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Mariana Breidenbach

UM ESTUDO PRELIMINAR SOBRE A POSSÍVEL ATUAÇÃO DA SINALIZAÇÃO ADRENÉRGICA NA MELHORA CARDÍACA VISTA EM RATOS SUBMETIDOS AO INFARTO AGUDO DO MIOCÁRDIO E TRATADOS COM N-ACETILCISTEÍNA E DEFEROXAMINA

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Trabalho de conclusão de curso de graduação apresentado ao Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de Bacharela em Biomedicina.

Orientador: Michael Everton Andrades

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‘’ A felicidade pode ser encontrada mesmo nas horas mais difíceis, se você se lembrar de acender a luz’’.

Harry Potter e o Prisioneiro de Azkaban.

RESUMO

O infarto agudo do miocárdio (IAM) é uma das doenças que mais acomete pacientes ao redor do mundo. Essa patologia é decorrente de um insulto isquêmico, o que diminui a chegada de nutrientes aos cardiomiócitos. Ainda, há a formação de radicais livres, inflamação, morte celular e fibrose, além da ativação excessiva da sinalização adrenérgica e do desenvolvimento de estresse oxidativo. Esses processos associados alteram a contração do músculo cardíaco através de modificações em proteínas essenciais para esse mecanismo, como o fosfolambam (PLB), impactando na mortalidade de pacientes. Assim, o uso de antioxidantes faz-se interessante em um ambiente marcado pela presença de espécies reativas de oxigênio. Dessa forma, este estudo investigou o potencial terapêutico da associação farmacológica antioxidante N-acetilcisteína (NAC) e Deferoxamina (DFX) bem como sua participação no sistema adrenérgico e na sinalização intracelular. Ratos Wistar machos (3 meses de idade) foram randomizados nos seguintes grupos: SHAM (PBS n = 16), IAM (PBS, n = 12) e IAM + NAC/DFX (NAC 25 mg/kg/dia durante todo o acompanhamento e DFX 40 mg/kg/dia por 7 dias, n = 16). Os animais foram acompanhados por 10 ou 28 dias, com análises ecocardiográficas aos 2, 10 e 28 dias após a indução do infarto. Após o seguimento, os animais foram mortos e o coração foi removido para as análises de imunoconteúdo dos receptores β1 adrenérgicos (β-AR) e a fosforilação do fosfolambam (PLB), por Western Blot. O grupo IAM apresentou uma área acinética de 40,5% e uma fração de ejeção de 50,1% aos 2 dias, com piora do quadro ao longo dos 28 dias (FE = 43,4%). O tratamento com NAC/DFX causou uma redução discreta na área de infarto (32,7%; p = 0,092) ao serem equiparados ao grupo infartado não tratado. Não houve diferença estatística entre os grupos na quantificação de β-AR e PLB. Esses resultados indicam que não há evidências da participação do sistema adrenérgico e da sinalização celular por PLB na melhora discreta causada por NAC/DFX.

Palavras-chave: estresse oxidativo. IAM. NAC/DFX. receptores β adrenérgicos.

ABSTRACT

The acute myocardial infarction is one of the diseases that most affects patients around the world. This pathology occurs due to an ischemic insult, which reduces the arrival of nutrients in the cardiomyocytes. Moreover, there is the formation of free radicals, inflammation, cell death and fibrosis, in addition to the excessive activation of adrenergic signaling and the development of oxidative stress. These associated processes alter the contraction of the heart muscle, through modifications in proteins essential for this mechanism, such as phospholambam (PLB), increasing mortality in patients. Thus, the use of antioxidants is interesting in an environment marked by the presence of reactive oxygen species. This paper investigated the therapeutic potential of N-acetylcysteine (NAC) associated with Deferoxamine (DFX) and their participation in the adrenergic system and in the intracellular pathway. Male Wistar rats (3 months old) were randomized into the following groups: SHAM (PBS, n = 16), AMI (PBS, n = 12) and AMI + NAC/DFX (NAC 25 mg/kg/day for 10 and 28 days; DFX 40 mg/kg/day for 7 days, n = 16). The animals were followed for 28 days, with echocardiographic analyses in 2, 10 and 28 days after the infarction induction. After the follow-up, the animals were killed and the heart was removed for immunocontent analysis of β1 adrenergic receptors (β-AR) and phosphorylation of phospholambam (PLB), by Western Blot. 2 days after the surgery, the AMI group had a 40.5% acinetic area and a 50.1% ejection fraction (EF) with worsening of the condition over the 28 days (43.4% EF). The treatment with NAC/DFX promoted a slight improvement in the acinectic area (32.7%, p = 0.092) when compared to the group AMI. There was no statistical difference between the groups in the β-AR and PLB analyses. These results indicate that there is no evidence of the participation of the adrenergic system and cell signaling by PLB in the slight improvement caused by NAC/ DFX.

Keywords: oxidative stress. AMI. NAC/DFX. β adrenergic receptors.

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1.0 INTRODUÇÃO

1.1 INFARTO AGUDO DO MIOCÁRDIO E INSUFICIÊNCIA CARDÍACA

As doenças cardiovasculares (DC) estão entre as principais causas de morte no mundo e, no Brasil, a taxa de mortalidade por esta causa encontra-se entre as maiores do mundo (SANTOS et al., 2018). Dentre as DC, o infarto agudo do miocárdio (IAM) e a insuficiência cardíaca (IC) causaram mais de 120 mil mortes no Brasil em 2017, sendo responsáveis por 9% de todas as mortes registradas naquele ano (DATASUS, 2017).

O IAM decorre de um insulto isquêmico, ou seja, da redução da perfusão no músculo cardíaco, o que diminui a chegada de nutrientes para as células desse tecido (FRANGOGIANNIS, 2015). A causa mais comum dessa doença é a formação de trombos nas artérias coronárias decorrente da ruptura de placas ateroscleróticas. Etiologias mais raras incluem trombos intracardíacos, uso de cocaína, dissecação de artérias coronárias, hipotensão e anemia (BOATENG; SANBORN, 2013).

No coração, essa patologia promove a formação de radicais livres, inflamação, morte celular e fibrose. Conjuntamente, estes processos levam a um remodelamento do órgão, com expansão do ventrículo esquerdo e diminuição da força contrátil. O avanço no remodelamento ventricular pode fazer com que o tecido cardíaco enfrente dificuldade em bombear o sangue, acarretando em uma diminuição geral da qualidade de vida do indivíduo, uma condição conhecida como insuficiência cardíaca (IC) (COHN *et al*., 2000).

Historicamente, a IC foi classificada como um distúrbio hemodinâmico, ou seja, uma patologia relacionada com a circulação (BRUM *et al*.; 2008). Entretanto, no início dos anos 1980, estudos demonstraram que a resposta do sistema simpático ativada pela diminuição do débito cardíaco gerava, a longo prazo, efeitos adversos na sobrevida dos pacientes (BRUM *et al*.; 2008). Esse resultado permitiu identificar a IC como uma síndrome neuro-humoral e defini-la como a incapacidade do coração de suprir demandas metabólicas dos tecidos periféricos, podendo gerar dispneia, fadiga, pressão jugular venosa elevada, taquicardia e edema periférico (TANAI; FRANTZ, 2015). Mesmo com os avanços farmacológicos, a taxa de mortalidade, em 5 anos após o diagnóstico, pode chegar a 50% e cerca de metade das mortes de pacientes com IC acontecem por morte cardíaca súbita ou por falência múltipla dos órgãos devido à hipoperfusão dos tecidos (TANAI; FRANTZ, 2015).

A lesão isquêmica do miocárdio ativa diversas cascatas de sinalização hormonal, como o sistema simpático, o qual libera componentes vasoativos que causam vasoconstrição e estimulam o aumento da concentração intracelular de cálcio nos cardiomiócitos, importante

para a melhorar da contração do músculo (TANAI; FRANTZ, 2015). Ainda, ocorre o aumento da pressão sanguínea, bem como da resistência periférica, contribuindo para a redistribuição do sangue para órgãos vitais (TANAI; FRANTZ, 2015).

Os principais mediadores do sistema simpático são as catecolaminas (adrenalina e noradrenalina), que tem sua concentração aumentada no sangue de pacientes com IC. Esses níveis elevados estão relacionados com maior mortalidade nestes indivíduos pois podem induzir hipertrofia e apoptose em cardiomiócitos (TANAI; FRANTZ, 2015). Conforme a doença progride, há uma diminuição nesses valores, possivelmente devido a uma exaustão do sistema (TANAI; FRANTZ, 2015). Além disso, a sinalização simpática estimula a redução do número e da densidade dos receptores cardíacos β adrenérgicos (TANAI; FRANTZ, 2015). A ativação inicial do sistema simpático pode ser considerada protetora, uma vez que dificulta o desenvolvimento de arritmias, apoptose e hipertrofia patológica. Entretanto, caso ela se torne crônica, pode diminuir a funcionalidade do coração acarretando em prejuízos para o indivíduo (TANAI; FRANTZ, 2015).

1.2 SISTEMA ADRENÉRGICO: CATECOLAMINAS E RECEPTORES

O sistema adrenérgico é composto por catecolaminas, que atuam como sinalizadores, e por seus dois tipos de receptores: α (α-AR) e β adrenérgico (β-AR). As catecolaminas são catecóis, o qual é um composto químico formado por grupamentos hidroxilas associados a um anel de benzeno (GOLDSTEIN, 2010). Elas são produzidas naturalmente no organismo através da hidroxilação do aminoácido tirosina e, de acordo com reações celulares específicas, são transformadas em dopamina (DA), norepinefrina (NE) e epinefrina (EPI) (ANDREIS; SINGER, 2016). Após sua síntese nas células cromafins da glândula adrenal, são armazenadas em grânulos no citoplasma, e liberadas em resposta a um estímulo simpático (GOLDSTEIN, 2010; MAHATA *et al*., 2016).

Em condições fisiológicas, a glândula adrenal secreta cerca de 80% de EPI e 20% de NE. Entretanto, em situações de estresse, tanto físico (exposição ao frio, exercício, baixa tensão de oxigênio) quanto psicológico (medo, ansiedade), as concentrações plasmáticas de NE e EPI tendem a aumentar drasticamente (MAHATA *et al.*, 2016). Essa ativação, caso mantida, pode acarretar em diversos prejuízos para os tecidos. Em pacientes com IC, o nível plasmático elevado sustentado de catecolaminas é um dos principais fatores de risco para a progressão e mortalidade da doença. Além disso, níveis altos de NE são característicos de piores prognósticos (MAHATA *et al.*, 2016).

Uma vez no plasma, as catecolaminas possuem diversos órgãos-alvo: EPI e NE irão se ligar a músculos lisos, células glandulares epiteliais e a outros locais através de α-AR e β-AR, os quais, no coração, correspondem a 10% e 90%, respectivamente. Os β-AR são mais relevantes para o controle da contratilidade cardíaca e são divididos em β1, β2 e β3 (O'CONNELL *et al.,* 2014). O receptor β1 atua ativando a hipertrofia cardíaca, apoptose, necrose e o remodelando cardíaco, principalmente em estágios iniciais da IC (GAO *et al.,* 2014) e corresponde a cerca de 80% da densidade de β-AR cardíacos (O'CONNELL *et al.,* 2014). Já os receptores β2 estão relacionados com propriedades antiapoptóticas (LUCIA *et al.,* 2018) e, por fim, os β3 são raros em células cardíacas humanas, presentes apenas quando há IC severa (GAO *et al.*, 2014).

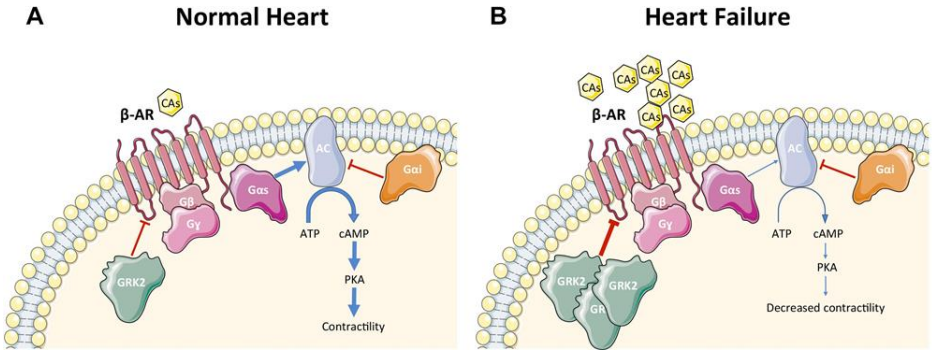
A ativação dos β-AR é responsável, na fase aguda da IC, pelo aumento da frequência e da contratilidade cardíaca, compensando a diminuição do débito cardíaco (SCHNEIDER *et al.*, 2011). Todavia, com a progressão da doença, ocorre uma diminuição da expressão dos receptores β1 para cerca de 50% (LUCIA *et al.*, 2018) e o grau redução está correlacionado com pior prognóstico (SPYROU *et al*., 2002). Além disso, essas alterações ocorrem de maneira diferente nas diversas regiões do coração afetado. Um estudo em animais infartados demonstrou que a densidade dos receptores β1 era menor na zona remota quando comparada à borda do epicárdio (STEINBERG *et al.,* 1995).

Ainda, as catecolaminas afetam o cronotropismo cardíaco através da estimulação dos receptores β1 do nó atrioventricular e sinoatrial, levando a fosforilação de canais de sódio e cálcio e, consequentemente, ao aumento do influxo desses íons. Essa alteração acarreta na elevação da frequência de disparo dos cardiomiócitos (ANDREIS; SINGER, 2016). Também, a entrada de cálcio nas células permite que haja um maior número de ligações actina-miosina, causando um aumento na força de contração (ANDREIS; SINGER, 2016).

O excesso de catecolaminas tem sido associado com a morte de cardiomiócitos, tanto em modelos animais quanto em humanos (ANDREIS; SINGER, 2016). O aumento crônico nos níveis de NE induz a diminuição da expressão dos β-AR, alterando a transmissão do sinal intracelular (GAEMPERLI *et al*., 2010). Mann *et al*. (1992) demonstraram que essa molécula é tóxica para miócitos de felinos em crescimento, sendo esse dano amenizado pelo bloqueio de receptores β. Também, a estimulação adrenérgica contínua levou a sobrecarga de cálcio intracelular no cardiomiócito (MANN *et al.,* 1992). O experimento mostrou que a ativação do sistema simpático após o evento do IAM pode causar dano progressivo às células, contribuindo para o remodelamento do ventrículo esquerdo e do coração como um todo (GAEMPERLI *et*

al., 2010). Além disso, o aumento na atividade do sistema simpático afeta todo órgão não se limitando apenas às regiões infartadas ou ao seu redor (GAEMPERLI *et al*.,2010).

Figura 1- Patofisiologia da IC comparada ao coração fisiológico.



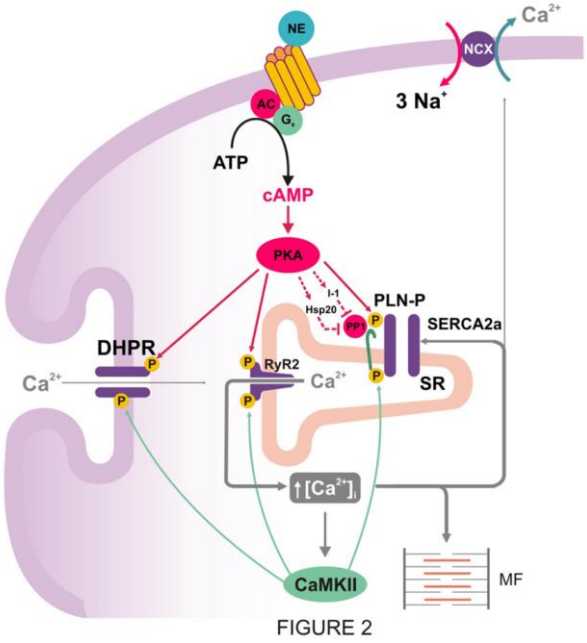
Aumento das catecolaminas circulantes leva à dessensibilização dos receptores acarretando na redução da contratilidade. Fonte: adaptado de Lucia *et al.* (2018).

1.3 SINALIZAÇÃO INTRACELULAR

Ao se ligarem aos β-AR, as catecolaminas estimulam um processo de sinalização intracelular que tem como finalidade realizar o processo de contração cardíaca (SOLARO, 2011). A proteína G ligada a esses receptores separa suas subunidades que levam ao aumento dos níveis de AMP cíclico (AMPc) (MATTIAZI; KRANIAS, 2014), o qual é um cofator necessário para a ativação da Proteína Cinase A (PKA) (SOLARO, 2011). A PKA, por sua vez, atua na fosforilação e regulação da proteína fosfolambam (PLB) e no receptor de Rianodina (RyR) importantes controladores da homeostase do cálcio (MATTIAZI; KRANIAS, 2014).

Os canais de RyR encontram-se na membrana do retículo sarcoplasmático (RS) e, após serem ativados, abrem, permitindo que os íons cálcio se dirijam do RS para o citoplasma (SOLARO, 2011). O cálcio citosólico interage com os miofilamentos, levando ao processo de contração muscular (SOLARO, 2011). Após, os íons cálcios são transportados de volta para o RS através de uma bomba proteica denominada cálcio-ATPase de retículo sarcoplasmático (SERCA), a qual induz o processo de relaxamento cardíaco (SOLARO, 2011). A SERCA pode ser inibida pela ligação com a PLB, que por sua vez é controlada pela PKA: a fosforilação da PLB no sítio Ser16 pela PKA encerra a interação PLB-SERCA permitindo que a atividade de internalização de cálcio ocorra (SOLARO, 2011; GORSKI *et al*.,2017). Curiosamente, apenas 40% da SERCA presente no miocárdio é regulada pela PLB (HAMSTRA, 2020). Entretanto, em algumas situações como envelhecimento e mutações gênicas, pode ocorrer uma alteração na concentração dessas proteínas, levando a uma superexpressão de PLB ou a uma diminuição da SERCA (HAMSTRA, 2020). Essa mudança ocasiona uma maior inibição da SERCA, o que, por sua vez, diminui o relaxamento e a contratilidade cardíaca (HAMSTRA, 2020).

Figura 2 -Sinalização Adrenérgica.

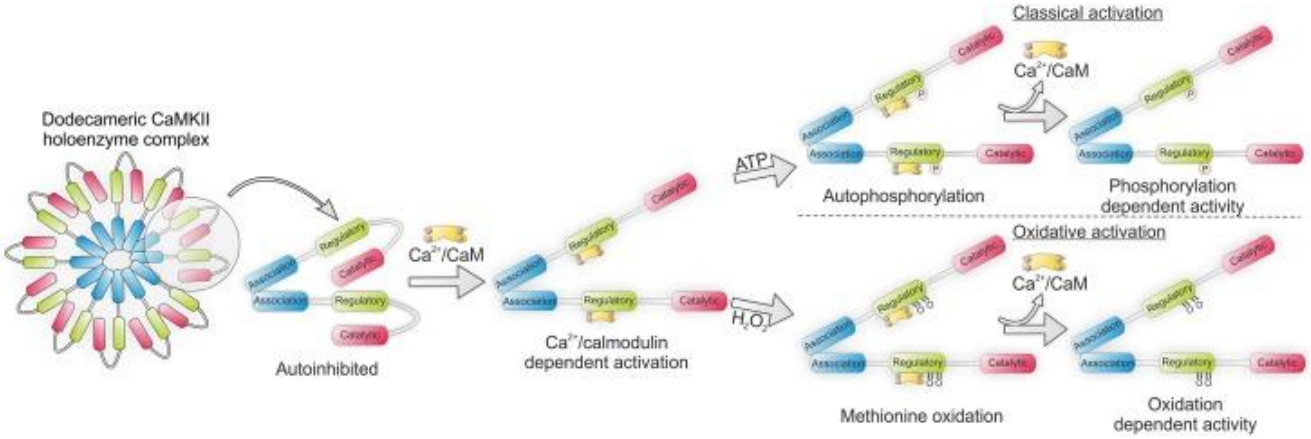


A sinalização adrenérgica é iniciada pela ligação do agonista (norepinefrina (NE), neste exemplo) e ativação da PKA, que por sua vez fosforila e ativa a RyR e o sistema PLB/SERCA, aumentando a contratilidade. O aumento sustentado do Ca^{2+} intracelular ativa a via da CaMKII. Fonte: Mattiazi; Kranias (2014).

Caso o estímulo dos β-AR mantenha-se prolongado, ocorre a ativação da proteína cinase dependente de cálcio/calmodulina II (CamKII). No coração, a isoforma predominante é a CamKIIδ que modula diversas moléculas essenciais no mecanismo de contração cardíaca, como a SERCA, os canais de cálcio do tipo L, RyR e PLB (WANG *et al.*, 2004). Nessas últimas proteínas, a CaMKII possui sítios específicos de fosforilação Thr17 (PLB) e Ser2814 (RyR), sendo que a sua hiperfosforilação leva a perda de homeostase do cálcio (DEWENTER *et al.*, 2017).

A CaMKII é ativada pela ligação de Calmodulina (CaM), que é acionada por cálcio, ao seu domínio autorregulatório. Caso a ativação se mantenha, e na presença de ATP, ocorre autofosforilação em seu resíduo Thr287 (LUCZAK; ANDERSON, 2014). Esse processo permite que a CaMKII permaneça funcional, mesmo na ausência de CaM. Além disso, a CaMKII pode sofrer oxidação por radicais livres em suas metioninas 281 e 282, também levando a sua ativação constante (LUCZAK; ANDERSON, 2014). O aumento de expressão da CaMKII, bem como de sua atividade, leva ao desbalanço na homeostase do cálcio, a arritmias, à insuficiência cardíaca, já vista tanto em pacientes quanto em modelos animais (LUCZAK; ANDERSON, 2014).

Figura 3 - Autofosforilação da CAMKII por suas duas vias.



Autofosforilação da CAMKII medida por CaM (Ativação clássica) e por oxidação em suas metioninas (Ativação por oxidação). A autofosforilação da CAMKII permite que ela mantenha-se ativa mesmo na ausência de CaM. Fonte: adaptado de Johnston *et al.* (2015).

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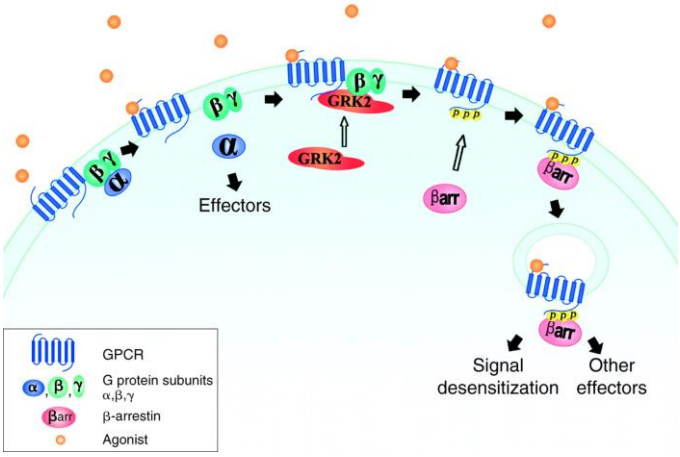
Na IC, os β1-AR e β2-AR encontram-se funcionalmente prejudicados uma vez que estão desacoplados e dessensibilizados devido a tentativa de proteger o coração do bombardeamento de catecolaminas (BECINVEGA *et al*., 2019). Internamente, o mecanismo desregulatório envolve a fosforilação dos receptores β adrenérgicos por uma família de proteínas cinases, denominadas Receptores de Cinase Acoplados à Proteína G (GRKs) (BECINVEGA *et al.*, 2019). Após a fosforilação do receptor, outras moléculas chamadas β-arrestinas se ligam a ele, dissociando-o das proteínas G e bloqueando a transmissão do sinal (BECINVEGA *et al.*, 2019).

A proteína GRK possui diferentes subfamílias, sendo, no coração, as mais expressas as subfamílias GRK2 e GRK5. Durante o desenvolvimento da IC, há um processo de aumento da expressão de GRK2 nos cardiomiócitos, acarretando na redução dos receptores β adrenérgicos (BECINVEGA *et al.*, 2019). Embora a superexpressão de GRK2 possa ser considerada um mecanismo protetor, diversos estudos demonstram que, na verdade, ela é prejudicial, por levar ao desacoplamento de β-AR funcionais, afetando o processo de contração e funcionamento cardíaco (BECINVEGA *et al.*, 2019). Também, a expressão de GRK2, durante a IC, encontra-se aumentada na medula adrenal, o que pode acarretar na disfunção de receptores α2 adrenérgicos e a uma hipersecreção de catecolaminas (CORBI *et al.,* 2013). Atualmente, o aumento de GRK2 no coração e no sangue representa um biomarcador para o diagnóstico e prognóstico de IC (BECINVEGA *et al*., 2019).

Além das GRKs, as β-arrestinas também atuam no processo de diminuição da expressão destes receptores. Após a fosforilação do receptor por GRKs, as β-arrestinas ligam-se a ele, levando a sua internalização para o citosol e acarretando em sua reciclagem de volta para a membrana celular ou na sua degradação (BECINVEGA *et al.*, 2019). O primeiro processo,

reciclagem, desfosforiza os receptores em vesículas ácidas e os carrega novamente para a membrana (BECINVEGA *et al.*, 2019). Já no segundo, degradação, os receptores são eliminados por lisossomos e enzimas (BECINVEGA *et al.*, 2019). Ainda, as β-arrestinas podem sofrer mudanças conformacionais, tornando-se capazes de formar um complexo estável com a proteína CaMKII, acarretando na ativação da sinalização intracelular mediada por essa molécula (BECINVEGA *et al.*, 2019). Essa alteração na via de sinalização ativada pode estar relacionada com a patogênese da IC e da hipertrofia patológica (BECINVEGA e*t al.*, 2019). Ainda, no coração, a β-arrestina 1 está associada com aumento da apoptose após o IAM, a ativação de vias inflamatórias, além de impactar negativamente a contratilidade do órgão (LYMPEROPOULOS *et al.*, 2019).

Figura 4 - Funcionamento da β-arrestina no processo de reciclagem e degradação dos receptores adrenérgicos.



Após sua internalização, os receptores podem ser encaminhados para o processo de reciclagem, terminando novamente na membrana dos cardiomiócitos. Ou, eles podem ser degradados por vesículas ácidas. Fonte: Ma; Pei (2007).

1.4 ESTRESSE OXIDATIVO

Umas das características da patofisiologia do IAM é a formação de espécies reativas de oxigênio (ERO), as quais são caracterizadas pela presença de elétrons desemparelhados, altamente reativos e são produzidos tanto por mecanismos patológicos quanto por fisiológicos, como, por exemplo, a respiração aeróbica celular mitocondrial. Essas moléculas atuam em vários processos de sinalização como na inflamação e no sistema imune e atuam, principalmente, nos resíduos cisteínas do centro catalítico de enzimas (CORBI *et al.*, 2013). Entretanto, sua produção excessiva pode causar danos aos componentes celulares como o DNA, proteínas, lipídios (CORBI *et al.*, 2013). Níveis aumentados de EROs têm sido associados com

desenvolvimento e manutenção de diversas enfermidades degenerativas, como câncer, diabetes, doenças neurodegenerativas e cardiovasculares (CORBI *et al.*, 2013).

O organismo possui formas de neutralizar a geração excessiva de ERO, permitindo a manutenção de níveis adequados e, consequentemente, a homeostase (CORBI *et al.*, 2013). Esse processo ocorre através de moléculas antioxidantes intracelulares ou de vias enzimáticas inibitórias, como da catalase, glutationa peroxidase e superóxido dismutase (FRANGOGIANNIS, 2015). Entretanto, quando ocorrem danos celulares graves, as defesas antioxidantes podem se encontrar sobrecarregadas ou diminuídas, o que aumenta a concentração de ERO e seus efeitos deletérios em macromoléculas importantes para o bom funcionamento do tecido (FRANGOGIANNIS, 2015). Nesta situação, ocorre a instauração do estresse oxidativo (EO) (JOHNSTON *et al.*, 2015).

No coração, as ERO são de vital importância para o reparo inicial do miocárdio após o infarto, porém, a estimulação sustentada promove morte celular por apoptose e induz a degradação da matriz extracelular (FRANGOGIANNIS, 2015). Eventos isquêmicos superiores a cinco minutos estão associados a disfunções sistólicas prolongadas mesmo após a reperfusão completa e esses danos estão relacionados com o desenvolvimento de EO (FRANGOGIANNIS, 2015).

O estabelecimento de EO leva a alterações na SERCA causando uma diminuição da recaptação de íons cálcio (GONZÁLEZ-MONTERO *et al.*, 2018). O aumento na concentração do cálcio citoplasmático pode levar a morte celular e a produção de mais ERO, contribuindo para a formação e manutenção de EO (GONZÁLEZ-MONTERO *et al.*, 2018). Outra proteína que pode ser afetada por EO é a RyR e sua desregulação poderia resultar na ativação de vias de sinalização pró-apoptóticas, necrose e alteração eletromecânicas (GONZÁLEZ-MONTERO *et al.*, 2018).

Os β-AR também possuem regiões susceptíveis (sítios de ligação O-glicosídica na Ser37 e Ser41) a oxidação e, consequentemente, a danos causados por ERO (STEINBERG, 2018). Park e Steinberg (2018) demonstraram que a adição de peróxido de hidrogênio, em cardiomiócitos de camundongos, levou a diminuição da expressão de β-AR1. Esse resultado indicou a possível relação entre o EO, proveniente do ambiente, e a diminuição da expressão dos receptores, característico da IC. Ainda, esses danos foram atenuados com a adição de Carvedilol, um conhecido antagonista adrenérgico, e seu efeito benéfico foi associado com a proteção de cisteínas da inativação por ERO (STEINBERG, 2018). Na mesma linha, Theccanat *et al.* (2016) demonstraram que o aumento na atividade de GRK2, estimulado pela ativação crônica de receptores adrenérgicos, leva a uma maior expressão de NAPDH oxidase 4 (Nox4),

a qual aciona a produção de ERO pelas mitocôndrias, promovendo danos ao DNA e induzindo a morte celular.

1.5 N-ACETILCISTEÍNA E DEFEROXAMINA

Em um ambiente marcado por ERO, o uso de moléculas antioxidantes se faz interessante no esforço de combatê-las e, consequentemente, diminuir os danos associados com o desenvolvimento de EO (ISMAI *et al.,* 2018). Fisiologicamente, os níveis de ERO são controlados por moléculas antioxidantes, como a glutationa (GSH), a qual atua na auto-oxidação de radicais livres envolvidos no processo de aterosclerose (ISMAI *et al.,* 2018). Ainda, a diminuição nos níveis de GSH tem sido relacionada com o aumento de EO no IAM e essa redução pode estar associada com uma dificuldade na recuperação da quantidade de GSH no tecido após isquemia (ISMAI *et al.,* 2018).

Uma das formas de aumentar o estado de GSH é através do uso de N-acetilcisteína (NAC) a qual atua como um precursor de cisteína, um aminoácido que inicia a síntese de GSH. Assim, a NAC indiretamente restaura os níveis de GSH, diminuídos devido ao ambiente oxidante (GONZÁLEZ-MONTERO *et al.*, 2018). Além disso, existem evidências que apontam que a NAC é capaz de causar uma inibição do fator de transcrição NF-κB, o qual tem um papel crítico nos processos de inflamação, imunidade, proliferação celular e diferenciação (GONZÁLEZ-MONTERO *et al.*, 2018). Entretanto, o mecanismo de ação pelo qual a NAC atua em diferentes moléculas e vias sinalizadoras ainda não é bem compreendido, sendo sua principal via de ação a estimulação da síntese de GSH (GONZÁLEZ-MONTERO *et al.*, 2018).

Em pacientes cardíacos, a NAC é empregada na tentativa de reduzir EO e inflamação (PEREIRA *et al*., 2019). Em modelo animal, Chakouri *et al*. (2018) demonstraram que o tratamento com NAC foi capaz de restaurar a habilidade da proteína PKA de modular a sensibilidade dos miofilamentos ao cálcio e, consequentemente, prevenir a disfunção cardíaca observada no grupo não tratado. Já Pereira *et al.* (2019) constataram que em 29 ensaios clínicos randomizados, o uso de NAC não resultou em uma redução significativa na mortalidade, insuficiência renal aguda, insuficiência cardíaca aguda e tempo de hospitalização.

A divergência entre os dois estudos sugere que há uma falta de consenso na comunidade científica sobre o uso de NAC e seus benefícios em pacientes com danos cardiovasculares, bem como, uma dificuldade na transposição dos resultados observados em animais para humanos. Uma das razões para esse fenômeno seria o papel pró-oxidante que a NAC possui em ambientes com presença de metais de transição, como o ferro, tornando-os capazes de reagir com peróxido de hidrogênio, gerando radicais hidroxilas, altamente reativos (SAGRISTÁ *et al.,* 2002) .

Portanto, o uso de moléculas que possam quebrar possíveis metais de transição presentes no ambiente cardíaco associado com NAC pode-se tornar necessário para evitar seu mecanismo pró-oxidante. A Deferoxamina (DFX), por exemplo, atua como um quelante de ferro através da formação de um complexo estável com o íon. Assim, a DFX diminui a presença desse metal no coração e contribui para uma melhora na recuperação do órgão após um evento isquêmico (GONZÁLEZ-MONTERO *et al.,* 2018). Entretanto, agudamente, as doses de DFX podem causar hipotensão e taquicardia. Caso o uso seja crônico, pode ocorrer aplasia, insuficiência renal, além de anemia e neurotoxicidade (BENTUR *et al.,* 1991).

De fato, essa combinação de fármacos tem sido utilizada em diversas patologias como estresse crônico, Distrofia Muscular de Duchenne e hemorragia hepática (ARENT *et al*., 2012; ARENT *et al.*, 2012; ORFANOS *et al.,* 2016). No tecido cardíaco, a associação de NAC e DFX melhorou a fração de ejeção em ratos submetidos a um modelo de IAM (PHAELANTE *et al.*, 2015).

1.1 JUSTIFICATIVA

A ausência de novas estratégias farmacológicas para o manejo do IAM e IC faz com que a mortalidade destes pacientes atinja até 50% em 5 anos. O uso da NAC/DFX tem se mostrado eficiente em diferentes modelos experimentais, inclusive no aumento da contratilidade cardíaca de ratos submetidos ao IAM. Porém, não temos informações sobre o mecanismo molecular pelo qual o NAC/DFX exerce os seus efeitos. Este trabalho tem a relevância de esclarecer um dos potenciais mecanismos da associação NAC/DFX sobre o coração infartado.

1.2 OBJETIVOS

1.2.1 OBJETIVO GERAL

Avaliar os níveis plasmáticos circulantes de catecolaminas e o conteúdo de receptores adrenérgicos β1 em modelo animal de IAM tratado com NAC/DFX.

1.2.2 OBJETIVOS ESPECÍFICOS

Objetivo específico 1: Avaliar os níveis plasmáticos de adrenalina e noradrenalina circulantes.

Objetivo específico 2: Avaliar o conteúdo de receptores β_1-adrenérgicos em homogenato de ventrículo esquerdo.

Objetivo específico 3: Avaliar o conteúdo de fosfolambam presente em homogenato de

ventrículo esquerdo.

2 ARTIGO CIENTÍFICO

Um estudo preliminar sobre a possível atuação da sinalização adrenérgica na melhora cardíaca vista em ratos submetidos ao infarto agudo do miocárdio e tratados com N-acetilcisteína e deferoxamina

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Resumo

O infarto agudo do miocárdio (IAM) induz a formação de espécies reativas de oxigênio (ERO), as quais podem levar ao desenvolvimento de estresse oxidativo. Ainda, o IAM causa a ativação excessiva da sinalização adrenérgica. Dessa forma, este estudo investigou o potencial terapêutico da associação farmacológica antioxidante N-acetilcisteína (NAC) e Deferoxamina (DFX) bem como sua participação no sistema adrenérgico e na sinalização intracelular. Ratos Wistar machos (3 meses de idade) foram randomizados nos seguintes grupos: SHAM (PBS, n = 16), IAM (PBS, n = 12) e IAM + NAC/DFX (NAC 25 mg/kg/dia durante todo o acompanhamento e DFX 40 mg/kg/dia por 7 dias, n = 16). Os animais foram acompanhados por 10 ou 28 dias, com análises ecocardiográficas aos 2, 10 e 28 dias após a indução do infarto. Após o seguimento, os animais foram mortos e o coração foi coletado para as análises de imunoconteúdo dos receptores β1 adrenérgicos (β-AR) e a fosforilação do fosfolambam (PLB), por Western Blot. O grupo IAM apresentou uma área acinética de 40,5% e uma fração de ejeção de 50,1% aos 2 dias, com piora do quadro ao longo dos 28 dias (FE = 43,4%). O tratamento com NAC/DFX causou uma melhora discreta na área de infarto (32,6%; p = 0,092), sem modificar a FE, ao serem equiparados ao grupo infartado não tratado. Não houve diferença estatística entre os grupos na quantificação de β-AR e PLB. Esses resultados indicam que há poucas evidências da participação do sistema adrenérgico e da sinalização celular por PLB na melhora discreta causada por NAC/DFX.

Introdução

Dentre as doenças cardiovasculares, o infarto agudo do miocárdio (IAM) e a insuficiência cardíaca (IC) causaram mais de 120 mil mortes no Brasil em 2017, 9% de todas as mortes registradas naquele ano [1]. O IAM promove a formação de radicais livres, inflamação, morte celular e fibrose no tecido cardíaco. Conjuntamente, estes processos levam a um remodelamento do órgão, com expansão do ventrículo esquerdo e diminuição da força contrátil. Com a progressão da doença, o coração torna-se incapaz de bombear o sangue de forma adequada, acarretando no desenvolvimento de insuficiência cardíaca (IC) [2].

O desequilíbrio da fisiologia cardiovascular no pós-IAM e durante o estabelecimento da IC está intimamente ligado à instabilidade do sistema adrenérgico. Inicialmente, sua hiperativação é essencial para o aumento da frequência e do débito cardíaco, de forma a compensar a diminuição do débito cardíaco [3]. Porém, caso a ativação seja sustentada, ela pode ocasionar fibrose e hipertrofia cardíaca [3]. Altos níveis circulantes de adrenalina estão associados com a maior mortalidade, o que dá suporte ao uso de bloqueadores desta sinalização na IC [4].

No coração, a adrenalina poderá ligar-se a dois tipos de receptores adrenérgicos: α e β, os quais respondem a 10% e 90% respectivamente, sendo os β receptores (β-AR) mais relevantes para o controle da contratilidade cardíaca [5]. O principal receptor envolvido nesse processo é o receptor β1, o qual atua ativando a hipertrofia cardíaca, apoptose, necrose e o remodelando cardíaco, principalmente em estágios iniciais da IC [6]. Todavia, com a progressão da doença, ocorre uma diminuição da expressão dos receptores β1 de 80% para cerca de 50% [7] e o grau redução está diretamente correlacionado com pior prognóstico [8].

Os β-receptores são acoplados à proteína G, que por sua vez regula sinais intracelulares, como o nível de AMP cíclico (cAMP) e a ativação da PKA, que irá fosforilar e ativar alvos

como o fosfolambam (PLB) e o Receptor de Rianodina (RyR) para regular a contratilidade muscular. Os canais de RyR, após serem ativados, abrem, permitindo que os íons cálcio se dirijam do retículo sarcoplasmático (RS) para o citoplasma [9]. O cálcio citosólico interage com os miofilamentos, levando ao processo de contração muscular [9]. Após, os íons cálcios são transportados de volta para o RS através de uma bomba proteica denominada cálcio-ATPase de retículo sarcoplasmático (SERCA). A SERCA pode ser inibida pela ligação com a PLB, o que impede que a atividade de internalização de cálcio ocorra [9-10]. Mudanças na concentração dessas proteínas podem ocasionar uma maior inibição da SERCA, o que, por sua vez, diminui o relaxamento e a contratilidade cardíaca [11].

O processo de sinalização adrenérgico e o controle do manejo do cálcio intracelular podem ser regulados por radicais livres [12-14], criando uma ligação interessante de ser explorada como alvo terapêutico. De fato, a produção excessiva de ROS tem sido associada ao remodelamento cardíaco patológico [15]. O uso da associação antioxidante N-acetilcisteína (NAC) e Deferoxamina (DFX) tem sido testada pelo nosso e por outros grupos de pesquisa em modelos animais de sepse [16], insuficiência hepática [17], renal [18], inflamação pulmonar [19], sempre com evidência de melhoras bioquímicas, morfológicas e funcionais. Assim, hipotetizamos que o tratamento com NAC/DFX pode melhorar a sinalização adrenérgica e os sinais intracelulares relacionados à contratilidade cardíaca vista em ratos IAM tratados com NAC/DFX.

Materiais e Métodos

DELINEAMENTO EXPERIMENTAL

Ratos *Wistar* machos (3 meses de idade, n = 44) foram alojados no biotério e mantidos em um regime de ciclo claro:escuro de 12h:12h, temperatura ambiente, com água e comida *ad*

libitum. 12 horas após a cirurgia de infarto, os animais foram randomizados para os seguintes grupos e tempo de seguimento: SHAM + PBS 10 dias (n = 8), SHAM + PBS 28 dias (n = 8), IAM + PBS 10 dias (n = 5), IAM + PBS (n = 7), IAM + NAC/DFX 10 dias (n = 7), IAM + NAC/DFX 28 dias (n = 9). A DFX foi administrada (40 mg/kg/dia) apenas nos 7 primeiros dias, enquanto que a NAC (25 mg/kg/dia) foi administrada ao longo de todo o seguimento conforme descrito anteriormente [20]. Os animais controle (SHAM e IAM) receberam PBS 20 mM no lugar do antioxidante. Todos os fármacos foram aplicados por via subcutânea. Estudo anterior demostrou que o uso isolado de NAC ou de DFX não promoveu melhora cardíaca e, por este motivo, não avaliaremos estes compostos isoladamente neste trabalho [20]. Após a eutanásia, os tecidos foram armazenados em freezer -80 ºC. Para garantir a homogeneidade dos grupos e o estabelecimento de um modelo consistente, animais do grupo SHAM com área acinética do ventrículo esquerdo ou animais dos grupos IAM com área acinética <25% na avaliação feita 2 dias após a cirurgia foram excluídos do estudo. O projeto encontra-se aprovado pelo Comitê de Ética em Pesquisa Animal do Hospital de Clínicas de Porto Alegre (HCPA), sob o número 2019-0589 e este manuscrito foi preparado seguindo as recomendações do ARRIVE Essential 10 [21].

CIRURGIA DE INDUÇÃO DO IAM

Os animais foram submetidos à isquemia cardíaca pela obstrução da coronária descendente anterior esquerda [22]. A anestesia ocorre através da injeção intraperitoneal de cetamina (100 mg/kg) e xilazina (10 mg/kg). Foi realizada uma incisão cutânea torácica na linha média e as costelas foram afastadas com o auxílio de um afastador para permitir uma melhor visualização do coração. A artéria coronária descendente anterior esquerda foi ligada por um fio mononylon de 6.0 e o infarto confirmado por posterior eletrocardiograma. O operador estava cegado para os grupos de tratamento.

ECOCARDIOGRAFIA

Os animais foram anestesiados com isoflurano (3% com 0,5 L/min de O_2), tricotomizados e colocados em decúbito lateral esquerdo para obter as imagens cardíacas em 2, 10 e 28 dias após a cirurgia. O sistema utilizado foi da Philips Systems – HD7 (Andover, MA, EUA), com um transdutor 12-3 MHz e profundidade de 2 centímetros. As medidas lineares, realizadas nas imagens obtidas pelo modo-M foram: diâmetros do VE ao final da diástole (DDVE) e ao final da sístole (DSVE). A extensão da área de infarto (%IAM) foi medida pelo comprimento das regiões acinética e/ou hipocinética (RAH) das paredes ventriculares e expressa como porcentagem do perímetro total do contorno endocárdico (PE) [23]. A função sistólica foi avaliada pela fração de ejeção (FE) do ventrículo esquerdo [24]. As análises foram realizadas por um operador treinado e cegado para todos os grupos de tratamento.

DENSIDADE RECEPTORES β_1-ADRENÉRGICOS

O conteúdo dos receptores β_1-adrenérgicos foi medido pela técnica de Western Blot. As amostras de coração (~50 mg de tecido) foram homogeneizadas, aplicadas em gel SDS-PAGE (12%) na quantidade de 24 µg por canaleta e separadas de acordo com o seu tamanho molecular. A transferência para membrana de PVDF ocorreu pelo sistema *semi-dry* e, em seguida, as membranas foram coradas com Solução de Comassie (comassie blue-G 250, metanol, ácido acético glacial), descoradas com solução descorante (metanol, ácido acético) até que o fundo ficasse claro e fotografadas. Após, as membranas foram bloqueadas com BSA 5% (sistema SNAP, Milllipore) e incubadas *overnight* com anticorpo para a proteína alvo (anti- β_1, Abcam, cód. ab3442; anti-PLB 24 kDa, Cell Signalling, cód 8495; anti-PLB 24 kDa fosforilada, Cell Signaling, cód 8496). Após, as membranas foram incubadas com anticorpo secundário conjugado com peroxidase. Ambos os anticorpos foram diluídos 1:1000,de acordo com a

orientação do fabricante. A análise da intensidade de cada banda foi determinada pelo software ImageJ. Os valores foram ponderados pela intensidade de coloração do Coomassie (*loading control*) e, posteriormente, analisados estatisticamente.

ANÁLISE ESTATÍSTICA

As variáveis foram testadas para a sua distribuição (Teste de Kolmogorov-Smirnov) e as comparações entre os grupos foram feitas pelo Teste de Kruskal-Wallis. As múltiplas comparações tiveram o valor de p corrigidos e valores abaixo de 0,05 foram considerados significantes. Os resultados estão expressos como mediana, percentis 25 e 75, mínimo e máximo.

Resultados

EFEITO DO TRATAMENTO COM NAC/DFX SOBRE OS PARÂMETROS ECOCARDIOGRÁFICOS

A quantificação dos parâmetros ecocardiográficos é mostrada na Tabela 1. O grupo IAM teve uma redução em sua FE quando comparado ao grupo SHAM, em todos os períodos de estudo ($p < 0,05$). Já o tratamento com NAC/DFX foi capaz de causar uma redução no tamanho de infarto quando comparado com o grupo IAM aos 28 dias, embora sem significância estatística ($p = 0,092$).

Tabela 1. Quantificação dos parâmetros ecocardiográficos. Quantificação dos parâmetros ecocardiográficos. Os resultados apresentados demonstram a média do grupo e entre parênteses os percentis 25 e 75, respectivamente. A letra n indica a quantidade de animais por grupo. FE

(Fração de ejeção), IAM (área de infarto), d (dias). *p < 0,05 *versus* SHAM; $^{+}$ p = 0,092 *versus* IAM.

EFEITO DO TRATAMENTO COM NAC/DFX SOBRE A DENSIDADE DE RECEPTORES β1 ADRENÉRGICOS

Não houve diferença estatística significativa entre todos os grupos experimentais tanto em 10 dias (Fig 1a) quanto em 28 dias (Fig 1b). No entanto, percebe-se um aumento nos β-AR no grupo IAM em 10 dias e uma pequena diminuição dos mesmos em 28 dias. O grupo infartado que recebeu o tratamento com NAC/DFX teve uma redução no aumento de β-AR aos 10 dias e uma diminuição da perda aos 28 dias, quando comparado ao grupo infartado.

Fig 1. Quantificação da densidade de receptores β1 adrenérgicos em 10 (a) e 28 (b)dias. Quantificação de receptores β1 adrenérgicos em 10 (a) e 28 (b) dias após IAM por Western Blot. As comparações entre os grupos foram realizadas pelo Teste de Kruskal-Wallis. As caixas das figuras representam os limites dos percentis 75 (superior) e 25 (inferior), com a mediana ao centro. Ainda, as barras verticais com um traço horizontal indicam os pontos mínimo e máximo.

EFEITO DO TRATAMENTO COM NAC/DFX SOBRE A SINALIZAÇÃO CELULAR

Não houve diferença estatística na fosforilação do PLB 24 kDa entre todos os grupos estudados em 10 (Fig 2a) e 28 dias (Fig 2b). Analisando graficamente, nota-se que houve uma grande diferença entre os valores de cada uma, indicando uma não homogeneidade no grupo IAM + NAC/DFX 28 dias (Fig 2b).

Fig 2. Quantificação da fosforilação do PLB 24 kDa em 10 (a) e 28 (b) dias. Quantificação da fosforilação do PLB 24 kDa em 10 (a) e 28 (b) dias após IAM por Western Blot. As

comparações entre os grupos foram realizadas pelo Teste de Kruskal-Wallis. As caixas das figuras representam os limites dos percentis 75 (superior) e 25 (inferior), com a mediana ao centro. Ainda, as barras verticais com um traço horizontal indicam os pontos mínimo e máximo.

Discussão

Embora o IAM seja uma das doenças cardiovasculares que mais acomete indivíduos no mundo todo, a ausência de novos tratamentos farmacológicos faz com que a mortalidade desses pacientes atinja até 50% em 5 anos. O objetivo desse estudo foi testar o potencial do tratamento com NAC/DFX em um modelo de infarto em ratos machos e avaliar a participação do sistema adrenérgico e da sinalização celular. Nossos resultados confirmaram a funcionalidade do modelo experimental devido à presença de área de infarto considerável e redução da FE. Todavia, não houve diferença significativa no grupo tratado tanto em relação a parâmetros ecocardiográficos quanto a análises protéicas.

Uma das principais formas de avaliar a função sistólica cardíaca é a fração de ejeção, a qual é medida através da quantidade de volume ejetado durante a sístole em relação a quantidade de volume sanguíneo no ventrículo ao final da diástole [25]. Nossos experimentos mostraram uma diminuição da FE de ratos infartados em 10 e 28 dias. Além disso, esses valores reduziram conforme o seguimento do estudo, imitando o curso natural da doença. Em animais infartados e tratados com NAC/DFX, houve uma discreta redução na área de infarto se comparados ao grupo que não recebeu tratamento.

Em um artigo publicado anteriormente pelo nosso laboratório, essa associação farmacológica causou uma melhora de 10% na FE em ratos infartados após 28 dias [20]. Porém, neste presente trabalho, o número amostral não foi o suficiente para garantir poder estatístico a fim de assegurar a presença dos desfechos previstos. De acordo com nossos estudos anteriores, o número amostral deveria ser 13 animais por grupo e devido a alta mortalidade ocorrida

durante o modelo experimental, material biológico degradado e exclusão de ratos com infarto menor que 25%, obtivemos cerca de 6 animais por grupo, número inferior ao desejado e que comprometeu os resultados.

Um dos fatores relacionados com o desenvolvimento do IAM é a ativação do sistema simpático. Nos primeiros momentos, ela pode ser considerada protetora, uma vez que dificulta o desenvolvimento de arritmias, apoptose e hipertrofia patológica [26]. Contudo, caso a ativação simpática torne-se crônica pode diminuir a funcionalidade do coração devido ao desenvolvimento de fibrose e hipertrofia cardíaca [3]. O excesso de catecolaminas circulantes irá ligar-se aos β-AR, os quais sofrem uma diminuição de sua expressão, alterando a força contrátil do miocárdio [7].

Nossos resultados não indicaram diferença estatística significativa entre os grupos avaliados, portanto, não houve redução na densidade de β-AR no grupo infartado, como era esperado. Esse desfecho sugere que não houve uma perda na quantidade desses receptores, mas sim, um desacoplamento da membrana, acarretando em uma dessensibilização e perda de funcionalidade dos mesmos [7].

O mecanismo desregulatório envolve a fosforilação dos β-AR por proteínas cinases, denominadas Receptores de Cinase Acoplados à Proteína G (GRKs) [4]. A superexpressão de GRK2 pode levar ao desacoplamento de β-AR funcionais, afetando o processo de contração e funcionamento cardíaco [4]. Após a fosforilação do receptor, moléculas denominadas β-arrestinas se ligam a ele, dissociando-o das proteínas G, bloqueando a transmissão do sinal e afetando a expressão dos β-AR [4]. A β-arrestina causa a internalização dos receptores para o citosol, onde eles podem serem devolvidos para a membrana ou degradados por lisossomos e enzimas [4]. Ainda, as β-arrestinas, através de mudanças conformacionais, tornam-se capazes de formar um complexo estável com a proteína cinase dependente de cálcio/calmodulina II

(CaMKII) [4]. A ativação expressiva dessa via de sinalização está relacionada com a patogênese da IC, da hipertrofia patológica e de arritmias, vistas tanto em pacientes quanto em modelos animais [4]. Dessa forma, ambas as proteínas, GRK2 e β-arrestina, explicariam os resultados obtidos, demonstrando que outras vias estariam agindo na manutenção vista da quantidade de β-AR e nas disfunções ecocardiográficas.

Além disso, a CaMKII modula diversas moléculas essenciais no mecanismo de contração cardíaca, como a SERCA, os canais de cálcio do tipo L, RyR e PLB [27]. Nessas últimas proteínas, a CaMKII possui sítios específicos de fosforilação Thr17 (PLB) e Ser2814 (RyR), sendo que a sua hiperfosforilação leva a perda de homeostase do cálcio [28]. Nossos experimentos não demonstraram alterações na quantidade de PLB fosforilada na comparação entre os grupos experimentais. Porém, o sítio de fosforilação avaliado foi a Ser16, local de ligação da PKA, e responsável pela inibição da SERCA [9-10]. Alterações nas concentrações dessas proteínas podem causar uma maior inibição da SERCA, diminuindo o relaxamento e a contratilidade cardíaca [11]. Nesse presente estudo, a ativação do PLB pela CamKII não foi avaliada bem como o funcionamento da SERCA. Assim, outros fatores, importantes na via de sinalização do cálcio, estariam envolvidos nas alterações fisiológicas presentes nos grupos e, baseado nos experimentos realizados nesse estudo prévio, não houve evidências da participação do sistema adrenérgico na melhora discreta vista nos animais tratados com NAC/DFX.

Conclusão

Nossos resultados preliminares indicam que o modelo IAM induz a perda da FE e não altera a modulação de β-AR e nem a fosforilação do PLB. O tratamento com NAC/DFX não modificou esses parâmetros, embora tenha causado uma redução na área de infarto aos 28 dias de seguimento (sem diferença estatística). É necessário investigar os sítios de fosforilação da PLB, visto que outros fatores podem estar atuando sobre essa molécula de maneira

independente. Além disso, deve-se averiguar o funcionamento dos β-AR através de proteínas que estão relacionadas com sua dessensibilização e desacoplamento da membrana.

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3 CONCLUSÕES E PERSPECTIVAS

Este trabalho possui poucas evidências para assegurar a participação do sistema adrenérgico no modelo de infarto proposto e nem para esclarecer o mecanismo de ação pelo qual o tratamento com NAC/DFX foi capaz de causar uma discreta melhora no tamanho de infarto ($p = 0,092$) de ratos submetidos ao modelo de IAM com 28 dias de acompanhamento.

Diversas razões podem explicar os resultados obtidos: durante a análise dos experimentos, animais com área de infarto menor que 25% foram excluídos, assim como, animais do grupo SHAM que sofreram infarto. Ainda, algumas amostras biológicas não foram suficientes para todos os experimentos necessários, reduzindo, ainda mais, o número final de animais por grupo. De acordo com o cálculo amostral realizado anteriormente e baseado em estudos do laboratório, para ser observada uma melhora de 10% na FE, deveriam ser alocados 13 animais por grupo. Infelizmente, devido a pandemia não foi possível aumentar o número amostral de forma a garantir o poder estatístico das amostras.

Como perspectiva, têm-se, além de aumentar o número amostral, avaliar as catecolaminas circulantes no plasma dos animais. Esse objetivo inicial do presente trabalho não pode ser realizado devido a crise sanitária que acomete o país. Ainda, gostaríamos de analisar outras proteínas relacionadas com o manejo do cálcio, como SERCA, RyR, CamKII, β-arrestina, e que poderiam explicar os resultados ecocardiográficos encontrados.

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ANEXO A – FIGURAS ARTIGO

Tabela 1. Quantificação dos parâmetros ecocardiográficos.

	Grupos		
	SHAM	**IAM**	**IAM + NAC/DFX**
FE 2d (%)	91,2 (86,2-95,5) n = 15	50,1* (44,5-57,8) n = 12	57,3* (46,9-63,3) n = 15
IAM 2d (%)	0,00 n = 15	40,5* (37,0-42,3) n = 12	37,0* (34,6-39,0) n = 15
FE 10d (%)	86,0 (83,5-89,0) n = 8	49,1* (44,3-57,6) n = 5	50,7* (46,2-61,4) n = 7
IAM 10d (%)	0,00 n = 8	45,1* (43,6-53,9) n = 5	40,0* (31,8-43,5) n = 7
FE 28d (%)	89,2 (84,4-89,9) n = 7	43,5* (29,6-52,6) n = 6	53,2* (45,6-58,8) n = 8
IAM 28d (%)	0,00 n = 7	49,4* (47,7-51,8) n = 6	32,7*$^{+}$ (29,0-37,9) n = 8

Quantificação dos parâmetros ecocardiográficos. Os resultados são apresentados como mediana e, entre parênteses, os percentis 25 e 75, respectivamente. A letra n indica a quantidade de animais por grupo. FE (Fração de ejeção), IAM (área de infarto), d (dias). *p < 0,05 *versus* SHAM; $^{+}$ p = 0,092 *versus* IAM.

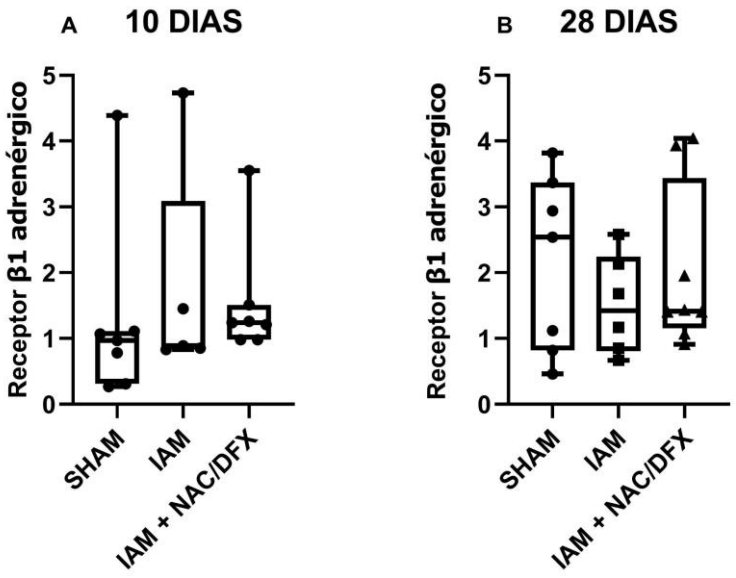

Fig 1. Quantificação da densidade de receptores β1 adrenérgicos em 10 (a) e 28 (b)dias. Quantificação de receptores β1 adrenérgicos em 10 (a) e 28 (b) dias após IAM por Western Blot. As comparações entre os grupos foram realizadas pelo Teste de Kruskal-Wallis. As caixas das figuras representam os limites dos percentis 75 (superior) e 25 (inferior), com a mediana ao centro. Ainda, as barras verticais com um traço horizontal indicam os pontos mínimo e máximo.

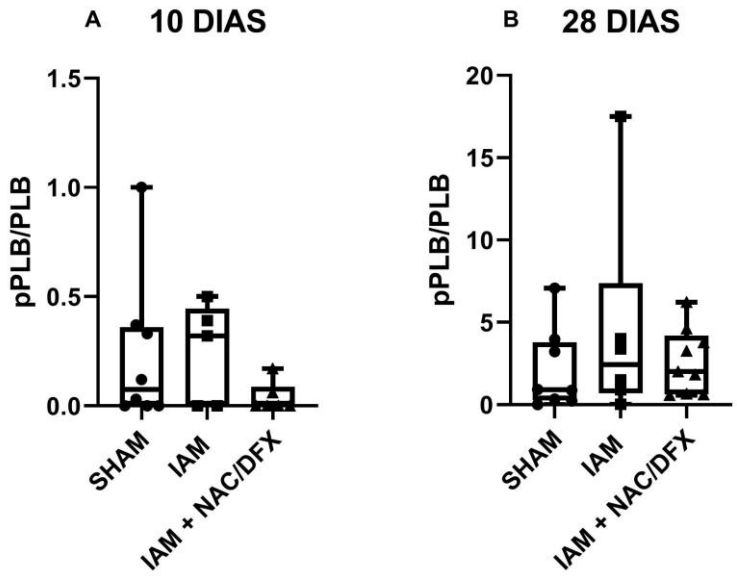

Fig 2. Quantificação da fosforilação do PLB 24 kDa em 10 (a) e 28 (b) dias. Quantificação da fosforilação do PLB 24 kDa em 10 (a) e 28 (b) dias após IAM por Western Blot. As comparações entre os grupos foram realizadas pelo Teste de Kruskal-Wallis. As caixas das figuras representam os limites dos percentis 75 (superior) e 25 (inferior), com a mediana ao centro. Ainda, as barras verticais com um traço horizontal indicam os pontos mínimo e máximo.

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ANEXO B – NORMAS DE PUBLICAÇÃO DA REVISTA PLOS ONE

STYLE AND FORMAT

File format	Manuscript files can be in the following formats: DOC, DOCX, or RTF. Microsoft Word documents should not be locked or protected. LaTeX manuscripts must be submitted as PDFs. Read the LaTeX guidelines.
Length	Manuscripts can be any length. There are no restrictions on word count, number of figures, or amount of supporting information. We encourage you to present and discuss your findings concisely.
Font	Use a standard font size and any standard font, except for the font named "Symbol". To add symbols to the manuscript, use the Insert → Symbol function in your word processor or paste in the appropriate Unicode character.
Headings	Limit manuscript sections and sub-sections to 3 heading levels. Make sure heading levels are clearly indicated in the manuscript text.
Layout and spacing	Manuscript text should be double-spaced. Do not format text in multiple columns.
Page and line numbers	Include page numbers and line numbers in the manuscript file. Use continuous line numbers (do not restart the numbering on each page).
Footnotes	Footnotes are not permitted. If your manuscript contains footnotes, move the information into the main text or the reference list, depending on the content.

Language	Manuscripts must be submitted in English. You may submit translations of the manuscript or abstract as supporting information. Read the supporting information guidelines.
Abbreviations	Define abbreviations upon first appearance in the text. Do not use non-standard abbreviations unless they appear at least three times in the text. Keep abbreviations to a minimum.
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.

Nomenclature Use correct and established nomenclature wherever possible.

Units of measurement	Use SI units. If you do not use these exclusively, provide the SI value in parentheses after each value. Read more about SI units.
Drugs	Provide the Recommended International Non-Proprietary Name (rINN).
Species names	Write in italics (e.g., *Homo sapiens*). Write out in full the genus and species, both in the title of the manuscript and at the first mention of an organism in a paper. After first mention, the first letter of the genus name followed by the full species name may be used (e.g., *H. sapiens*).
Genes, mutations, genotypes, and alleles	Write in italics. Use the recommended name by consulting the appropriate genetic nomenclature database (e.g., HGNC for human genes; we strongly recommend using this tool to check against previously approved names). It is sometimes advisable to indicate the synonyms for the gene the first time it appears in the text. Gene prefixes such as those used for oncogenes or cellular localization should be shown in roman typeface (e.g., v-fes, c-MYC).
Allergens	The systematic allergen nomenclature of the World Health Organization/International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee should be used for manuscripts that include the description or use of allergenic proteins. For manuscripts describing new allergens, the systematic name of the allergen should be approved by the WHO/IUIS Allergen Nomenclature Sub-Committee prior to manuscript publication. Examples of the systematic allergen nomenclature can be found at the WHO/IUIS Allergen Nomenclature site.

Copyediting manuscripts

Prior to submission, authors who believe their manuscripts would benefit from professional editing are encouraged to use language-editing and copyediting services. Obtaining this service is the responsibility of the

author, and should be done before initial submission. These services can be found on the web using search terms like “scientific editing service” or “manuscript editing service.”

Submissions are not copyedited before publication.

Submissions that do not meet the *PLOS ONE* publication criterion for language standards may be rejected.

MANUSCRIPT ORGANIZATION

Manuscripts should be organized as follows. Instructions for each element appear below the list.

MANUSCRIPT ORGANIZATION

Manuscripts should be organized as follows. Instructions for each element appear below the list.

Beginning section	*The following elements are required, in order:* Title page: List title, authors, and affiliations as first page of manuscript Abstract Introduction
Middle section	*The following elements can be renamed as needed and presented in any order:* Materials and Methods Results Discussion Conclusions (optional)
Ending section	*The following elements are required, in order:* Acknowledgments References Supporting information captions (if applicable)

Other elements	Figure captions are inserted immediately after the first paragraph in which the figure is cited. Figure files are uploaded separately. Tables are inserted immediately after the first paragraph in which they are cited. Supporting information files are uploaded separately.

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PARTS OF A SUBMISSION

Title

Include a full title and a short title for the manuscript.

Title	Length	Guidelines	Examples
Full title	250 characters	Specific, descriptive, concise, and comprehensible to readers outside the field	Impact of cigarette smoke exposure on innate immunity: A *Caenorhabditis elegans* model Solar drinking water disinfection (SODIS) to reduce childhood diarrhoea in rural Bolivia: A cluster-randomized, controlled trial
Short title	100 characters	State the topic of the study	Cigarette smoke exposure and innate immunity SODIS and childhood diarrhoea

Titles should be written in sentence case (only the first word of the text, proper nouns, and genus names are capitalized). Avoid specialist abbreviations if possible. For clinical trials, systematic reviews, or meta-analyses, the subtitle should include the study design.

Author list

Authorship requirements

All authors must meet the criteria for authorship as outlined in the authorship policy. Those who contributed to the work but do not meet the criteria for authorship can be mentioned in the Acknowledgments. Read more about Acknowledgments.

The corresponding author must provide an ORCID iD at the time of submission by entering it in the user profile in the submission system. Read more about ORCID.

Author names and affiliations

Enter author names on the title page of the manuscript and in the online submission system.

On the title page, write author names in the following order:

First name (or initials, if used)
Middle name (or initials, if used)
Last name (surname, family name)

Each author on the list must have an affiliation. The affiliation includes department, university, or organizational affiliation and its location, including city, state/province (if applicable), and country. Authors have the option to include a current address in addition to the address of their affiliation at the time of the study. The current address should be listed in the byline and clearly labeled “current address.” At a minimum, the address must include the author’s current institution, city, and country.

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Author names will be published exactly as they appear in the manuscript file. Please double-check the information carefully to make sure it is correct.

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The submitting author is automatically designated as the corresponding author in the submission system. The corresponding author is the primary contact for the journal office and the only author able to view or change the manuscript while it is under editorial consideration.

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Consortia and group authorship

If a manuscript is submitted on behalf of a consortium or group, include its name in the manuscript byline. Do not add it to the author list in the submission system. You may include the full list of members in the Acknowledgments or in a supporting information file.

PubMed only indexes individual consortium or group author members listed in the article byline. If included, these individuals must qualify for authorship according to our criteria.

Read the group authorship policy.

Author contributions

Provide at minimum one contribution for each author in the submission system. Use the CRediT taxonomy to describe each contribution. Read the policy and the full list of roles.

Contributions will be published with the final article, and they should accurately reflect contributions to the work. The submitting author is responsible for completing this information at submission, and we expect that all authors will have reviewed, discussed, and agreed to their individual contributions ahead of this time.

PLOS ONE will contact all authors by email at submission to ensure that they are aware of the submission.

Cover letter

Upload a cover letter as a separate file in the online system. The length limit is 1 page.

The cover letter should include the following information:

- Summarize the study's contribution to the scientific literature
- Relate the study to previously published work
- Specify the type of article (for example, research article, systematic review, meta-analysis, clinical trial)
- Describe any prior interactions with PLOS regarding the submitted manuscript
- Suggest appropriate Academic Editors to handle your manuscript (see the full list of Academic Editors)
- List any opposed reviewers

IMPORTANT: Do not include requests to reduce or waive publication fees in the cover letter. This information will be entered separately in the online submission system.

Read about publication fee assistance.

Title page

The title, authors, and affiliations should all be included on a title page as the first page of the manuscript file.

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Abstract

The Abstract comes after the title page in the manuscript file. The abstract text is also entered in a separate field in the submission system.

The Abstract should:

- Describe the main objective(s) of the study
- Explain how the study was done, including any model organisms used, without methodological detail
- Summarize the most important results and their significance
- Not exceed 300 words

Abstracts should not include:

- Citations
- Abbreviations, if possible

Introduction

The introduction should:

- Provide background that puts the manuscript into context and allows readers outside the field to understand the purpose and significance of the study
- Define the problem addressed and why it is important
- Include a brief review of the key literature
- Note any relevant controversies or disagreements in the field
- Conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved

Materials and Methods

The Materials and Methods section should provide enough detail to allow suitably skilled investigators to fully replicate your study. Specific information and/or protocols for new methods should be included in detail. If materials, methods, and protocols are well established, authors may cite articles where those protocols are described in detail, but the submission should include sufficient information to be understood independent of these references.

Supporting reproducibility with protocols

To enhance the reproducibility of your results, we recommend and encourage you to make your protocols public. There are several options:

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Protocol documents may be uploaded as Supporting Information or linked from the Methods section of the article. For laboratory protocols, we recommend protocols.io. Include the DOI link in the Methods section of your manuscript using the following format: http://dx.doi.org/10.17504/protocols.io.[PROTOCOL DOI]. This allows editors and reviewers to consult the detailed step-by-step protocol when evaluating your manuscript. You can choose to keep the protocol private on the protocols.io platform until your article is published—at which time it will be published automatically.

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PLOS ONE offers two options for publishing stand-alone protocol articles: Lab Protocols that describe verified methodologies and Study Protocols that describe detailed plans and proposals for research projects. Specific guidelines apply to the submission of Lab Protocol and Study Protocol manuscripts. Read the detailed instructions for submitting Lab Protocols and Study Protocols.

Results, Discussion, Conclusions

These sections may all be separate, or may be combined to create a mixed Results/Discussion section (commonly labeled "Results and Discussion") or a mixed Discussion/Conclusions section (commonly labeled "Discussion"). These sections may be further divided into subsections, each with a concise subheading, as appropriate. These sections have no word limit, but the language should be clear and concise.

Together, these sections should describe the results of the experiments, the interpretation of these results, and the conclusions that can be drawn.

Authors should explain how the results relate to the hypothesis presented as the basis of the study and provide a succinct explanation of the implications of the findings, particularly in relation to previous related studies and potential future directions for research.

PLOS ONE editorial decisions do not rely on perceived significance or impact, so authors should avoid overstating their conclusions. See the *PLOS ONE* Criteria for Publication for more information.

Acknowledgments

Those who contributed to the work but do not meet our authorship criteria should be listed in the Acknowledgments with a description of the contribution.

Authors are responsible for ensuring that anyone named in the Acknowledgments agrees to be named.

PLOS journals publicly acknowledge the indispensable efforts of our editors and reviewers on an annual basis. To ensure equitable recognition and avoid any appearance of partiality, do not include editors or peer reviewers—named or unnamed—in the Acknowledgments.

Do not include funding sources in the Acknowledgments or anywhere else in the manuscript file. Funding information should only be entered in the financial disclosure section of the submission system.

References

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- Manuscripts on preprint servers, providing the manuscript has a citable DOI or arXiv URL.

Do not cite the following sources in the reference list:

- Unavailable and unpublished work, including manuscripts that have been submitted but not yet accepted (e.g., "unpublished work," "data not shown"). Instead, include those data as supplementary material or deposit the data in a publicly available database.
- Personal communications (these should be supported by a letter from the relevant authors but not included in the reference list)

References are listed at the end of the manuscript and numbered in the order that they appear in the text. In the text, cite the reference number in square brackets (e.g., "We used the techniques developed by our colleagues [19] to analyze the data"). PLOS uses the numbered citation (citation-sequence) method and first six authors, et al.

Do not include citations in abstracts.

Make sure the parts of the manuscript are in the correct order *before* ordering the citations.

Formatting references

Because all references will be linked electronically as much as possible to the papers they cite, proper formatting of the references is crucial.

PLOS uses the reference style outlined by the International Committee of Medical Journal Editors (ICMJE), also referred to as the "Vancouver" style. Example formats are listed below. Additional examples are in the ICMJE sample references.

A reference management tool, EndNote, offers a current style file that can assist you with the formatting of your references. If you have problems with any reference management program, please contact the source company's technical support.

Journal name abbreviations should be those found in the National Center for Biotechnology Information (NCBI) databases.

Source	**Format**

Published articles	Hou WR, Hou YL, Wu GF, Song Y, Su XL, Sun B, et al. cDNA, genomic sequence cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda (Ailuropoda melanoleuca). Genet Mol Res. 2011;10: 1576-1588. Devaraju P, Gulati R, Antony PT, Mithun CB, Negi VS. Susceptibility to SLE in South Indian Tamils may be influenced by genetic selection pressure on TLR2 and TLR9 genes. Mol Immunol. 2014 Nov 22. pii: S0161-5890(14)00313-7. doi: 10.1016/j.molimm.2014.11.005. Note: A DOI number for the full-text article is acceptable as an alternative to or in addition to traditional volume and page numbers. When providing a DOI, adhere to the format in the example above with both the label and full DOI included at the end of the reference (doi: 10.1016/j.molimm.2014.11.005). Do not provide a shortened DOI or the URL.
Accepted, unpublished articles	Same as published articles, but substitute “Forthcoming” for page numbers or DOI.
Online articles	Huynen MMTE, Martens P, Hilderlink HBM. The health impacts of globalisation: a conceptual framework. Global Health. 2005;1: 14. Available from: http://www.globalizationandhealth.com/content/1/1/14
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.
Deposited articles (preprints, e-prints, or arXiv)	Krick T, Shub DA, Verstraete N, Ferreiro DU, Alonso LG, Shub M, et al. Amino acid metabolism conflicts with protein diversity. arXiv:1403.3301v1 [Preprint]. 2014 [cited 2014 March 17]. Available from: https://128.84.21.199/abs/1403.3301v1 Kording KP, Mensh B. Ten simple rules for structuring papers. BioRxiv [Preprint]. 2016 bioRxiv 088278 [posted 2016 Nov 28; revised 2016 Dec 14; revised 2016 Dec 15; cited 2017 Feb 9]: [12 p.]. Available from: https://www.biorxiv.org/content/10.1101/088278v5 doi: 10.1101/088278

Published media (print or online newspapers and magazine articles)	Fountain H. For Already Vulnerable Penguins, Study Finds Climate Change Is Another Danger. The New York Times. 2014 Jan 29 [Cited 2014 March 17]. Available from: http://www.nytimes.com/2014/01/30/science/earth/climate-change-taking-toll-on-penguins-study-finds.html
New media (blogs, web sites, or other written works)	Allen L. Announcing PLOS Blogs. 2010 Sep 1 [cited 17 March 2014]. In: PLOS Blogs [Internet]. San Francisco: PLOS 2006 - . [about 2 screens]. Available from: http://blogs.plos.org/plos/2010/09/announcing-plos-blogs/.
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
Databases and repositories (Figshare, arXiv)	Roberts SB. QPX Genome Browser Feature Tracks; 2013 [cited 2013 Oct 5]. Database: figshare [Internet]. Available from: http://figshare.com/articles/QPX_Genome_Browser_Feature_Tracks/701214
Multimedia (videos, movies, or TV shows)	Hitchcock A, producer and director. Rear Window [Film]; 1954. Los Angeles: MGM.

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Authors can submit essential supporting files and multimedia files along with their manuscripts. All supporting information will be subject to peer review. All file types can be submitted, but files must be smaller than 20 MB in size.

Authors may use almost any description as the item name for a supporting information file as long as it contains an “S” and number. For example, “S1 Appendix” and “S2 Appendix,” “S1 Table” and “S2 Table,” and so forth.

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Example caption

S1 Text. Title is strongly recommended. Legend is optional.

In-text citations

We recommend that you cite supporting information in the manuscript text, but this is not a requirement. If you cite supporting information in the text, citations do not need to be in numerical order.

Read the supporting information guidelines for more details about submitting supporting information and multimedia files.

Figures and tables

Figures

Do not include figures in the main manuscript file. Each figure must be prepared and submitted as an individual file.

Cite figures in ascending numeric order at first appearance in the manuscript file.

Read the guidelines for figures and requirements for reporting blot and gel results.

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Figure captions must be inserted in the text of the manuscript, immediately following the paragraph in which the figure is first cited (read order). Do not include captions as part of the figure files themselves or submit them in a separate document.

At a minimum, include the following in your figure captions:

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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.
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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.

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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.

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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.

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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.

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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

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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

Registered Reports

Submission and format requirements for Registered Report Protocols and Registered Reports are similar to those for a regular submission and may be specific to your study type. For instance, if your Registered Report Protocol submission is about a Clinical Trial or a Systematic Review, follow the appropriate guidelines.

For Registered Report Protocols:

- Provide enough methodological detail to make the study reproducible and replicable
- Confirm that data will be made available upon study completion in keeping with the PLOS Data policy
- Include ethical approval or waivers, if applicable
- Preliminary or pilot data may be included, but only if necessary to support the feasibility of the study or as a proof of principle
- For meta-analyses or Clinical Trials, use the protocol-specific reporting guidelines PRISMA-P or SPIRIT respectively

For more guidance on format and presentation of a protocol, consult the sample template hosted by the Open Science Framework. Discipline-specific and study-specific templates are also available.

If data need to be collected, modified or processed specifically for your study, or if participants need to be recruited specifically for your study, then it should occur only after your Registered Report Protocol is accepted for publication.

For Registered Report Research Articles:

- Report the results of all planned analyses and, if relevant, detail and justify all deviations from the protocol.
- The manuscript may also contain exploratory, unplanned analyses.

Read more about Registered Report framework.

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:

> The name of the registry and the registration number must be included in the Abstract.
> 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 name of the registry and the registry number must be provided in the Abstract. If the trial is registered in more than one location, please provide all relevant registry names and numbers.

Lab Protocols

Lab Protocols consist of two interlinked components: a protocol hosted on the protocols.io platform and a peer-reviewed article on *PLOS ONE* that contextualises the protocol.

protocols.io is a secure open access platform that specializes in laboratory protocols. It allows scientists to share, discover and reuse up-to-date protocol knowledge. The platform provides specialist tools and guidance on how to add each element of the protocol, including the title, abstract, steps, files, links, reagents, measurements, formulae, videos, charts and more.

The *PLOS ONE* article component must comply with the general submission guidelines (detailed above in this article).

The *PLOS ONE* article component must also comply with the general *PLOS ONE* criteria for publication and in addition it should:

> Present a step-by-step protocol that adds value to the published literature.
> Link, in the Introduction section, to at least one supporting peer-reviewed publication in which the protocol was applied to generate data.
> Link, in the Materials and Methods section, to the protocol.io component, using the digital object identifier (DOI) and format provided by protocols.io, for example *https://dx.doi.org/10.17504/protocols.io[....]*.
> Describe the appropriate controls, sample sizes and replication needed to ensure that the data are robust and reproducible.
> Provide the protocol as a supporting information (S1) file for printing purposes. You can download a PDF from protocols.io for this purpose.
> Optionally, provide minimal new data relevant to the development of the protocol e.g., for additional benchmarking, validation or troubleshooting purposes.

Download a sample Lab Protocol template

Lab Protocols describing routine methods, or extensions or modifications of routine methods, add little or no value to the published literature and will not be considered for publication.

Manuscripts that report new methods should be submitted as research articles, not as Lab Protocols

Lab Protocols are subject to the same editorial and peer review process as all other articles, except that the peer review process may be expedited and carried out by one internal Academic Editor and one external reviewer.

Lab Protocols are eligible for both signed and published peer review.

We encourage you to post your protocol to the protocols.io platform before submitting your manuscript to *PLOS ONE*, or at the latest, before the editorial and peer review process. This approach is optional, but beneficial, because:

> Your DOI is assigned on the protocols.io platform. You need this identifier to link out from the Material and Methods section of your manuscript.

You can keep your protocol private on the protocols.io platform (until you are satisfied that it is ready for publication), but still assign a DOI.
The protocol will be accessible to editors and reviewers during the editorial and peer review process.

If you prefer to submit your manuscript to *PLOS ONE* before uploading your protocol to protocols.io, please provide your protocol as a supporting information (S1) file. You can use protocols.io's editorial service at no cost: they will check and publish your protocol for you t. As part of *PLOS ONE*'s partnership with protocols.io, your waiver code for this purpose will be provided in the first decision letter.

Preprint posting is not available for Lab Protocols and bioRxiv does not accept them.

Study Protocols

Study Protocols describe plans for conducting research projects and consist of a single article on *PLOS ONE*.

Study Protocols must comply with the *PLOS ONE* general submission guidelines (detailed above in this article) and any guidelines specific to the related research study type. In addition, the protocol must:

Relate to a research study that has not yet generated results.
Be submitted before recruitment of participants or collection of data for the study is complete.
Meet the same standards for ethics of experimentation and research integrity as the research study. If it involves human or animal subjects, cell lines or field sampling, or has potential biosafety implications, prior approval from the relevant ethics body must be obtained prior to submission. Please contact us if you have a valid reason for not obtaining approval.

Additional prerequisites apply for these study types:

Clinical trials:

The trial must be registered prior to submission of your protocol in one of the publicly accessible registries approved by the WHO or ICMJE (International Committee of Medical Journal Editors).
The name of the registry and the trial or study registration number must be included in the Abstract.
A copy of the protocol that was approved by the ethics committee must be submitted as a supplementary information file. Please provide an addtional English translation if the original document is not in English.
A SPIRIT schedule of enrollment, interventions, and assessments must be included as the manuscript's Figure 1, and a completed SPIRIT checklist must be uploaded as Supporting Information file S1.

Systematic reviews and meta-analyses:

A completed PRISMA-P checklist must be provided as a supporting information (SI) file. See PRISMA-P Explanation and Elaboration for more information on completing your checklist.

Study Protocols must also comply with general *PLOS ONE* criteria for publication and in addition you should:

include the word “Protocol” in your Title.
include a detailed description of the planned study in the Materials and Methods section.This should provide sufficient methodological detail for the protocol to be reproducible and replicable. Your description should cover all relevant and applicable facts and hypothesis, including:

- the aim, design, and settling
- the sample size calculation
- how data saturation will be determined (for qualitative studies)
- the characteristics of participants e.g., inclusion and exclusion criteria, sample selection criteria, variables to be measured, randomization and blinding criteria (where applicable), and how informed consent will be obtained
- how materials will be selected and used e.g., where and how they will be sourced, the processes, interventions, or comparisons to be used, the outcomes to be measured, and when and how they will be measured
- the data management plan
- safety considerations
- the type of data and statistical analyses to be used
- the status and timeline of the study, including whether participant recruitment or data collection has begun
- where and when the data will be made available. See our Data Availability policy for more.

include an analysis of preliminary or pilot data, only if it is necessary to support the feasibility of the study or as a proof of principle. This is optional.
we encourage authors you to register with OSF and provide the your registration number in the Materials and Methods section. This is optional.
optionally add any other SI files, figures or tables that elaborate or authenticate the protocol: e.g., any reporting checklists applicable to your study type.

Read the supporting information guidelines for more details about adding SI files.

Download our sample Study Protocol template or an OSF discipline or study-specific template.
Study Protocols are subject to the same editorial and peer review process as all other articles, and are eligible for both signed and published peer review.

You can expedite the review process by providing:

proof of external funding. This is typically your funding approval letter and a list of the names and credentials of the funders who conducted the external peer review of the protocol. Include an English translation if needed.
proof of ethics approval (if required). This is typically the approval or waiver letter from the relevant ethics body and a copy of the protocol approved by this body.

These documents are used for internal purposes and do not form part of the published Study Protocol.

Expedited review is conducted by an internal Staff Editor only and bypasses the external review process.

If the Study Protocol describes a replication study or involves re-analysis of published work, we will invite the author of the initial or replicated study to provide a signed review.

We encourage you to share your Study Protocol with other researchers, either before or after submission. You can publish it on your website or protocols.io, or submit it for posting on medRxiv or another preprint server.

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

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New taxon names

Zoological names

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Anochetus boltoni *Fisher* ***sp. nov.*** *urn:lsid:zoobank.org:act:B6C072CF-1CA6-40C7-8396-534E91EF7FBB*
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Please also insert the following text into the **Methods** section, in a sub-section to be called "Nomenclatural Acts":

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature, and hence the new names contained herein are available under that Code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix "http://zoobank.org/". The LSID for this publication is: urn:lsid:zoobank.org:pub: XXXXXXX. The electronic edition of this work was published in a journal with an ISSN, and has been archived and is available from the following digital repositories: PubMed Central, LOCKSS [author to insert any additional repositories].
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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]).
Journal staff will contact IPNI to obtain the GUID (LSID) after your manuscript is accepted for publication, and this information will then be added to the manuscript during the production phase

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In addition, new names contained in this work have been submitted to IPNI, from where they will be made available to the Global Names Index. The IPNI LSIDs can be resolved and the associated information viewed through any standard web browser by appending the LSID contained in this publication to the prefix http://ipni.org/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)].

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Effective January 2012, the description or diagnosis of a new taxon can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

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Hymenogaster huthii. Stielow et al. 2010, sp. nov. [urn:lsid:indexfungorum.org:names:518624]
You will need to contact either Mycobank or Index Fungorum to obtain the 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. Effective January 2013, all papers describing new fungal species must reference the identifier issued by a recognized repository in the protologue in order to be considered effectively published.

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The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

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