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CUBOSSOMAS DE MONOLEÍNA PARA ENTREGA DE TEMOZOLOMIDA E SILENCIAMENTO DE NEK4 COMO ESTRATÉGIAS TERAPÊUTICAS PARA SUPERAR A RESISTÊNCIA TUMORAL DO GLIOBLASTOMA

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Cubossomas de monoleína para entrega de temozolomida e silenciamento de Nek4 como estratégias terapêuticas para superar a resistência tumoral do glioblastoma

Trabalho de Conclusão de Curso de graduação apresentado ao curso de Biomedicina - Noturno da Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, como requisito parcial para a obtenção do grau de Bacharel em Biomedicina.

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RESUMO

O câncer é uma importante preocupação de saúde pública devido a sua complexidade e impacto social. Entre os tumores do sistema nervoso central, os gliomas — especialmente o glioblastoma (GBM) — são agressivos e apresentam baixa taxa de sobrevivência, mesmo com os tratamentos padrão de cirurgia, radioterapia e quimioterapia. O GBM é difícil de tratar devido a sua localização no cérebro e à resistência às terapias convencionais. O tratamento atual com temozolomida (TMZ) e radioterapia enfrenta desafios pela resistência das células tumorais. Assim, há necessidade urgente de novas estratégias terapêuticas para melhorar o prognóstico dos pacientes. A proteína Nek4 surge como um alvo promissor, pois regula mitose, senescência, resposta a danos no DNA e função mitocondrial. Além disso, novos sistemas de liberação de fármacos (DDS), como os cubossomas de monoleína, têm sido estudados para melhorar a entrega, eficácia e segurança da TMZ. Este estudo desenvolveu e caracterizou cubossomas de monoleína com e sem TMZ, avaliando a citotoxicidade na linhagem de glioblastoma U87-WT e investigando o potencial terapêutico da Nek4. Os cubossomas foram analisados por DLS e cryo-TEM. Células U87 com knockout de Nek4 (Nek4-KO) foram construídas e validadas por qRT-PCR. Ensaios de citotoxicidade foram realizados utilizando: cubossomas brancos e carregados com TMZ em células de glioblastoma U87-WT; os agentes indutores de dano ao DNA, TMZ e zeocina, em células U87-WT e Nek4-KO; e, adicionalmente, a presença ou ausência de Spautin-1 em células U87-WT. A presença de TMZ aumentou a polidispersidade e agregação dos cubossomas. Cubossomas brancos e carregados mostraram citotoxicidade dependente de tempo e dose, com janela terapêutica estreita. O pré-tratamento com Spautin-1 sensibilizou as células WT à TMZ e zeocina, efeito ligado à inibição de Nek4 e autofagia. Embora o knockout de Nek4 não tenha alterado o IC_{50}, ensaios de viabilidade indicaram respostas diferentes ao longo do tempo, sugerindo mecanismos compensatórios. Esses resultados destacam a importância de estratégias moleculares e nanotecnológicas para combater a quimiorresistência no glioblastoma e apoiam o desenvolvimento de terapias direcionadas a Nek4.

Palavras-chave: Glioblastoma; Quinases Relacionadas a NIMA; Cubossomas

ABSTRACT

Cancer is a global public health concern due to its complexity and societal impact. Among central nervous system tumors, gliomas—especially glioblastoma (GBM)—are highly aggressive with low patient survival rates despite standard treatments like surgery, radiotherapy, and chemotherapy. GBM's location in the brain and resistance to conventional therapies complicate treatment. Current therapies using the chemotherapeutic agent temozolomide (TMZ) and radiotherapy face challenges due to tumor cell resistance. Thus, new therapeutic strategies are urgently needed to improve outcomes. The Nek4 protein is a promising target because of its role in mitosis regulation, senescence, DNA damage response, and mitochondrial function. Additionally, novel Drug Delivery Systems (DDS), such as monolein cubosomes, have gained interest to enhance TMZ delivery, efficacy, and safety. This study developed and characterized monolein cubosomes with and without TMZ, assessing their cytotoxicity in U87-WT glioblastoma cells and evaluating Nek4 as a therapeutic target. Cubosomes were analyzed by DLS and cryo-TEM. Nek4 knockout (Nek4-KO) U87 glioma cells were created and validated via qRT-PCR. Cytotoxicity assays were performed using blank and TMZ-loaded cubosomes in U87-WT cells; DNA-damaging agents (TMZ and zeocin) in both WT and Nek4-KO cells; and Spautin-1, applied with or without co-treatment, in WT cells. Additionally, TMZ increased cubosome polydispersity and aggregation, suggesting drug-induced destabilization. Both blank and TMZ-loaded cubosomes showed time- and dose-dependent cytotoxicity within a narrow therapeutic window. Spautin-1 pre-treatment sensitized WT cells to TMZ and zeocin, linked to Nek4 and autophagy inhibition. Although Nek4 knockout didn't significantly change IC_{50} values, viability assays showed different responses over time, suggesting compensatory mechanisms in Nek4-deficient cells. These results highlight the importance of combining molecular targeting and nanotechnology to combat glioblastoma chemoresistance and support further Nek4-targeted therapy development.

Keywords: Glioblastoma; NIMA-Related Kinases; Cubosomes

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1 INTRODUÇÃO

O câncer é uma preocupação de saúde pública e a compreensão de todos os mecanismos envolvidos nesta doença ainda é um desafio para a comunidade científica, devido a sua complexidade intrínseca. Dessa forma, há também um enorme empecilho em desenvolver abordagens terapêuticas eficazes, de baixo custo e com reduzidos efeitos colaterais. De acordo com a Organização Mundial da Saúde (OMS), houveram, em 2022, 20 milhões de novos casos de câncer. O órgão estima que 1 em cada 5 pessoas desenvolverá câncer durante a vida. Ainda, há a previsão de mais de 35 milhões de novos casos de câncer em 2050 (OPAS, 2024).

Estimativas da Agência Internacional de Pesquisa em Câncer (IARC) apontam que, em 2045, o número de mortes por câncer será de, aproximadamente, 18,5 milhões, o que representa quase o dobro do número de mortes que ocorreu em 2022, 9,7 milhões (IARC, 2024). Desse modo, há um aumento da incidência e da mortalidade por câncer com o passar dos anos e isso ocorre devido ao crescimento e envelhecimento populacional, além da prevalência crescente de fatores de risco para desenvolvimento da doença, como tabagismo, consumo de álcool, dieta inadequada e obesidade (Rezende et al, 2019).

No contexto dos tumores que acometem exclusivamente o sistema nervoso central (SNC), os gliomas são particularmente notáveis, representando aproximadamente 80% das neoplasias malignas deste sistema. Além disso, a taxa de sobrevida associada a essas neoplasias é extremamente baixa, com uma média de cerca de 15 meses (Chahal et al, 2022; Ma et al, 2024). Vale ressaltar que o termo glioma compreende uma série de tumores gliais, os quais são classificados de acordo com a histopatologia e, nesse cenário, o astrocitoma grau IV, ou glioblastoma (GBM), é o tumor mais agressivo, com fenótipo mais severo e pior prognóstico, ao mesmo tempo, é o tipo mais comuns desses tumores (Ma et al, 2024; Gimenez et al, 2015). A recorrência do tumor ocorre de 75 a 90% dos pacientes com GBM e apenas de 3 a 5% sobrevivem por mais de três anos com esse diagnóstico (Tykocki e Eltayeb, 2018), mostrando a alta letalidade desta doença.

Apesar do tratamento oncológico ser complexo em qualquer tecido, o do GBM representa um desafio ainda maior, pois afeta o órgão mais sofisticado do corpo humano (Perkins e Liu, 2016; Adalpe et al, 2019) e, devido à localização do

tumor e às características do tecido neural, ele prolifera em um microambiente único. Portanto, tratamentos efetivos ainda são difíceis, outros fatores complicadores são a heterogeneidade intratumoral e a sua natureza invasiva (Tetzlaff et al, 2025). Tendo em vista os empecilhos apresentados, é destacada mais uma vez a dificuldade de seu tratamento e, de certo modo, explicando o porquê de não terem ocorrido maiores avanços na prevenção, detecção precoce e no seu tratamento nas últimas quatro décadas (Miller et al, 2019).

Atualmente, seu tratamento consiste na tríade: ressecção cirúrgica, radioterapia e quimioterapia (Perkins e Liu, 2016; Goenka et al, 2021). A ressecção cirúrgica é de alta complexidade devido à localização do tumor e às características do tecido neural, como já mencionado, portanto, qualquer intervenção cirúrgica deve ser bem considerada e analisada, pois lesões no SNC levariam a consequências catastróficas (Adalpe et al, 2019; Perkins e Liu, 2016). Além disso, há uma alta necessidade de habilidades do neurocirurgião para que a ressecção traga mais benefícios do que prejuízos à saúde do paciente. Ademais, pode haver recorrência do tumor, porque a sua ressecção completa é extremamente difícil, o que leva aos outros dois tipos de tratamento da tríade (Xiong et al, 2019; Bush et al, 2016).

Em relação à radioterapia, esta é empregada para erradicar células tumorais residuais após a intervenção cirúrgica. Contudo, similarmente à cirurgia, a delimitação precisa das margens tumorais continua sendo um desafio significativo no tratamento por radiação. Outro ponto a ser levado em consideração é que a radioterapia pode ter muitos efeitos colaterais, que incluem inchaço do cérebro e acúmulo de células mortas, dificultando a avaliação da redução do tumor durante exames de rotina (Bush et al, 2019), por isso, é necessário que haja uma dosagem estrita e específica, a fim de diminuir esses efeitos, principalmente os que causam disfunção neurocognitiva (Lawrie et al, 2019). Em termos moleculares, o tratamento radioterápico é responsável por danos diretos ao DNA, uma vez que rompe ligações fosfodiéster, e danos indiretos, através do aumento de espécies reativas de oxigênio, que levam a um estresse oxidativo no DNA (Tan et al, 2020; Goenka et al, 2021). Esse dano de quebra dupla causado pela radiação pode ser simulado in vitro com o uso da zeocina (Chankova et al., 2007)

A quimioterapia como tratamento do GBM é baseada no uso da TMZ (Tan et al, 2020; Strobel et al, 2019). A TMZ é um pró-fármaco lipofílico, portanto é capaz de

atravessar a barreira hematoencefálica (BHE), tendo a via oral como via de administração (Lee, 2016). O mecanismo de ação da TMZ é baseado na alquilação do DNA, pela adição de um grupo metila na posição O-6 da guanina, desse modo, há um acúmulo de dano e indução de mutações (Khabibov et al, 2022). Com a adição dessa metila na posição O-6 da guanina, que ativa o sistema de reparo por erro de pareamento (MMR, do inglês *mismatch repair*), em replicações posteriores, há a troca da citosina oposta a metilguanina por uma timina, levando a danos de quebra-dupla no DNA e, consequentemente, à apoptose (Lee, 2016; Hiddingh et al, 2013).

Todavia, o GBM pode ser insensível à TMZ logo no momento do seu diagnóstico ou adquirir essa resistência com o passar do tratamento (Hiddingh et al, 2013). A via de reparo de excisão de base (BER), através de uma glicosilase de DNA, remove os sítios de metilação da TMZ e pode ocorrer também a desalquilação do DNA pela enzima O6-metilguanina metiltransferase (MGMT), ambos pontos determinantes na resistência do GBM (Hiddingh et al, 2013; Lee, 2016; Khabibov et al, 2022). Dessa forma, os mecanismos de resposta aos danos ao DNA (DDR) são fatores cruciais no contexto do tratamento com TMZ, pois são responsáveis tanto pela eficácia do tratamento assim como pela sua resistência. Devido a todos esses obstáculos encontrados e à resistência tumoral do GBM, urge a necessidade de novas estratégias terapêuticas, incluindo alvos moleculares e melhora na entrega de drogas antitumorais.

A Nek4 (*NIMA-related Kinase 4*) é uma proteína da família das Neks, cinases serinas/treoninas, que atua na regulação dos microtúbulos, na senescência replicativa, na DDR, na invasão e migração celular no contexto tumoral e na regulação da respiração e morfologia mitocondrial (Doles e Hemann, 2010; Coene et al, 2011; Huo et al, 2017; Ding et al, 2018; Nguyen et al, 2018; Basei et al, 2022). Os microtúbulos têm papel fundamental na mitose, promovendo a formação do fuso mitótico e a segregação do DNA replicado subsequentemente, o que os tornam alvos interessantes no tratamento do câncer (Doles e Hemann, 2010; Jordan e Wilson, 1998; Martin e Cotter, 1990), pois o impedimento da mitose resulta na morte celular em células cancerígenas de alta proliferação. A supressão da Nek4 sensibiliza a célula a agentes tóxicos microtubulares (Doles e Hemann, 2010). Além disso, a Nek4 é necessária para a entrada normal na senescência replicativa e a

sua supressão atrasa essa entrada, reduz as resposta ao dano ao DNA de fita dupla, tanto no recrutamento de proteínas de reparo quanto na interrupção de divisões celulares adicionais, e também reduz a atividade da proteína supressora de tumores p53 (Nguyen et al, 2018; Huo et al, 2017), sinalizadora de danos de quebra dupla no DNA. Ainda, foi demonstrado em células de câncer de pulmão que a supressão da Nek4 inibe a migração e invasão celular, além de diminuir a expressão de proteínas de adesão celular (Ding et al, 2018), e estar relacionada com estágios de câncer colorretal (Huo et al, 2017). A sua expressão aumenta a produção de espécies reativas de oxigênio e promove a integridade do mtDNA (Basei et al, 2022). A sua inibição pode ser feita farmacologicamente pelo uso do composto N-[2-(substituted-phenyl)ethyl]-6-fluoro-4-quinazolinamines, sendo o primeiro inibidor farmacológico de boa atividade e seletividade dessa proteína já descrito (Elsocht et al, 2021), comercialmente, o composto é o spautin-1. Desse modo, essa proteína demonstra ser um interessante alvo terapêutico no tratamento do câncer, em especial do glioblastoma, já que a sua maior taxa de expressão no corpo humano é no cérebro (Coene et al, 2011).

Apesar da descoberta de novos alvos terapêuticos, sejam eles genéticos ou protéicos, o desenvolvimento clínico de novas abordagens terapêuticas para tratar o GBM ainda falha em fornecer um melhor prognóstico aos pacientes. Isso ocorre pela incapacidade dos vetores convencionais atingirem o local do tumor, pela presença da barreira hematoencefálica (BHE) ou pela resistência ao tratamento convencional (Westphal e Lamszus, 2011).

Nesse contexto, os *Drug Delivery Systems* (DDS) podem ser empregados principalmente para obter controle preciso sobre o tempo e o local de administração de fármacos e/ou agentes biológicos. Através deles, as moléculas têm um perfil de liberação modulados de modo sítio-específico, de tamanhos variados entre 1 a 500 nm. Dessa forma, há uma melhora na segurança e na eficácia do tratamento, pois há uma minimização dos efeitos colaterais e um melhor manejo dos esquemas de administração dos fármacos (Wakaskar, 2017). Pela alteração do perfil farmacocinético das moléculas e das propriedades das matrizes desse sistema, os DDS permitem a administração de fármacos potentes, mas que são instáveis naturalmente, garantindo a concentração plasmática aliada à uma menor incidência de toxicidade (Wang et al, 2016).

No esforço pelo desenvolvimento de novos DDS, as nanopartículas têm aparecido com destaque nesse cenário, pois aumentam a solubilidade de drogas hidrofóbicas e reduzem efeitos colaterais, ao mesmo tempo que possuem um bom perfil de liberação da droga (Allen e Cullis, 2004). Nesse sentido, nanopartículas lipídicas de estrutura cúbica, cubossomas, estão em destaque, devido a possibilidade de escalonamento de produção (Ragini et al, 2024). Os cubossomas são dispersões nanométricas de fases cúbicas bicontínuas lipídicas em água, compostas por um interior lipídico e domínios aquosos organizados numa estrutura cúbica (Oliveira et al, 2022), portanto, possuem uma estrutura cúbica cristalina única e propriedade anfifílica, o que permite o encapsulamento e liberação de moléculas hidrofóbicas, hidrofílicas ou anfifílicas, podendo conter mais de uma ao mesmo tempo (Gao et al, 2012; Ragini et al, 2024; Steichen et al, 2013). Toda essa configuração estrutural, faz com que haja uma liberação sustentada da droga e que ele tenha a capacidade de proteger tecidos adjacentes e entregar moléculas com segurança (Oliveira et al, 2022), ou seja, ela reduz significativamente os efeitos negativos dos medicamentos ao permitir o acúmulo seletivo do medicamento tóxico na área desejada, diminuindo a dose recebida pelos tecidos saudáveis (Anselmo e Mitragotri, 2019). Por fim, devido à sua biocompatibilidade, biodegradabilidade e, no contexto do glioblastoma, tão importante quanto, capacidade de atravessar a BHE, os cubossomas têm demonstrado ótima capacidade como DDS para drogas antitumorais (Jablonowska et al, 2021; Ragini et al, 2024).

Diversas estratégias podem ser tomadas para preparação do cubossoma, mas todas elas são feitas a partir de um monoglicerídeo, comumente, a monoleína. A monoleína, ou monooleato de glicerila, é uma mistura lipídica que consiste em glicerídeos de ácido oleico e outros ácidos graxos (principalmente monooleato) (Garg et al, 2007), e, em contato com a água, molda-se em diferentes estruturas, o que garante versatilidade à nanopartícula e a torna ideal para uso em diversas aplicações, incluindo tratamentos para o câncer (Oliveira et al, 2022; Varghese et al, 2022). Ainda, a biocompatibilidade, biodegradabilidade, não toxicidade e a habilidade de formar várias estruturas cristalinas líquidas tornam a monoleína uma molécula ideal e de grande interesse para a formulação de nanopartículas, como cubossomas (Kulkarni et al, 2011). Ademais, cubossomas de monoleína encapsulando diferentes compostos já foram testados para tratamento de diferentes

tipos de câncer e houve um sucesso devido à melhora na eficácia desse tratamento (Gajda et al, 2020; Zhai et al, 2020; Zhang et al, 2020; Bazylinska et al, 2018), além de não apresentarem citotoxicidade quando não carregadas com fármacos (Zhai et al, 2020).

Atentando-se à necessidade de novos alvos terapêuticos para o GBM, tendo em vista que seu tratamento quase não avançou nas últimas quatro décadas (Miller et al, 2021), e a outros fatores já mencionados, como a resistência aos agentes da terapêutica atual e as características do tecido cerebral, justifica-se a busca por novas estratégias de tratamento envolvendo a proteína Nek4, como alvo molecular para tratamento, assim como, os cubossomas, como estratégia de entrega. Desse modo, busca-se a promoção de um melhor prognóstico ao paciente, através do uso de tecnologias e recursos disponíveis, visando a exploração dos mecanismos da Nek4 e desse novo DDS, para entender melhor sobre as interações celulares *in vitro* e toxicidade dos cubossomas de monoleína, a fim de diminuir a resistência ao tratamento do tumor mais letal do sistema nervoso central.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar a citotoxicidade de cubossomas de monoleína contendo o quimioterápico temozolomida (TMZ) e o potencial da proteína Nek4 como alvo terapêutico no contexto do glioblastoma.

2.2 OBJETIVOS ESPECÍFICOS

1. Obter os cubossomas de monoleína contendo TMZ.
2. Analisar a citotoxicidade dos cubossomas de monoleína contendo TMZ na linhagem U87-WT após exposição aguda e crônica.
3. Definir as doses de IC_{50} de TMZ e zeocina (radiomimético) na sublinhagem U87 Nek4-KO.
4. Compreender o efeito da inibição e do silenciamento da Nek4 nas células de glioblastoma.

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5 CONCLUSÃO

Este estudo demonstra que não houveram diferenças no tamanho e no índice de polidispersão dos cubossomas em branco ou com TMZ Além disso, ambos exibiram efeitos citotóxicos dependentes da dose e do tempo, com uma janela terapêutica estreita, porém promissora. A toxicidade inesperada dos cubossomas brancos ressalta a importância de uma caracterização físico-química e biológica rigorosa, mesmo para *Drug Delivery Systems* geralmente considerados biocompatíveis.

A construção e validação bem-sucedidas da linha celular U87 Nek4-KO permitiram a investigação funcional do papel da Nek4 na resposta celular ao estresse genotóxico. Embora os valores de IC_{50} para TMZ e zeocina tenham sido semelhantes entre as células selvagens (WT) e Nek4-KO, os ensaios de viabilidade revelaram diferenças na suscetibilidade ao longo do tempo, sugerindo que a perda de Nek4 influencia a adaptação ao estresse e pode ativar mecanismos compensatórios durante tratamentos prolongados.

Além disso, o pré-tratamento com Spautin-1 sensibilizou as células de glioblastoma WT tanto para TMZ quanto para zeocina. Esse efeito de sensibilização provavelmente reflete não apenas a inibição da Nek4, mas também a supressão da autofagia, um alvo secundário conhecido do Spautin-1. Esses achados sugerem um mecanismo duplo de ação, onde a inibição combinada da resposta a danos no DNA e da autofagia aumenta a vulnerabilidade celular à quimioterapia.

Em conjunto, este trabalho destaca o potencial terapêutico de direcionar as vias de Nek4 e autofagia para superar a quimiorresistência no glioma. Estudos adicionais são necessários para desvendar as contribuições específicas da inibição farmacológica de Nek4 *versus* a supressão da autofagia e para otimizar formulações de cubossomas visando maior eficácia e segurança no tratamento do glioblastoma.

6 ANEXOS

6.1 Diretrizes de submissão para a revista “Cellular Signaling”

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Authors are encouraged to carefully check all images before submission and to connect all the data in any figures to the original, unprocessed data.

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- Institutional affiliations: Authors should use either the full, standard title of their institution or the standard abbreviation of the institutional name so that the institutional name can be independently verified for research integrity purposes.

Studies in humans

Authors must follow ethical guidelines for studies carried out in humans.

Work which involves the use of human subjects should be carried out in accordance with the World Medical Association Declaration of Helsinki: Ethical principles for medical research involving human subjects.

Manuscripts should follow the International Committee of Medical Journal Editors (ICMJE) recommendations for the conduct, reporting, editing and publication of scholarly work in medical journals and aim to be representative of human populations in terms of sex, age and ethnicity. Sex and gender terms should be used correctly, as outlined by WHO (World Health Organization).

Manuscripts must include a statement that all procedures were performed in compliance with relevant laws and institutional guidelines and have been approved by the appropriate institutional committee(s). The statement should contain the date and reference number of the ethical approval(s) obtained.

Manuscripts must also include a statement that the privacy rights of human subjects have been observed and that informed consent was obtained for experimentation with human subjects.

This journal will not accept manuscripts that contain data derived from unethically sourced organs or tissue, including from executed prisoners or prisoners of conscience, consistent with recommendations by Global Rights Compliance on Mitigating Human Rights Risks in Transplantation Medicine. For all studies that use human organs or tissues, sufficient evidence must be provided that these were procured in line with WHO Guiding Principles on Human Cell, Tissue and Organ Transplantation. For clinical studies, a statement of informed consent having been obtained from a patient or their nominated representative, paired with ethical approval for the study from a suitable institution, as required by the policies of the journal, may be considered sufficient evidence, but the journal reserves the right to request additional evidence in cases where it feels this is not sufficient. The source of the organs or tissues used in clinical research must be transparent and traceable. If your manuscript describes organ transplantation you must additionally declare within the manuscript that:

- autonomous consent free from coercion was obtained from the donor(s) or their next of kin.
- organs and/or tissues were not sourced from executed prisoners or prisoners of conscience.

Writing and formatting

File format

We ask you to provide editable source files for your entire submission (including figures, tables and text graphics). Some guidelines:

- Save files in an editable format, using the extension .doc/.docx for Word files and .tex for LaTeX files. A PDF is not an acceptable source file.
- Lay out text in a single-column format.
- Remove any strikethrough and underlined text from your manuscript, unless it has scientific significance related to your article.
- Use spell-check and grammar-check functions to avoid errors.

We advise you to read our Step-by-step guide to publishing with Elsevier.

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You are required to include the following details in the title page information:

- Article title. Article titles should be concise and informative. Please avoid abbreviations and formulae, where possible, unless they are established and widely understood, e.g., DNA).
- Author names. Provide the given name(s) and family name(s) of each author. The order of authors should match the order in the submission system. Carefully check that all names are accurately spelled. If needed, you can add your name between parentheses in your own script after the English transliteration.
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You are required to provide a concise and factual abstract which does not exceed 250 words. The abstract should briefly state the purpose of your research, principal results and major conclusions. Some guidelines:

- Abstracts must be able to stand alone as abstracts are often presented separately from the article.

- Avoid references. If any are essential to include, ensure that you cite the author(s) and year(s).
- Avoid non-standard or uncommon abbreviations. If any are essential to include, ensure they are defined within your abstract at first mention.

Keywords

You are required to provide 1 to 7 keywords for indexing purposes. Keywords should be written in English. Please try to avoid keywords consisting of multiple words (using "and" or "of").

We recommend that you only use abbreviations in keywords if they are firmly established in the field.

Highlights

You are required to provide article highlights at submission.

Highlights are a short collection of bullet points that should capture the novel results of your research as well as any new methods used during your study. Highlights will help increase the discoverability of your article via search engines. Some guidelines:

- Submit highlights as a separate editable file in the online submission system with the word "highlights" included in the file name.
- Highlights should consist of 3 to 5 bullet points, each a maximum of 85 characters, including spaces.

We encourage you to view example article highlights and read about the benefits of their inclusion.

Graphical abstract

You are encouraged to provide a graphical abstract at submission.

The graphical abstract should summarize the contents of your article in a concise, pictorial form which is designed to capture the attention of a wide readership. A graphical abstract will help draw more attention to your online article and support readers in digesting your research. Some guidelines:

- Submit your graphical abstract as a separate file in the online submission system.
- Ensure the image is a minimum of 531 x 1328 pixels (h x w) or proportionally more and is readable at a size of 5 x 13 cm using a regular screen resolution of 96 dpi.
- Our preferred file types for graphical abstracts are TIFF, EPS, PDF or MS Office files.

We encourage you to view example graphical abstracts and read about the benefits of including them.

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Tables must be submitted as editable text, not as images. Some guidelines:

- Place tables next to the relevant text or on a separate page(s) at the end of your article.
- Cite all tables in the manuscript text.
- Number tables consecutively according to their appearance in the text.
- Please provide captions along with the tables.
- Place any table notes below the table body.
- Avoid vertical rules and shading within table cells.

We recommend that you use tables sparingly, ensuring that any data presented in tables is not duplicating results described elsewhere in the article.

Figures, images and artwork

Figures, images, artwork, diagrams and other graphical media must be supplied as separate files along with the manuscript. We recommend that you read our detailed artwork and media instructions. Some excerpts:

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- Cite all images in the manuscript text.
- Number images according to the sequence they appear within your article.
- Submit each image as a separate file using a logical naming convention for your files (for example, Figure_1, Figure_2 etc).

- Please provide captions for all figures, images, and artwork.
- Text graphics may be embedded in the text at the appropriate position. If you are working with LaTeX, text graphics may also be embedded in the file.

Artwork formats

When your artwork is finalized, "save as" or convert your electronic artwork to the formats listed below taking into account the given resolution requirements for line drawings, halftones, and line/halftone combinations:

- Vector drawings: Save as EPS or PDF files embedding the font or saving the text as "graphics."
- Color or grayscale photographs (halftones): Save as TIFF, JPG or PNG files using a minimum of 300 dpi (for single column: min. 1063 pixels, full page width: 2244 pixels).
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- Combinations bitmapped line/halftones (color or grayscale): Save as TIFF, JPG or PNG files using a minimum of 500 dpi (for single column: min. 1772 pixels, full page width: 3740 pixels).

Please do not submit:

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All images must have a caption. A caption should consist of a brief title (not displayed on the figure itself) and a description of the image. We advise you to keep the amount of text in any image to a minimum, though any symbols and abbreviations used should be explained.

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If you submit usable color figures with your accepted article, we will ensure that they appear in color online.

Please ensure that color images are accessible to all, including those with impaired color vision. Learn more about color and web accessibility.

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- The use of generative AI or AI-assisted tools in the production of artwork such as for graphical abstracts is not permitted. The use of generative AI in the production of cover art may in some cases be allowed, if the author obtains prior permission from the journal editor and publisher, can demonstrate that all necessary rights have been cleared for the use of the relevant material, and ensures that there is correct content attribution.

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We encourage the use of supplementary materials such as applications, images and sound clips to enhance research. Some guidelines:

- Supplementary material should be accurate and relevant to the research.
- Cite all supplementary files in the manuscript text.
- Submit supplementary materials at the same time as your article. Be aware that all supplementary materials provided will appear online in the exact same file type as received. These files will not be formatted or typeset by the production team.

- Include a concise, descriptive caption for each supplementary file describing its content.
- Provide updated files if at any stage of the publication process you wish to make changes to submitted supplementary materials.
- Do not make annotations or corrections to a previous version of a supplementary file.
- Switch off the option to track changes in Microsoft Office files. If tracked changes are left on, they will appear in your published version.

Video

This journal accepts video material and animation sequences to support and enhance your scientific research. We encourage you to include links to video or animation files within articles. Some guidelines:

- When including video or animation file links within your article, refer to the video or animation content by adding a note in your text where the file should be placed.
- Clearly label files ensuring the given file name is directly related to the file content.
- Provide files in one of our recommended file formats. Files should be within our preferred maximum file size of 150 MB per file, 1 GB in total.
- Provide "stills" for each of your files. These will be used as standard icons to personalize the link to your video data. You can choose any frame from your video or animation or make a separate image.
- Provide text (for both the electronic and the print version) to be placed in the portions of your article that refer to the video content. This is essential text, as video and animation files cannot be embedded in the print version of the journal.

We publish all video and animation files supplied in the electronic version of your article.

For more detailed instructions, we recommend that you read our guidelines on submitting video content to be included in the body of an article.

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We are committed to supporting the storage of, access to and discovery of research data, and our research data policy sets out the principles guiding how we work with the research community to support a more efficient and transparent research process.

Research data refers to the results of observations or experimentation that validate research findings, which may also include software, code, models, algorithms, protocols, methods and other useful materials related to the project.

Please read our guidelines on sharing research data for more information on depositing, sharing and using research data and other relevant research materials.

For this journal, the following instructions from our research data guidelines apply.

Option C: Research data deposit, citation and linking

You are required to:

- Deposit your research data in a relevant data repository.
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Data statement

To foster transparency, you are required to state the availability of any data at submission.

Ensuring data is available may be a requirement of your funding body or institution. If your data is unavailable to access or unsuitable to post, you can state the reason why (e.g., your research data includes sensitive or confidential information such as patient data) during the submission process. This statement will appear with your published article on ScienceDirect.

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Linking to the data underlying your work increases your exposure and may lead to new collaborations. It also provides readers with a better understanding of the described research.

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- For some data repositories, a repository banner will automatically appear next to your published article on ScienceDirect.
- You can also link relevant data or entities within the text of your article through the use of identifiers. Use the following format: Database: 12345 (e.g. TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN).

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Article sections

- Divide your article into clearly defined and numbered sections. Number subsections 1.1 (then 1.1.1, 1.1.2, ...), then 1.2, etc.
- Use the numbering format when cross-referencing within your article. Do not just refer to "the text."
- You may give subsections a brief heading. Headings should appear on a separate line.
- Do not include the article abstract within section numbering.

Glossary

Please provide definitions of field-specific terms used in your article, in a separate list.

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Include any individuals who provided you with help during your research, such as help with language, writing or proof reading, in the acknowledgements section. Acknowledgements should be placed in a separate section which appears directly before the reference list. Do not include acknowledgements on your title page, as a footnote to your title, or anywhere else in your article other than in the separate acknowledgements section.

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It is not necessary to include detailed descriptions on the program or type of grants, scholarships and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

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Appendices

We ask you to use the following format for appendices:

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- Give separate numbering to formulae and equations within appendices using formats such as Eq. (A.1), Eq. (A.2), etc. and in subsequent appendices, Eq. (B.1), Eq. (B. 2) etc. In a similar way, give separate numbering to tables and figures using formats such as Table A.1; Fig. A.1, etc.

References

References within text

Any references cited within your article should also be present in your reference list and vice versa. Some guidelines:

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- We recommend that you do not include unpublished results and personal communications in your reference list, though you may mention them in the text of your article.
- Any unpublished results and personal communications included in your reference list must follow the standard reference style of the journal. In substitution of the publication date add "unpublished results" or "personal communication."
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Linking to cited sources will increase the discoverability of your research.

Before submission, check that all data provided in your reference list are correct, including any references which have been copied. Providing correct reference data allows us to link to abstracting and indexing services such as Scopus, Crossref and PubMed. Any incorrect surnames, journal or book titles, publication years or pagination within your references may prevent link creation.

We encourage the use of Digital Object Identifiers (DOIs) as reference links as they provide a permanent link to the electronic article referenced.

Reference format

This journal does not set strict requirements on reference formatting at submission. Some guidelines:

- References can be in any style or format as long as the style is consistent.
- Author names, journal or book titles, chapter or article titles, year of publication, volume numbers, article numbers or pagination must be included, where applicable.
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Reference style

Indicate references by adding a number within square brackets in the text. You can refer to author names within your text, but you must always give the reference number, e.g., "as demonstrated [3,6]. Barnaby and Jones [8] obtained a different result ....".

Number references in the order they appear in your article.

Abbreviate journal names according to the List of Title Word Abbreviations (LTWA).

Examples:

Reference to a journal publication:

[1] J. van der Geer, T. Handgraaf, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2020) 51 – 59. https://doi.org/10.1016/j.sc.2020.00372.

Reference to a journal publication with an article number:

[2] J. van der Geer, T. Handgraaf, R.A. Lupton, 2022. The art of writing a scientific article. Heliyon. 19, e00205. https://doi.org/10.1016/j.heliyon.2022.e00205.

Reference to a book:

[3] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.

Reference to a chapter in a book:

[4] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2020, pp. 281 - 304.

Reference to a website:

[5] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/, 2023 (accessed 13 March 2023).

Reference to a dataset:

[6] M. Oguro, S. Imahiro, S. Saito, T. Nakashizuka, Mortality data for Japanese oak wilt disease and surrounding forest compositions [dataset], Mendeley Data, v1, 2015. https://doi.org/10.1234/abc12nb39r.1.

Reference to software:

[7] E. Coon, M. Berndt, A. Jan, D. Svyatsky, A. Atchley, E. Kikinzon, D. Harp, G. Manzini, E. Shelef, K. Lipnikov, R. Garimella, C. Xu, D. Moulton, S. Karra, S. Painter, E. Jafarov, S. Molins, Advanced Terrestrial Simulator (ATS) v0.88 [software], Zenodo, March 25, 2020. https://doi.org/10.1234/zenodo.3727209.

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When listing web references, as a minimum you should provide the full URL and the date when the reference was last accessed. Additional information (e.g. DOI, author

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We encourage you to cite underlying or relevant datasets within article text and to list data references in the reference list.

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If a template is not available for this journal, follow the format given in examples in the reference style section of this Guide for Authors.

Submitting your manuscript

Submission checklist

Before completing the submission of your manuscript, we advise you to read our submission checklist:

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- Spelling and grammar checks have been carried out.
- All references in the article text are cited in the reference list and vice versa.
- Permission has been obtained for the use of any copyrighted material from other sources, including the Web.
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