peroxinitrito ($ONOO^-$) as principais EROs produzidas. A síntese de EROS é mediada por enzimas específicas que podem atuar utilizando outra molécula considerada ERO como substrato, ou a própria a produção de uma ERO pode desencadear a produção de outra por meio de sinalização celular (RIBEIRO et al., 2005).

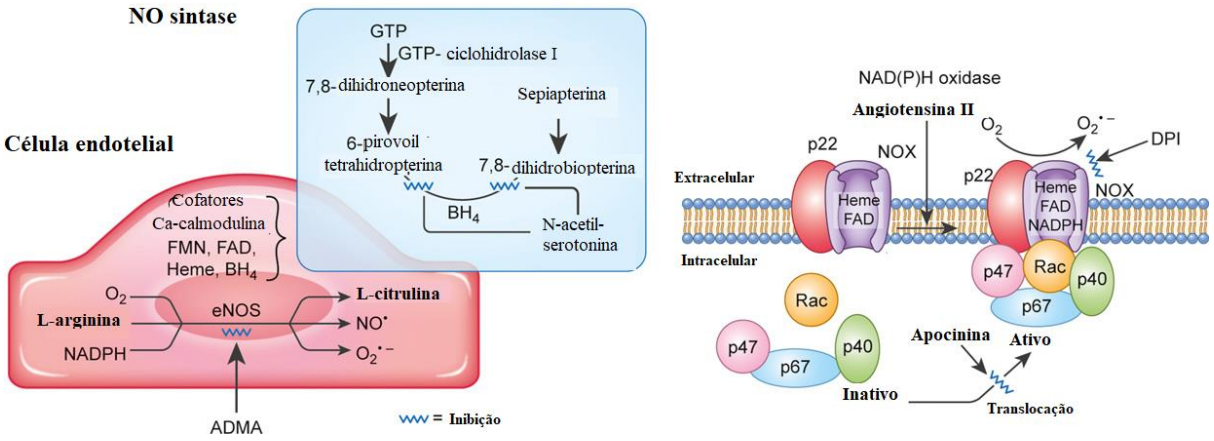
A síntese de EROs pode ocorrer em diversos tipos celulares (RIBEIRO et al., 2005). Em especial, nas células endoteliais, as principais enzimas envolvidas na síntese de EROs são as enzimas mitocondriais, as NADPH oxidases (NOX) e a eNOS desacoplada (INCALZA et al., 2018) (Figura 4). As enzimas mitocondriais são de grande importância para a respiração celular nos seres aeróbicos e são as principais fontes geradoras de EROs nos mamíferos (SILVA; FERRARI, 2011). Essas enzimas geram principalmente $O_2^{\circ-}$ em uma taxa que varia de 0,01 a 1% em uma mitocôndria saudável. Porém essa produção pode ser alterada por mecanismos fisiopatológicos, sendo que o aumento da produção de EROs pelas mitocôndrias pode ser prejudicial à integridade celular (KOWALTOWSKI, 2004).

As NOX são as únicas enzimas que possuem como função primária a geração de EROs. Elas são um complexo enzimático constituído por cinco componentes principais, sendo dois associados à membrana, $gp91^{phox}$ e $p22^{phox}$, e três citosólicos, $p47^{phox}$, $p67^{phox}$ e Rac1 ou Rac2. Esses componentes são importantes para a funcionalidade dessas enzimas, porém, em alguns tipos celulares, um ou mais componentes podem não ser expressos, tendo sua função suprida por outro componente (BABIOR, 2000). A NOX4, por exemplo, necessita apenas do componente $p22^{phox}$ para estar ativa em células endoteliais (NISIMOTO et al., 2010). As NOX geram principalmente H_2O_2 e $O_2^{\circ-}$ e são mais expressas em células fagocitárias (DRUMMOND; SOBEY, 2014). Nessas células, a produção de EROs é de grande importância para a resposta imune, uma vez que tais moléculas possuem efeito microbicida (DRUMMOND; SOBEY, 2014). As NOX também são expressas, em menor concentração, em outros tipos celulares, como por exemplo nas CE, nas quais NOX1, NOX2 e NOX5 geram $O_2^{\circ-}$, e NOX4 gera H_2O_2. Essas enzimas são de grande importância para o endotélio, uma vez que a superativação das isoformas NOX1, NOX2 e NOX5 está relacionada com o desencadeamento de um processo inflamatório, que pode culminar na DE e até mesmo na apoptose. A NOX4 é a mais expressa

nas células endoteliais e apontada como uma enzima de efeito vasoprotetor (DRUMMOND; SOBEY, 2014; LANGBEIN et al., 2015; LIAO et al., 2018).

A alteração na função da eNOS, conhecida por desacoplamento, faz com que essa enzima produza $O_2^{\bullet-}$ ao invés de NO nas CE (SENA; PEREIRA; SEIÇA, 2013). O desacoplamento da eNOS pode ocorrer devido à ausência de L-arginina, oxidação de tetraidrobiopterina (BH_4) (Figura 4), processo inflamatório ou estresse oxidativo (SENA; PEREIRA; SEIÇA, 2013). Esse desacoplamento causa a perda da conformação dimérica da enzima, que passa a ser um monómero que sintetiza mais $O_2^{\bullet-}$ do que NO. Essa condição aumenta as concentrações de EROs, o que afeta ainda mais a atividade endotelial (COUTO et al., 2014; GHIMIRE et al., 2016; VARADHARAJ et al., 2015).

Figura 4- Representação da produção de Espécies Reativas de Oxigênio (EROs) na parede vascular pela eNOS e pela NOX. *No lado esquerdo, tem-se a representação da produção de ânion superóxido via eNOS e a via de síntese de tetra-hidrobiopterina (BH_4), um importante cofator. À direita, a encontra-se representada a ativação da NAD(P)H oxidase (NOX). (FMN: mononucleotídeo de flavina; GTP: guanosina 5'-trifosfato; ADMA: inibidor de eNOS; DPI: difenilenoiodônio inibidor inespecífico das NOX).*



Fonte: Adaptado de VANHOUTE et al., 2017

É importante ressaltar que as EROs regulam a expressão de genes sensíveis ao sinais redox e também controlam a homeostase vascular por serem moléculas vasoativas (BATLOUNI, 2001; INCALZA et al., 2018; KHADDAJ MALLAT et al., 2017; RIBEIRO et al., 2005). Contudo, em concentrações supra-fisiológicas, essas moléculas podem reagir com lipídeos e carboidratos, conduzindo à sua oxidação; e com o DNA, causando danos genéticos (BONOMINI; RODELLA; REZZANI, 2015; RIBEIRO et al., 2005). Nesse sentido, a célula possui um sistema de controle da biodisponibilidade de EROs, que pode incluir mecanismos antioxidantes enzimáticos e não enzimáticos. O excesso de EROs ativa e/ ou induz a expressão

de enzimas antioxidantes como superóxido dismutases (SOD), catalase (CAT), glutationa peroxidase (GSH-Px), peroxirredoxinas e tioredoxina (RIBEIRO et al., 2005). O mecanismo antioxidante não enzimático, por sua vez, é uma proteção das biomoléculas realizada por moléculas (como carotenoides, compostos fenólicos e ascorbato) que podem evitar a produção de radicais livres ou causar a redução das EROs comprometendo sua reatividade (RIBEIRO et al., 2005).

Os mecanismos de controle antioxidantes existem para evitar que ocorra o estresse oxidativo (INCALZA et al., 2018), caracterizado pela produção excessiva e sustentada de EROs e/ou à baixa disponibilidade de agentes antioxidantes (FÖRSTERMANN; XIA; LI, 2017; WEFERS; SIES, 1983). Tal situação encontra-se associada a condições patológicas e ao próprio envelhecimento celular (SIES, 2015). Em especial, evidências apontam que, nas doenças cardiovasculares, a produção de EROs é aumentada, contribuindo para a sintomatologia das doenças (HARRISON; GONGORA, 2009; SINHA; KUMAR DABLA, 2015) e para os danos vasculares (MONTEZANO et al., 2015).

2.4. Disfunção endotelial (DE) e patologias associadas

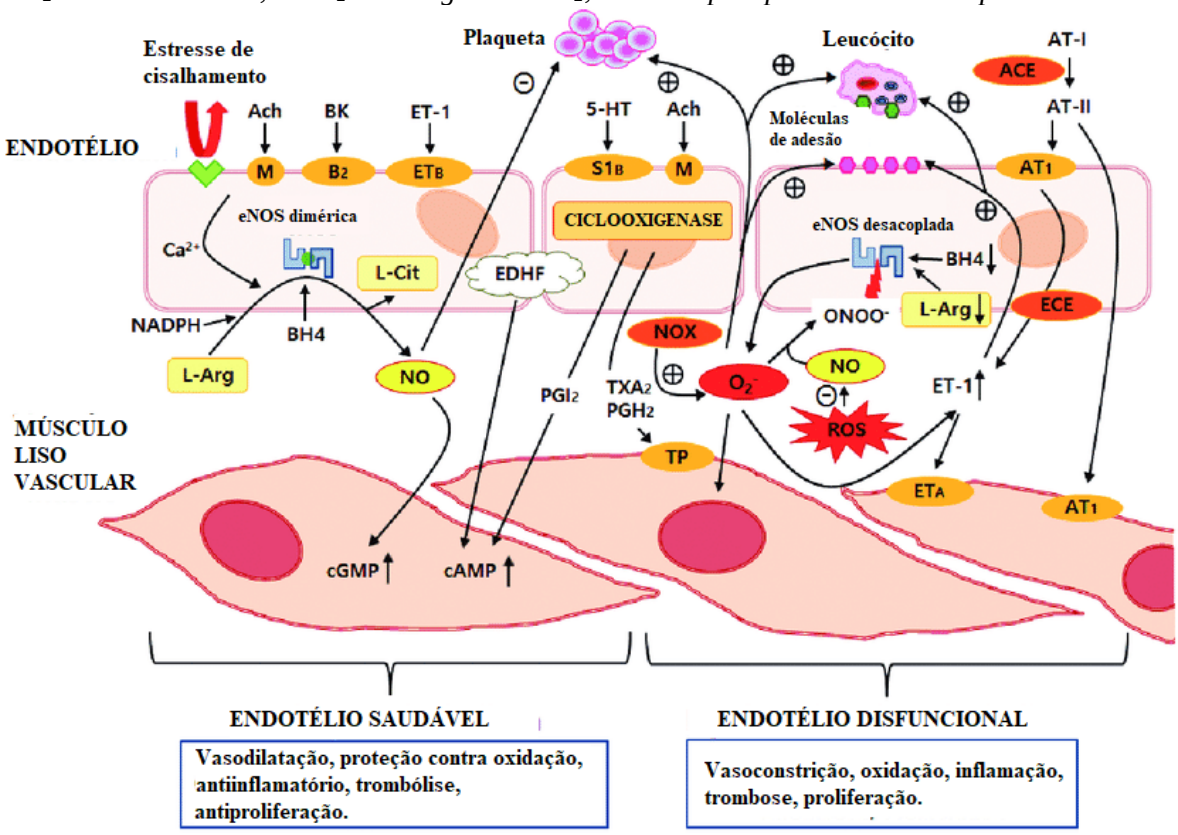
A DE é uma condição caracterizada por um processo inflamatório concomitante ao estresse oxidativo, o que ocasiona a perda da função endotelial decorrente do dano celular (DAIBER et al., 2017). Diante do importante papel realizado pelo NO, a DE é geralmente relacionada a uma redução da biodisponibilidade dessa biomolécula, a qual é o primeiro e um dos mais significativos eventos que caracterizam a DE (INCALZA et al., 2018; RUDIC; SESSA, 1999; VANHOUTTE et al., 2017).

Adicionalmente, a DE está intimamente ligada ao aumento da concentração de EROs (DAVEL; BRUM; ROSSONI, 2014; INCALZA et al., 2018), sendo esse aumento uma das causas do desacoplamento da eNOS, da redução de sua expressão (DAIBER et al., 2019) e do aumento da degradação de NO (SENA; PEREIRA; SEIÇA, 2013). Em conjunto, esses eventos ocasionam um comprometimento na regulação do tônus vascular e outras alterações fenotípicas nas CE (INCALZA et al., 2018), tais como redução da vasodilatação, aumento da inflamação e indução de um estado pró-trombótico (ENDEMANN; SCHIFFRIN, 2004).

A DE e os sintomas relacionados, entretanto, não incluem apenas a alteração no metabolismo do NO e da consequente vasoconstrição, mas também a oxidação das lipoproteínas com alteração da permeabilidade da membrana plasmática, acúmulo de leucócitos, alteração no metabolismo extracelular (GIMBRONE JÚNIOR; GARCÍA-

CARDEÑA, 2013; KONUKOGLU; UZUN, 2016), agregação e adesão plaquetária, proliferação das CMLVs e inflamação vascular (YUYUN; NG; NG, 2018) (Figura 5). A DE representa, portanto, um mecanismo patológico relacionado a diferentes fatores e é apontada como uma condição presente em várias doenças metabólicas e cardiovasculares como aterosclerose, doença arterial periférica, diabetes, hipercolesterolemia e HAS (DAIBER et al., 2019; FALCONER et al., 2018; HONG et al., 2019).

Figura 5 - ***Produção de biomoléculas em um endotélio saudável e em um endotélio disfuncional***. *Ach: Acetilcolina; BK: Bradicinina; ET-1:Endotelina 1; M: Receptor muscarínico; B_2: Receptor beta-2; ET_B: Receptor de Endotelina; 5-HT: 5-hidroxitriptamina; AT-I: Angiotensina I; AT-II: Angiotensina II; TXA_2: Tromboxano A2; PGH_2: Prostaglandina H_2; TP: Receptor para tromboxano-prostanóide.*



Fonte: Adaptado de PARK; PARK, 2015.

A aterosclerose tem a DE como um importante marcador. A produção ineficiente e anormal de NO, concomitante ao aumento dos níveis de EROs, desencadeiam o dano no endotélio, que, por sua vez, causa agregação plaquetária, oxidação de lipídeos, inflamação vascular e progressão da doença (GHOSH et al., 2017; YUYUN; NG; NG, 2018). Pesquisas atuais buscam estratégias de tratamento que visam atuar na via do NO, assim como na sua síntese, com o intuito de substituir as drogas tradicionais. Essas novas terapias visam principalmente aumentar a expressão de eNOS e a inibição de seu desacoplamento (HONG et al., 2019; KHADDAJ MALLAT et al., 2017).

A doença arterial periférica é caracterizada pela oclusão progressiva de vasos grandes e médios dos membros inferiores (WILLIANS et al., 2012). Nessa patologia, a DE também é uma condição primária e pode predizer o prognóstico dos pacientes. Portanto, drogas capazes de aumentar os níveis de NO podem ser potentes no tratamento da doença arterial periférica (FALCONER et al., 2018).

O diabetes tipo 2 está comumente associado ao estresse oxidativo na vasculatura, somado a uma baixa concentração de antioxidantes naturais, acarretando na disfunção endotelial, a qual, em um sistema de cascata, agrava a condição do paciente (KHADDAJ MALLAT et al., 2017). É comum que pacientes diabéticos apresentem uma redução na elasticidade dos vasos e hipertensão arterial secundária (KHADDAJ MALLAT et al., 2017). De fato, o diabetes causa a ativação da enzima aldose redutase, que afeta a fosforilação e a expressão da eNOS (JAMWAL; SHARMA, 2018).

Outra patologia importante é a hipercolesterolemia, uma condição que estimula a oxidação do colesterol, a liberação de ET e a geração de EROs (JAMWAL; SHARMA, 2018). Todos esses fatores estão relacionados à uma redução na expressão de eNOS e à perturbação da integridade do endotélio vascular, ou seja, estão associados com a DE (JAMWAL; SHARMA, 2018).

Finalmente, na HAS, a DE atua diretamente no quadro da doença, sendo o NO uma molécula chave para a adequada manutenção dos níveis de pressão arterial. Dessa forma, em pacientes com HAS essencial ou primária observam-se baixos níveis de NO circulante, edema subendotelial causado por aumento da permeabilidade vascular, aumento da aderência leucocitária à parede vascular, aumento da agregação plaquetária e aumento da proliferação de CMLVs (BATLOUNI, 2001; CARVALHO et al., 2001; DAVEL et al., 2011; HAMILTON et al., 2001). Além disso, pacientes com HAS apresentam altos níveis de liberação de ET, prostanóides vasoconstritores, AT-II, citocinas inflamatórias e xantina oxidase, culminando com o aumento na degradação de NO (JAMWAL; SHARMA, 2018). Nesse sentido, moléculas doadoras de NO atuam compensando a redução ocorrida durante a DE e têm se mostrado potentes agentes terapêuticos para a HAS (GHEIBI et al., 2018; KONUKOGLU; UZUN, 2016; WENCESLAU; ROSSONI, 2014).

Compreende-se, portanto, que a DE é uma condição presente em diversas doenças cardiovasculares e metabólicas. Levando em conta que tais patologias podem coexistir em um mesmo indivíduo e que a DE é um evento comum nessas doenças, a DE pode tratada com um potencial alvo para o desenvolvimento de novas estratégias terapêuticas (JAMWAL; SHARMA, 2018). Faz-se necessário, então, o estudo mais aprofundado da disfunção endotelial

e dos mecanismos e vias envolvidos no seu estabelecimento e progressão. Nesse sentido, abordagens metodológicas capazes de avaliar a função endotelial *in vitro* e *in vivo* se fazem necessárias.

2.5. Estratégias para o teste da função endotelial

Diferentes abordagens são adotadas para avaliar a função endotelial e identificar características gerais da DE. Esses métodos possibilitam o desenvolvimento de métodos diagnósticos para diversas doenças relacionadas à DE (GIRIBELA et al., 2011). Nesse contexto, algumas análises permitem identificar CE inteiras ou apoptóticas na circulação, marcadores circulantes da função endotelial e a dilatação fluxo-mediada (DILA) da artéria braquial (GIRIBELA et al., 2011; INCALZA et al., 2018).

Durante a DE, o estresse oxidativo, concomitante com a inflamação crônica, causa a lesão celular, formando corpos apoptóticos ou até mesmo a liberação de CE inteiras na circulação. Essas células podem ser identificadas pela coleta e análise do sangue (GIRIBELA et al., 2011; INCALZA et al., 2018). Além disso, marcadores da função endotelial circulantes, como o NO, moléculas de adesão celular, citocinas inflamatórias, agentes trombóticos e antitrombóticos, podem ser identificados (INCALZA et al., 2018). Vale ressaltar, no entanto, que alguns desses marcadores são de difícil identificação e dosagem, como o NO que possui uma meia-vida muito curta, o que limita a viabilidade dessa técnica (GIRIBELA et al., 2011; PREMER et al., 2019).

In vivo, a dosagem de NO em amostras biológicas é um processo complexo e com uma alta margem de erro. A integridade funcional do endotélio pode ser observada por meio da DILA, em que utiliza-se uma ultrassonografia Doppler para observar a dilatação ou não da artéria após uma isquemia induzida pela inflação de um manguito colocado no membro superior (CELERMAJER et al., 1992). Esse estímulo é suficiente para a liberação de NO pelo endotélio, caso esse esteja íntegro, causando a dilatação da artéria (FALCONER et al., 2018; GIRIBELA et al., 2011; PREMER et al., 2019).

Entretanto, é importante ressaltar que ainda existe a necessidade de desenvolvimento de novos métodos e estratégias, pois muitas das ferramentas disponíveis são de difícil execução e/ou de alto custo (GIRIBELA et al., 2011). Dessa forma, a concepção de condições experimentais que se assemelham à DE *in vitro* possibilita maior compreensão dessa condição, facilitando a análise *in vivo* e o desenvolvimento de técnicas de diagnóstico e terapia para essa doença.

2.6. Inibição da síntese de NO e indução de disfunção endotelial

O L-NAME é um pró-fármaco com capacidade inibitória da síntese de NO (PFEIFFER et al, 1996), pois é um análogo de L-arginina, o substrato para a síntese de NO. A inibição da NOS pelo L-NAME ocorre por meio da ligação desse inibidor à enzima no sítio catalítico, competindo com o substrato e impedindo a ligação da L-arginina (PETERSON et al, 1992; REES, et al., 1990). Além disso, o L-NAME é um inibidor reversível e não seletivo das NOSs comumente utilizado em experimentos a longo e curto prazo, seja *in vitro* ou *in vivo*, com o objetivo de identificar os eventos associados à restrição da produção de NO (KOPINCOVÁ; PÚZSEROVÁ; BERNÁTOVÁ, 2012). Dados da literatura descrevem os efeitos do L-NAME em modelos animais. A administração de L-NAME, seja injetável ou na água de beber, conduz ao aumento na vasoconstrição, de pressão arterial e bradicardia (REES, et al., 1990); além do aumento da resistência vascular periférica, hipertrofia do ventrículo esquerdo do coração, redução da resposta relaxante nos vasos calibrosos, aumento da resposta vascular à estímulos adrenérgicos e inflamação perivascular nos indivíduos experimentais (KOPINCOVÁ; PÚZSEROVÁ; BERNÁTOVÁ, 2012; TÖRÖK, 2008).

Sabendo-se que a DE é comum em pacientes com hipertensão essencial e que está intimamente relacionada à redução da biodisponibilidade de NO, um modelo animal de indução de hipertensão foi previamente desenvolvido (REES, et al., 1990). Este consiste na administração de L-NAME de forma crônica em doses altas, induzindo uma hipertensão denominada hipertensão por deficiência de NO em ratos que antes não eram hipertensos (REES, et al., 1990). *In vitro*, por sua vez, resultados demonstraram que a inibição causada pelo L-NAME da produção de NO em anéis de aorta é dose-dependente e leva a um aumento da contração dependente do endotélio, redução de vasodilatação dependente de acetilcolina (Ach) (REES, et al., 1990), desestabilização da membrana lisossomal (FOMINA et al., 2018) e a redução da biodisponibilidade de NO (KOPINCOVÁ; PÚZSEROVÁ; BERNÁTOVÁ, 2012). Até o presente, todavia, não foi descrito um modelo de indução de condição semelhante à DE em cultura de células.

Dessa forma, sabendo-se que CE em cultura são expostas a diferentes situações e estímulos próprios da DE, esse tipo celular se torna mais suscetível ao desacoplamento da eNOS e à indução de um estado semelhante à DE. Estudos apontam que o aumento da oxidação de lipoproteínas de baixa densidade, alta concentração de glicose, homocisteína e angiostatina são capazes de induzir tal condição (INCALZA et al., 2018). Por isso, buscou-se observar nesse

trabalho se o tratamento com o L-NAME, e consequente diminuição na biodisponibilidade de NO, também seria capaz de realizar essa indução.

CAPÍTULO 2

ENDOTHELIAL DYSFUNCTION BY INHIBITING NITRIC OXIDE SYNTHESIS: PROPOSAL AND CHARACTERIZATION OF AN *IN VITRO* CELLULAR MODEL

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Endothelial dysfunction by inhibiting nitric oxide synthesis: Proposal and characterization of an *in vitro* cellular model

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Abstract

Vascular endothelium plays an important role in the maintenance of vascular homeostasis, mediated by vasoactive molecules produced by endothelial cells. The balance between vasoconstrictor and vasodilator biomolecules is what guarantees this equilibrium. Therefore, an increase in the bioavailability of vasoconstrictors together with a reduction in vasodilators may indicate a condition called endothelial dysfunction. Endothelial dysfunction is mainly characterized by a reduction in nitric oxide (NO) bioavailability and an increase in the production of reactive oxygen species (ROS), triggering an inflammatory process. This condition is a predictive marker of several cardiovascular diseases (e.g. atherosclerosis, hypertension and diabetes), but research is affected by the scarcity of suitable *in vitro* models that simulates such condition. In this context, the goal of this study was to induce an *in vitro* condition that mimics endothelial dysfunction by inhibiting NO synthesis. Thymus-derived endothelial cells (tEnd.1) were treated with different concentrations of L-NAME (1µM, 10 µM, 100 µM and 1mM) for different times (12, 24, 48, 72, 96 and 120 hours with and without retreatment every 24 hours). Cell viability, nitrite concentration, $p22^{phox}$ gene expression and lipid peroxidation were evaluated. Results indicated that treatment with 100 µM L-NAME for 96 hours with retreatment was able to trigger a reduction in NO concentration by 94% and reduce $p22^{phox}$ expression by 65%. We thus propose that 100 µM L-NAME treatment for 96 hours caused a condition similar to endothelial dysfunction. Besides, our results show for the first time, the $p22^{phox}$ expression in tEnd.1 cell line. However, additional studies aiming to determine the role of ROS in cells submitted to treatment with L-NAME are necessary for the improvement and characterization of the proposed experimental model.

Introduction

The endothelium consists of a cell monolayer, that not only acts as a tissue barrier, but also has important biological functions. In this sense, endothelial cells are very active in the vasculature [1] and essential for the integrity and maintenance of vascular homeostasis. Also, they perform important functions as regulator of angiogenesis, vascular tone, and cellular permeability, influencing in capillary transport [2].

To perform such diverse functions, endothelial cells produce a range of important biomolecules. The main biomolecules produced by the endothelium are endothelium-derived hyperpolarizing factor (EDHF), nitric oxide (NO) and prostacyclins (PGI_2), which have vasodilatory and antiproliferative effects on vascular smooth muscle cells. On the other hand, endothelial cells also generate endothelin-1 (ET-1), angiotensin II (AT-II), and reactive oxygen species (ROS), which present vasoconstrictor effects and promote vascular smooth muscle cells proliferation [1].

Endothelial dysfunction is a condition characterized by an inflammatory process concomitant with oxidative stress, which causes loss of endothelial function and consequent

imbalance in the production of biomolecules [2]. Two of the most important events that characterize endothelial dysfunction are the reduction in NO bioavailability and a considerable increase in the production of ROS, such as superoxide anion [3-5]. This condition represents a pathological mechanism that is related to a variety of factors and is pointed as a predictor of distinct cardiovascular and metabolic diseases [2].

It is thus clear that endothelial dysfunction is a conserved target in metabolic disorders. In line with that, given that different metabolic and cardiovascular diseases may coexist in the same patient and that endothelial dysfunction is a common event in these diseases, endothelial dysfunction may also be used as a target for new therapies [6]. It is therefore necessary to deepen the studies regarding endothelial dysfunction to better understand the mechanisms and pathways involved in this condition.

Different *in vitro* and *in vivo* approaches may be used to access endothelial function. At present, however, there is no specific protocol to mimic *in vitro* endothelial dysfunction, which limits the study of this condition at the cellular level and to search for biomolecules targeting diagnosis and treatment. Therefore, this study aimed to develop a protocol to simulate endothelial dysfunction *in vitro* by inhibiting NO synthesis in thymus-derived endothelial cells (tEnd.1).

Materials and methods

Cell Culture

Murine thymus-derived endothelial cell line tEnd.1 (RRID: CVCL_6272) was cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO®), enriched with 10% fetal bovine serum (FBS, GIBCO®), 100 U/mL penicillin and 100 µg/mL streptomycin (GIBCO®) at 37 °C, 5% CO_2 in an humidified incubator until reaching 80 to 90% confluence. Cell morphology during cultivation is shown in Supplementary Figure 1. Cells were used between the 3rd and 8th passages. Depending on the experiment, cells were plated in 6, 12 or 96-well plates, with 2 x 10^5 cells/ well, 4 x 10^4 cells/ well and 1 x 10^3 cells/ well, respectively.

L-NAME Treatment

After 24 hours of plating and prior to treatments, cells were made quiescent by fetal bovine serum deprivation (0.5%) for 3 hours and subsequently subjected to L-NAME

(SIGMA®) treatment (1µM, 10 µM, 100 µM and 1mM) or 100 µM L-arginine (SIGMA®) treatment, as a negative control [7] for 12, 24, 48, 72, 96 and 120 hours with and without retreatment every 24 hours. Retreatments were performed every 24 hours by replacing the "old culture medium" by the "new culture medium" with the same treatment.

Cell Viability

Following cell treatments, 25 µL of Tetrazolium Blue Thiazolyl Bromide (MTT) (Ludwig Biotec®) was added at a concentration of 5 mg/ mL in PBS (w/v) to each well and plates were left for 4 hours in the incubator. The culture medium with MTT excess was then aspirated, followed by the addition of dimethyl sulfoxide (DMSO) to dissolve formazan crystals [8]. MTT method is based on the ability of living cells to reduce the yellow tetrazolium salt to the purple insoluble formazan, which precipitates thanks to the action of the mitochondrial enzyme succinyl dehydrogenase, active only on living cells [8]. Optical reading was performed on an automatic plate reader at 560 nm (Readwell PLATE, ROBONIK®). Cell viability results were obtained according to Equation 1.

$$\%\ Cell\ viability = \frac{(A_t - A_b)}{(A_c - A_b)} \times 100 \quad (1)$$

Where:

A_t: Absorbance at 560 nm of treated cells (cells + treatment)

A_b : Absorbance at 560 nm of blank wells (only DMSO)

A_c: Absorbance at 560 nm of control cells (culture medium + cells)

Nitrite Quantification

Nitrite quantification was performed as an indirect measurement of NO levels. The treatments were performed as described before, but using phenol red free DMEM (GIBCO, Grand Island, New York, USA) to not influence the readings. Nitrite content was determined using a Griess reagent kit (Thermo Fisher Scientific®) according to the manufacturer's instructions. The culture medium from each well was collected, centrifuged at 16,000 rpm, 4°C (Hermle Labor Technik, Z 36 HK) for 10 minutes and the supernatant was kept for further analysis. In a 96-well microplate, 20 µL Griess reagent, 150 µL of nitrite-containing sample

and 130 µL deionized water were mixed. After 30 minutes of incubation in the dark at room temperature, the plate was read on an ELISA plate reader (Readwell PLATE, ROBONIK®) at 560 nm. Nitrite concentration in the samples was calculated based on a standard curve of different sodium nitrite concentrations (1, 5, 10, 30, 50 and 100 µM).

Real-Time PCR Gene Expression Analysis

After treatments, total cellular RNAs were extracted with Trizol® reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The quality of the extracted RNAs is represented in Supplementary Figure 2. Reverse transcription was performed as previously described [9] (Supplementary Figure 3). The reference gene B-actin was used (5'GGGAAATCGTGCGTGACATC3' and 5'GCCACAGGATTCCATACCCAA3') to validate RNA quality of each sample and for normalization of qPCR assays. For validation, conventional PCR reactions were performed as follows: 2.0 µL of cDNA amplicons, 1.0 U of Taq DNA Polymerase Platinum (Invitrogen), 50 mM KCl; 10 mM Tris– HCl pH 8.3, 2.0 mM MgCl2, 200 µM dNTPs, 5.0 pmol of each primer, all components incubated for 25 cycles at 94 °C 30 s, 60 °C 1 min, 72 °C 1 min, preceded by an initial denaturation at 95 °C for 5 min (Supplementary Figure 3).

For qPCR, each 2 µL aliquot of cDNA was amplified with 5 pmol of each specific primer (EXXTEND®) for eNOS [10], $p47^{phox}$ (5'ATCCCCAGCCAGCACTATGTG3' and 5'GAGATCCACACAAGAGAACAGAG3') and $p22^{phox}$(5'CCAGTGTGATCTATCTGCTGGCA3' and 5'GCCTCCTCTTCACCCTCACTC3'). The reactions were conducted in six replicates in a total volume of 10 µL containing Power SYBR_ Green PCR Master Mix (Applied Biosystems - Carlsbad, CA, USA) in a thermal cycler (StepOnePlus™ Real-Time PCR System, Applied Biosystems). Standard relative curves for all primers were constructed and expression of each gene was quantified through comparative Cq method.

Western Blotting Protein Expression Analysis

Following cells treatment, total proteins were obtained using an extraction buffer (1% Triton X-100, 135 mM NaCl, 20 mM Tris, pH 8.0 and 10% glycerol). The crude lysate was centrifuged (14000 x g, 10 minutes, 4 °C). The supernatant was then collected, and 20 µg of

total protein was separated by 10% SDS-polyacrylamide gel electrophoresis. After separation, proteins were transferred to nitrocellulose membranes (Amersham Protran Premium - GE Healthcare, Life Science, USA). Firstly, membranes were blocked with 3% bovine serum albumin (SIGMA®) for 1 hour at room temperature. Primary antibodies to proteins of interest eNOS (Purified Mouse Anti-eNOS / NOS Type III, BD transduction Laboratories™) and actin (Anti-Actin Antibody, clone C4, MERCK®) were incubated overnight at 4 °C. Peroxidase-conjugated IgG secondary antibodies were used to detect the primary antibody (Jackson Immunoresearch, West Grove, PA, USA). Bands were revealed by chemiluminescence on X-ray films (T-MAT G/ RA Film, KODAK, Sao Jose dos Campos, Sao Paulo, Brazil) using the ECL detection system (WESTAR SUN, Cyanagen, Bologna, Italy).

Determination of Lipid Peroxidation

The quantification of lipid peroxidation is essential to evaluate oxidative stress in pathophysiological processes. One of the main products of lipid peroxidation is malondialdehyde (MDA), the most abundant aldehyde generated by the attack of reactive species on polyunsaturated fatty acids in cell membranes [11]. MDA levels were determined by testing thiobarbituric acid reactive substances using the method of Buege & Aust (1978) [11], which is based on the ability of thiobarbituric acid (TBA) to bind to oxidized lipids. Briefly, 2 x 10^7 cells submitted to different treatments were homogenized in 1 mL of cold 20mM Tris HCL (pH 7.4) buffer. All homogenate was mixed with trichloroacetic acid (28% w/v in 0.25 N HCl), TBA (1% in 0.25 M acetic acid) and BHT (125 mM in ethanol), heated for 1 hour at 95 °C and then placed in an ice bath. The precipitate was then removed by centrifugation at 10,000 x g for 15 minutes at 4 °C, and the supernatant absorbance was determined at 535 nm in a spectrophotometer (Gehaka, UV-340 G). MDA levels were calculated using 1,1,3,3-tetramethoxypropane as standard for constructing the calibration curve (12.5, 6.25, 3.125, 1.562, 0.781 and 0.390 µmoL/L).

Statistical Analysis

Data are presented as mean ± standard deviation for each of the measurements performed. Sample number (n) represents the number of experiments performed with different treatments in the cell line culture. For the comparison between groups, Two-way and One-way

analysis of variance (ANOVA) was applied and Tukey's multiple comparisons test was used as *post-hoc* test, because the distribution was normal. Differences between groups were considered significant at $p < 0.05$. Data were analyzed using GraphPad Prism software, version 7.00 for Windows.

Results

Cell Viability

The MTT assay results show how different treatments affected the viability of the tEnd.1 cells. In groups without retreatment, 100 µM L-arginine increased cell viability (18.67%) after 48 hours, while reduced cell viability (28.77% and 37.52%) after 96 and 120 hours, respectively (Fig.1). In general, L-NAME increased cell viability after 24, 48, 96 and 120 hours of treatment. Differences were most evident in L-NAME-treated cells at any concentration for 24 hours (cell viability approximately 140%); 10 µM and 100 µM L-NAME for 48 hours (cell viability 133.85% and 134.83%, respectively); 100 µM for 96 hours (cell viability 122.88%); 10 µM, 100 µM and 1 mM for 120 hours (cell viability 163.52%, 121.76% and 122.29%, respectively) (Fig.1). Treatments performed for 72 hours without retreatment had the least influence on cell viability when compared to control (Fig.1).

Fig. 1. Cell viability of the thymus-derived endothelial cell line tEnd.1 after treatment with L-arginine (100 µM) and L-NAME (1 µM, 10 µM, 100 µM, 1 mM) for 12, 24, 48, 72, 96 and 120 hours without retreatment. Data (mean ± standard deviation, n=5) were analyzed by Two-way ANOVA, followed by Tukey's multiple comparisons test. (*represents significant difference between groups, for $p < 0.05$; **represents significant difference between groups, for $p < 0.01$; ***represents significant difference between groups, for $p < 0.001$; ****represents significant difference from the control group, for $p < 0.0001$).

In groups that experienced retreatment every 24 hours (Fig.2), 100 µM L-arginine generally does not affect cell viability, except after 72 hours with reduction of 26,04% of cell viability. Once again, L-NAME increased cell viability after 24, 48 and 72 hours of treatment,

in most L-NAME treated (Fig.2). Comparing to untreated cells, after 24 hours, L-NAME at any concentration increased cell viability by approximately 40%. In a same manner, 100 µM and 1 mM L-NAME after 48 hours also increased cell viability around 38.22% and 27.51%, respectively. The treatments for 96 and 120 hours with retreatment every 24 hours were the ones that least influenced cell viability when compared to control cells (Fig.2).

Fig. 2. Cell viability of the thymus-derived endothelial cell line tEnd.1 after treatment with L-arginine (100 µM) and L-NAME (1 µM, 10 µM, 100 µM, 1 mM) for 48, 72, 96 and 120 hours with retreatment. Data (mean ± standard deviation, n=5) were analyzed by Two-way ANOVA, followed by Tukey's multiple comparisons test. (*represents significant difference between groups, for $p < 0.05$; **represents significant difference between groups, for $p < 0.01$; ***represents significant difference between groups, for $p < 0.001$; ****represents significant difference between groups, for $p < 0.0001$).

Nitrite Quantification

Results presented in Figures 3 and 4 show that in both groups (without and with retreatment) nitrite concentration increases in control group until it reaches a peak after 72 hours, and then decreases.

Fig. 3. Nitrite concentration in culture medium of the thymus-derived endothelial cell line tEnd.1after treatment with L-arginine (100 µM) and L-NAME (1 µM, 10 µM, 100 µM, 1 mM) for 12, 24 , 48, 72, 96 and 120 hours without retreatment. Data (mean ± standard deviation, n=5) were compared by Two-way ANOVA, followed by Tukey's test. (*represents statistically significant difference compared between groups for $p < 0.01$; **represents statistically significant difference compared between groups for $p < 0.01$; ***represents significant difference compared between groups, for $p < 0.001$; ****represents significant difference compared between groups, for $p < 0.0001$).

Fig. 4. Nitrite concentration in culture medium of the thymus-derived endothelial cell line tEnd.1 after treatment with L-arginine (100 µM) and L-NAME (1 µM, 10 µM, 100 µM, 1 mM) for 48, 72, 96 and 120 hours with retreatment every 24 hours. Data (mean ± standard deviation, n=5) were

compared by Two-way ANOVA, followed by Tukey's test (*represents statistically significant difference compared between groups for p <0.01; **represents statistically significant difference compared between groups for p <0.01; ***represents significant difference between groups, for p <0.001; ****represents significant difference between groups, for p <0.0001).

Treatment with L-arginine increased nitrite concentration in the culture medium, which was more evident after 72, 96 and 120 hours, with an increase of 21.98%, 61.90% and 62.68% respectively, when compared to untreated cells (Fig.3.). L-NAME at different concentrations tend to reduce nitrite concentration in the culture medium in a dose-dependent manner. This reduction was more evident in 10 µM, 100 µM and 1 mM L-NAME after 24, 48, 72, 96 and 120 hours without retreatment. The largest reductions were observed with 1 mM L-NAME (91.37%), 100 µM L-NAME (70.83%) and 10 µM L-NAME (70.35%) after 72 hours of treatment, when compared to untreated cells (Fig.3.).

In groups with retreatment (Fig.4), L-NAME significantly reduced nitrite concentrations as follows: with reductions of 62.05% at 100 µM and 92.60% at 1mM after 48 hours; 24.10% at 10 µM, 69.55% at 100 µM and 95.14% at 1 mM after 72 hours; and 86.01% at 1 mM after 120 hours. After 96 hours with retreatment, results were more uniform and consistent with the literature, with reductions of 32.62% at 10 µM, 51.47% at 100 µM and 94.30% at 1 mM L-NAME (Fig.4).

As endothelial dysfunction is characterized by reduced NO bioavailability, cells treated with 10 µM and 100 µM L-NAME for 72 hours without retreatment and for 96 hours with retreatment were chosen for subsequent experiments since they significantly reduced nitrite concentration and did not affect cell viability. Cells treated with 100 µM L-arginine were used as negative control at the same times of treatment.

Real-Time PCR Gene Expression Analysis

The results showed that relative levels of $p22^{phox}$ mRNA did not differ after different treatments for 72 hours (Fig.5 A). On the other hand, the treatment with 100 µM L-NAME for 96 hours with retreatment exhibited a significant reduction of approximately 65% in $p22^{phox}$ mRNA levels when compared with control group (Fig. 5B). It is noteworthy that the expression of $p22^{phox}$ gene in tEnd.1 cells was firstly reported in the present study. No amplification for the $p47^{phox}$ and eNOS genes were obtained with the selected primers.

Fig. 5. Relative levels of $p22^{phox}$ gene mRNA in the thymus-derived endothelial cell line tEnd.1 after treatment with L-arginine (100 µM) and L-NAME (10 µM and 100 µM) for (A) 72 hours without retreatment (B) for 96 hours with retreatment. Data (mean ± standard deviation, n=3) were compared by One-way ANOVA, followed by Tukey's test. (**represents statistically significant difference from the control group for $p < 0.01$).

Western Blotting Protein Expression Analysis

Once we observed a peak of nitrite concentration after 72 hours cells treated with 100 µM L-arginine, 10 µM and 100 µM L-NAME the goal of this experiment was to determine eNOS protein expression levels at this time point. Unfortunately, it was not possible to visualize eNOS expression in tEnd.1 cells by the method used (Supplementary Fig. 4).

Determination of Lipid Peroxidation

Thiobarbituric acid reactive substances (TBARS) were quantified to see if lipid peroxidation, a common event in endothelial dysfunction, was occurring in cells submitted to different treatments. The results obtained showed that there was no significant difference between the different treatments (Fig.6).

Fig. 6 - Concentration of thiobarbituric acid reactive substances (TBARS) given in µmol/ mL PTN in the thymus-derived endothelial cell line tEnd.1 medium after treatment with L-arginine (100 µM) and L-NAME (10 µM and 100 µM) for 72 hours without retreatment. Data (mean ± standard deviation, n=3) were compared by One-way ANOVA. No statistical differences were observed between groups.

Discussion

Endothelial dysfunction is a primary condition of many cardiovascular diseases but is still little explored as a target for diagnosis and treatment [12-14]. Given this, the interest in the study of this condition has gradually grown over the years and studies focusing on the evaluation of endothelial function have shown to be very promising [15].

Researchers have already proposed that certain treatments may induce a condition similar to endothelial dysfunction in cell culture when they mimic the metabolic changes inherent to this pathological state. It is well known that treatment with native and oxidized low density lipoproteins, angiostatin, homocysteine and high glucose are capable of causing eNOS uncoupling, inducing a state similar to endothelial dysfunction [3]. However, this is the first time that endothelial dysfunction has been proposed using a NO synthesis inhibitor (L-NAME) as an inducer of this condition.

Most research regarding endothelial dysfunction is performed *in vivo* and little is known about what happens at the cellular level. *In vitro* research is also of great importance for a better understanding of this condition and to elucidate the pathways involved in endothelial cells. For these reasons, there is a crescent need for a standardization of a method of inducing *in vitro* endothelial dysfunction [16].

Throughout the present research, we proposed the development of a protocol to mimic endothelial dysfunction *in vitro* by inhibiting NO synthesis using L-NAME. Initially, we evaluated how treatments affected endothelial cells viability. It is already known that NO affects the viability of endothelial cells and inhibits cell apoptosis induced by inflammation or atherosclerotic factors [17]. In addition, when NO synthesis is induced by vasoactive agents, there is autocrine regulation of microvascular events, causing neovascularization, resulting from endothelial cell angiogenesis, growth and migration [18]. This inhibition of apoptosis is what contributes to NO having a significant effect as an anti-inflammatory and pro-angiogenic molecule [17]. L-arginine is an important amino acid that is considered versatile due to the fact that it is the substrate for the synthesis of many molecules, including NO. Studies indicate that L-arginine supplementation increases endometrial cell proliferation by a NO-dependent mechanism [19]. In endothelial cells, L-arginine is known to play an important role in cell survival during oxidative stress [20].

Considering the above mentioned, a reduction in cell viability in L-NAME-treated cells and an increase in L-arginine-treated cells was expected, but this did not occur. Cell viability was increased in most L-NAME-treated cells. This can be justified by the fact that NOS isoforms can also generate superoxide anion [21], an important ROS, which is cytotoxic and capable of affecting the organization of cellular plasma membrane, leading to apoptosis or even

necrosis [22-23]. Thus, treatment with L-NAME inhibits both NO and superoxide production, and inhibition of superoxide synthesis may contribute to greater cell proliferation, as noted in the results [24].

Despite the fact that endothelial dysfunction may reduce cell viability, *in vitro* studies should pay attention to treatments that affect cell survival, since the viability may be affected directly by the drug used in the treatment and not by the pathological condition. In addition, it is known that numerous pathways are involved in cell proliferation and death processes [25]. Therefore, for a better study of endothelial dysfunction we proposed the use of concentrations and treatment times that least altered cell viability which were 72 hours without L-NAME retreatment and at 96 hours with L-NAME retreatment.

NO plays an important role in protecting the endothelium against abnormal constrictions and atherosclerosis of the larger caliber arteries [5], and is, therefore, considered one of the essential biomolecules for the maintenance of vascular homeostasis [3]. One of the striking features of endothelial dysfunction is the reduction in NO bioavailability, which can occur either by a reduction in its synthesis or by an increase in its degradation [5]. A reduction in NO bioavailability could be achieved by treatment with L-NAME because it reduces NO synthesis in a dose-dependent manner, as observed from nitrite quantification results. This is due to the fact that L-NAME, one of the first synthetic inhibitors of NOS, has good experimental application and is already widespread in investigating NO involvement in different processes [26].

Treatment with L-arginine in most of time points showed an increase in nitrite concentration, indicating a possible NO synthesis. NO is known to be synthesized from L-arginine as a substrate, and the absence or impairment of L-arginine could reduce the synthesis of NO, characterizing a classic endothelial dysfunction. L-arginine supplementation has been shown to be beneficial for patients with vascular disease, as it contributes to increase NO synthesis [5]. In addition, this increase in NO synthesis caused by L-arginine indicates good NOS activity in these cells [27].

Based on the results of cell viability and indirect NO quantification, it was hypothesized that cells treated with 10µM and 100µM L-NAME for 72 hours without retreatment and for 96 hours with retreatment would be able to mimic a condition similar to endothelial dysfunction. However, endothelial dysfunction is not only characterized by reduced NO bioavailability and another important feature of this condition is the increased synthesis of ROS, and consequent oxidative stress [3, 28]. In endothelial cells it is known that the main sources of ROS are NADPH oxidases (NOX), xanthine oxidase, COXs and eNOS itself, when uncoupled [5]. NOX

and uncoupled eNOS are pointed as the main sources of ROS in an endothelial dysfunction condition [29].

Endothelial NOS can be uncoupled due to lack of L-arginine substrate or lack of tetrahydrobiopterin (BH_4) cofactor [5], as well as constant hyperactivation [30]. Enzyme uncoupling produces superoxide instead of NO. However, the protocol performed in this study by treatment with L-NAME did not allow the observation of superoxide production by eNOS, since L-NAME also inhibits superoxide synthesis as mentioned before, being used in experiments aimed at identifying the source of superoxide [31-32].

Therefore, we investigated the expression of NOX isoforms components, $p22^{phox}$ and $p47^{phox}$, already described as expressed in endothelial cells [33-34] and as a component of the main isoforms present in these cells, NOX1, NOX2, NOX4 and NOX5 [29]. NOX1, NOX2 and NOX5 are characterized by their direct involvement in the onset of inflammation, apoptosis and endothelial dysfunction, while NOX4, by contrast, is characterized as an important vasoprotective agent, involved in suppression of cell death pathways and increased NO bioavailability [29, 35].

Langbein and collaborators (2015) observed in *in vivo* studies that the endothelial function was compromised in the thoracic aorta of mice knockout for the Nox4 gene and in a hyperlipidic diet, causing an endothelial dysfunction [36]. NOX isoforms are generally characterized by synthesizing $O_2^{\circ-}$, with the exception of NOX4 which synthesizes H_2O_2 [37]. While $O_2^{\circ-}$ can react with NO and cause the formation of another ROS, peroxynitrite, reducing the bioavailability of NO, H_2O_2 does not interact with NO. In addition, research highlights the importance of producing H_2O_2, which has a vasodilating action in pathological conditions, acting as a hyperpolarizing factor derived from the endothelium [38-39].

Our results pointed to a significant reduction of $p22^{phox}$ in cells treated with 100 µM L-NAME for 96 hours with retreatment, which may be an indicative of a reduction in NOX4 expression. Since NOX4 is the most expressed NADPH oxidase in endothelial cells [29], the only isoform that is constitutively expressed and that requires only the $p22^{phox}$ component to be active [37; 40], our results may indicate a possible reduction in the expression and activity of this enzyme, causing a reduction in vasoprotection, reinforcing the signals of a condition of endothelial dysfunction [36]. Importantly, we must highlight that the expression of $p22^{phox}$ is being demonstrated for the first time in tEnd.1 cells.

Reduction of eNOS protein expression or in the dimer/ monomer eNOS ratio may be an indicative of eNOS uncoupling with consequent endothelial dysfunction [41]. Therefore, this ratio is often checked by Western Blotting analysis in studies searching for endothelial

dysfunction, as in the research developed by Couto and collaborators (2018) [42]. Some authors have already demonstrated the expression of eNOS in tEnd.1 cells [27;43], but we could not reproduce such results in our experimental design. Therefore, higher protein loading per well or prior immunoprecipitation may be necessary, as the expression of this enzyme seems to be low in our conditions [27; 43].

In addition to checking gene expression, we sought to visualize another indicative of oxidative stress, which is lipid peroxidation. No significant differences in TBARS test were observed after 72 hours without retreatment. Importantly, the absence of lipid peroxidation does not exclude oxidative stress, and other antioxidant enzymes, such as glutathione peroxidase may have acted, preventing lipid peroxidation [44]. Glutathione peroxidase is an important enzyme that acts by minimizing the effects of ROS on endothelial cells. Several studies indicate how supplementation with different antioxidants can activate the expression/ activity of this enzyme and reduce the damage caused by oxidative stress, preventing endothelial dysfunction [45-46]. Our results suggest that after treatment with L-NAME, ROS production has increased, but antioxidant enzymes may have acted to prevent lipid peroxidation [47].

Conclusion

Treatment of endothelial cells with 100µM L-NAME for 96 hours with retreatment was able to decrease NO bioavailability that was verified by diminished nitrite concentrations and reduced $p22^{phox}$ expression. We thus propose for the first time a protocol to simulate endothelial dysfunction based in NO synthesis inhibition, using a cellular *in vitro* approach. Finally, we understand that additional studies specially aiming to determine the role of ROS in cells submitted to treatment with L-NAME are necessary for the improvement and characterization of the proposed experimental model.

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Supporting information

S1 Fig. Morphology of the thymus-derived endothelial cell line tEnd.1 in Culture. In (A) and (B) are the images recorded by a camera attached to the optical microscope with an augmentation of 100 and 200 x, respectively. Cells were 80 to 90% confluent.

S2 Fig. Quality of total RNA extraction. Three microliters of diluted RNA, 3 µL of loading buffer, 3 µL of formamide and 1 µL of 20 x gel red were added to each well. The run was performed at 90 V for 30 minutes + 100 V for 20 min. The image was taken by a transluminator.

S3 Fig. β-actin amplification through conventional PCR (203 pb) to validate total RNA extraction. Five microliters of sample, 3 µL of loading buffer and 1 µL of 20 x gel red were added to each well. The run was performed at 100 V for 30 minutes. The image was taken by a transluminator.

S4 Fig. Protein expression of eNOS in tEnd.1 cells after treatment with L-arginine (100 µM) and L-NAME (10 µM and 100 µM) for 72 hours without retreatment. About 20 µg of protein were addet to each well as follows: 1) Control; 2) L-arginine (100µM); 3) L-NAME (10 µM); and 4) L-NAME (100 µM).

LIST OF FIGURES

Fig. 1

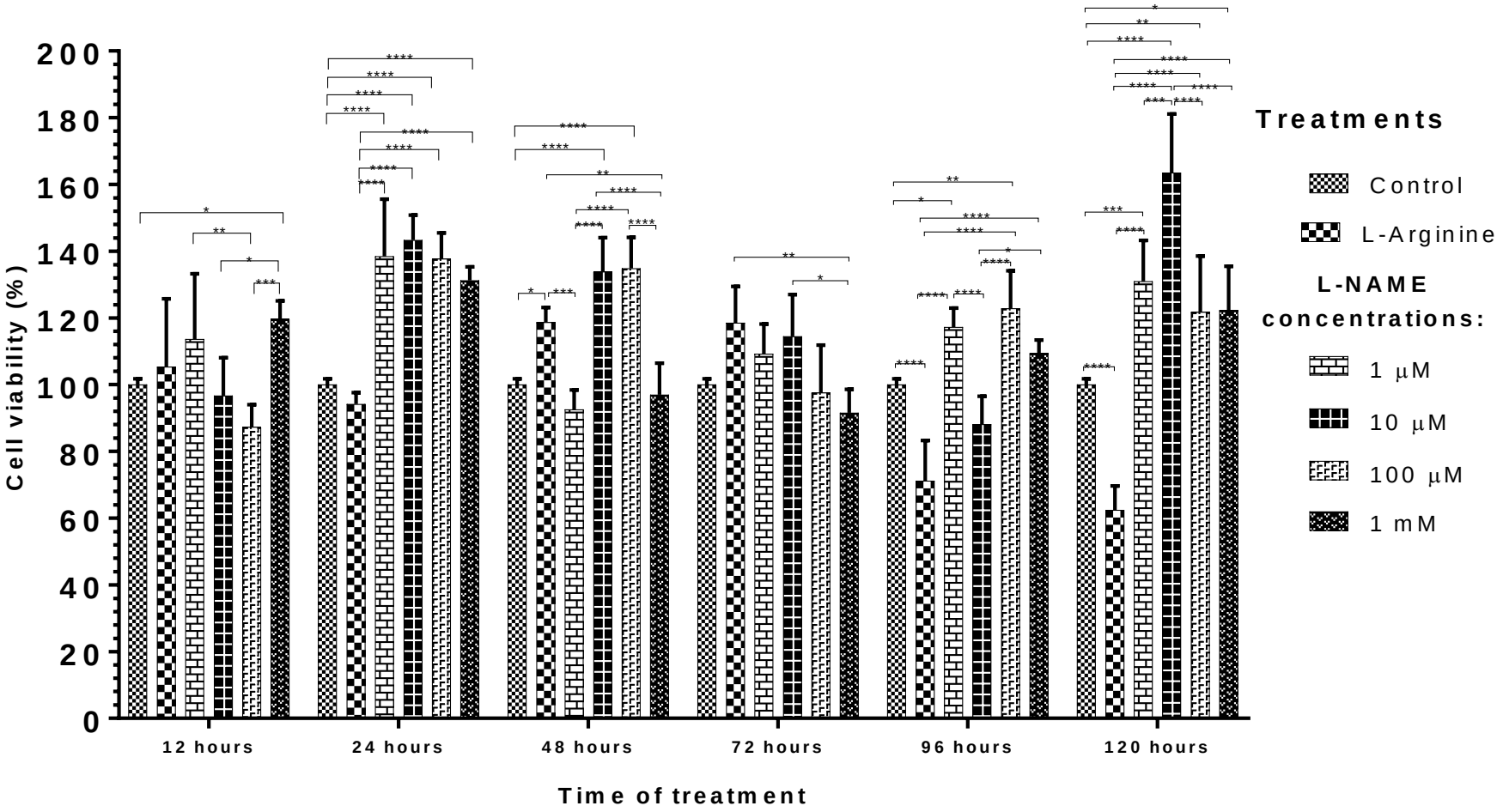


Fig. 2

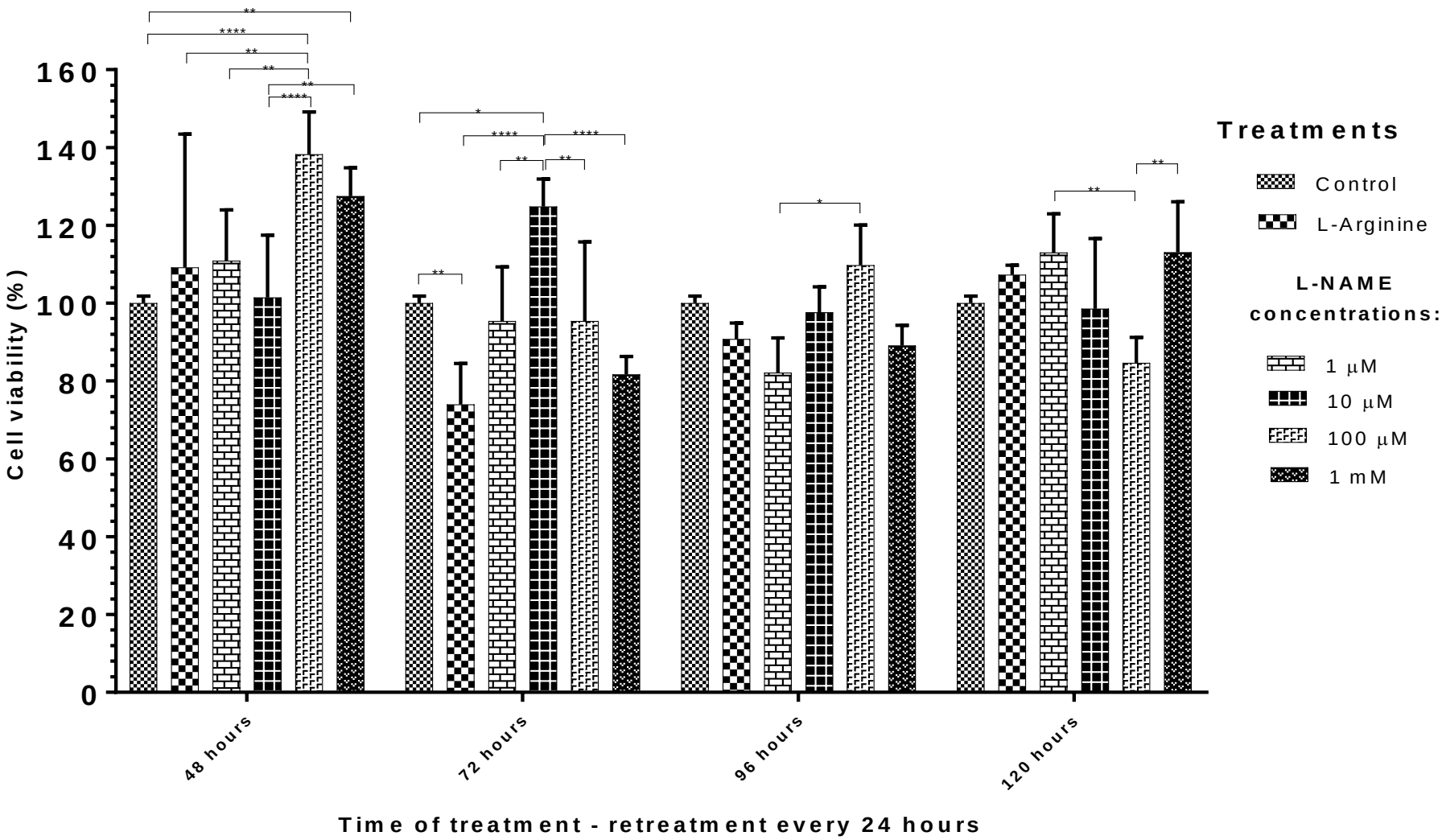

Cell viability (%)
160
140
120
100
80
60
40
20
0
48 hours
72 hours
96 hours
120 hours
Time of treatment - retreatment every 24 hours
Treatments
Control
L-Arginine
L-NAME concentrations:
1 μM
10 μM
100 μM
1 mM

Fig. 3

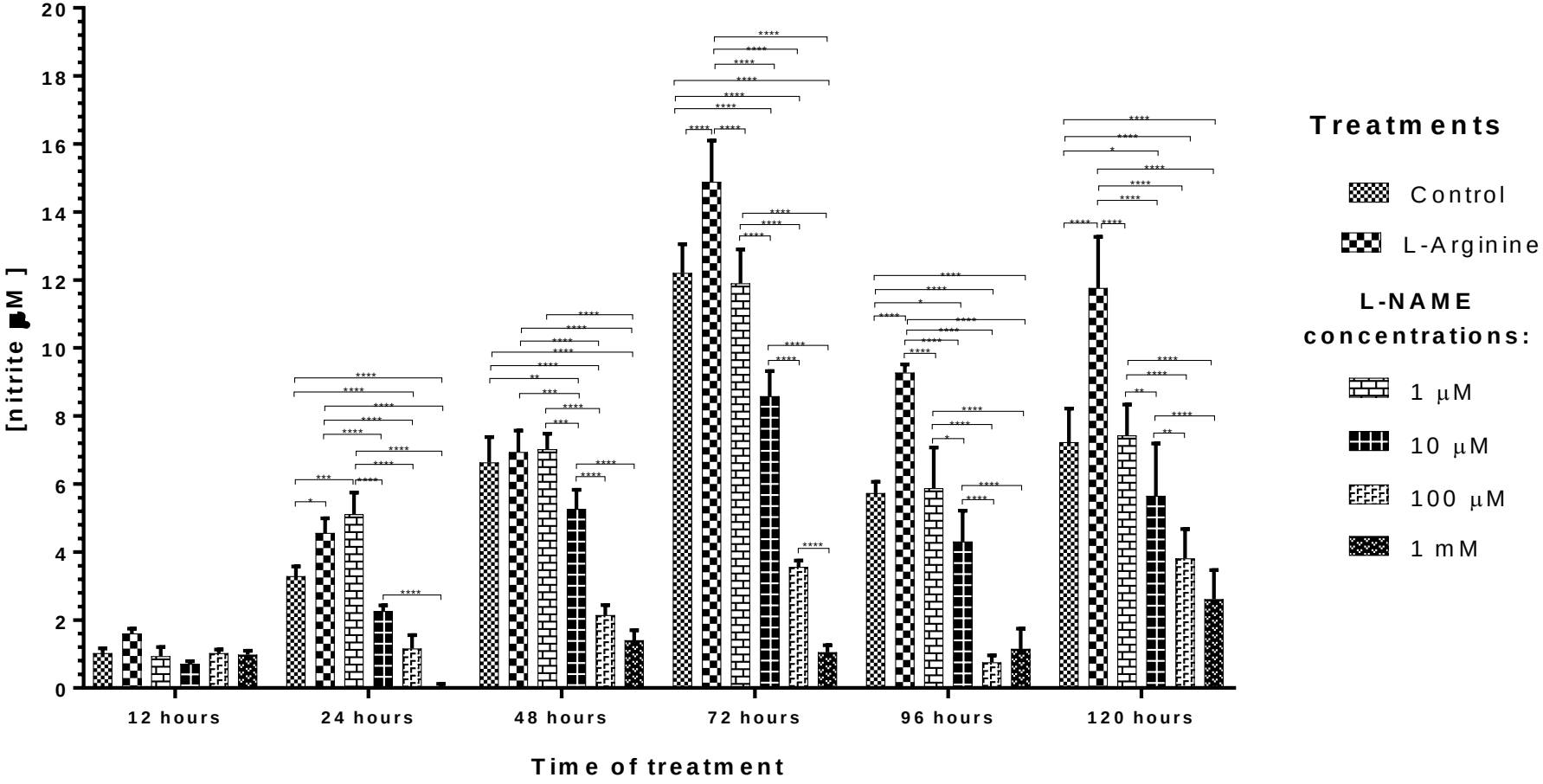

[nitrite μM]
20
18
16
14
12
10
8
6
4
2
0
12 hours
24 hours
48 hours
72 hours
96 hours
120 hours
Time of treatment
Treatments
Control
L-Arginine
L-NAME concentrations:
1 μM
10 μM
100 μM
1 mM

Fig. 4

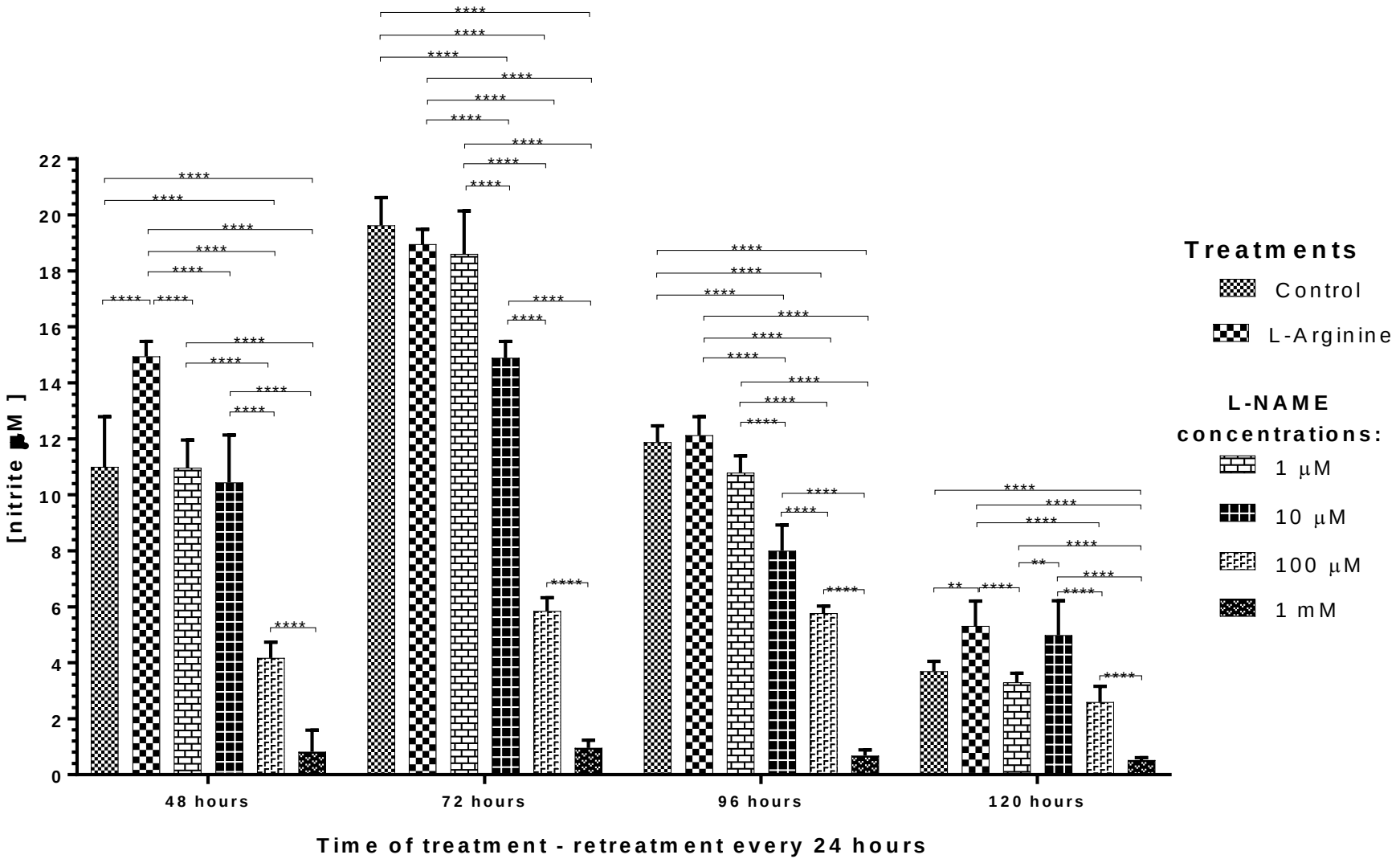

[nitrite μM]
0
2
4
6
8
10
12
14
16
18
20
22
48 hours
72 hours
96 hours
120 hours
Time of treatment - retreatment every 24 hours
Treatments
Control
L-Arginine
L-NAME concentrations:
1 μM
10 μM
100 μM
1 mM

Fig. 5

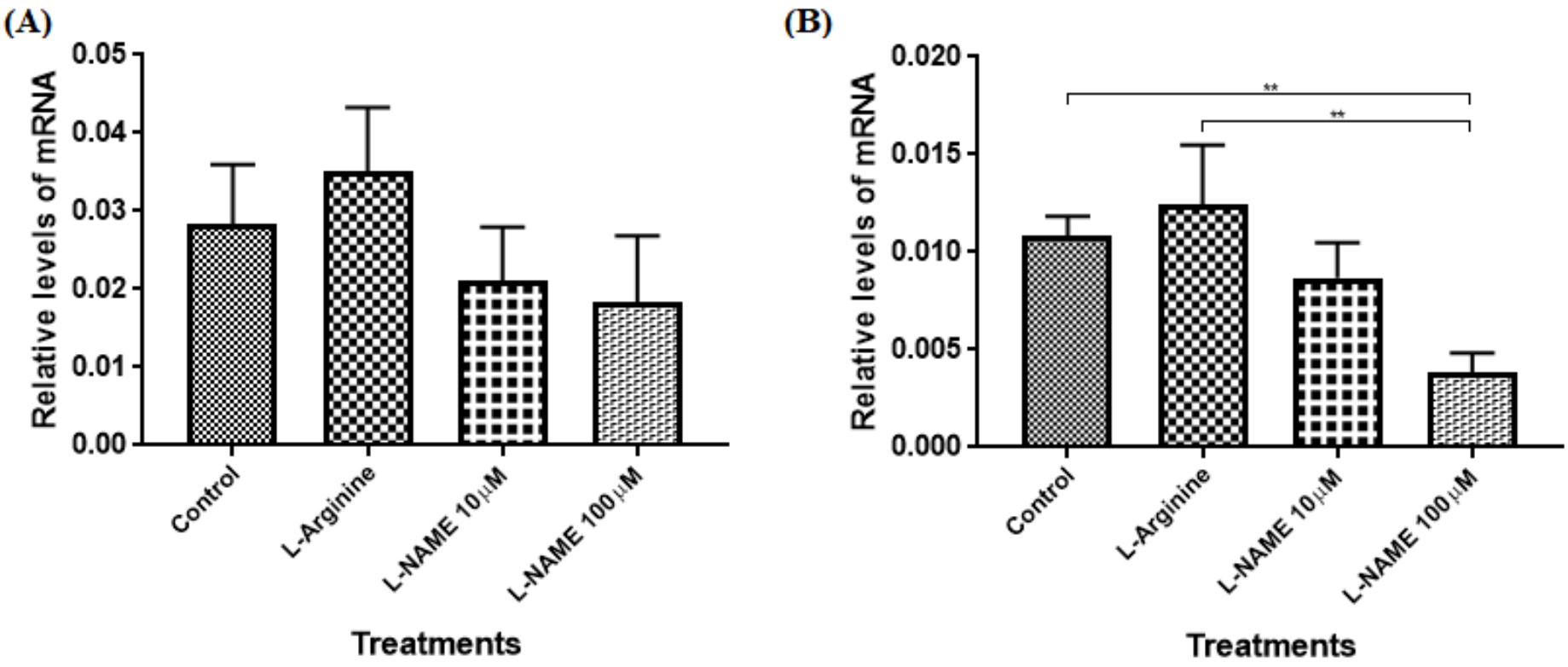
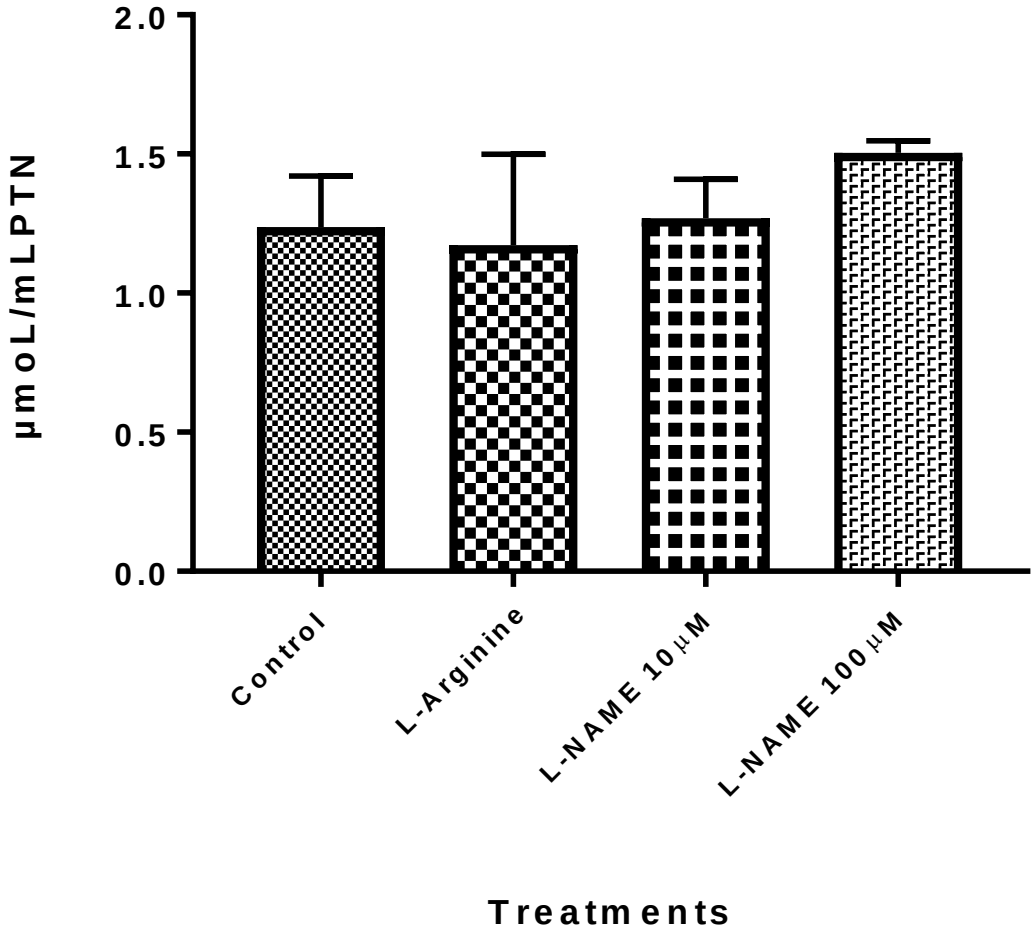
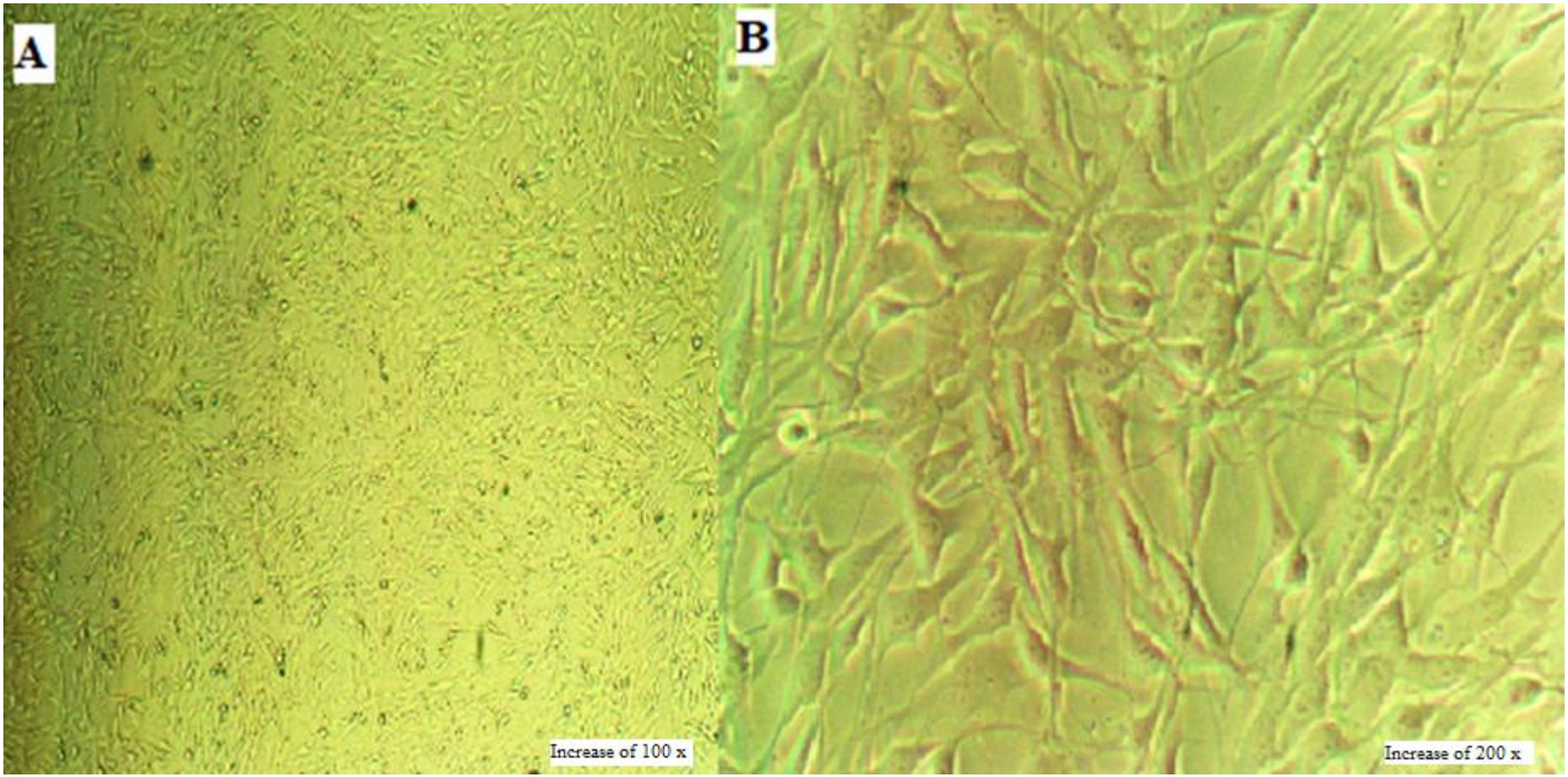

(A)
Relative levels of mRNA
0.05
0.04
0.03
0.02
0.01
0.00
Control
L-Arginine
L-NAME 10μM
L-NAME 100μM
Treatments
(B)
Relative levels of mRNA
0.020
0.015
0.010
0.005
0.000
**
**
Control
L-Arginine
L-NAME 10μM
L-NAME 100μM
Treatments

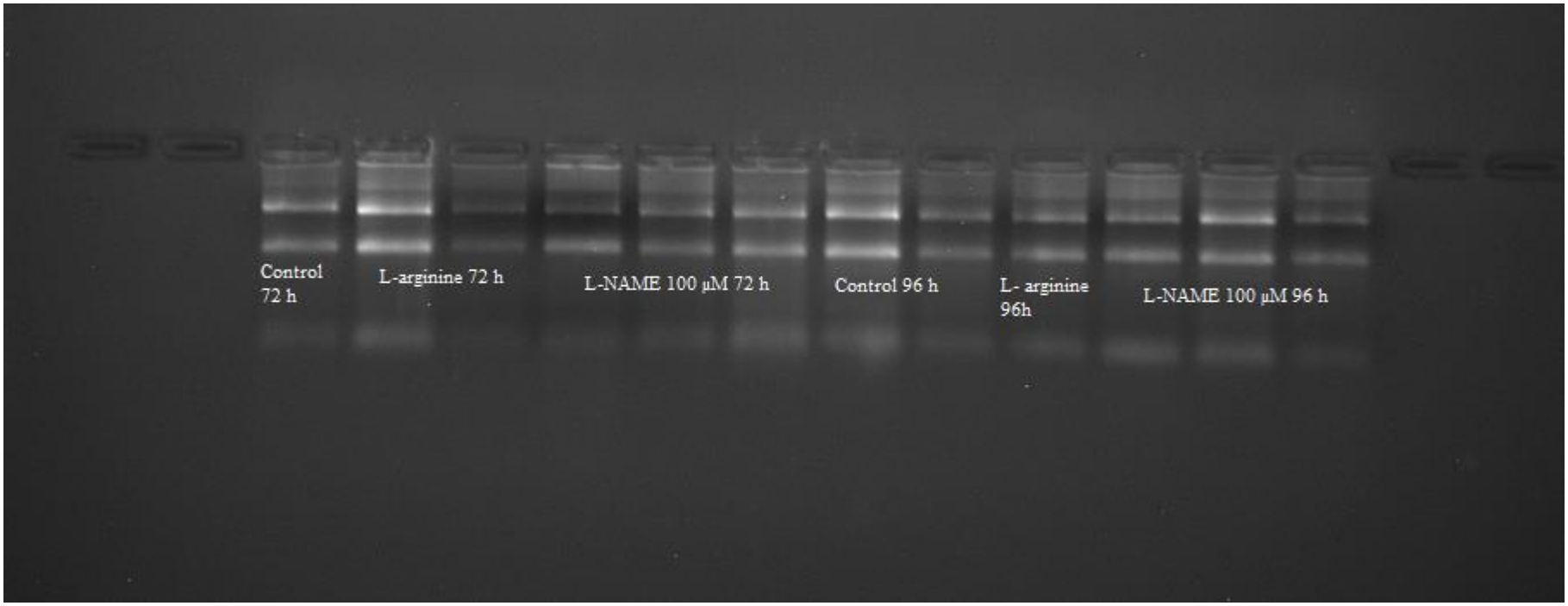
Fig. 6


2.0
1.5
1.0
0.5
0.0
µmoL/mLPTN
Control
L-Arginine
L-NAME 10µM
L-NAME 100µM
Treatments

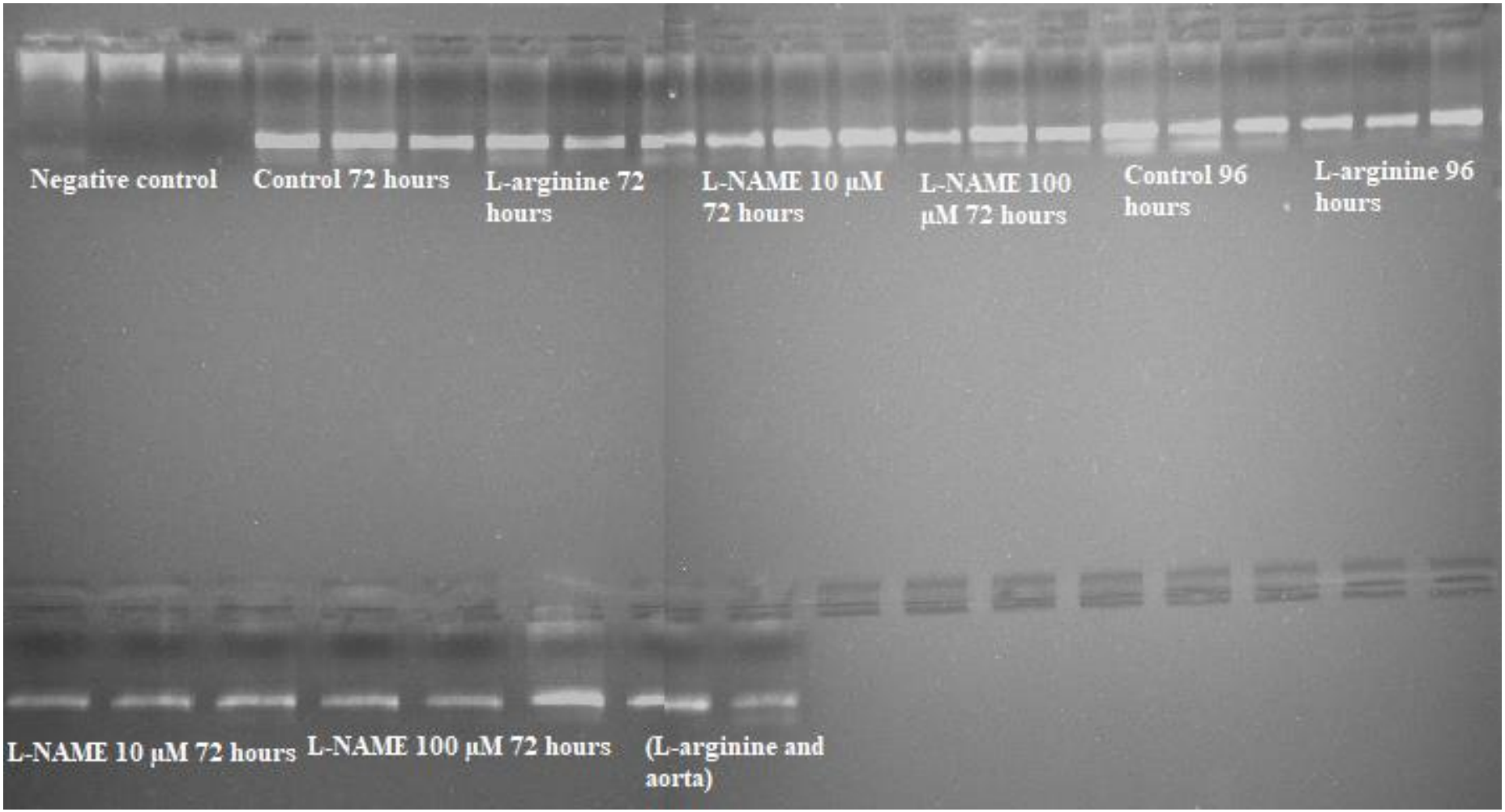
S1 Fig.



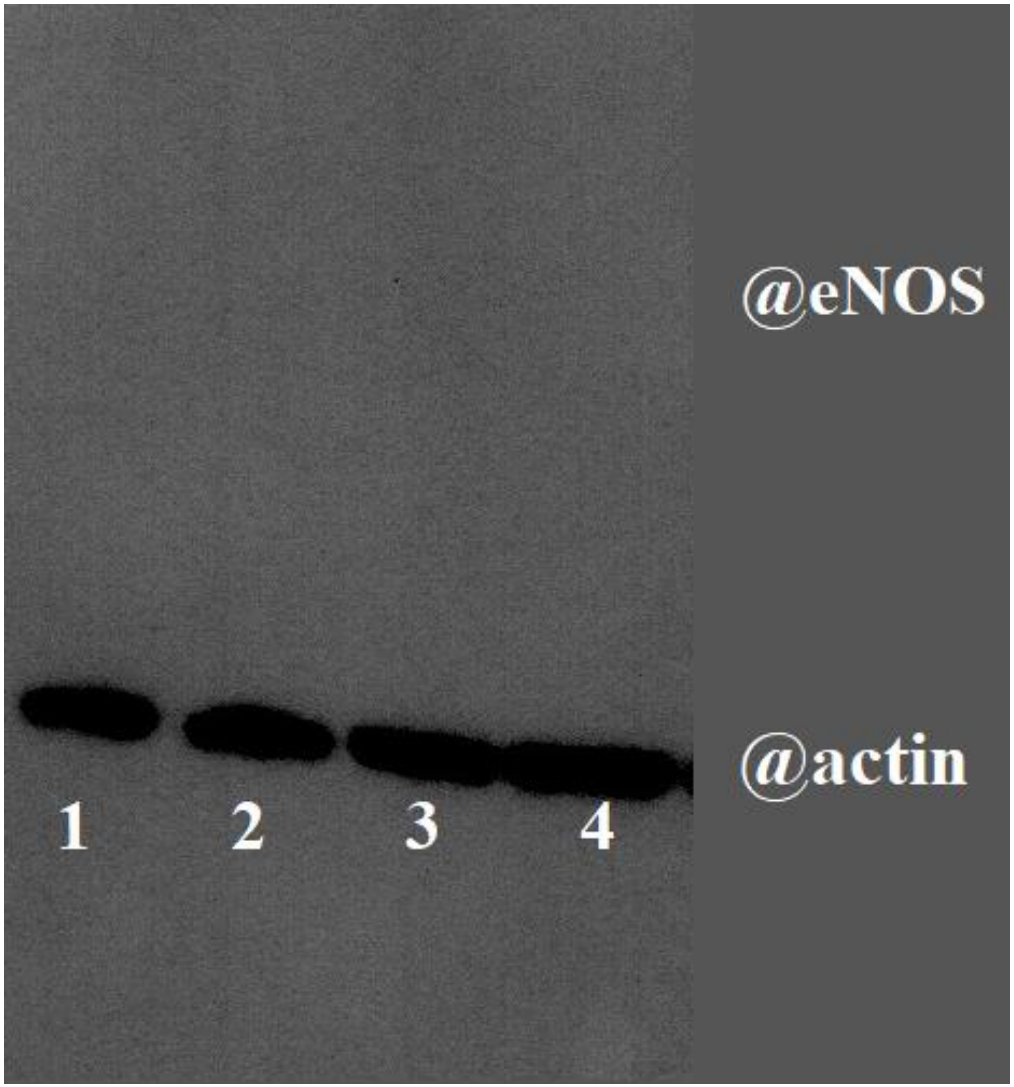
S2 Fig.


Control
72 h
L-arginine 72 h
L-NAME 100 µM 72 h
Control 96 h
L- arginine
96h
L-NAME 100 µM 96 h

S3 Fig.


Negative control
Control 72 hours
L-arginine 72 hours
L-NAME 10 µM 72 hours
L-NAME 100 µM 72 hours
Control 96 hours
L-arginine 96 hours
L-NAME 10 µM 72 hours
L-NAME 100 µM 72 hours
(L-arginine and aorta)

S4 Fig.



CONCLUSÃO

De acordo com os resultados aqui apresentados, pode-se concluir que o tratamento de células endoteliais com o L-NAME parece ser capaz de induzir nessas células um estado semelhante ao que ocorre na disfunção endotelial, reduzindo a biodisponibilidade de NO e, aparentemente, aumentando o estresse oxidativo e a degradação de NO. Sendo assim, os dados apontaram que o tratamento com L-NAME 100µM por 96 horas com retratamento foi capaz de reduzir a concentração de nitritos no meio de cultura celular e reduzir a expressão de $p22^{phox}$, que pode estar relacionado à uma redução na expressão e atividade da NOX4, a qual tem função protetora na vasculatura.

Dessa forma, propomos aqui, pela primeira vez, um método que permite a investigação de algumas das vias envolvidas na disfunção endotelial *in vitro* e mostramos a expressão do gene $p22^{phox}$ na linhagem tEnd.1. Outros experimentos são necessários para uma melhor caracterização da abordagem proposta, especialmente visando esclarecer o papel das EROs nas células endoteliais tratadas com L-NAME. Reforçamos, entretanto, que os resultados obtidos são bastante promissores e contribuem para o entendimento da DE e para pesquisas voltadas para sua utilização como alvo para o tratamento de patologias cardiovasculares e metabólicas, como a HAS, aterosclerose e diabetes.

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ANEXO 1

Normas para publicação de artigo no periódico *PLOS ONE*

PUBLISH WITH PLOS ONE

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Related information for authors

- **Submission system**
- **Journal scope and publication criteria**
- **Getting started guide**
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Reference style		PLOS uses "Vancouver" style, as outlined in the ICMJE sample references. See reference formatting examples and additional instructions below.
Equations		We recommend using MathType for display and inline equations, as it will provide the most reliable outcome. If this is not possible, Equation Editor or Microsoft's Insert→Equation function is acceptable. Avoid using MathType, Equation Editor, or the Insert→Equation function to insert single variables (e.g., "$a^2 + b^2 = c^2$"), Greek or other symbols (e.g., β, Δ, or ′ [prime]), or mathematical operators (e.g., x, ≥, or ±) in running text. Wherever possible, insert single symbols as normal text with the correct Unicode (hex) values. Do not use MathType, Equation Editor, or the Insert→Equation function for only a portion of an equation. Rather, ensure that the entire equation is included. Equations should not contain a mix of different equation tools. Avoid "hybrid" inline or display equations, in which part is text and part is MathType, or part is MathType and part is Equation Editor.
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Books	Bates B. Bargaining for life: A social history of tuberculosis. 1st ed. Philadelphia: University of Pennsylvania Press; 1992.
Book chapters	Hansen B. New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health; 1991. pp. 21-28.
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Masters' theses or doctoral dissertations	Wells A. Exploring the development of the independent, electronic, scholarly journal. M.Sc. Thesis, The University of Sheffield. 1999. Available from: http://cumincad.scix.net/cgi-bin/works/Show?2e09
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Source	Format
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Read the guidelines for tables.

- Statistical reporting

Manuscripts submitted to *PLOS ONE* are expected to report statistical methods in sufficient detail for others to replicate the analysis performed. Ensure that results are rigorously reported in accordance with community standards and that the statistical methods employed are appropriate for the study design.

Consult the following resources for additional guidance:

- SAMPL guidelines, for general guidance on statistical reporting
- *PLOS ONE* guidelines, for clinical trials requirements
- *PLOS ONE* guidelines, for systematic review and meta-analysis requirements
- EQUATOR, for specific reporting guidelines for a range of other study types

Reporting of statistical methods

In the methods, include a section on statistical analysis that reports a detailed description of the statistical methods. In this section:

- List the name and version of any software package used, alongside any relevant references
- Describe the technical details or procedures required to reproduce the analysis
- Provide the repository identifier for any code used in the analysis (See our code-sharing policy.)

Statistical reporting guidelines:

- Identify research design and independent variables as being between- or within-subjects
- For pre-processed data:
    - Describe any analysis carried out to confirm the data meets the assumptions of the analysis performed (e.g. linearity, co-linearity, normality of the distribution).
    - If data were transformed include this information, with a reason for doing so and a description of the transformation performed
- Provide details of how outliers were treated and your analysis, both with the full dataset and with the outliers removed
- If relevant, describe how missing/excluded data were handled
- Define the threshold for significance (alpha)
- If appropriate, provide sample sizes, along with a description of how they were determined. If a sample size calculation was performed, specify the inputs for power, effect size and alpha. Where relevant, report the number of independent replications for each experiment.
- For analyses of variance (ANOVAs), detail any post hoc tests that were performed
- Include details of any corrections applied to account for multiple comparisons. If corrections were not applied, include a justification for not doing so
- Describe all options for statistical procedures. For example, if t-tests were performed, state whether these were one- or two-tailed. Include details of the type of t-test conducted (e.g. one sample, within-/between-subjects).
- For step-wise multiple regression analyses:
    - Report the alpha level used
    - Discuss whether the variables were assessed for collinearity and interaction
    - Describe the variable selection process by which the final model was developed (e.g., forward-stepwise; best subset). See SAMPL guidelines.

- For Bayesian analysis explain the choice of prior trial probabilities and how they were selected. Markov chain Monte Carlo settings should be reported.

Reporting of statistical results

Results must be rigorously and appropriately reported, in keeping with community standards.

- Units of measurement. Clearly define measurement units in all tables and figures.
- Properties of distribution. It should be clear from the text which measures of variance (standard deviation, standard error of the mean, confidence intervals) and central tendency (mean, median) are being presented.
- Regression analyses. Include the full results of any regression analysis performed as a supplementary file. Include all estimated regression coefficients, their standard error, p-values, and confidence intervals, as well as the measures of goodness of fit.
- Reporting parameters. Test statistics (F/t/r) and associated degrees of freedom should be provided. Effect sizes and confidence intervals should be reported where appropriate. If percentages are provided, the numerator and denominator should also be given.
- P-values. Report exact p-values for all values greater than or equal to 0.001. P-values less than 0.001 may be expressed as $p < 0.001$, or as exponentials in studies of genetic associations.
- Displaying data in plots. Format plots so that they accurately depict the sample distribution. 3D effects in plots can bias and hinder interpretation of values, so avoid them in cases where regular plots are sufficient to display the data.
- Open data. As explained in PLOS's Data Policy, be sure to make individual data points, underlying graphs and summary statistics available at the time of publication. Data can be deposited in a repository or included within the Supporting Information files.
- Data reporting

All data and related metadata underlying the findings reported in a submitted manuscript should be deposited in an appropriate public repository, unless already provided as part of the submitted article.

See instructions on providing underlying data to support blot and gel results

Read our policy on data availability.

Repositories may be either subject-specific (where these exist) and accept specific types of structured data, or generalist repositories that accept multiple data types. We recommend that authors select repositories appropriate to their field. Repositories may be subject-specific

(e.g., GenBank for sequences and PDB for structures), general, or institutional, as long as DOIs or accession numbers are provided and the data are at least as open as CC BY. Authors are encouraged to select repositories that meet accepted criteria as trustworthy digital repositories, such as criteria of the Centre for Research Libraries or Data Seal of Approval. Large, international databases are more likely to persist than small, local ones.

See our list of recommended repositories.

To support data sharing and author compliance of the PLOS data policy, we have integrated our submission process with a select set of data repositories. The list is neither representative nor exhaustive of the suitable repositories available to authors. Current repository integration partners include Dryad and FlowRepository. Please contact data@plos.org to make recommendations for further partnerships.

Instructions for PLOS submissions with data deposited in an integration partner repository:

- Deposit data in the integrated repository of choice.
- Once deposition is final and complete, the repository will provide you with a dataset DOI (provisional) and private URL for reviewers to gain access to the data.
- Enter the given data DOI into the full Data Availability Statement, which is requested in the Additional Information section of the PLOS submission form. Then provide the URL passcode in the Attach Files section.

If you have any questions, please email us.

- Accession numbers

All appropriate data sets, images, and information should be deposited in an appropriate public repository. See our list of recommended repositories.

Accession numbers (and version numbers, if appropriate) should be provided in the Data Availability Statement. Accession numbers or a citation to the DOI should also be provided when the data set is mentioned within the manuscript.

In some cases authors may not be able to obtain accession numbers of DOIs until the manuscript is accepted; in these cases, the authors must provide these numbers at acceptance. In all other cases, these numbers must be provided at full submission.

Identifiers

As much as possible, please provide accession numbers or identifiers for all entities such as genes, proteins, mutants, diseases, etc., for which there is an entry in a public database, for example:

- Ensembl
- Entrez Gene

- FlyBase
- InterPro
- Mouse Genome Database (MGD)
- Online Mendelian Inheritance in Man (OMIM)
- PubChem

Identifiers should be provided in parentheses after the entity on first use.

- Striking image

You can choose to upload a "Striking Image" that we may use to represent your article online in places like the journal homepage or in search results.

The striking image must be derived from a figure or supporting information file from the submission, i.e., a cropped portion of an image or the entire image. Striking images should ideally be high resolution, eye-catching, single panel images, and should ideally avoid containing added details such as text, scale bars, and arrows.

If no striking image is uploaded, we will designate a figure from the submission as the striking image.

Striking images should not contain potentially identifying images of people. Read our policy on identifying information.

The PLOS licenses and copyright policy also applies to striking images.

Additional Information Requested at Submission

- Financial Disclosure Statement

This information should describe sources of funding that have supported the work. It is important to gather these details prior to submission because your financial disclosure statement cannot be changed after initial submission without journal approval. If your manuscript is published, your statement will appear in the Funding section of the article. Enter this statement in the Financial Disclosure section of the submission form. Do not include it in your manuscript file.

The statement should include:

- Specific grant numbers
- Initials of authors who received each award
- Full names of commercial companies that funded the study or authors
- Initials of authors who received salary or other funding from commercial companies
- URLs to sponsors' websites

Also state whether any sponsors or funders (other than the named authors) played any role in:

- Study design
- Data collection and analysis
- Decision to publish
- Preparation of the manuscript

If they had no role in the research, include this sentence: “The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.”
If the study was unfunded, include this sentence as the Financial Disclosure statement: “The author(s) received no specific funding for this work."
Read our policy on disclosure of funding sources.

- Competing interests

This information should not be in your manuscript file; you will provide it via our submission system.
All potential competing interests must be declared in full. If the submission is related to any patents, patent applications, or products in development or for market, these details, including patent numbers and titles, must be disclosed in full.
Read our policy on competing interests.

- Manuscripts disputing published work

For manuscripts disputing previously published work, it is *PLOS ONE* policy to invite a signed review by the disputed author during the peer review process. This procedure is aimed at ensuring a thorough, transparent, and productive review process.
If the disputed author chooses to submit a review, it must be returned in a timely fashion and contain a full declaration of all competing interests. The Academic Editor will consider any such reviews in light of the competing interest.
Authors submitting manuscripts disputing previous work should explain the relationship between the manuscripts in their cover letter, and will be required to confirm that they accept the conditions of this review policy before the manuscript is considered further.

- Related manuscripts

Upon submission, authors must confirm that the manuscript, or any related manuscript, is not currently under consideration or accepted elsewhere. If related work has been submitted to *PLOS ONE* or elsewhere, authors must include a copy with the submitted article.
Reviewers will be asked to comment on the overlap between related submissions.
We strongly discourage the unnecessary division of related work into separate manuscripts, and we will not consider manuscripts that are divided into “parts.” Each submission to *PLOS ONE* must be written as an independent unit and should not rely on any work that has not

already been accepted for publication. If related manuscripts are submitted to *PLOS ONE*, the authors may be advised to combine them into a single manuscript at the editor's discretion. Read our policies on related manuscripts.

- Preprints

PLOS encourages authors to post preprints as a way to accelerate the dissemination of research and supports authors who wish to share their work early and receive feedback before formal peer review. Deposition of manuscripts with preprint servers does not impact consideration of the manuscript at any PLOS journal.

Authors posting on bioRxiv or medRxiv may submit directly to relevant PLOS journals through the direct transfer to journal service.

Authors submitting manuscripts in the life sciences to *PLOS ONE* may opt-in to post their work on bioRxiv during the *PLOS ONE* initial submission process.

Read more about preprints.

Learn how to post a preprint to bioRxiv during *PLOS ONE* initial submission.

Guidelines for Specific Study Types

- Human subjects research

All research involving human participants must have been approved by the authors' Institutional Review Board (IRB) or by equivalent ethics committee(s), and must have been conducted according to the principles expressed in the Declaration of Helsinki. Authors should be able to submit, upon request, a statement from the IRB or ethics committee indicating approval of the research. We reserve the right to reject work that we believe has not been conducted to a high ethical standard, even when formal approval has been obtained.

Subjects must have been properly instructed and have indicated that they consent to participate by signing the appropriate informed consent paperwork. Authors may be asked to submit a blank, sample copy of a subject consent form. If consent was verbal instead of written, or if consent could not be obtained, the authors must explain the reason in the manuscript, and the use of verbal consent or the lack of consent must have been approved by the IRB or ethics committee.

All efforts should be made to protect patient privacy and anonymity. Identifying information, including photos, should not be included in the manuscript unless the information is crucial and the individual has provided written consent by completing the Consent Form for

Publication in a PLOS Journal (PDF). Download additional translations of the form from the Downloads and Translations page. More information about patient privacy, anonymity, and informed consent can be found in the International Committee of Medical Journal Editors (ICMJE) Privacy and Confidentiality guidelines.

Manuscripts should conform to the following reporting guidelines:

- Studies of diagnostic accuracy: STARD
- Observational studies: STROBE
- Microarray experiments: MIAME
- Other types of health-related research: Consult the EQUATOR web site for appropriate reporting guidelines

Methods sections of papers on research using human subjects or samples must include ethics statements that specify:

- The name of the approving institutional review board or equivalent committee(s). If approval was not obtained, the authors must provide a detailed statement explaining why it was not needed
- Whether informed consent was written or oral. If informed consent was oral, it must be stated in the manuscript:
    - Why written consent could not be obtained
    - That the Institutional Review Board (IRB) approved use of oral consent
    - How oral consent was documented

For studies involving humans categorized by race/ethnicity, age, disease/disabilities, religion, sex/gender, sexual orientation, or other socially constructed groupings, authors should:

- Explicitly describe their methods of categorizing human populations
- Define categories in as much detail as the study protocol allows
- Justify their choices of definitions and categories, including for example whether any rules of human categorization were required by their funding agency
- Explain whether (and if so, how) they controlled for confounding variables such as socioeconomic status, nutrition, environmental exposures, or similar factors in their analysis

In addition, outmoded terms and potentially stigmatizing labels should be changed to more current, acceptable terminology. Examples: “Caucasian” should be changed to “white” or “of [Western] European descent” (as appropriate); “cancer victims” should be changed to “patients with cancer.”

For papers that include identifying, or potentially identifying, information, authors must download the Consent Form for Publication in a PLOS Journal, which the individual, parent, or guardian must sign once they have read the paper and been informed about the terms of PLOS open-access license. The signed consent form should not be submitted with the manuscript, but authors should securely file it in the individual's case notes and the methods section of the manuscript should explicitly state that consent authorization for publication is on file, using wording like:

The individual in this manuscript has given written informed consent (as outlined in PLOS consent form) to publish these case details.

For more information about *PLOS ONE* policies regarding human subjects research, see the Publication Criteria and Editorial Policies.

- Clinical trials

Clinical trials are subject to all policies regarding human research. *PLOS ONE* follows the World Health Organization's (WHO) definition of a clinical trial:

A clinical trial is any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes [...] Interventions include but are not restricted to drugs, cells and other biological products, surgical procedures, radiologic procedures, devices, behavioural treatments, process-of-care changes, preventive care, etc.

All clinical trials must be registered in one of the publicly-accessible registries approved by the WHO or ICMJE (International Committee of Medical Journal Editors). Authors must provide the trial registration number. Prior disclosure of results on a clinical trial registry site will not affect consideration for publication. We reserve the right to inform authors' institutions or ethics committees, and to reject the manuscript, if we become aware of unregistered trials.

PLOS ONE supports prospective trial registration (i.e. before participant recruitment has begun) as recommended by the ICMJE's clinical trial registration policy. Where trials were not publicly registered before participant recruitment began, authors must:

- Register all related clinical trials and confirm they have done so in the Methods section
- Explain in the Methods the reason for failing to register before participant recruitment

Clinical trials must be reported according to the relevant reporting guidelines, i.e. CONSORT for randomized controlled trials, TREND for non-randomized trials, and other specialized guidelines as appropriate. The intervention should be described according to the

requirements of the TIDieR checklist and guide. Submissions must also include the study protocol as supporting information, which will be published with the manuscript if accepted.
Authors of manuscripts describing the results of clinical trials must adhere to the CONSORT reporting guidelines appropriate to their trial design, available on the CONSORT Statement web site. Before the paper can enter peer review, authors must:

- Provide the registry name and number in the methods section of the manuscript
- Provide a copy of the trial protocol as approved by the ethics committee and a completed CONSORT checklist as supporting information (which will be published alongside the paper, if accepted). This should be named S1 CONSORT Checklist.
- Include the CONSORT flow diagram as the manuscript's “Fig 1”

Any deviation from the trial protocol must be explained in the paper. Authors must explicitly discuss informed consent in their paper, and we reserve the right to ask for a copy of the patient consent form.
The methods section must include the name of the registry, the registry number, and the URL of your trial in the registry database for each location in which the trial is registered.

- Animal research

All research involving vertebrates or cephalopods must have approval from the authors' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee(s), and must have been conducted according to applicable national and international guidelines. Approval must be received prior to beginning research.
Manuscripts reporting animal research must state in the Methods section:

- The full name of the relevant ethics committee that approved the work, and the associated permit number(s).
- Where ethical approval is not required, the manuscript should include a clear statement of this and the reason why. Provide any relevant regulations under which the study is exempt from the requirement for approval.
- Relevant details of steps taken to ameliorate animal suffering.

Example ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Minnesota (Protocol Number: 27-2956). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Authors should always state the organism(s) studied in the Abstract. Where the study may be confused as pertaining to clinical research, authors should also state the animal model in the title.

To maximize reproducibility and potential for re-use of data, we encourage authors to follow the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines for all submissions describing laboratory-based animal research and to upload a completed ARRIVE Guidelines Checklist to be published as supporting information.

Non-human primates

Manuscripts describing research involving non-human primates must report details of husbandry and animal welfare in accordance with the recommendations of the Weatherall report, *The use of non-human primates in research*, including:

- Information about housing, feeding, and environmental enrichment.
- Steps taken to minimize suffering, including use of anesthesia and method of sacrifice, if appropriate.

Random source animals

Manuscripts describing studies that use random source (e.g. Class B dealer-sourced in the USA), shelter, or stray animals will be subject to additional scrutiny and may be rejected if sufficient ethical and scientific justification for the study design is lacking.

Unacceptable euthanasia methods and anesthetic agents

Manuscripts reporting use of a euthanasia method(s) classified as unacceptable by the American Veterinary Medical Association or use of an anesthesia method(s) that is widely prohibited (e.g., chloral hydrate, ether, chloroform) must include at the time of initial submission, scientific justification for use in the specific study design, as well as confirmation of approval for specific use from their animal research ethics committee. These manuscripts may be subject to additional ethics considerations prior to publication.

Humane endpoints

Manuscripts reporting studies in which death of a regulated animal (vertebrate, cephalopod) is a likely outcome or a planned experimental endpoint, must comprehensively report details of study design, rationale for the approach, and methodology, including consideration of humane endpoints. This applies to research that involves, for instance, assessment of survival, toxicity, longevity, terminal disease, or high rates of incidental mortality.

Definition of a humane endpoint

A humane endpoint is a predefined experimental endpoint at which animals are euthanized

when they display early markers associated with death or poor prognosis of quality of life, or specific signs of severe suffering or distress. Humane endpoints are used as an alternative to allowing such conditions to continue or progress to death following the experimental intervention ("death as an endpoint"), or only euthanizing animals at the end of an experiment. Before a study begins, researchers define the practical observations or measurements that will be used during the study to recognize a humane endpoint, based on anticipated clinical, physiological, and behavioral signs. Please see the NC3Rs guidelines for more information. Additional discussion of humane endpoints can be found in this article: Nuno H. Franco, Margarida Correia-Neves, I. Anna S. Olsson (2012) How "Humane" Is Your Endpoint? — Refining the Science-Driven Approach for Termination of Animal Studies of Chronic Infection. PLoS Pathog 8(1): e1002399 doi.org/10.1371/journal.ppat.1002399.

Full details of humane endpoints use must be reported for a study to be reproducible and for the results to be accurately interpreted.

For studies in which death of an animal is an outcome or a planned experimental endpoint, authors should include the following information in the Methods section of the manuscript:

- The specific criteria (i.e. humane endpoints) used to determine when animals should be euthanized.
- The duration of the experiment.
- The numbers of animals used, euthanized, and found dead (if any); the cause of death for all animals.
- How frequently animal health and behavior were monitored.
- All animal welfare considerations taken, including efforts to minimize suffering and distress, use of analgesics or anaesthetics, or special housing conditions.

If humane endpoints were not used, the manuscript should report:

- A scientific justification for the study design, including the reasons why humane endpoints could not be used, and discussion of alternatives that were considered.
- Whether the institutional animal ethics committee specifically reviewed and approved the anticipated mortality in the study design.
- Observational and field studies

Methods sections for submissions reporting on any type of field study must include ethics statements that specify:

- Permits and approvals obtained for the work, including the full name of the authority that approved the study; if none were required, authors should explain why

- Whether the land accessed is privately owned or protected
- Whether any protected species were sampled
- Full details of animal husbandry, experimentation, and care/welfare, where relevant
- Paleontology and archaeology research

Manuscripts reporting paleontology and archaeology research must include descriptions of methods and specimens in sufficient detail to allow the work to be reproduced. Data sets supporting statistical and phylogenetic analyses should be provided, preferably in a format that allows easy re-use. Read the policy.

Specimen numbers and complete repository information, including museum name and geographic location, are required for publication. Locality information should be provided in the manuscript as legally allowable, or a statement should be included giving details of the availability of such information to qualified researchers.

If permits were required for any aspect of the work, details should be given of all permits that were obtained, including the full name of the issuing authority. This should be accompanied by the following statement:

All necessary permits were obtained for the described study, which complied with all relevant regulations.

If no permits were required, please include the following statement:

No permits were required for the described study, which complied with all relevant regulations.

Manuscripts describing paleontology and archaeology research are subject to the following policies:

- Sharing of data and materials. Any specimen that is erected as a new species, described, or figured must be deposited in an accessible, permanent repository (i.e., public museum or similar institution). If study conclusions depend on specimens that do not fit these criteria, the article will be rejected under *PLOS ONE*'s data availability criterion.
- Ethics. *PLOS ONE* will not publish research on specimens that were obtained without necessary permission or were illegally exported.
- Systematic reviews and meta-analyses

A systematic review paper, as defined by The Cochrane Collaboration, is a review of a clearly formulated question that uses explicit, systematic methods to identify, select, and critically appraise relevant research, and to collect and analyze data from the studies that are included in the review. These reviews differ substantially from narrative-based reviews or synthesis

articles. Statistical methods (meta-analysis) may or may not be used to analyze and summarize the results of the included studies.

Reports of systematic reviews and meta-analyses must include a completed PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) checklist and flow diagram to accompany the main text. Blank templates are available here:

- Checklist: PDF or Word document
- Flow diagram: PDF or Word document

Authors must also state in their "Methods" section whether a protocol exists for their systematic review, and if so, provide a copy of the protocol as supporting information and provide the registry number in the abstract.

If your article is a systematic review or a meta-analysis you should:

- State this in your cover letter
- Select "Research Article" as your article type when submitting
- Include the PRISMA flow diagram as Fig 1 (required where applicable)
- Include the PRISMA checklist as supporting information
- Meta-analysis of genetic association studies

Manuscripts reporting a meta-analysis of genetic association studies must report results of value to the field and should be reported according to the guidelines presented in *Systematic Reviews of Genetic Association Studies* by Sagoo *et al.*

On submission, authors will be asked to justify the rationale for the meta-analysis and how it contributes to the base of scientific knowledge in the light of previously published results. Authors will also be asked to complete a checklist (DOCX) outlining information about the justification for the study and the methodology employed. Meta-analyses that replicate published studies will be rejected if the authors do not provide adequate justification.

- Personal data from third-party sources

For all studies using personal data from internet-based and other third-party sources (e.g., social media, blogs, other internet sources, mobile phone companies), data must be collected and used according to company/website Terms and Conditions, with appropriate permissions. All data sources must be acknowledged clearly in the Materials and Methods section.

Read our policy on data availability.

In the Ethics Statement, authors should declare any potential risks to individuals or individual privacy, or affirm that in their assessment, the study posed no such risks. In addition, the following Ethics and Data Protection requirements must be met.

For interventional studies, which impact participants' experiences or data, the study design must have been prospectively approved by an Ethics Committee, and informed consent is required. The Ethics Committee may waive the requirement for approval and/or consent.
For observational studies in which personal experiences and accounts are not manipulated, consultation with an Ethics or Data Protection Committee is recommended. Additional requirements apply in the following circumstances:

- If information used could threaten personal privacy or damage the reputation of individuals whose data are used, an Ethics Committee should be consulted and informed consent obtained or specifically addressed.
- If authors accessed any personal identifying information, an Ethics or Data Protection Committee should oversee data anonymization. If data were anonymized and/or aggregated before access and analysis, informed consent is generally not required.

Note that Terms of Use contracts do not qualify as informed consent, even if they address the use of personal data for research.
See our reporting guidelines for human subjects research.

- Cell lines

Authors reporting research using cell lines should state when and where they obtained the cells, giving the date and the name of the researcher, cell line repository, or commercial source (company) who provided the cells, as appropriate.
Authors must also include the following information for each cell line:
For *de novo* (new) cell lines, including those given to the researchers as a gift, authors must follow our policies for human subjects research or animal research, as appropriate. The ethics statement must include:

- Details of institutional review board or ethics committee approval; AND
- For human cells, confirmation of written informed consent from the donor, guardian, or next of kin

For established cell lines, the Methods section should include:

- A reference to the published article that first described the cell line; AND/OR
- The cell line repository or company the cell line was obtained from, the catalogue number, and whether the cell line was obtained directly from the repository/company or from another laboratory

Authors should check established cell lines using the ICLAC Database of Cross-contaminated or Misidentified Cell Lines to confirm they are not misidentified or contaminated. Cell line

authentication is recommended – e.g., by karyotyping, isozyme analysis, or short tandem repeats (STR) analysis – and may be required during peer review or after publication.

- Blots and gels

Please review *PLOS ONE's* requirements for reporting blot and gel results and providing the underlying raw images.

- Antibodies

Manuscripts reporting experiments using antibodies should include the following information:

- The name of each antibody, a description of whether it is monoclonal or polyclonal, and the host species.
- The commercial supplier or source laboratory.
- The catalogue or clone number and, if known, the batch number.
- The antigen(s) used to raise the antibody.
- For established antibodies, a stable public identifier from the Antibody Registry.

The manuscript should also report the following experimental details:

- The final antibody concentration or dilution.
- A reference to the validation study if the antibody was previously validated. If not, provide details of how the authors validated the antibody for the applications and species used.

We encourage authors to consider adding information on new validations to a publicly available database such as Antibodypedia or CiteAb.

- Small and macromolecule crystal data

Manuscripts reporting new and unpublished three-dimensional structures must include sufficient supporting data and detailed descriptions of the methodologies used to allow the reproduction and validation of the structures. All novel structures must have been deposited in a community endorsed database prior to submission (please see our list of recommended repositories).

Small molecule single crystal data

Authors reporting X-Ray crystallographic structures of small organic, metal-organic, and inorganic molecules must deposit their data with the Cambridge Crystallographic Data Centre (CCDC), the Inorganic Crystal Structure Database (ICSD), or similar community databases providing a recognized validation functionality. Authors are also required to include the relevant structure reference numbers within the main text (e.g. the CCDC ID number), as well as the crystallographic information files (.cif format) as Supplementary Information, along

with the checkCIF validation reports that can be obtained via the International Union of Crystallography (IUCr).

Macromolecular structures

Authors reporting novel macromolecular structures must have deposited their data prior to initial submission with the Worldwide Protein Data Bank (wwPDB), the Biological Magnetic Resonance Data Bank (BMRB), the Electron Microscopy Data Bank (EMDB), or other community databases providing a recognized validation functionality. Authors must include the structure reference numbers within the main text and submit as Supplementary Information the official validation reports from these databases.

- Methods, software, databases, and tools

PLOS ONE will consider submissions that present new methods, software, databases, or tools as the primary focus of the manuscript if they meet the following criteria:

Utility

The tool must be of use to the community and must present a proven advantage over existing alternatives, where applicable. Recapitulation of existing methods, software, or databases is not useful and will not be considered for publication. Combining data and/or functionalities from other sources may be acceptable, but simpler instances (i.e. presenting a subset of an already existing database) may not be considered. For software, databases, and online tools, the long-term utility should also be discussed, as relevant. This discussion may include maintenance, the potential for future growth, and the stability of the hosting, as applicable.

Validation

Submissions presenting methods, software, databases, or tools must demonstrate that the new tool achieves its intended purpose. If similar options already exist, the submitted manuscript must demonstrate that the new tool is an improvement over existing options in some way. This requirement may be met by including a proof-of-principle experiment or analysis; if this is not possible, a discussion of the possible applications and some preliminary analysis may be sufficient.

Availability

If the manuscript's primary purpose is the description of new software or a new software package, this software must be open source, deposited in an appropriate archive, and conform to the Open Source Definition. If the manuscript mainly describes a database, this database must be open-access and hosted somewhere publicly accessible, and any software

used to generate a database should also be open source. If relevant, databases should be open for appropriate deposition of additional data. Dependency on commercial software such as Mathematica and MATLAB does not preclude a paper from consideration, although complete open source solutions are preferred. In these cases, authors should provide a direct link to the deposited software or the database hosting site from within the paper. If the primary focus of a manuscript is the presentation of a new tool, such as a newly developed or modified questionnaire or scale, it should be openly available under a license no more restrictive than CC BY.

Software submissions

Manuscripts whose primary purpose is the description of new software must provide full details of the algorithms designed. Describe any dependencies on commercial products or operating system. Include details of the supplied test data and explain how to install and run the software. A brief description of enhancements made in the major releases of the software may also be given. Authors should provide a direct link to the deposited software from within the paper.

Database submissions

For descriptions of databases, provide details about how the data were curated, as well as plans for long-term database maintenance, growth, and stability. Authors should provide a direct link to the database hosting site from within the paper.

Read the PLOS policy on sharing materials and software.

- New taxon names

Zoological names

When publishing papers that describe a new zoological taxon name, PLOS aims to comply with the requirements of the International Commission on Zoological Nomenclature (ICZN). Effective 1 January 2012, the ICZN considers an online-only publication to be legitimate if it meets the criteria of archiving and is registered in ZooBank, the ICZN's official registry.

For proper registration of a new zoological taxon, we require two specific statements to be included in your manuscript.

In the Results section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

Anochetus boltoni Fisher sp. nov. urn:lsid:zoobank.org:act:B6C072CF-1CA6-40C7-8396-534E91EF7FBB

You will need to contact Zoobank to obtain a GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your

responsibility to provide us with this information so we can include it in the final published paper.

Please also insert the following text into the Methods section, in a sub-section to be called "Nomenclatural Acts":

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Solanum aspersum S.Knapp, sp. nov. [urn:lsid:ipni.org:names:77103633-1] Type: Colombia. Putumayo: vertiente oriental de la Cordillera, entre Sachamates y San Francisco de Sibundoy, 1600-1750 m, 30 Dec 1940, J. Cuatrecasas 11471 (holotype, COL; isotypes, F [F-1335119], US [US-1799731]).
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Hymenogaster huthii. Stielow et al. 2010, sp. nov.
[urn:lsid:indexfungorum.org:names:518624]
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