UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA

Amanda Lopes

EFEITO DA ANGIOTENSINA 1-7 EM LINHAGEM DE CARDIOMIÓCITOS H9C2 TRATADOS COM METILGLIOXAL

Porto Alegre
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Trabalho de Conclusão de Curso, apresentado ao curso de Farmácia da Universidade Federal do Rio Grande do Sul, como requisito para obtenção do título de Bacharel em Farmácia.

Orientadora: Dra. Nadine Oliveira Clausell
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Dedico este trabalho aos meus pais, Darci e Ada, minha base, meu porto seguro.

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Isaac Newton

RESUMO

Pacientes diabéticos tem elevadas chances de desenvolver complicações cardiovasculares. O metilglioxal (MGO), aumentado na diabetes, é um subproduto tóxico da glicólise que parece ter papel importante na fisiopatologia das doenças cardiovasculares. Esta molécula é responsável por modificar biomoléculas, formar produtos finais de glicação avançada (AGEs) e desencadear respostas intracelulares por receptores específicos, como ativar o sistema renina-angiotensina tecidual que, pela angiotensina II (AngII), pode causar efeitos deletérios nas células. O objetivo do trabalho foi avaliar o efeito cardioprotetor da angiotensina 1-7 (Ang1-7), um peptídeo com efeito antagônico à AngII, sobre viabilidade, autofagia e apoptose de cardiomiócitos H9c2 tratados com MGO. A concentração de MGO utilizada neste estudo foi determinada pelo teste MTT, que analisa viabilidade celular. A DL_{50} para o MGO foi 272 µM. Utilizamos 200 µM, próximo à concentração sub-letal e capaz de alterar o fluxo autofágico celular. O efeito cardioprotetor da Ang1-7 foi testado em duas concentrações (0,1 µM e 1,0 µM) que foram comparadas aos grupos MGO e controle. Utilizamos um grupo controle positivo de morte celular, tratado com 500 µM de MGO. Os grupos receberam o tratamento por 24 horas. As análises de autofagia e apoptose foram realizadas por Western Blot, através da detecção das proteínas LC3B, p62 e caspase-3 clivada. Os dados preliminares indicam maior mortalidade das células que receberam o co-tratamento (MGO + Ang1-7) quando comparadas aos grupos de tratamento único. Observamos maior acúmulo de p62 no grupo controle positivo co-tratado. Ao avaliar apoptose, não observamos ativação em nenhum outro grupo, apenas no controle positivo. A maior mortalidade no grupo co-tratado pode estar associada à possível reação do MGO com Ang1-7, que originaria um produto tóxico, com efeitos negativos semelhantes aos AGEs, inviabilizando a molécula de Ang1-7 de desempenhar cardioproteção. O projeto prevê um número amostral maior que permitirá concluir a consistência destes dados.

Palavras chaves: Autofagia. Cardiomiócitos. Metilglioxal. Sistema renina-angiotensina.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

AGEs	Produtos finais de glicação avançada
Ang1-5	Angiotensina 1-5
Ang1-7	Angiotensina 1-7
Ang1-9	Angiotensina 1-9
AngI	Angiotensina I
AngII	Angiotensina II
AngIII	Angiotensina III
AngIV	Angiotensina IV
Atg	Autophagy-related genes/proteins
DCV	Doenças cardiovasculares
DHAP	Fosfato de diihidroxiacetona
DL_{50}	Dose letal 50%
DMSO	Dimetilsulfóxido
ECA	Enzima conversora de angiotensina
ECA2	Enzima conversora de angiotensina 2
G3P	Gliceraldeido-3-fosfato
MGO	Metilglioxal
MTT	Brometo de [3-(4,5-dimetiltiazol-2yl)-2,5-difenil tetrazolium]
NEP	Neprilisina
POP	Prolil-oligopeptidase
SBF	Soro bovino fetal
SRA	Sistema renina – angiotensina
TOP	Thimet-oligopeptidase

SUMÁRIO

Efeito da angiotensina 1-7 em linhagem de cardiomiócitos H9c2 tratados com metilglioxal

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¶ Esses autores contribuíram igualmente para este trabalho.

Resumo

Pacientes diabéticos tem elevadas chances de desenvolver complicações cardiovasculares. O metilglioxal (MGO), aumentado na diabetes, é um subproduto tóxico da glicólise que parece ter papel importante na fisiopatologia das doenças cardiovasculares. Esta molécula é responsável por modificar biomoléculas, formar produtos finais de glicação avançada (AGEs) e desencadear respostas intracelulares por receptores específicos, como ativar o sistema renina-angiotensina tecidual que, pela angiotensina II (AngII), pode causar efeitos deletérios nas células. O objetivo do trabalho foi avaliar o efeito cardioprotetor da angiotensina 1-7 (Ang1-7), um peptídeo com efeito antagônico à AngII, sobre viabilidade, autofagia e apoptose de cardiomiócitos H9c2 tratados com MGO. A concentração de MGO utilizada neste estudo foi determinada pelo teste MTT, que analisa viabilidade celular. A DL_{50} para o MGO foi 272 µM. Utilizamos 200 µM, próximo à concentração sub-letal e capaz de alterar o fluxo autofágico celular. O efeito cardioprotetor da Ang1-7 foi testado em duas concentrações (0,1 µM e 1,0 µM) que foram comparadas aos grupos MGO e controle. Utilizamos um grupo controle positivo de morte celular, tratado com 500 µM de MGO. Os grupos receberam o tratamento por 24 horas. As análises de autofagia e apoptose foram realizadas por Western Blot, através da detecção das proteínas LC3B, p62 e caspase-3 clivada. Os dados preliminares indicam maior mortalidade das células que receberam o co-tratamento (MGO + Ang1-7) quando comparadas aos grupos de tratamento único. Observamos maior acúmulo de p62 no grupo controle positivo co-tratado. Ao avaliar apoptose, não observamos ativação em nenhum outro grupo, apenas no controle positivo. A maior mortalidade no grupo co-tratado pode estar associada à possível reação do MGO com Ang1-7, que originaria um produto tóxico, com efeitos negativos semelhantes aos AGEs, inviabilizando a molécula de Ang1-7

de desempenhar cardioproteção. O projeto prevê um número amostral maior que permitirá concluir a consistência destes dados.

Introdução

Doenças cardiovasculares

As doenças cardiovasculares (DCV) são um grupo de doenças que inclui arritmias, cardiomiopatias, hipertensão arterial, doenças arteriais coronarianas, entre outras. A progressão dessas doenças pode resultar na insuficiência cardíaca, que é a principal causa de morte no mundo segundo dados da Organização Mundial da Saúde (1). As DCV têm causas multifatoriais, com componentes genéticos e ambientais, tais como hipercolesterolemia, obesidade e *diabetes mellitus* (2). Pacientes com diabetes, por exemplo, têm 2 a 3 vezes mais chances de desenvolver insuficiência cardíaca (3).

Metilglioxal e efeitos celulares

O MGO é um intermediário natural do metabolismo da glicose que se encontra em níveis elevados em pacientes com diabetes (4). Esta molécula é formada, principalmente, na via glicolítica como um subproduto da degradação não enzimática do gliceraldeído-3-fosfato (G3P – do inglês *glyceraldehyde 3-phosphate*) e de seu isômero fosfato de diidroxiacetona (DHAP – do inglês *dihydroxyacetone phosphate*) (5).

Quimicamente, é um dicarbonil altamente reativo e, no organismo, seus alvos mais importantes são resíduos de proteínas e ácidos nucleicos (6). O MGO é considerado o mais potente precursor dos produtos finais de glicação avançada

(AGEs, do inglês *Advanced Glycation end Products*) ao carbonilar, principalmente, resíduos de arginina e lisina de proteínas, pela reação de Maillard (6). Os AGEs constituem uma gama de moléculas heterogêneas que exercem um importante papel na indução do processo inflamatório e no estresse oxidativo e, desta forma, participam no desenvolvimento e progressão de DCV (7).

Os AGEs podem desempenhar sua ação diretamente sobre as biomoléculas, ao serem internalizados, ou através de receptores específicos, chamados RAGEs. A interação dos AGEs com RAGES ativa inúmeras vias de sinalização, que, frequentemente, convergem para a ativação do fator de transcrição nuclear *kappa-B* (NF-κB) (8). Além da progressão das DCV, os AGEs também estão implicados em muitos outros mecanismos fisiopatológicos, incluindo aqueles associados as demais complicações diabéticas (catarata, retinopatia, nefropatia, angiopatia), envelhecimento e distúrbios neurodegenerativos (9, 10).

Conjuntamente com os efeitos negativos causados pela interação do MGO com proteínas celulares, o dicarbonil é um importante agente citotóxico, capaz de modificar estruturas de DNA através de rupturas de cadeia (11), transversões de nucleotídeos (12) e ligações cruzadas (13-15), comprometendo a replicação celular e induzindo a apoptose (15). Muitos efeitos deletérios do MGO também são relacionados à sua contribuição com o estresse oxidativo, por depletar defesas antioxidantes (16). Estudos indicam que o dicarbonil, em certo nível, também é responsável por disparar o processo autofágico celular (17, 18).

Evidências sugerem que a exposição aos AGEs e ao MGO atue como promotor da ativação da via clássica do sistema renina-angiotensina (SRA) (19-26). Pacientes com diabetes possuem, de fato, um desequilíbrio do sistema, o que contribui diretamente no processo de remodelamento cardíaco, fibrose miocárdica e

morte de cardiomiócitos, elevando as chances do desenvolvimento da insuficiência cardíaca. Em razão disto, inibidores da enzima conversora de angiotensina (ECA) e bloqueadores de receptores de AngII representam terapias importantíssimas na prevenção de doenças e complicações cardiovasculares (27).

Rota clássica e alternativa do sistema renina-angiotensina

O SRA é um sistema enzimático-peptídico que atua no controle homeostático dos sistemas cardiovascular e renal e na regulação do volume de fluidos extracelulares.

O SRA sistêmico, assim como o tecidual, é ativado pela ação da proteinase renina sobre o angiotensinogênio, produzindo a decapeptídeo angiotensina I (AngI) que pode sofrer hidrólises sequenciais para gerar diferentes produtos ativos. Didaticamente, o SRA pode ser dividido em duas rotas principais: rota clássica e rota alternativa.

A rota clássica se caracteriza pela produção de AngII a partir da AngI por meio de ação catalítica da ECA (28). A AngII tem ação parácrina, autócrina ou atua como hormônio circulante e exerce sua ação biológica ao interagir com os receptores AT1 e AT2 (29) gerando uma grande variedade de respostas (30).

Já a rota alternativa se caracteriza pela ação da Ang1-7, um peptídeo biologicamente ativo que apresenta efeitos opostos aos da AngII e regula negativamente a rota clássica (31). A Ang1-7 pode ser derivada diretamente da AngII pela ação da enzima conversora de angiotensina 2 (ECA2) ou ainda, da AngI através das endopeptidases tecido específicas como a neprilisina (NEP), prolil-oligopeptidase (POP), e thimet-oligopeptidase (TOP) (32, 33).

A homeostase cardiovascular é resultado do equilíbrio das rotas do SRA, portanto, quando ocorre um desequilíbrio em favor da rota clássica (AngII/AT1) observam-se efeitos nocivos como aumento do processo inflamatório, estresse oxidativo e hipertensão. Por outro lado, o eixo alternativo (Ang1-7/AT2 ou Ang1-7/MAS) inibe a rota clássica produzindo vasodilatação e efeitos anti-inflamatórios (Fig 1) (32).

Fig 1. Rota clássica e alternativa do sistema renina-angiotensina. Em azul: enzimas e moléculas associadas à rota clássica, e em vermelho: enzimas e moléculas associadas à rota alternativa. Adaptado de Farag E, Maheshwari K, Morgan J, Sakr Esa WA, Doyle DJ, 2015.

Em inúmeros estudos a Ang1-7 tem demonstrado efeitos biológicos benéficos, considerados cardioprotetores: propriedades anti-hipertróficas, anti-apoptóticas, e de controle de fluxo autofágico (22, 34-36). Em estudos de exposição de cardiomiócitos a AGEs, pesquisadores encontraram uma expressão gênica diminuída de ECA2, o que potencialmente diminuiria os níveis de Ang1-7 e, desta forma, poderia estar associado a um pior prognóstico para doenças cardiovasculares (25).

Autofagia

A autofagia é um processo celular que permite a reciclagem de organelas ou moléculas danificadas a fim de manter o correto funcionamento das células (37). Este processo se caracteriza pelo sequestro de proteínas ou organelas por vacúolos autofágicos que se fundem com lisossomos, originando autofagolisossomos (38). Os

produtos da reciclagem autofágica servirão como substrato energético ou serão utilizados para síntese de novas proteínas (39-41). Em níveis basais, o processo tem um importante papel na sobrevivência celular, no entanto quando sua ativação é sustentada e excessiva, a autofagia pode causar a morte celular devido à perda de grande quantidade de proteínas e organelas necessárias para o metabolismo celular (42).

A ativação da autofagia pode ocorrer em resposta a diferentes estímulos que, por meio de mensageiros secundários, irão ativar proteínas relacionadas conhecidas como Atgs (do inglês *Autophagy-related genes/proteins*). Essas proteínas irão participar do recrutamento de lipídeos para a expansão do fagóforo. O processo inicia com a formação do complexo Atg1/Atg13/Atg17. A proteína Atg8, mais conhecida como LC3B, por sua vez, é necessária para o alongamento e maturação do fagóforo. Sua forma citosólica (LC3B-I) é clivada e conjugada com fosfatisdiletanolamina, sendo então incorporada na membrana em formação (LC3B-II). A LC3B é um conhecido marcador de autofagia e seu nível é correlacionado diretamente com o número de autofagolisossomos (43). Após formado, o recrutamento de proteínas para degradação no interior do fagóforo é realizado pela p62, que se liga diretamente na LC3B presente na membrana. Finalmente, a proteína recrutada é degradada, conjuntamente com a p62, e o conteúdo resultante fica disponível para reciclagem (Fig 2) (44, 45).

Fig 2. Ativação da via autofágica e formação do autofagossoma. Adaptado de Baek KH, Park J, Shin I, 2012.

Quando as células estão em condições normais, as proteínas sofrem controle pelo complexo TOR cinase (mTOR em mamíferos) que ao fosforilar o Atg13 impede sua interação com o Atg1, desta forma inibindo o início do processo autofágico (46).

O complexo mTOR cinase é um sensor energético que é ativado quando as células possuem substratos energéticos suficientes para promover crescimento e proliferação celular como, por exemplo, na hipertrofia cardíaca. Sabe-se que a ligação da AngII ao receptor AT1 é um importante ativador do complexo mTOR e essa ação está associada ao desenvolvimento da hipertrofia cardíaca patológica. No entanto, estudos são contraditórios ao relacionar diretamente AngII e autofagia. Numerosos pesquisadores têm demonstrado indução na autofagia, enquanto alguns, a inibição do processo (47). Isso pode ocorrer devido ao controle do processo por vias m-TOR independentes. Pouco se sabe sobre o papel da rota alternativa (Ang1-7/AT2 ou Ang1-7/MAS) no complexo mTOR e autofagia, no entanto, já foi visto que a rota está mais ativa em processos fisiológicos do que patológicos, dessa maneira agiria como um regulador do fluxo autofágico.

Como o eixo alternativo do SRA tem efeitos protetores, é possível que o co-tratamento com Ang1-7 em cardiomiócitos tratados com o MGO possa diminuir a mortalidade de cardiomiócitos, controlando os processos de autofagia e apoptose e mantendo a viabilidade das células em cultura. O objetivo deste trabalho foi avaliar a ação do co-tratamento de MGO e Ang1-7 em linhagem de cardiomiócitos H9c2, sobre a viabilidade celular e ativação das vias autofágica e apoptótica.

Metodologia

Desenho experimental

Foi realizado um estudo experimental *in vitro.* O cálculo amostral foi realizado a partir do desvio padrão das análises de *Western Blot* para a proteína autofágica p62, no qual foi considerada uma diferença de até 20% entre os grupos, com um poder de 80% e nível de significância de 5%. Neste sentido, o número de 6 replicatas são necessárias por grupo. Os cardiomiócitos foram divididos em grupos conforme o tratamento recebido: 1) grupo controle, sem tratamento (n = 6); 2) grupo tratamento com MGO (n = 6); 3) grupo co-tratamento com MGO + Ang1-7 0,1 µM (n = 6); 4) grupo co-tratamento om MGO + Ang1-7 1,0 µM (n = 6); 5) grupo tratamento com Ang1-7 0,1 µM (n = 6); 6) grupo tratamento com Ang1-7 1,0 µM (n = 6); 7) grupo controle positivo de morte celular (n = 6) e, 8) grupo controle positivo de morte celular + Ang1-7 1,0 µM (n = 6).

Tabela 1. Grupos de tratamento.

Grupos	**Tratamento**		**Número amostral**
	MGO	**Ang1-7**	
Controle	-	-	6
Tratamento com MGO	200 µM	-	6
Co-tratamento com MGO + Ang1-7	200 µM	0,1 µM	6
	200 µM	1,0 µM	6
Tratamento com Ang1-7	-	0,1 µM	6
	-	1,0 µM	6
Controle positivo (morte celular)	500 µM	-	6
Controle positivo – co-tratamento (morte celular)	500 µM	1,0 µM	6

A preparação das células e tratamento dos grupos foi realizado conforme segue:

Dia 1) Plaqueamento: células foram plaqueadas em placas de 96 (1 x 10^4 células/poço) e 6 poços (30 x 10^4 células/poço);

Dia 2) Jejum (*Starving*): o meio de cultura dos cardiomiócitos foi substituído por meio de cultura DMEM sem soro bovino fetal (SBF);

Dia 3) Tratamento: as células receberam o tratamento diluído em meio DMEM sem SBF, conforme o grupo designado (tabela 1);

Dia 4) Preparação da amostra para análise: após 24 horas de tratamento, as células foram destinadas aos testes da seguinte maneira:

- Viabilidade – As células em placas de 96 poços foram utilizadas para o teste de MTT.
- Autofagia e apoptose – As células em placas de 6 poços foram raspadas em tampão de lise e preparadas para quantificação de proteínas e análise de *Western Blot.*

Até o encerramento deste manuscrito 80% do trabalho foi concluído, com aproximadamente 50% do número amostral.

Cultura celular

Foi utilizada a linhagem comercial H9c2 (#0098, Banco de Células do Rio de Janeiro), que é constituída de mioblastos provenientes de miocárdio de rato (*Rattus norvegicus*). As células foram cultivadas em meio DMEM (#D5523, Sigma Aldrich) suplementado com 1% de aminoácidos não-essenciais (#M7145, Sigma Aldrich), 1500 mg/L de bicarbonato de sódio (S5761, Sigma Aldrich), 10% SBF (#F6178, Sigma

Aldrich) e 1% de penicilina/estreptomicina (#P4333, Sigma Aldrich). Para a passagem das células, esperou-se uma confluência de aproximadamente 75%. As células foram mantidas em ambiente controlado (5% de CO2, 37° C e 95% umidade) (48, 49).

Definição da concentração de MGO

A dose de MGO (#M0252, Sigma Aldrich) foi definida através de uma curva de concentrações (0, 1, 10, 100, 200, 300, 400, 500 e 1000 µM) com a análise de viabilidade celular e autofagia após 24 horas de tratamento (17, 18, 50, 51).

Co-tratamento MGO + Ang1-7

O co-tratamento de MGO com Ang1-7 ocorreu com diferentes concentrações de Ang1-7 (#H-1715, Bachem) (0,10; 1,0 µM) durante 24 horas (22, 36, 52).

Viabilidade celular

Foi utilizado o ensaio colorimétrico de MTT, que mede a atividade da redutase mitocondrial em células vivas. Ele se baseia na clivagem do sal amarelo tetrazólio em cristais azuis/púrpura de formazan por células metabolicamente ativas, servindo como um indicador direto de citotoxicidade. Uma solução de MTT (0,05% em meio DMEM suplementado sem SBF) (#M5655, Sigma Aldrich) foi adicionada ás células de uma placa de 96 poços, e incubada por 2 horas. O sobrenadante foi removido e os cristais de formazan foram dissolvidos em DMSO (0,5 mg/mL) (#D2650, Sigma Aldrich). A absorbância foi lida a 560 nm em espectrofotômetro, possibilitando uma correlação direta com o número total de células viáveis (53).

Autofagia

A autofagia foi avaliada por *Western Blot*. Foram utilizados anticorpos policlonais contra LC3B-I, LC3B-II (#2775S, Cell Signaling Technology) e p62/SQSTM1 (#5114S, Cell Signaling Technology). Inicialmente, células de uma placa de 6 poços foram lavadas com tampão fosfato-salino (PBS 1X) e raspadas com tampão de lise (tris 50 mM, Triton™ 1%, fluoreto de sódio 5mM, ortovanadato de sódio 1 mM, fluoreto de fenil sulfonil 1 mM, pastilha inibidora de protease Sigma Fast™). Os fragmentos celulares foram removidos em 5 minutos de centrifugação a uma velocidade de 10000 x *g* e temperatura de 4° C. A concentração proteica do sobrenadante foi determinada pelo método de Bradford (54). Para a preparação das amostras, 10 µg de proteína foram diluídos em tampão de Laemmli (tris 250 mM, SDS 8%, glicerol 40%, β-mercaptoetanol 20%, azul de bromofenol 0,008, pH 6,8) e a mistura aquecida a 70°C por 10 minutos. A eletroforese foi realizada em gel 15 % de poliacrilamida–SDS por 150 minutos a 120 V e a transferência em membrana de fluoreto de polivinilideno (PVDF) por sistema semi-úmido. Após o bloqueio com 5% de leite desnatado, as membranas foram incubadas com os anticorpos primários (1:1000) *overnight* em temperatura controlada de 4 a 8° C e então, incubadas com o anticorpo secundário anti IgG de coelho conjugado com peroxidase (1:1000 – LC3B, 1:10000 - p62) por 2 horas em temperatura ambiente. As bandas foram reveladas com o substrato para a peroxidase (Immobilon - Western Chemiluminescent HRP Substrate - Millipore Corporation Inc.) e as imagens captadas em fotodocumentador (ImageQuant LAS500, GEHealthcare Life Sciences, Washington, EUA). A quantificação das bandas foi feita com o software ImageJ e a normalização realizada pela densidade de proteínas totais da membrana corada com *Commassie blue* (55).

Apoptose

A apoptose foi avaliada por *Western Blot,* pela detecção da caspase-3 clivada, utilizando o anticorpo policlonal Asp 175 (#9661, Cell Signaling Technology). A técnica utilizada seguiu as informações previamente descritas. Os anticorpos foram utilizados na diluição 1:1000, sendo o secundário já descrito.

Análise estatística

Os dados preliminares não foram analisados estatisticamente, pois não foram previstas análises interinas. Portanto, o comportamento dos grupos foi analisado descritivamente pela mediana e distribuição interquartis, utilizando gráficos de *boxplot* criados pelo software GraphPad Prism 6 (GraphPad Software, Inc., California, EUA).

Quando todas as replicatas estiverem concluídas, os dados serão avaliados quanto à normalidade de distribuição pelo teste de Shapiro-Wilk. Para amostras com distribuição normal serão utilizados os dados de média e desvio padrão, e as comparações serão realizadas com o teste t Independente ou ANOVA *one-way* com *post-hoc* de Bonferroni. Para amostras com distribuição não normal será utilizada a mediana de intervalos interquartis, e as comparações serão realizadas utilizando o teste U de Wilcoxon ou Kruskal Wallis.

Resultados

Efeitos do MGO sobre viabilidade celular e autofagia

Para definir a concentração de MGO a ser utilizada no estudo, os cardiomiócitos foram incubados com concentrações crescentes de 1 a 1000 µM do

agente glicante durante 24 horas. A viabilidade celular e a capacidade de interferência no nível autofágico das células das diferentes concentrações foram avaliadas. A partir de dados do MTT, a dose letal que matou 50% da cultura (DL_{50}) foi definida como 272,0 ± 1,24 µM (Fig 3). As concentrações de 100 e 200 µM, abaixo da DL_{50}, foram escolhidas para avaliação de autofagia. Ambas foram capazes de interferir no nível autofágico das células, quando comparadas com o grupo controle, principalmente a concentração mais elevada (Fig 4). Utilizando estes dados, foi escolhida a concentração de 200 µM para prosseguir com os experimentos, e a concentração de 500 µM como controle positivo de morte celular.

Fig 3. Curva de sobrevivência celular ao MGO. Percentual de sobrevivência das células quando incubadas com crescentes concentrações de MGO por 24 horas.

Fig 4. Interferência do MGO no fluxo autofágico. Nível de expressão de p62 nos grupos MGO 100 µM e MGO 200 µM após tratamento por 24 horas. A) Análise gráfica; B) eletroforese em gel de poliacrilamida-SDS. Dados expressos como mediana, valor mínimo e máximo.

Efeitos do co-tratamento de MGO e Ang1-7 sobre viabilidade celular

Os grupos que receberam somente MGO seguiram o padrão da curva de sobrevivência previamente estabelecida. Os grupos que receberam somente Ang1-7 mantiveram-se viáveis, não diferindo do grupo controle. No entanto, quando foi realizado o co-tratamento (MGO + Ang1-7) observou-se um aumento na mortalidade

celular em relação aos grupos que receberam tratamento único (MGO ou Ang1-7), sugerindo possível citotoxicidade da combinação MGO + Ang1-7 (Fig 5).

Fig 5. Sobrevivência celular ao co-tratamento. Percentual de sobrevivência dos grupos após tratamento com MGO e/ou Ang1-7 por 24 horas.

Efeitos do co-tratamento de MGO e Ang1-7 sobre autofagia

O grupo controle e os grupos de tratamento tiveram o fluxo autofágico avaliado por *Western Blot*, pela análise da LC3B-I, LC3B-II, sua razão LC3B-II/LC3B-I e pela proteína p62.

Analisando graficamente o comportamento das amostras, nota-se um alto valor para a razão LC3B-II/LC3B-I, o que pode indicar que todos os grupos estavam com o processo autofágico ativo. No entanto, o grupo controle positivo do co-tratamento (500 µM de MGO + 1,0 µM de Ang1-7) apresentou os níveis mais altos e destoantes para a expressão da proteína p62, quando comparado aos demais grupos de tratamento (Fig 6).

Fig 6. Interferência do co-tratamento na autofagia. A) Níveis de expressão de LC3B-I e LC3B-II; B) razão LC3B-II/LC3B-I; C) nível de expressão de p62 dos grupos após tratamento com MGO e/ou Ang-7 por 24 horas; D) eletroforese em gel de poliacrilamida-SDS. Dados expressos como mediana, valor mínimo e máximo.

Efeitos do co-tratamento de MGO e Ang1-7 sobre apoptose

A apoptose foi avaliada pelo nível da caspase-3 clivada por *Western Blot.* Os grupos experimentais não apresentaram bandas detectáveis, demostrando não haver

ativação do processo apoptótico nestes grupos, sendo comprovado pela presença da banda nos grupos de controle positivos (500 µM MGO; e 500 µM MGO + 1,7 Ang 1-7 µM) (Fig 7).

Fig 7. Interferência do co-tratamento na apoptose. Nível de expressão de caspase-3 clivada nos grupos MGO 500 µM e MGO 500 µM + Ang 1-7 1,0 µM após tratamento por 24 horas. A) Análise gráfica; B) eletroforese em gel de poliacrilamida-SDS. Dados expressos como mediana, valor mínimo e máximo.

Discussão

O teste de viabilidade demonstrou que o co-tratamento com Ang1-7 acentuou a morte celular causada pelo MGO. Quando avaliamos a autofagia pela razão LC3B-II/LC3B-I, podemos observar a ativação do processo em todos os grupos, porém não é possível observar um comportamento diferenciado entre eles, devido à elevada variação entre as replicatas. No entanto, ao analisarmos a expressão de p62, nota-se um acúmulo da proteína no grupo controle positivo de morte celular co-tratado (500 µM MGO + 1,0 µM Ang1-7) em comparação aos demais grupos, evidenciando um processo autofágico não concluído. Esse resultado concorda com o fato de que no mesmo grupo também houve uma elevada expressão da proteína caspase-3 clivada (indicadora de apoptose). Dessa forma, a viabilidade celular estaria sendo afetada diretamente pelo processo de morte celular programada, sem a participação eficiente do processo autofágico. Ao considerarmos que o grupo de células que recebeu tratamento único com MGO 500 µM também expressou a proteína indicadora de apoptose, sugere-se que grande parte da ativação do processo ocorreu devido à ação da alta dose do agente glicante, o qual foi acentuada pelo co-tratamento. Já os grupos

de co-tratamento que não apresentaram expressão de caspase-3 clivada detectável, e mesmo assim tiveram redução da viabilidade, podem estar sofrendo processo de morte celular por necrose ou ainda morte celular autofágica (56).

O MGO é um dicarbonil altamente reativo cujos principais alvos são resíduos de arginina de proteínas. Através da reação de Maillard é responsável pela formação de AGEs, moléculas que desempenham inúmeros efeitos deletérios nas células, inclusive em cardiomiócitos. Muitos estudos avaliando as consequências da exposição ao MGO e AGEs sobre a fisiologia celular encontraram aumento do estresse oxidativo, aumento da apoptose, ativação da autofagia e redução da viabilidade celular (16, 57, 58). A Ang1-7 por sua vez, é um peptídeo do SRA, cuja importância na regulação do sistema foi recentemente reconhecida. É responsável por causar efeitos antagônicos à AngII, sendo considerados benéficos em casos de super ativação do SRA, como ocorre no diabetes. O estudo de Lin e colaboradores (35), por exemplo, demonstrou a proteção da Ang1-7 sobre o remodelamento cardíaco causado pela AngII, e o estudo de Alzayadneh e colaboradores (22) demonstrou a proteção do peptídeo sobre a hipertrofia celular causada por AGEs.

No presente estudo, o comportamento evidenciado na linhagem H9c2 em relação à viabilidade, autofagia e apoptose vai de encontro à hipótese inicial, de que os grupos co-tratados com Ang1-7 apresentariam redução nos danos moleculares causados pelo MGO. O comportamento dos cardiomiócitos pode indicar que no co-tratamento proposto aconteça uma reação direta do MGO com grupos amina da Ang1-7, especialmente com a arginina presente na sua estrutura. Essa reação direta, formaria uma molécula citotóxica, com efeitos celulares semelhantes aos AGEs produzidos endogenamente. Os efeitos deletérios do produto estariam levando as células à morte, sem um processo autofágico eficiente, como tentativa de recuperação

de danos, conforme descrito por Hu e colaboradores (59). Ainda, a reação consumiria a Ang 1-7, impedindo que a molécula desempenhe seu papel cardioprotetor, papel revisado largamente por Wastermeier e colaboradores (60), e confirmado na cardiomiopatia diabética por Papinska e colaboradores (61).

Se a análise estatística com um número amostral adequado confirmar os dados descritivos aqui apresentados, faz-se necessária a alteração do desenho experimental, de co-tratamento para pré-tratamento com Ang 1-7, para atender o objetivo deste trabalho, e assim, analisarmos sua potencial ação protetora sobre os danos causados pelo MGO aos cardiomiócitos.

Agradecimentos

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ANEXO A – Figuras

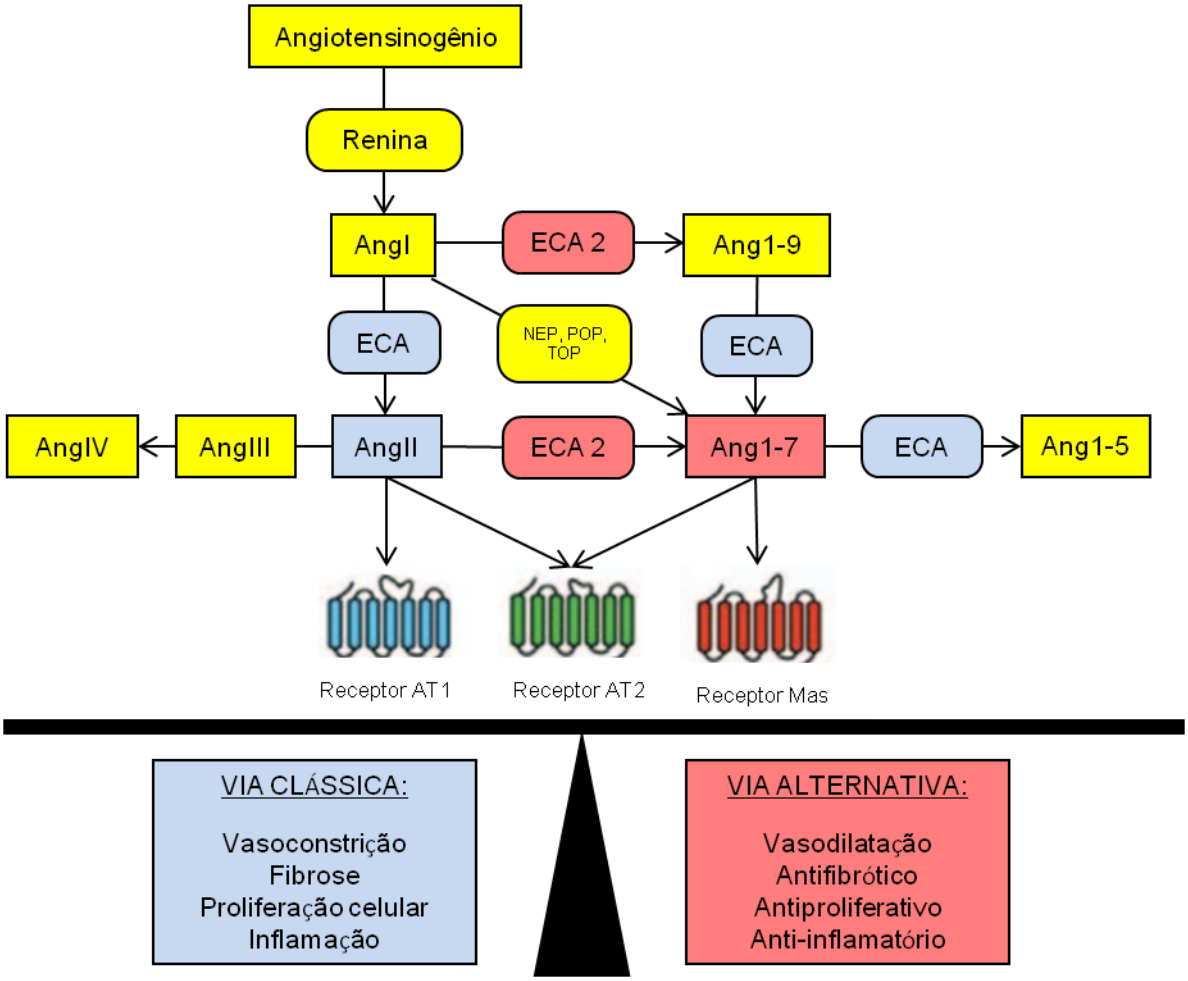


Fig 1. Rota clássica e alternativa do sistema renina-angiotensina. Em azul: enzimas e moléculas associadas à rota clássica, e em vermelho: enzimas e moléculas associadas à rota alternativa. Adaptado de Farag E, Maheshwari K, Morgan J, Sakr Esa WA, Doyle DJ, 2015.

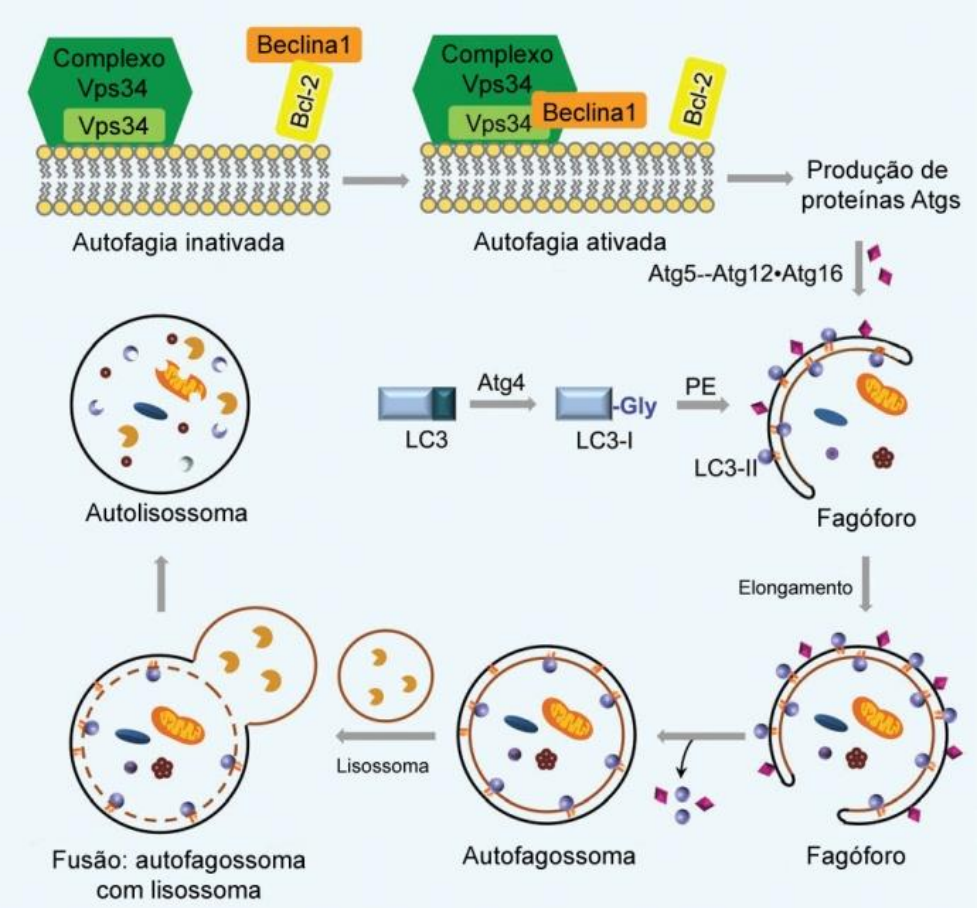


Fig 2. Ativação da via autofágica e formação do autofagossoma. Adaptado de Baek KH, Park J, Shin I, 2012.

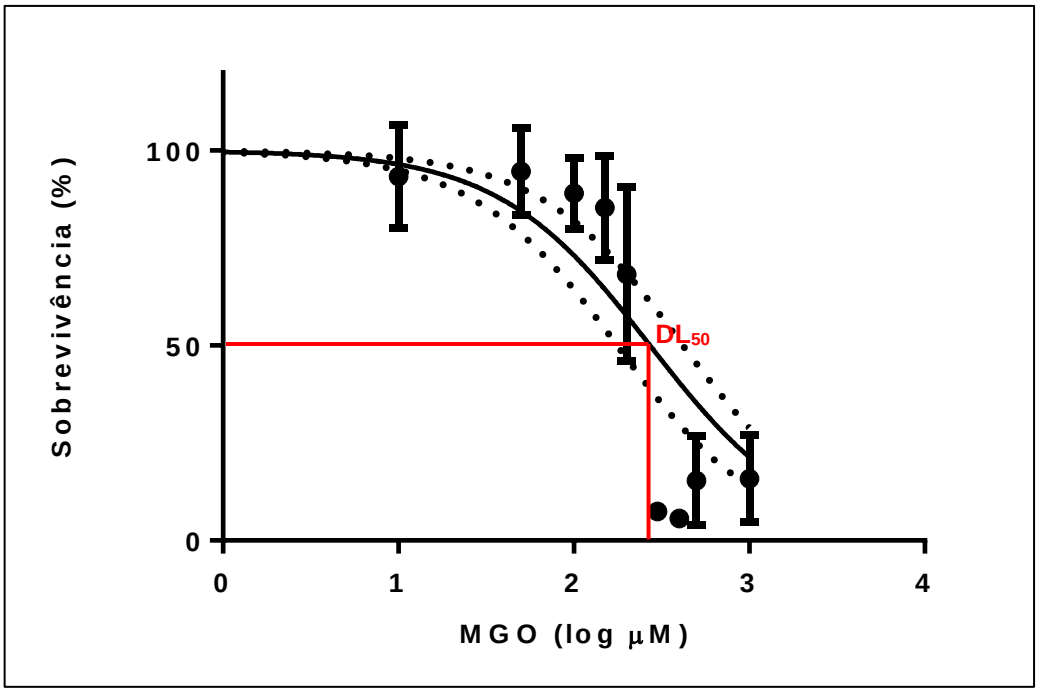


Fig 3. Curva de sobrevivência celular ao MGO. Percentual de sobrevivência das células quando incubadas com crescentes concentrações de MGO por 24 horas.

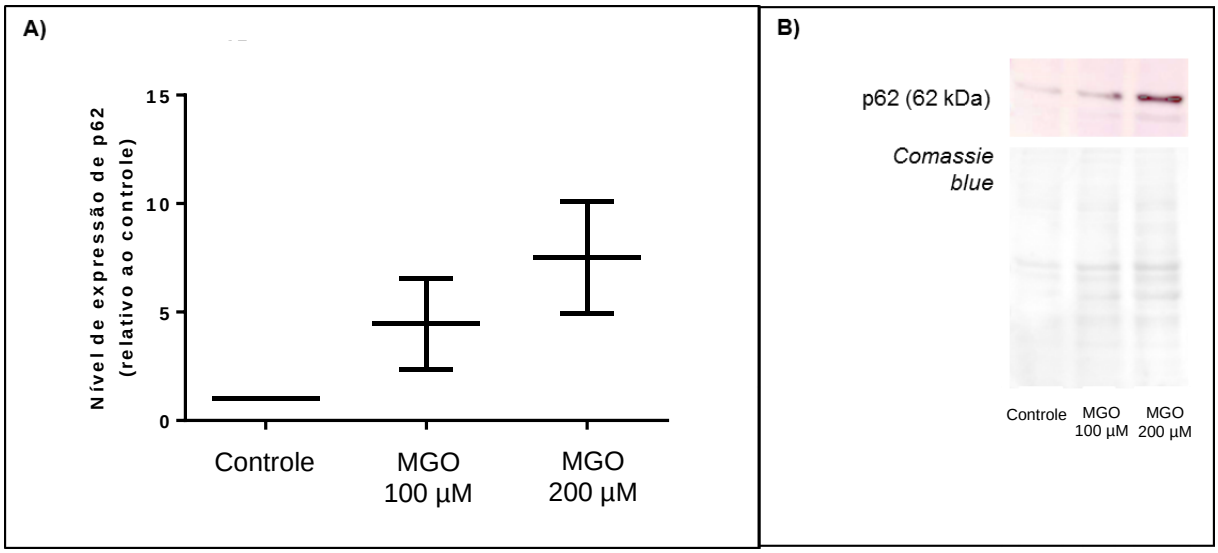


Fig 4. Interferência do MGO no fluxo autofágico. Nível de expressão de p62 nos grupos MGO 100 µM e MGO 200 µM após tratamento por 24 horas. A) Análise gráfica; B) eletroforese em gel de poliacrilamida-SDS. Dados expressos como mediana, valor mínimo e máximo.

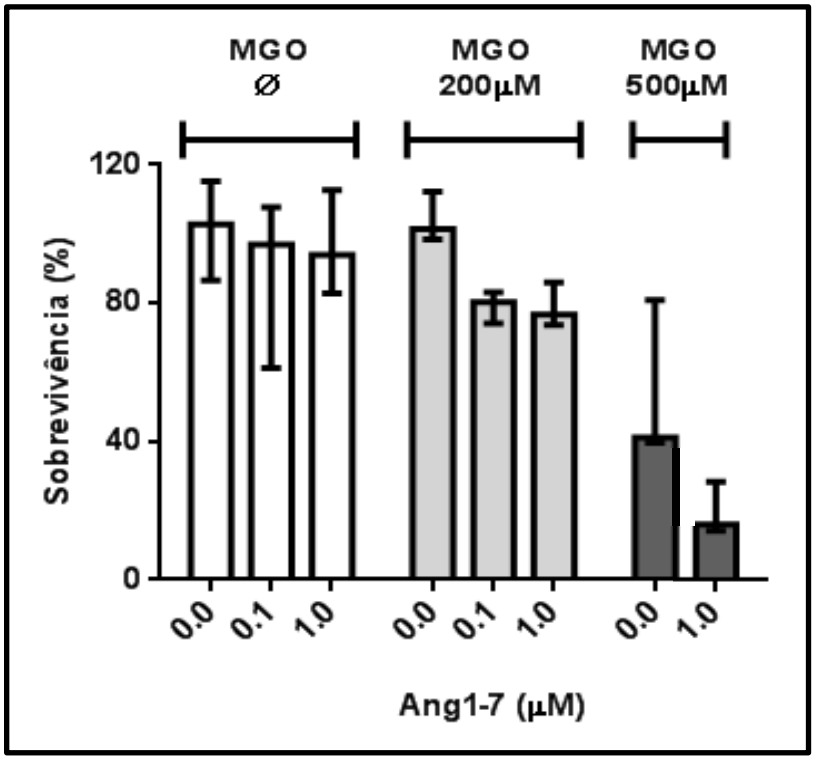


Fig 5. Sobrevivência celular ao co-tratamento. Percentual de sobrevivência dos grupos após tratamento com MGO e/ou Ang1-7 por 24 horas.

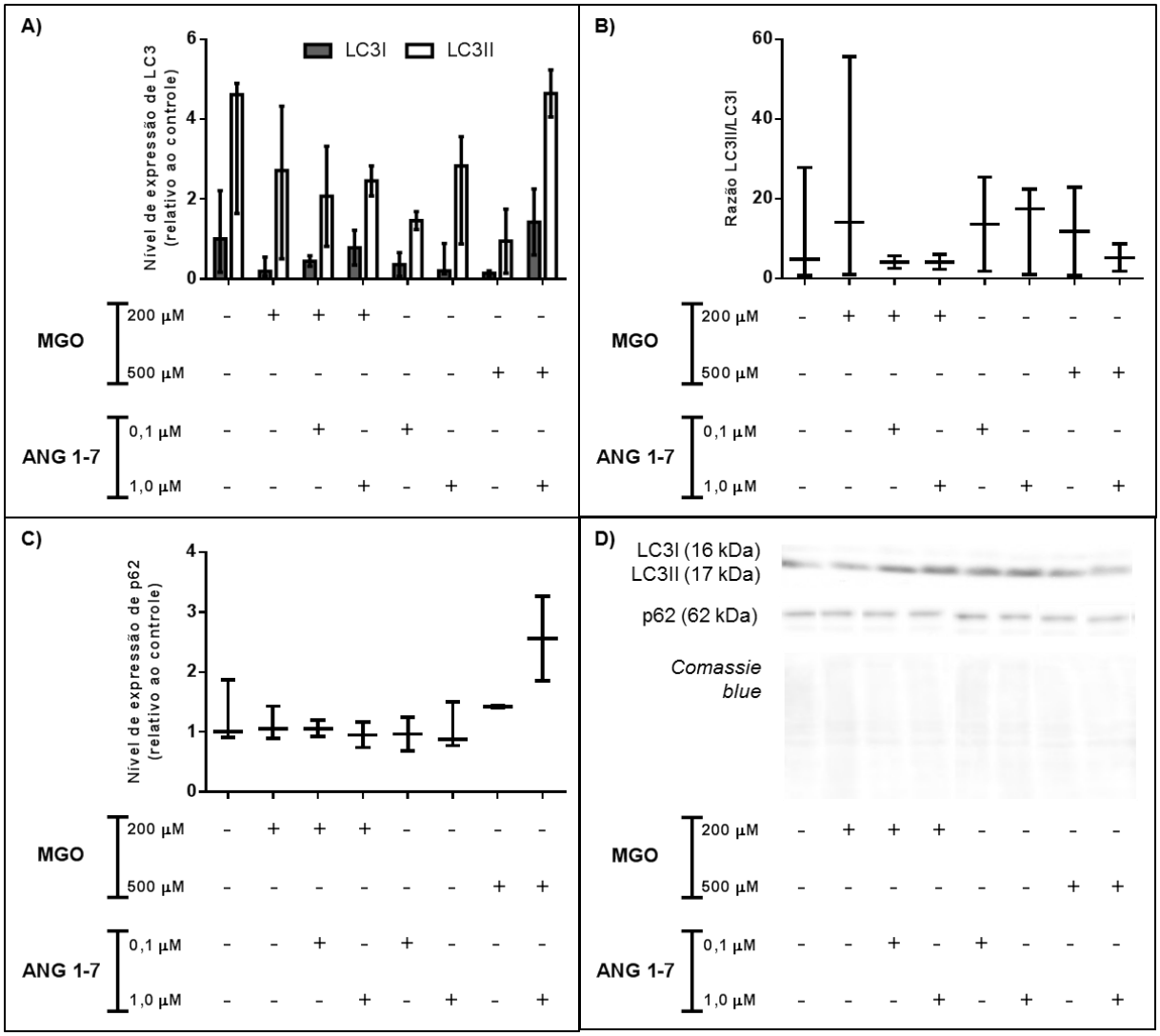


Fig 6. Interferência do co-tratamento na autofagia. A) Níveis de expressão de LC3B-I e LC3B-II; B) razão LC3B-II/LC3B-I; C) nível de expressão de p62 dos grupos após tratamento com MGO e/ou Ang-7 por 24 horas; D) eletroforese em gel de poliacrilamida-SDS. Dados expressos como mediana, valor mínimo e máximo.

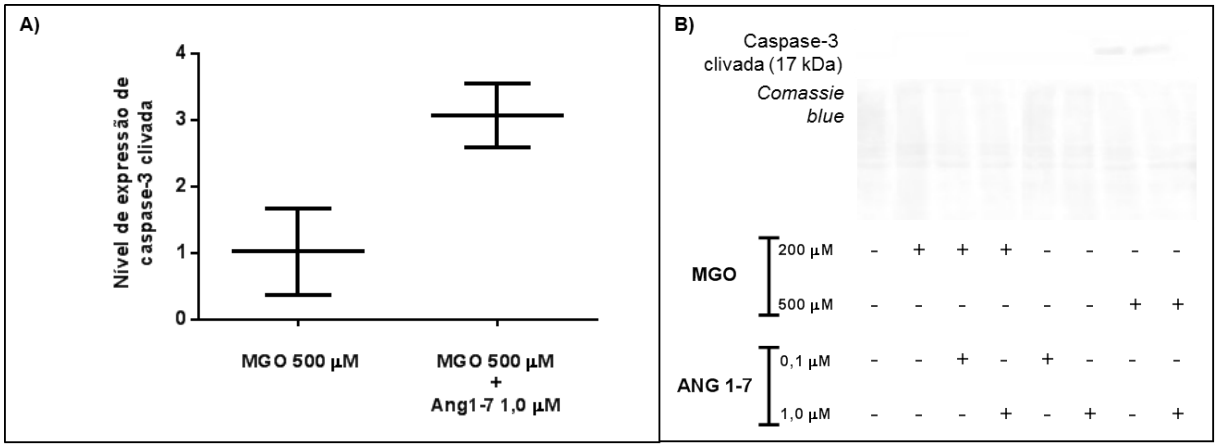


Fig 7. Interferência do co-tratamento na apoptose. Nível de expressão de caspase-3 clivada nos grupos MGO 500 µM e MGO 500 µM + Ang 1-7 1,0 µM após tratamento por 24 horas. A) Análise gráfica; B) eletroforese em gel de poliacrilamida-SDS. Dados expressos como mediana, valor mínimo e máximo.

ANEXO B – Sobre a revista

A PlosOne é uma revista científica muldisciplinar de acesso livre, publicada pela editora norte americana Public Library of Science, disponível apenas em plataforma online: www.plosone.org. A revista cobre, principalmente, pesquisa primária de qualquer disciplina dentro das áreas de ciência e medicina. O fator de impacto em 2016 foi de 3,54.

Critérios para publicação:

- O estudo apresenta os resultados da pesquisa científica primária, e esses não foram publicados em outro lugar;
- Experimentos, estatísticas e outras análises foram realizadas com um alto padrão técnico e são descritas em detalhes suficientes;
- As conclusões são apresentadas de forma adequada e são suportadas pelos dados;
- O artigo é apresentado de forma inteligível e está escrito em lingua inglesa padrão;
- A pesquisa atende a todos os padrões aplicáveis à ética da experimentação e à integridade da pesquisa;
- O artigo adere às diretrizes de relatório apropriadas e aos padrões da comunidade para a disponibilidade de dados.

ANEXO C – Normas técnicas para submissão de artigos científicos

Submission Guidelines

Read the Chinese translation of the PLOS policies referred to in this page. PLOS编辑与出版规定

Submitting a revision? Read our Revision Guidelines.

Style and Format

File format	Manuscript files can be in the following formats: DOC, DOCX, RTF, or PDF. 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 Symbol font.
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 is acceptable. Avoid using MathType or Equation Editor 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 or Equation Editor for only a portion of an equation. Rather, ensure that the entire equation is included. 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., HUGO for human genes). 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).

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.

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.

Please refer to our downloadable sample files to ensure that your submission meets our formatting requirements:

› Download sample title, author list, and affiliations page (PDF)

- Download sample manuscript body (PDF)

Viewing Figures and Supporting Information in the compiled submission PDF
The compiled submission PDF includes low-resolution preview images of the figures after the reference list. The function of these previews is to allow you to download the entire submission as quickly as possible. Click the link at the top of each preview page to download a high-resolution version of each figure. Links to download Supporting Information files are also available after the reference list.

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.

If an author has multiple affiliations, enter all affiliations on the title page only. In the submission system, enter only the preferred or primary affiliation. Author affiliations will be listed in the typeset PDF article in the same order that authors are listed in the submission.

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.

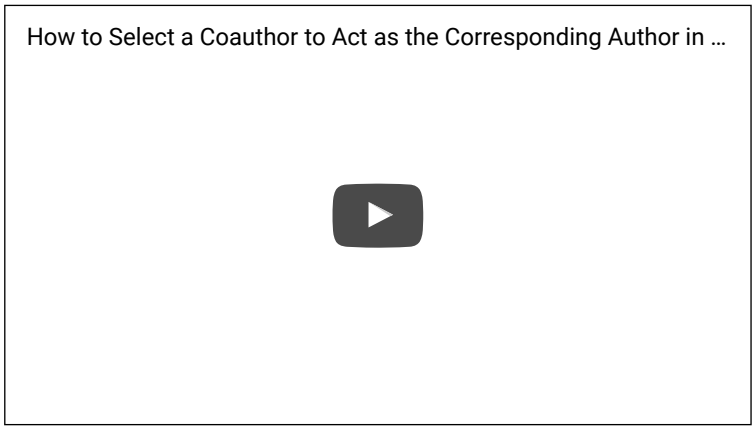
Corresponding author

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.

The corresponding author role may be transferred to another coauthor. However, note that transferring the corresponding author role also transfers access to the manuscript. (To designate a new corresponding author while the manuscript is still under consideration, watch the video tutorial below.)

Only one corresponding author can be designated in the submission system, but this does not restrict the number of corresponding authors that may be listed on the article in the event of publication. Whoever is designated as a corresponding author on the title page of the manuscript file will be listed as such upon publication. Include an email address for each corresponding author listed on the title page of the manuscript.

How to select a new corresponding author in Editorial Manager



Consortia and group authorship

If a manuscript is submitted on behalf of a consortium or group, include the consortium or group name in the author list, and include the full list of members in the Acknowledgments or in a supporting information file. 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.

Download sample title, author list, and affiliations page (PDF)

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.

Protocol documents for clinical trials, observational studies, and other **non-laboratory** investigations may be uploaded as supporting information. Read the supporting information guidelines for formatting instructions. We recommend depositing **laboratory protocols** at protocols.io. Read detailed instructions for depositing and sharing your laboratory protocols.

Human or animal subjects and/or tissue or field sampling

Methods sections describing research using human or animal subjects and/or tissue or field sampling must include required ethics statements. See the reporting guidelines for human research, clinical trials, animal research, and observational and field studies for more information.

Data

PLOS journals require authors to make all data underlying the findings described in their manuscript fully available without restriction, with rare exception.

Large data sets, including raw data, may be deposited in an appropriate public repository. See our list of recommended repositories.

For smaller data sets and certain data types, authors may provide their data within supporting information files accompanying the manuscript. Authors should take care to maximize the accessibility and reusability of the data by selecting a file format from which data can be efficiently extracted (for example, spreadsheets or flat files should be provided rather than PDFs when providing tabulated data).

For more information on how best to provide data, read our policy on data availability. PLOS does not accept references to “data not shown.”

Cell lines

Methods sections describing research using cell lines must state the origin of the cell lines used. See the reporting guidelines for cell line research for more information.

Laboratory Protocols

To enhance the reproducibility of your results, we recommend and encourage you to deposit laboratory protocols in protocols.io, where protocols can be assigned their own persistent digital object identifiers (DOIs).

To include a link to a protocol in your article:

1. Describe your step-by-step protocol on protocols.io
2. Select **Get DOI** to issue your protocol a persistent digital object identifier (DOI)
3. Include the DOI link in the Methods section of your manuscript using the following format provided by protocols.io: http://dx.doi.org/10.17504/protocols.io.[PROTOCOL DOI]

At this stage, your protocol is only visible to those with the link. This allows editors and reviewers to consult your protocol when evaluating the manuscript. You can make your protocols public at any time by selecting **Publish** on the protocols.io site. Any referenced protocol(s) will automatically be made public when your article is published.

New taxon names

Methods sections of manuscripts adding new taxon names to the literature must follow the reporting guidelines below for a new zoological taxon, botanical taxon, or fungal taxon.

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.

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

Any and all available works can be cited in the reference list. Acceptable sources include:

- Published or accepted manuscripts
- Manuscripts on preprint servers, if the manuscript is submitted to a journal and also publicly available as a preprint

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 or author summaries.

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.*
Accepted, unpublished articles	Same as published articles, but substitute “Forthcoming” for page numbers or DOI.
Web sites or 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; 1991. Preprint. Available from: arXiv:1403.3301v1. Cited 17 March 2014.
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. 29 Jan 2014. Available from: http://www.nytimes.com/2014/01/30/science/earth/climate-change-taking-toll-on-penguins-study-finds.html. Cited 17 March 2014.
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.

Supporting Information

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 10 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.

Supporting information files are published exactly as provided, and are not copyedited.

Supporting information captions

List supporting information captions at the end of the manuscript file. Do not submit captions in a separate file.

The file number and name are required in a caption, and we highly recommend including a one-line title as well. You may also include a legend in your caption, but it is not required.

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

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

Read the guidelines for figures.

Figure captions

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:

- A figure label with Arabic numerals, and “Figure” abbreviated to “Fig” (e.g. Fig 1, Fig 2, Fig 3, etc). Match the label of your figure with the name of the file uploaded at submission (e.g. a figure citation of “Fig 1” must refer to a figure file named “Fig1.tif”).
- A concise, descriptive title

The caption may also include a legend as needed.

Read more about figure captions.

Tables

Cite tables in ascending numeric order upon first appearance in the manuscript file.

Place each table in your manuscript file directly after the paragraph in which it is first cited (read order). Do not submit your tables in separate files.

Tables require a label (e.g., “Table 1”) and brief descriptive title to be placed above the table. Place legends, footnotes, and other text below the table.

Read the guidelines for tables.

Data reporting

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

Read our policy on data availability.

Repositories may be either subject-specific (where these exist) and accept specific types of structured data, or generalist repositories that accept multiple data types. We recommend that authors select repositories appropriate to their field. Repositories may be subject-specific (e.g., GenBank for sequences and PDB for structures), general, or institutional, as long as DOIs or accession numbers are provided and the data are at least as open as CC BY. Authors are encouraged to select repositories that meet accepted criteria as trustworthy digital repositories, such as criteria of the Centre for Research Libraries or Data Seal of Approval. Large, international databases are more likely to persist than small, local ones.

See our list of recommended repositories.

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

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

- Deposit data in the integrated repository of choice.
- Once deposition is final and complete, the repository will provide you with a dataset DOI (provisional) and private URL for reviewers to gain access to the data.

- Enter the given data DOI into the full Data Availability Statement, which is requested in the Additional Information section of the PLOS submission form. Then provide the URL passcode in the Attach Files section.

If you have any questions, please email us.

Accession numbers

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

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

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

Identifiers

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

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

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

Striking image

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

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

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

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

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

Additional Information Requested at Submission

Funding statement

This information should not be in your manuscript file; you will provide it via our submission system.

This information will be published with the final manuscript, if accepted, so please make sure that this is accurate and as detailed as possible. You should not include this information in your manuscript file, but it is important to gather it prior to submission, because your financial disclosure statement cannot be changed after initial submission.

Your statement should include relevant grant numbers and the URL of any funder's web site. Please also state whether any individuals employed or contracted by the funders (other than the named authors) playe any role in: study design, data collection and analysis, decision to publish, or preparation of the manuscript If so, please name the individual and describe their role.

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.

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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 no 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.

PLOS does support 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 choosing bioRxiv may now concurrently submit directly to select PLOS journals through bioRxiv’s direct transfer to journal service.

Read our policies on related manuscripts and preprint servers.

Guidelines for Specific Study Types

Human subjects research

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

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

All efforts should be made to protect patient privacy and anonymity. Identifying information, including photos, should not be included in the manuscript unless the information is crucial and the individual has provided written consent by completing the Consent Form for Publication in a PLOS Journal (PDF). Download additional translations of the form from the Downloads and Translations page. More information about patient privacy, anonymity, and informed consent can be found in the International Committee of Medical Journal Editors (ICMJE) Privacy and Confidentiality guidelines.

Manuscripts should conform to the following reporting guidelines:

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

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

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

- That the Institutional Review Board (IRB) approved use of oral consent
- How oral consent was documented

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

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

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

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

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

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

Clinical trials

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

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

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

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

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

Clinical trials must be reported according to the relevant reporting guidelines, i.e. CONSORT for randomized controlled trials, TREND for non-randomized trials, and other specialized guidelines as appropriate. The intervention should be described according to the requirements of the TIDieR checklist and guide. Submissions must also include the study protocol as supporting information, which will be published with the manuscript if accepted.

Authors of manuscripts describing the results of clinical trials must adhere to the CONSORT reporting guidelines appropriate to their trial design, available on the CONSORT Statement web site. Before the paper can enter peer review, authors must:

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

Any deviation from the trial protocol must be explained in the paper. Authors must explicitly discuss informed consent in their paper, and we reserve the right to ask for a copy of the patient consent form.

The methods section must include the name of the registry, the registry number, and the URL of your trial in the registry database for each location in which the trial is registered.

Animal research

We work in consultation with the *PLOS ONE* Animal Research Advisory Group to develop policies. Animal Research Advisory Group members may also be consulted on individual submissions.

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.

If we note differences between an IACUC-approved protocol and the methods reported in a submitted manuscript, we may report these discrepancies to the relevant institution or committee.

Methods sections of manuscripts reporting results of animal research must include required ethics statements that specify:

- 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.
- Relevant details for efforts 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 (Permit Number: 27-2956). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

The organism(s) studied should always be stated in the abstract. Where research may be confused as pertaining to clinical research, the animal model should also be stated in the title.

Where unregulated animals are used or ethics approval is not required, authors should make this clear in submitted articles and explain why ethical approval was not required. Relevant regulations that grant exemptions should be cited in full. It is the authors' responsibility to understand and comply with all relevan regulations.

We reserve the right to reject work that the editors believe has not been conducted to a high ethical standard, even if authors have obtained formal approval or approval is not required under local regulations

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. Please note that inclusion of a completed ARRIVE Checklist may be a formal requirement for publication at a later date.

Non-human primates

Manuscripts describing research involving non-human primates must include details of animal welfare, including information about housing, feeding, and environmental enrichment, and steps taken to minimize suffering, including use of anesthesia and method of sacrifice if appropriate, in accordance with the recommendations of the Weatherall report, *The use of non-human primates in research* (PDF).

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 ethics consideration and may be rejected if sufficient ethical and scientific justification for the study design is lacking.

Humane endpoints

For studies in which death of a regulated animal (vertebrate, cephalopod) is a likely outcome or a planned experimental endpoint, *PLOS ONE* asks authors to report additional details related to the study design. This applies to research that involves, for instance, assessment of survival, toxicity, longevity, terminal disease, or high rates of incidental mortality. These studies may be subject to additional ethical considerations, and *PLOS ONE* may reject submissions if they lack sufficient reporting, appropriate justification for the study design, or adequate consideration of humane endpoints, regardless of study-specific institutional animal ethics committee approval.

Definition of a humane endpoint

A humane endpoint is an 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. These may include, for instance, body temperature or weight changes, tumor size or appearance, abnormal behaviors, pathological changes, ruffled fur, reduced mobility, body posture, or expression of specific body fluid markers. Please see the NC3Rs guidelines for more information.

Authors of these studies should report all of the following information in the Methods section:

1. Describe whether humane endpoints were used for all animals involved in the study

If humane endpoints were used, report the following:

- The specific criteria used to determine when animals should be euthanized
- Once animals reached endpoint criteria, the amount of time elapsed before euthanasia
- Whether any animals died before meeting criteria for euthanasia

If humane endpoints were not used, report the following:

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

2. Include the following details of the study design and outcomes:

- 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
- Any special training in animal care or handling provided for research staff

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.

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 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

Manuscripts reporting results from blots (including Western blots) and electrophoretic gels should follow these guidelines:

- In accordance with our policy on image manipulation, the image should not be adjusted in any way that could affect the scientific information displayed, e.g. by modifying the background or contrast.
- All blots and gels that support results reported in the manuscript should be provided.
- Original uncropped and unadjusted blots and gels, including molecular size markers, should be provided in either the figures or the supplementary files.
- Lanes should not be overcropped around the bands; the image should show most or all of the blot or gel. Any non-specific bands should be shown and an explanation of their nature should be given.
- The image should include all relevant controls, and controls should be run on the same blot or gel as the samples.
- A figure panel should not include composite images of bands originating from different blots or gels. If the figure shows non-adjacent bands from the same blot or gel, this should be clearly denoted by vertical black lines and the figure legend should provide details of how the figure was made.

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.

Methods, software, databases, and tools

PLOS ONE will consider submissions that present new methods, software, or databases 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
Software should be open source, deposited in an appropriate archive, and conform to the Open Source Definition. Databases 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. Authors should provide a direct link to the deposited software or the database hosting site from within the paper.

Software submissions

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

Database submissions

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

New taxon names

Zoological names

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

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

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

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

You will need to contact Zoobank to obtain a GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper.

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

> 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].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Botanical names

When publishing papers that describe a new botanical taxon, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). The following guidelines for publication in an online-only journal have been agreed such that any scientific botanical name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature, and apply only to seed plants, ferns, and lycophytes.

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.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found here.

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

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

> **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

In the **Methods** section, include a sub-section called “Nomenclature” using the following wording:

> 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.
>
> 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)].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Fungal names

When publishing papers that describe a new botanical taxon, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). The following guidelines for publication in an online-only journal have been agreed such that any scientific botanical name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature.

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.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found here.

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

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

> ***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.

In the **Methods** section, include a sub-section called “Nomenclature” using the following wording (this example is for taxon names submitted to MycoBank; please substitute appropriately if you have submitted to Index Fungorum):

> 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.
>
> In addition, new names contained in this work have been submitted to MycoBank from where they will be made available to the Global Names Index. The unique MycoBank number can be resolved and the associated information viewed through any standard web browser by appending the MycoBank number contained in this publication to the prefix http://www.mycobank.org/MB/. 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)].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Qualitative research

Qualitative research studies use non-quantitative methods to address a defined research question that may not be accessible by quantitative methods, such as people's interpretations, experiences, and perspectives The analysis methods are explicit, systematic, and reproducible, but the results do not involve numerical values or use statistics. Examples of qualitative data sources include, but are not limited to, interviews, text documents, audio/video recordings, and free-form answers to questionnaires and surveys.

Qualitative research studies should be reported in accordance to the Consolidated criteria for reporting qualitative research (COREQ) checklist. Further reporting guidelines can be found in the Equator Network's Guidelines for reporting qualitative research.

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