PONTIFÍCIA UNIVERSIDADE CATÓLICA DO PARANÁ
ESCOLA DE MEDICINA E CIÊNCIAS DA VIDA
PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA
ÁREA DE CONCENTRAÇÃO CLÍNICA ODONTOLÓGICA INTEGRADA

BRUNO EDUARDO SANT'ANNA FALCE DE MACEDO

AVALIAÇÃO DO EFEITO DE DIFERENTES CONCENTRAÇÕES DE RESVERATROL EM CÉLULAS-TRONCO DA POLPA DENTAL HUMANA: ESTUDO DA CITOTOXICIDADE E ESTABILIDADE GENÉTICA

Curitiba
2024

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO PARANÁ
ESCOLA DE MEDICINA E CIÊNCIAS DA VIDA
PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA
ÁREA DE CONCENTRAÇÃO CLÍNICA ODONTOLÓGICA INTEGRADA

BRUNO EDUARDO SANT'ANNA FALCE DE MACEDO

AVALIAÇÃO DO EFEITO DE DIFERENTES CONCENTRAÇÕES DE RESVERATROL EM CÉLULAS-TRONCO DA POLPA DENTAL HUMANA: ESTUDO DA CITOTOXICIDADE E ESTABILIDADE GENÉTICA

Dissertação apresentada ao Programa de Pós-Graduação em Odontologia da Pontifícia Universidade Católica do Paraná, como parte dos requisitos para obtenção do título de Mestre em Odontologia, Área de Concentração em Clínica Odontológica Integrada (Sub-área Estomatologia).

Orientadora: Profª. Drª. Luciana Reis Azevedo Alanis
Coorientadora: Drª. Patrícia Tolentino da Rosa de Souza

Curitiba
2024

Dados da Catalogação na Publicação
Pontifícia Universidade Católica do Paraná
Sistema Integrado de Bibliotecas – SIBI/PUCPR
Biblioteca Central
Luci Eduarda Wielganczuk – CRB 9/1118

M141a
2024

Macedo, Bruno Eduardo Sant'Anna Falce de
Avaliação do efeito de diferentes concentrações de resveratrol em células-tronco da polpa dentária humana : estudo da citotoxidade e estabilidade genética / Bruno Eduardo Sant'Anna Falce de Macedo ; orientadora: Luciana Reis Azevedo Alanis ; coorientadora: Patrícia Tolentino da Rosa de Souza. – 2024.
78 f. : il. ; 30 cm

Dissertação (mestrado) – Pontifícia Universidade Católica do Paraná, Curitiba, 2024
Inclui bibliografias

1. Odontologia. 2. Resveratrol. 3. Células-tronco. 4. Polpa dentária. I. Alanis, Luciana Reis Azevedo. II. Souza, Patrícia Tolentino da Rosa de. III. Pontifícia Universidade Católica do Paraná. Programa de Pós-Graduação em Odontologia. IV. Título.

CDD. 20. ed. – 617.6

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO PARANÁ
Escola de Medicina e Ciências da Vida

TERMO DE APROVAÇÃO

BRUNO EDUARDO SANT'ANNA FALCE DE MACEDO

AVALIAÇÃO DE DIFERENTES CONCENTRAÇÕES DE RESVERATROL EM CÉLULAS-TRONCO DA POLPA DENTAL HUMANA

Dissertação apresentada ao Programa de Pós-Graduação em Odontologia da Pontifícia Universidade Católica do Paraná, como parte dos requisitos parciais para a obtenção do Título de **Mestre em Odontologia**, Área de Concentração em **Clínica Odontológica Integrada - Estomatologia**.

Orientador: Profª Drª Luciana Reis Azevedo Alanis
Programa de Pós-Graduação em Odontologia, PUCPR

Prof. Dr. Edvaldo Antonio Ribeiro Rosa
Programa de Pós-Graduação em Odontologia, PUCPR

Prof. Dr. Heliton Gustavo de Lima
Programa de Pós-Graduação em Odontologia, UFPR

Curitiba, 03 de dezembro de 2024

SUMÁRIO

ARTIGO 1 - VERSÃO EM PORTUGUÊS

Página título

Título: Avaliação do efeito de diferentes concentrações de resveratrol em células-tronco da polpa dental humana: estudo da citotoxicidade e estabilidade genética

Autores: Bruno Eduardo Sant'Anna Falce de Macedo, Patrícia Tolentino da Rosa de Souza, Luciana Reis Azevedo Alanis

Pontifícia Universidade Católica do Paraná, Escola de Medicina e Ciências da Vida, Programa de Pós-Graduação em Odontologia, Área Clínica Odontológica Integrada – Sub-área Estomatologia

Resumo

Objetivo: avaliar o efeito de diferentes concentrações de resveratrol no cultivo de células tronco da polpa dental humana (DPSCs) em diferentes tempos, por meio de testes de citotoxicidade e estabilidade genética. *Material e método*: o tecido pulpar dental foi obtido após exodontias de terceiros molares de três pacientes. As células foram isoladas e cultivadas em diferentes concentrações de resveratrol. Os testes da viabilidade celular por citometria de fluxo foram realizados após 24 e 96 horas no grupo controle (sem resveratrol) e no grupo teste (cultivo em concentrações de 12,5 µM, 25 µM, 50 µM e 75 µM de resveratrol). O bandeamento G foi utilizado para detecção de estabilidade genética. Os testes não paramétrico de Kruskal-Wallis e de comparações múltiplas de Dunn foram empregados para comparar a viabilidade celular entre grupos. *Resultados:* as amostras de DPSCs apresentaram resultados da caracterização celular segundo os critérios da ISCT (*International Society for Cell and Gene Therapy*). Após 96 horas de cultivo, as concentrações de 50 µM e 75 µM de resveratrol foram citotóxicas (< 70%) para as amostras avaliadas. A viabilidade celular na concentração de 50µM foi significativamente menor do que em 12,5 µM (p=0,045), e em comparação ao grupo controle (p=0,018). A concentração de 75 µM apresentou viabilidade celular significativamente menor comparado ao grupo controle (p=0,022). Ao comparar a viabilidade celular nos grupos avaliados entre os dois tempos, 24 horas e 96 horas, não houve diferenças significativas (p>0,05). Não foram encontradas alterações clonais no grupo controle e nas concentrações de 12,5 µM e 25 µM. *Conclusão*: houve citotoxicidade nas DPSCs cultivadas em

concentrações de 50 µM e 75 µM de resveratrol. O resveratrol, nas concentrações de 12,5 µM e 25 µM, não se mostrou citotóxico e manteve a estabilidade genética das DPSCs tanto em 24 horas quanto em 96 horas de cultivo. Estas concentrações se demonstraram seguras e promissoras para futuros ensaios que avaliem seu uso terapêutico *in vivo*.

Palavras-chave: trans Resveratrol, Dental Pulp, Pluripotent Stem Cells, Mesenchymal Stem Cells, Karyotyping.

Introdução

O resveratrol é um polifenol natural obtido de alguns alimentos, como vinho tinto, nozes, amendoim, cacau, frutas e algumas ervas (TOU, 2014). Tem sido amplamente empregado no manejo de doenças crônicas inflamatórias (TIMMERS et al., 2011; TOMÉ-CARNEIRO et al., 2012; MALAGUARNERA, 2019; HUANG et al., 2020; FANTACUZZI et al., 2022; SUBHAN, SIDDIQUE, 2024). A resposta inflamatória pode ser regulada pelo resveratrol através de diferentes vias de sinalização, como os metabólitos do ácido araquidônico (MAGRONE et al., 2019). Além disso, a ativação da sirtruína 1 (SIRT1), uma deacetilase envolvida em diversos eventos moleculares, através do resveratrol inibe a produção de fatores inflamatórios (SAQIB et al., 2018; MALAGUARNERA, 2019). No entanto, o uso do resveratrol como agente anti-inflamatório ou facilitador no processo de reparo através de aplicação tópica seja em pele (CHAN 2002; SHEVELEV et al., 2020; HECKER et al., 2022; YANG et al., 2022; JIA et al., 2022) ou em mucosa ainda tem sido objeto de discussão (MARTINS et al., 2018; PACZKOWSKA-WALENDOWSKA et al., 2021), assim como sua ação frente a lesões potencialmente malignas e ao carcinoma de células escamosas (JAYARAMAN et al., 2022; YANG et al., 2024).

Previamente à veiculação de cosméticos ou medicamentos que contenham resveratrol, é necessário investigar o potencial para causar dano às células, seja pela toxicidade ou por desencadear o processo da oncogênese (ICH, 2012; OMS, 2013). A avaliação toxicológica de substâncias *in vitro* em células humanas substitui o uso de animais, tem demonstrado ampla aceitação e é uma abordagem emergente (FRACARO et al., 2023). Durante o cultivo de células, o acúmulo de mutações genéticas e epigenéticas, bem como a ocorrência da senescência celular, as quais prejudicam a biologia celular e as propriedades terapêuticas, são fatores de risco para a transformação celular (KIM et al., 2015; NERI., 2019). É necessário que as respostas terapêuticas de medicamentos naturais e seus compostos bioativos sejam claramente identificadas, para garantir eficácia terapêutica e segurança (RAO et al., 2019).

Células estromais mesenquimais (Mesenchymal Stromal Cells - MSCs) são células-tronco pluripotentes não hemopoiéticas derivadas da mesoderme, com capacidade de auto-renovação (BARYAWNO et al., 2019; PITTENGER et al.,

1999). MSCs são isoladas de diversas fontes, como tecidos dentais, tais como polpa dental, ligamento periodontal, folículo dental, papila apical, gengiva, germe dentário, dente decíduo esfoliado e osso alveolar (GRONTOS et al., 2000; DAVE, TOMAR., 2018; LI et al., 2021). Em MSCs cultivadas, o resveratrol pode otimizar os efeitos terapêuticos, de modo a garantir sua sobrevivência, autorrenovação, comprometimento com a linhagem e efeitos anti-envelhecimento (HU & LI., 2019).

A realização de testes citogenéticos deve ocorrer a fim de garantir que não tenha ocorrido transformação celular durante o cultivo. Dentre os testes, a cariotipagem pela técnica do bandeamento G é o padrão-ouro para detecção de estabilidade genética (LIEHR, 2017, ISSCR 2008). O potencial tumorigênico de MSCs cultivadas é uma questão crítica e está estritamente correlacionada à instabilidade genômica (NERI., 2019). Comparadas às células tronco pluripotentes induzidas e células tronco embrionárias, MSCs apresentam menor potencial tumorigênico (JUNG, BAUER, NOLTA., 2012).

Ensaios de viabilidade celular permitem avaliar a potência ou toxicidade de fármacos, os efeitos na proliferação celular e efeitos citotóxicos diretos, além de serem uma importante ferramenta para pesquisa e desenvolvimento de medicamentos (LUZAK, SIARKIEWICZ, BONCLER., 2022; ADAN, KIRAZ, BARAN., 2016). Geralmente, dados não-clínicos sobre toxicidade são necessários para embasar testes clínicos; dependendo do uso pretendido, estudos adicionais sobre citotoxicidade podem ser necessários, como genotoxicidade, tumorigenicidade, toxicidade reprodutiva e de desenvolvimento, e estudos de imunotoxicidade (EMA, 2024). Os estudos de toxicidade devem ser planejados para gerar dados clinicamente significativos e relevantes que suportem o uso seguro do produto na indicação clínica pretendida, como estimativa de dose inicial segura e regime de dosagem (EMA, 2024).

Diante da limitação de investigações que avaliaram a citotoxicidade do resveratrol no cultivo de células-tronco da polpa dental (DPSCs) e a estabilidade genética de DPSCs cultivadas, o objetivo do presente estudo foi avaliar o efeito de diferentes concentrações de resveratrol no cultivo de DPSCs em diferentes tempos, por meio de testes de citotoxicidade e de estabilidade genética.

Material e Métodos

O projeto de pesquisa foi aprovado pelo Comitê de Ética em Pesquisa da Pontifícia Universidade Católica do Paraná (PUCPR), com parecer número 38458920.4.0000.0020.

Três pacientes do sexo feminino, com idades entre 20 e 23 anos, atendidas na Clínica de Odontologia da PUCPR, cederam seus terceiros molares extraídos, para o presente estudo, mediante assinatura do Termo de Consentimento Livre e Esclarecido (TCLE).

Foram utilizados terceiros molares irrompidos ou inclusos, que estivessem hígidos, e apresentassem indicações para exodontia. Os terceiros molares inclusos foram extraídos sem necessidade de odontossecção. O cultivo de células foi realizado a partir do tecido pulpar de três terceiros molares, cada dente proveniente de uma paciente, dos quais dois dentes eram molares superiores direito e um molar superior esquerdo.

Previamente às exodontias, as pacientes realizaram bochecho com digluconato de clorexidina 0,12% para remover possíveis contaminantes (PUPO et al., 2021). Imediatamente após as cirurgias, em campo estéril, os dentes foram limpos com soro fisiológico e seccionados transversalmente ao longo eixo, na junção cemento-esmalte (GRONTHOS et al., 2000), com broca zekrya estéril. Durante a odontosecção, os dentes foram irrigados com soro fisiológico. Após essa etapa, os dentes foram acondicionados em um meio de coleta, Meio de Dulbecco Modificado de Iscove (IMDM) (Gibco, ThermoFisher Scientific, Grand Island, NY), suplementado com soro bovino fetal (FBS) a 20% (Gibco, ThermoFisher Scientific, Grand Island, NY), penicilina (100 unidades/mL, Sigma-Aldrich, Saint Louis, MO), estreptomicina (100 µg/mL, Sigma-Aldrich, Saint Louis, MO), fluconazol (2 mg/mL, Sanobiol, Pouso Alegre, MG) e heparina sódica (5.000 UI/mL, Cristália, Pouso Alegre, MG), e levados ao Núcleo de Tecnologia Celular da PUCPR.

Isolamento de células do tecido pulpar dental e cultivo

O tecido pulpar foi removido com lima endodôntica do tipo H, dissociado mecanicamente, com lâmina de bisturi em uma placa de petri e submetido à digestão enzimática pela ação da colagenase tipo II a 4 mg/mL (Gibco, ThermoFisher Scientific, Grand Island, NY) por 1 h, a 37 °C, sob agitação contínua. (CORRÊA et al., 2018). Em seguida, a amostra foi filtrada através de filtros de

células de 100 µm para obtenção de suspensão celular e depois centrifugada a 400 × G por 10 min. A suspensão celular foi semeada em frascos de 25 cm^2 e cultivada em IMDM suplementado com FBS a 20%, penicilina 100 unidades/mL e estreptomicina 100 µg/mL (CORRÊA et al. 2018).

As culturas foram mantidas em incubadora a 37 °C em atmosfera de 5% de CO_2, com troca do meio a cada três dias. Quando as células atingiram a confluência (80%), foram dissociadas por tripsina/EDTA a 0,25% (Gibco, ThermoFisher Scientific, Grand Island, NY) e ressemeadas em frascos de 75 cm^2 (CORRÊA et al. 2018).

Caracterização celular por imunofenotipagem

Para a caracterização celular por imunofenotipagem, foi feito acompanhamento diário da amostra e análise da morfologia celular e aderência ao plástico, além da diferenciação para adipócitos, condroblastos e osteoblastos, conforme diretrizes da ISCT (DOMINICI et al., 2006). A caracterização foi conduzida após as células atingirem P3.

A etapa da marcação consistiu na lavagem das células com PBS (Gibco, ThermoFisher Scientific, Grand Island, NY) e incubação no escuro durante 30 min (REBELATTO et al., 2008; REBELATTO et al., 2023) com anticorpos anti CD29-APC (1:20), CD14-FITC (1:20), CD45-FITC (1:20), CD19-FITC (1:20), CD34-APC (1:20), CD105-APC (1:20), CD73-APC (1:33), CD90-PE (1:100), (1:20) (Becton Dickinson, San Diego, CA, EUA). Em seguida, as células foram lavadas com solução tampão e ressuspensas em 500 µL de uma solução contendo 1% de paraformaldeído (SigmaAldrich, MO, USA) (PUPO et al., 2021). Anticorpos isotípicos IgG1 de camundongo foram utilizados como controle (FRACARO et al., 2023).

Aproximadamente 100.000 células marcadas foram adquiridas por um citômetro de fluxo FACS Calibur (BD Bioscience, San Jose, CA) e analisadas por meio do software FlowJo (FlowJo, Ashland, OR, EUA - versão 8.1) (PUPO et al., 2021).

Cultivo das DPSCs com Resveratrol

Após a terceira passagem (P3), as DPSCs foram colocadas em frascos de 75 cm^2 e mantidas a 37 °C em atmosfera de 5% de CO_2 por 24 e 96 horas com resveratrol (Trans-Resveratrol - Pharmaceutical Secondary Standard; Certified Reference Material, Sigma-Aldrich, Saint Louis, MO). O resveratrol foi diluído com

DMSO (Dimetil Sulfóxido) até obtenção das concentrações 12,5 µM, 25 µM, 50 µM e 75 µM.

Teste da viabilidade celular por citometria de fluxo

A citotoxicidade foi quantificada através da viabilidade celular por citometria de fluxo, após 24 e 96 horas, no grupo teste - DPSCs cultivadas em diferentes concentrações de resveratrol (12,5 µM, 25 µM, 50 µM, e 75 µM) e no grupo controle – DPSCs cultivadas sem resveratrol.

As células foram incubadas com Anexina PE (marcador de apoptose celular) e 7-AAD (corante vital). As incubações foram realizadas em temperatura ambiente por 30 min. Anticorpos isotípicos conjugados com fluorocromos PE e PE-CY5 foram utilizados como controle. Após a incubação, as células foram lavadas com PBS e fixadas com PBS contendo 1% de paraformaldeído. As amostras foram adquiridas no citômetro de fluxo FACSCalibur e analisadas no software FlowJo. Os testes foram realizados em triplicata para cada grupo de amostras.

As concentrações de resveratrol foram consideradas citotóxicas quando o valor da viabilidade celular foi inferior a 70% (Food and Drug Administration, 2020).

Avaliação por citogenética através do bandeamento G

Foram avaliados o grupo teste, nas concentrações de 12,5 µM, 25 µM e 50 µM de resveratrol, e o grupo controle.

O bandeamento G foi utilizado para detecção de estabilidade genética e foi executado após o cultivo (P3). A cultura celular, ao atingir a confluência de 80%, sofreu adição de Colchicina 0,1 µg/mL, e então os frascos foram incubados a 37°C por um período de 2 a 6 horas. Mudanças na morfologia da célula foram monitoradas por meio de um microscópio invertido (BORGONOVO et al., 2014).

As DPSCs foram dissociadas do frasco utilizando 2 mL de tripsina-EDTA a 0,25% aquecida. Após 3 min de monitoramento, duas gotas de FBS foram adicionadas e as células foram transferidas para um tubo de centrífuga contendo o meio. As amostras foram centrifugadas a 400 × G por 10 min (BORGONOVO et al., 2014).

Para o tratamento hipotônico, foram adicionados lenta e cuidadosamente 5 mL de KCl 0,075 M com Hepes, seguido de incubação a 37ºC por 30 min e fixação com solução de metanol:ácido acético (3:1). Para melhorar a visualização das metáfases, as amostras foram lavadas duas vezes em solução fria (5°C) de

metanol:ácido acético fresco (2:1). Antes da preparação da lâmina, as lâminas limpas foram colocadas em vapor enquanto as células foram ressuspensas em solução fixadora fresca e gotejadas sobre a lâmina (BORGONOVO et al., 2014).

Para obtenção das bandas G, as lâminas foram submetidas a 60ºC por no mínimo 16h. Em seguida, foram imersas em solução de tripsina (0,002 g/mL) por 5 s, lavadas em solução salina e rapidamente enxaguadas em água destilada. A coloração foi realizada com Giemsa (1:20) ou solução de Wright (1:6), de modo a produzir bandas de tripsina e Giemsa (GTG) ou tripsina e Wright (GTW), respectivamente. A qualidade das bandas do cromossomo foi avaliada ao microscópio sob a magnificação de 1000 × e os tempos de ação da tripsina e para o tingimento foram ajustados para produzir bandas claras e bem coradas (BORGONOVO et al., 2014).

Em cada amostra de DPSCs foram observadas 20 metáfases ao microscópio óptico, registradas e interpretadas com o auxílio de um sistema de cariotipagem (MCGOWAN-JORDAN et al., 2020).

Análise estatística

Foram calculadas média, mediana e desvio padrão entre os valores de viabilidade celular da amostra de DPSCs (n=3) para cada concentração de resveratrol avaliada no grupo teste e no grupo controle. O teste não paramétrico de Kruskal-Wallis para amostras independentes foi empregado para comparar a viabilidade celular entre o grupo controle e as quatro concentrações do grupo teste em 24 e em 96 horas. O teste de comparações múltiplas de Dunn foi utilizado para comparação dois a dois entre concentrações de resveratrol com diferenças significativas na viabilidade celular (tempo de 96 horas). O teste não paramétrico de Wilcoxon para amostras emparelhadas foi empregado para comparar os valores da viabilidade celular entre os dois tempos (24 e 96 horas) dentro de cada grupo. O poder do teste para a variável viabilidade celular em 96 horas segundo os grupos foi 0,938, o que indica elevada confiabilidade dos resultados. Em todos os testes, foi adotado nível de significância de 5% ($p < 0,05$).

Resultados

Caracterização celular por imunofenotipagem

A amostra de DPSCs das três pacientes apresentou resultados da caracterização celular segundo os critérios sugeridos pela ISCT (tabela 1).

Tabela 1: Valores referentes à caracterização celular de amostra de DPSCs de terceiros molares de três pacientes.

Paciente	CD14	CD34	CD45	HLA-DR	CD19	CD73	CD90	CD105	CD29
I	1,60%	0,37%	0,22%	1,50%	0,27%	98,40%	99,00%	98,30%	98,20%
II	1,15%	0,25%	0,23%	1,78%	0,17%	98,70%	98,90%	98,20%	99,20%
III	0,62%	0,08%	0,44%	0,38%	0,19%	99,70%	99,70%	99,50%	99,00%

Teste da viabilidade celular por citometria de fluxo

As Tabelas 2 e 3 mostram os resultados do teste da viabilidade celular da cultura de DPSCs por citometria de fluxo no grupo teste e no grupo controle, após 24 e 96 horas, respectivamente. A viabilidade celular da cultura de DPSCs da amostra III após 24 horas foi 66,20%, indicando citotoxicidade (Tabela 2). Após 96 horas de cultivo, as concentrações de 50 μM e 75 μM de resveratrol foram citotóxicas para as três amostras avaliadas (Tabela 3).

Tabela 2: Valores da viabilidade celular da cultura de DPSCs por citometria de fluxo após 24 horas no grupo teste (em concentrações de resveratrol de 12,5 µM, 25 µM, 50 µM e 75 µM) e no grupo controle (sem resveratrol).

Paciente	Tempo	Variáveis	Controle	12,5µM	25µM	50µM	75µM
		Anexina (%)	0,36	0,29	0,43	0,29	0,39
		7-AAD (%)	5,23	14,10	5,08	9,88	20,20
I	24 horas	Viabilidade celular (%)	94,77	85,90	94,92	90,12	79,80
		Anexina (%)	0,39	0,27	0,20	0,09	0,14
		7-AAD (%)	4,92	5,86	7,15	9,48	12,40
II	24 horas	Viabilidade celular (%)	95,08	94,14	92,85	90,52	87,60
		Anexina (%)	0,89	1,27	0,98	0,60	0,72
		7-AAD (%)	8,71	12,50	7,91	21,20	33,80
III	24 horas	Viabilidade celular (%)	91,29	87,50	92,09	78,80	66,20

Tabela 3: Valores da viabilidade celular da cultura de DPSCs por citometria de fluxo após 96 horas no grupo teste (em concentrações de resveratrol de 12,5 µM, 25 µM, 50 µM e 75 µM) e no grupo controle (sem resveratrol).

Paciente	Tempo	Variáveis	Controle	12,5µM	25µM	50µM	75µM
		Anexina (%)	0,18	0,35	0,28	0,18	0,47
		7-AAD (%)	6,22	6,34	6,67	37,00	45,20
I	96 horas	Viabilidade celular (%)	93,78	93,66	93,33	63,00	54,80
		Anexina (%)	0,35	0,61	0,39	0,48	0,42
		7-AAD (%)	2,71	5,17	6,84	58,40	83,40
II	96 horas	Viabilidade celular (%)	97,29	94,83	93,16	41,60	16,60
		Anexina (%)	0,88	0,94	0,37	1,07	0,31
		7-AAD (%)	9,69	8,55	5,57	56,10	28,70
III	96 horas	Viabilidade celular (%)	93,31	91,45	94,43	43,90	71,30

A Tabela 4 mostra valores médios das porcentagens, desvios padrão e valores de p da viabilidade celular em 24 e 96 horas nos grupos teste e controle.

Tabela 4: Valores médios das porcentagens e desvio-padrão da viabilidade celular da cultura de DPSCs das amostras avaliadas por citometria de fluxo após 24 e 96 horas no grupo teste (em concentrações de resveratrol de 12,5µM, 25µM, 50µM e 75µM) e no grupo controle (sem resveratrol).

Tempo de cultivo	**Controle X(%)±DP**	**12,5 µM X(%)±DP**	**25 µM X(%)±DP**	**50 µM X(%)±DP**	**75 µM X(%)±DP**	**Valor p**
24 horas	93,713±2,104^{A}	89,180±4,369^{A}	93,287±1,465^{A}	86,480 ± 6,654^{A}	77,867±10,830^{A}	0,595
96 horas	94,793±2,175^{a,A}	93,313±1,716^{a,c,A}	93,640 ± 0,689^{b,A}	49,500 ± 11,748^{b,A}	47,567±28,058^{b,c,A}	0,035*

Letras maiúsculas sobreescritas iguais em coluna indicam que não houve diferenças significantes entre grupos (p>0,05). Teste de Wilcoxon.

* letras minúsculas sobreescritas diferentes em linha indicam que houve diferença significante entre grupos (p<0,05). Letras minúsculas sobreescritas iguais em linha indicam que não houve diferenças significantes entre grupos (p>0,05). Teste de Kruskal-Wallis e Teste de Dunn.

Não houve diferenças significantes dos valores de viabilidade celular da cultura de DPSCs das amostras avaliadas por citometria de fluxo após 24 horas entre o grupo controle e as concentrações de resveratrol do grupo teste (p=0,595). Houve diferenças significantes entre o grupo controle e as concentrações do grupo teste em 96 horas (p=0,035). A viabilidade celular na concentração de 50 µM de resveratrol foi significativamente menor do que em 12,5 µM (p=0,045) e em comparação ao grupo controle (p=0,018). A concentração de 75 µM de resveratrol apresentou viabilidade celular significativamente menor em comparação ao controle (p=0,022) (Tabela 4).

Ao comparar a viabilidade celular nos grupos avaliados entre os dois tempos, 24 horas e 96 horas, não houve diferenças estatisticamente significantes (p>0,05, tabela 4).

Os valores da mediana da viabilidade celular entre os grupos e tempos após 24 e 96 horas são expressos nas figuras 1 e 2, respectivamente.

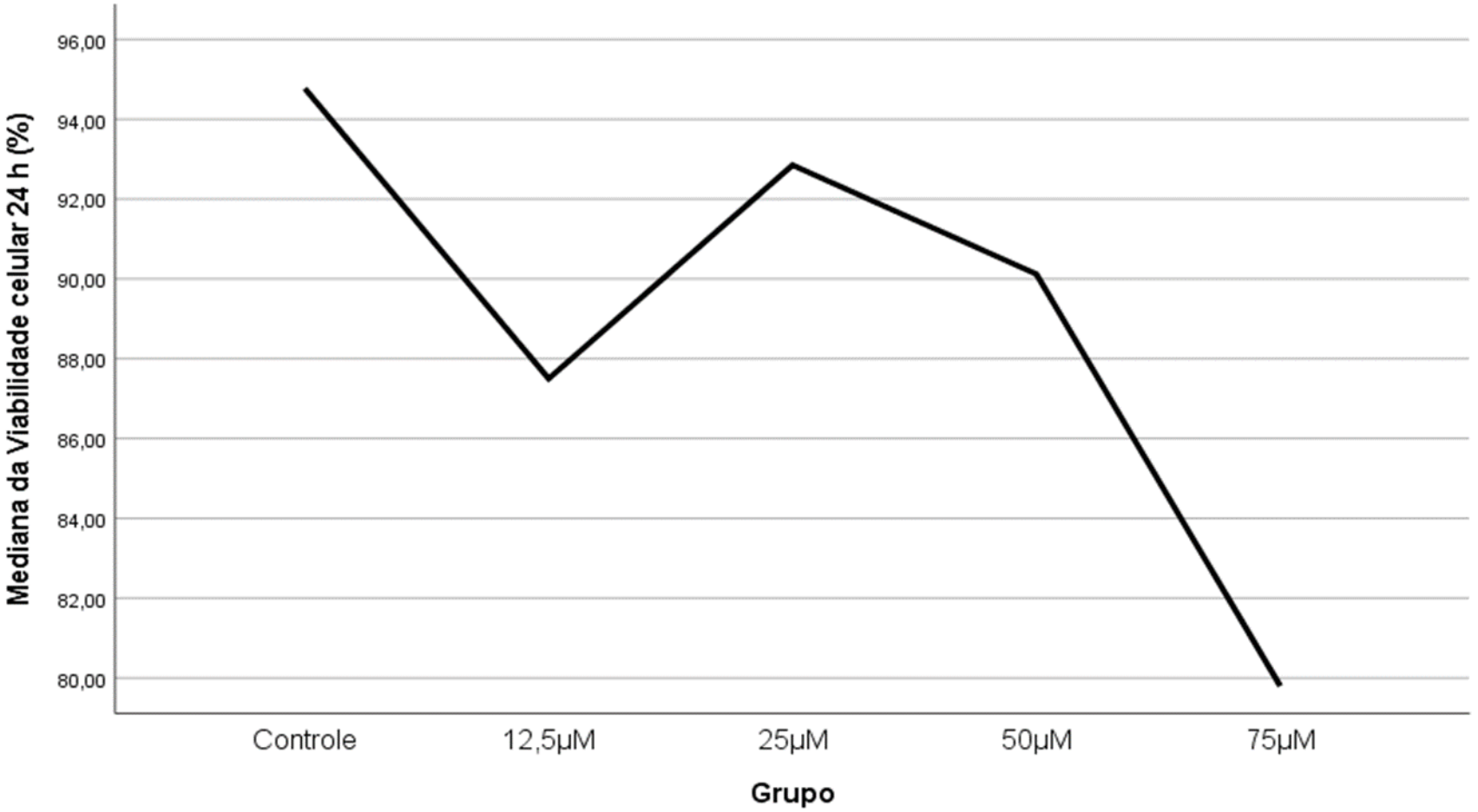

Figura 1: Valores da mediana da viabilidade celular no grupo controle e nas diferentes concentrações de resveratrol em 24 horas.

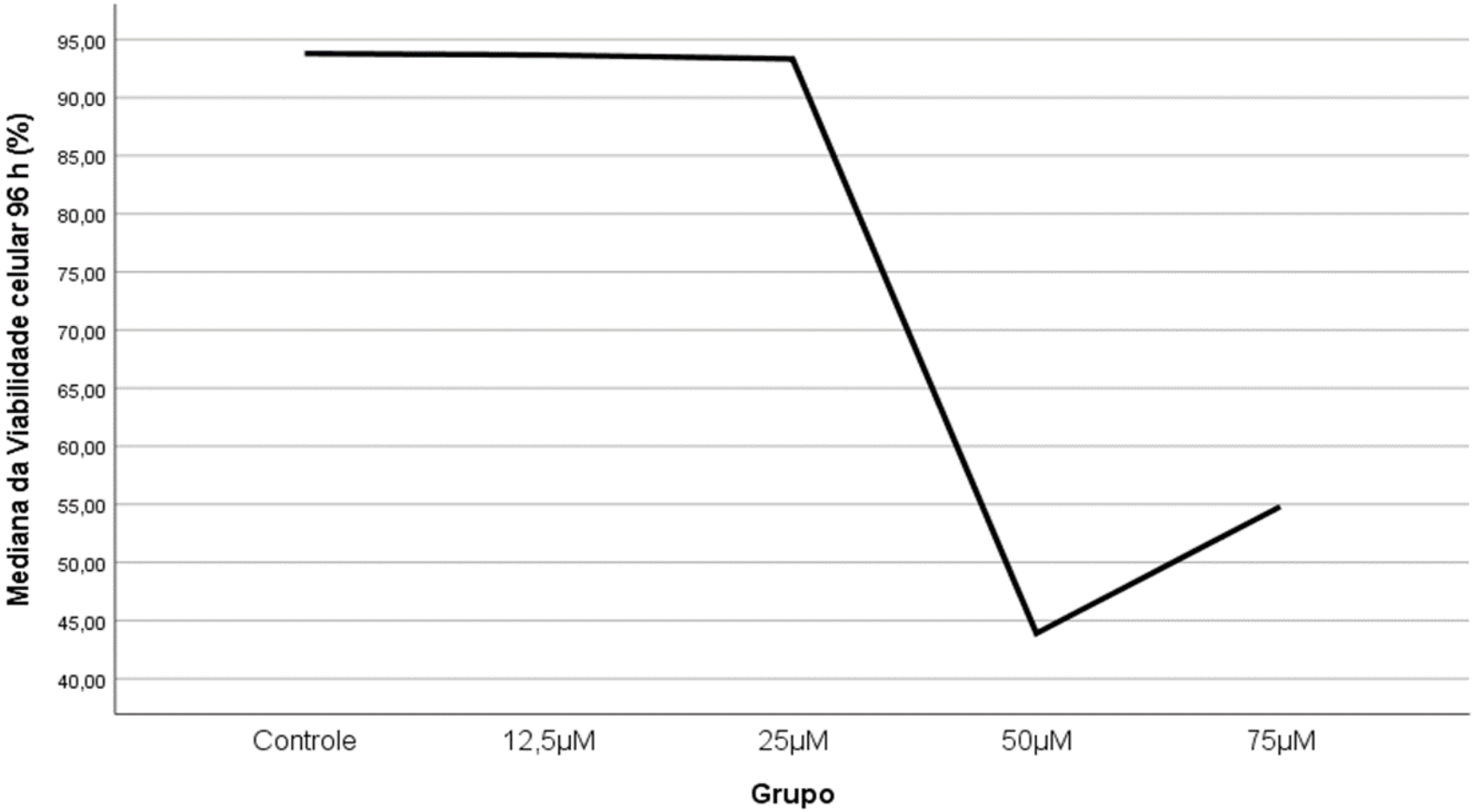

Figura 2: Valores da mediana da viabilidade celular no grupo controle e nas diferentes concentrações de resveratrol em 96 horas.

Avaliação por citogenética através do bandeamento G

Não foram encontradas alterações clonais no grupo controle e nas concentrações de 12,5 μM e 25 μM de resveratrol, após 96 horas. Em 50 μM e 75 μM de resveratrol, não foi possível realizar o bandeamento G (Tabela 5).

Tabela 5: Cariótipos das DPSCs cultivadas sem resveratrol (controle) e nas concentrações de 12,5 μM e 25 μM de resveratrol após 96 horas.

Paciente	Controle	12,5 μM	25 μM
I	46,XX[20]	46,XX[20]	46,XX[20]
II	46,XX[20]	46,XX[20]	46,XX[20]
III	46,XX[20]	46,XX[20]	46,XX[20]

Legenda: o número 46 indica a quantidade de cromossomos em cada célula, XX indica os cromossomos sexuais, e [20] a quantidade de metáfases analisadas

A Figura 3 mostra cariogramas de amostras de DPSCs.

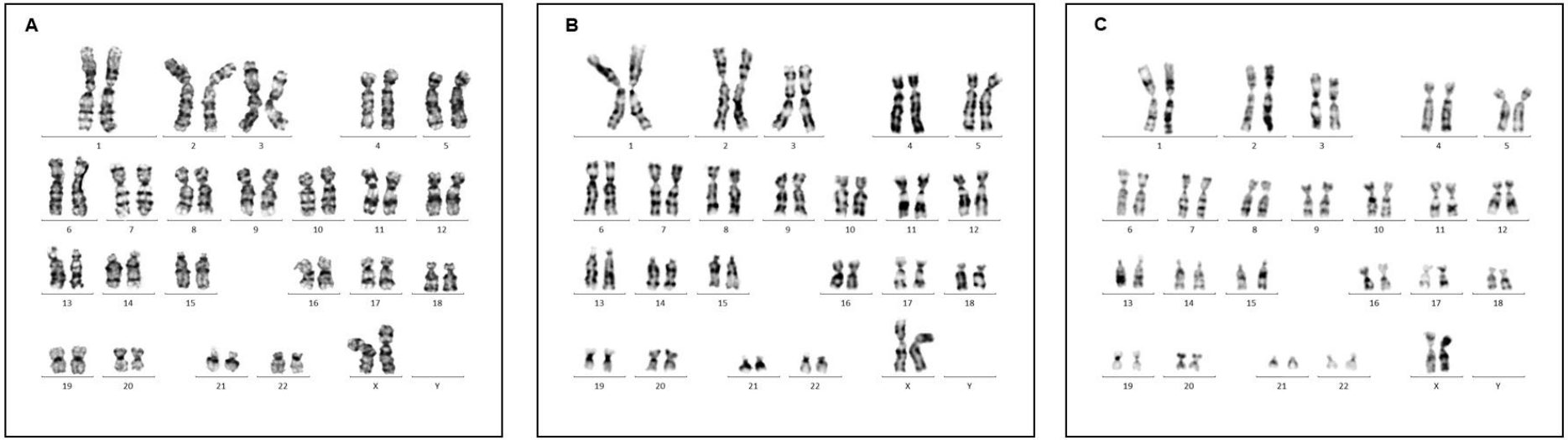

Figura 3: Cariogramas de amostras de DPSCs da paciente II. A) cultivadas sem resveratrol (grupo controle), B) cultivadas na concentração de 12,5 µM de resveratrol, C) cultivadas na concentração de 25 µM de resveratrol.

Discussão

Frente a limitação de estudos que avaliaram a estabilidade genética de DPSCs e a indicação destas células para o estudo de propriedades promissoras do resveratrol como agente anti-inflamatório e/ou facilitador no processo de reparo, os resultados do presente estudo mostraram que houve citotoxicidade nas DPSCs cultivadas em concentrações de 50 µM e 75 µM de resveratrol após 96 horas. Além disso, as DPSCs cultivadas em concentrações de resveratrol de 12,5 µM e 25 µM apresentaram elevada viabilidade celular e estabilidade genética, após 96 horas de cultivo.

Os parâmetros de citotoxicidade adotados no presente estudo basearam-se nos critérios sugeridos pela FDA, nos quais a viabilidade celular mínima aceitável é 70%. Assim, as concentrações de 50 e 75 µM de resveratrol mostraram-se citotóxicas para as DPSCs após 96 horas de cultivo. Resultados similares foram encontrados em estudo que avaliou o processo de diferenciação das DPSCs para neuronal; o resveratrol nas concentrações de um, cinco, 10, 15, 20, 25 e 50 µM não foi citotóxico durante o cultivo das DPSCs, enquanto a concentração de 100 µM foi. Além disso, a concentração de 15 µM durante 12 horas foi considerada ótima para o processo de diferenciação (GENG, ZHENG, LIU, HU, 2017). Em outro estudo, a concentração de 10 µM de resveratrol aumentou significativamente a viabilidade celular e reduziu o estresse oxidativo em DPSCs (ZHANG et al., 2022). Em MSCs do endométrio cultivadas em concentrações de 10, 20, 40 e 60 µM de resveratrol, o efeito antioxidante foi avaliado; nas concentrações mais baixas houve redução nas espécies reativas de oxigênio, enquanto em 40 e 60 µM, foram observados danos ao DNA e indução à senescência prematura (KORNIENKO et al., 2019). O resveratrol pode interagir especificamente com as membranas mitocondriais, privar as células de ATP (adenosina trifosfato) e aumentar as espécies reativas de oxigênio; dependendo do grau de privação de ATP ocasionado pelo resveratrol, as células podem sofrer apoptose ou necrose. Tais mecanismos parecem ser a base molecular da toxicidade do resveratrol (OLIVEIRA et al., 2016; OLIVARES-MARIN, GONZÁLEZ-HERNÁNDEZ, MADRIGAL-PEREZ 2019).

Dependendo das concentrações de resveratrol, sua atuação é antioxidante ao nível fisiológico ou citotóxica pró-oxidativa, a qual ocasiona leve estresse

oxidativo e ativação dos sistemas antioxidantes, como um mecanismo de defesa celular. Além disso, o estado energético da célula também influencia no efeito citotóxico do resveratrol; ao alterar o estado energético da célula, é possível potencializar o efeito citotóxico do resveratrol. É sugerido ainda, que o efeito antioxidante desempenhado pelo resveratrol pode ser atribuído a um fenótipo adaptativo de células capazes de neutralizar o efeito pró-oxidante (OLIVARES-MARIN, GONZÁLEZ-HERNÁNDEZ, MADRIGAL-PEREZ 2019).

No presente estudo, não foram detectadas alterações clonais em DPSCs cultivadas nas concentrações de 12,5 e 25 μM de resveratrol e sem resveratrol. Em uma proposta de um possível *guidelines* com os critérios de qualidade mínimos para o uso clínico de MSCs cultivadas, a análise do cariótipo deve ser realizada para demonstrar que há manutenção da estabilidade genômica durante a expansão (TORRE et al., 2015). O estabelecimento da análise do cariótipo permitiu a avaliação de genotoxicidade de produtos químicos (SATO et al., 2019). Dentre as possíveis alterações clonais em células-tronco estão aneuploidia, mutações em oncogenes, genes supressores de tumor e mutações epigenéticas (ISSCR, 2008).

A polpa dental se destaca por ser um tecido com notável resistência à oncogênese, embora DPSCs saudáveis compartilhem características em comum com fenótipos de CTCs (células-tronco cancerígenas) identificados em neoplasias malignas (CRENDE et al., 2020). A elevada expressão de genes supressores de tumor e a capacidade de interromper a proliferação celular em resposta a danos no DNA (CRENDE et al., 2020; BADIOLA et al., 2015) são hipóteses para explicar parcialmente a resistência à oncogênese das DPSCs. Em estudo com fibroblastos embrionários de camundongos cultivados, o uso de resveratrol contribuiu para manutenção da estabilidade genômica e as células foram protegidas contra mutações na via ARF/p53 (MATSUNO et al., 2020).

O bandeamento G permite detecção de anomalias numéricas e estruturais, tais como translocações, inversões e baixos níveis de mosaicismo (VAZ et al., 2021; BEN-DAVID, BENVENISTY., 2012). O limiar de detecção dessa técnica é >5% (BEN-DAVID, BENVENISTY, 2012). Uma limitação do bandeamento G é impossibilidade de detectar pequenas alterações, podendo ser necessária associação com métodos complementares (VAZ et al., 2021), tais como Fluorescência por Hibridização in Situ (FISH), que pode ser aplicada tanto para intérfases quanto para metáfases (SILVA et al., 2019; GRANADA et al., 2020).

Apesar disso, a análise a partir do bandeamento dos cromossomos permanece como o método mais compreensivo para avaliar anormalidades cromossômicas (GRANADA et al., 2020).

Neste estudo, as concentrações de 12,5 μM e 25 μM de resveratrol não apresentaram citotoxicidade às DPSCs e mantiveram sua estabilidade genética, o que sugere que o resveratrol é seguro às DPSCs do ponto de vista oncogênico. Este resultado é promissor para estudos futuros que avaliem ação anti-inflamatória e de facilitador do processo de reparo do resveratrol, principalmente em tecidos do sistema estomatognático.

A ação do resveratrol frente a prevenção e cura de lesões inflamatórias na mucosa bucal foi avaliada com o uso de mucoadesivos, os quais parecem ser uma alternativa (MARTINS et al., 2018). Um estudo com células do epitélio bucal induzidas à transformação maligna demonstrou que o resveratrol pode promover significativamente apoptose em células cancerígenas (SUN, et al., 2024). A suplementação de resveratrol inibiu a atividade de miofibroblastos e suprimiu a expressão de genes fibrogênicos em pacientes com fibrose submucosa oral, uma lesão potencialmente maligna (CHANG et al., 2016).

Tanto a rápida metabolização quanto a rápida excreção do resveratrol são os principais fatores que implicam em seu uso clínico; a melhora na biodisponibilidade tem sido investigada (HECKER et al., 2021). Ao analisar o metabolismo dos isômeros do resveratrol em modelo celular, o resveratrol di-hidratado apresentou melhor absorção (99%) quando comparado ao trans-reveratrol e cis-resveratrol (20% e 62%, respectivamente) (JAROSOVA et al., 2020).

A aplicação de medicamentos em mucosa tem recebido grande atenção no campo das pesquisas, pois apresenta vantagens como controle preciso da dose e prevenção da rápida metabolização pela administração oral. Apesar das vantagens, uma dificuldade nessa via de administração é a presença de saliva, que pode dissolver o medicamento e liberá-lo para vias não desejadas (YOU et al., 2015). Um estudo que avaliou as propriedades antifúngicas do resveratrol frente à candidíase bucal demonstrou que a incorporação de alginato de sódio a um sistema cristalino líquido aumentou significativamente a mucoadesividade e melhorou a permanência do resveratrol na cavidade bucal (MIYASHIRO et al., 2020).

No presente estudo, a amostra de DPSCs apresentou resultados da caracterização celular de acordo com os parâmetros sugeridos pela ISCT. Os resultados obtidos para CD 29 (98,2%; 99,2%, e 99%) são similares aos valores médios encontrados por outro estudo – 98,8% (SUCHANEK et al., 2009). O marcador de superfície CD 29, proveniente da família das integrinas β1 (STANIOWSKI, KNEFEL, MALINOWSKA, 2021), é comumente utilizado em MSCs e, quando avaliado em DPSCs, apresenta perfil positivo (SUCHANEK et al., 2009; MARCHIONNI et al., 2009). Em DPSCs, a presença do CD 29 caracteriza propriedades odonto-osteogênicas (SEIFRTOVÁ et al., 2012).

Marcadores de superfície permitem a distinção entre os tipos de células-tronco; são proteínas específicas que atuam como receptores capazes de vincular ou aderir-se à outra molécula, seletivamente (STANIOWSKI, KNEFEL, MALINOWSKA, 2021). Células-tronco provenientes de diferentes tecidos apresentam marcadores de superfície ligeiramente diferentes (LAN, LUO, WEI 2021). No entanto, não há marcadores específicos para DPSCs (MACRIN et al., 2019), assim como não existe consenso sobre qual seria o único marcador de superfície capaz de identificar MSCs de diferentes tecidos (LAN, LUO, WEI 2021; JUAN, TUAN, CHEUNG, LEUNG, 2014). A ISCT (DOMINICI et al., 2006) fornece parâmetros para caracterização de MSCs, as quais estão presentes em diferentes órgãos e tecidos. O estabelecimento de uma diretriz com os parâmetros para caracterização celular de DPSCs especificamente permitiria aos pesquisadores realizar comparações, de modo apurado, e não subjetivo. Com o estabelecimento de um padrão, existe garantia de que células cultivadas de fato pertençam à linhagem celular em questão e apresentem propriedades e características esperadas.

As potenciais limitações do estudo são ausência de outras concentrações de resveratrol (>12,5 µM, diferente de 25 µM, e <50 µM), a não execução de diferentes técnicas para quantificação da citotoxicidade e ausência da realização de um ensaio funcional. Além disso, não foi possível realizar o bandeamento G nas concentrações de 50 µM e 75 µM; devido à citotoxicidade houve apoptose generalizada, e consequentemente, comprometimento na qualidade do material a ser analisado, uma vez que não houve número mínimo disponível de 20 metáfases para serem registradas e interpretadas. Sistemas *ex-vivo* humanos apresentam potencial no campo da farmacologia translacional, pois intensificam o processo

geral de desenvolvimento de medicamentos e pode ser um passo importante na determinação da eficácia antes da realização de ensaios clínicos (SILVA, HAGGARTY., 2020). Por outro lado, o uso de modelos animais ainda é essencial a fim de compreender a administração do medicamento, a passagem do medicamento pela barreira hematoencefálica, a farmacocinética e a farmacodinâmica em um organismo por completo (SILVA, HAGGARTY., 2020).

Conclusão

Houve citotoxicidade nas DPSCs cultivadas em concentrações de 50 µM e 75 µM de resveratrol após 96 horas. O resveratrol, nas concentrações de 12,5 µM e 25 µM, não se mostrou citotóxico e manteve a estabilidade genética das DPSCs tanto em 24 horas quanto em 96 horas de cultivo. Estas concentrações se demonstraram seguras e promissoras para futuros ensaios que avaliem seu uso terapêutico *in vivo*.

Referências

1. Adan A, Kiraz Y, Baran Y. Cell Proliferation and Cytotoxicity Assays. Current Pharmaceutical Biotechnology. 2016;17(14).
2. Badiola I, Santaolalla F, Garcia-Gallastegui P, Ana S del R, Unda F, Ibarretxe G. Biomolecular bases of the senescence process and cancer. A new approach to oncological treatment linked to ageing. Vol. 23, Ageing Research Reviews. 2015.
3. Baryawno N, Przybylski D, Kowalczyk MS, Kfoury Y, Severe N, Gustafsson K, et al. A Cellular Taxonomy of the Bone Marrow Stroma in Homeostasis and Leukemia. Cell. 2019;177(7).
4. Ben-David U and Benvenisty N (2012) Analyzing the genomic integrity of stem cells. In: StemBook. Harvard Stem Cell Instite, Cambridge, MA.
5. Borgonovo T, Vaz IM, Senegaglia AC, Rebelatto CLK, Brofman PRS. Genetic evaluation of mesenchymal stem cells by G-banded karyotyping in a Cell Technology Center. Revista Brasileira de Hematologia e Hemoterapia. 2014;36(3).
6. Chan MMY. Antimicrobial effect of resveratrol on dermatophytes and bacterial pathogens of the skin. Biochemical Pharmacology. 2002;63(2).
7. Chang YC, Lin CW, Yu CC, Wang BY, Huang YH, Hsieh YC, et al. Resveratrol suppresses myofibroblast activity of human buccal mucosal fibroblasts through the epigenetic inhibition of ZEB1 expression. Oncotarget. 2016;7(11).
8. Corrêa NCR, Kuligovski C, Paschoal ACC, Abud APR, Rebelatto CLK, Leite LMB, et al. Human adipose-derived stem cells (ADSC) and human periodontal ligament stem cells (PDLSC) as cellular substrates of a toxicity prediction assay. Regulatory Toxicology and Pharmacology. 2018;92.
9. Crende O, García-Gallastegui P, Luzuriaga J, Badiola I, de la Hoz C, Unda F, et al. Is there such a thing as a genuine cancer stem cell marker? Perspectives from the gut, the brain and the dental pulp. Vol. 9, Biology. 2020.
10. Dave JR, Tomar GB. Dental tissue–derived mesenchymal stem cells: Applications in tissue engineering. Critical Reviews in Biomedical Engineering. 2018;46(5).
11. de Oliveira MR, Nabavi SF, Manayi A, Daglia M, Hajheydari Z, Nabavi SM. Resveratrol and the mitochondria: From triggering the intrinsic apoptotic

pathway to inducing mitochondrial biogenesis, a mechanistic view. Vol. 1860, Biochimica et Biophysica Acta - General Subjects. 2016.

12. Dominici M, le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8(4).
13. European Medicines Agency. Draft guideline on the quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials (second version). Amsterdam: European Medicines Agency; 2024.
14. Fantacuzzi M, Amoroso R, Carradori S, de Filippis B. Resveratrol-based compounds and neurodegeneration: Recent insight in multitarget therapy. Vol. 233, European Journal of Medicinal Chemistry. 2022.
15. Fracaro L, Hochuli AHD, Selenko AH, Capriglione LGA, Brofman PRS, Senegaglia AC. Mesenchymal stromal cells derived from exfoliated deciduous teeth express neuronal markers before differentiation induction. Journal of Applied Oral Science. 2023;31.
16. Geng YW, Zhang Z, Liu MY, Hu WP. Differentiation of human dental pulp stem cells into neuronal by resveratrol. Cell Biology International. 2017;41(12).
17. Granada I, Palomo L, Ruiz-Xivillé N, Mallo M, Solé F. Cytogenetics in the genomic era. 2020 [cited 2024 May 27]; Available from: https://doi.org/10.1016/j.beha.2020.101196
18. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proceedings of the National Academy of Sciences of the United States of America. 2000;97(25).
19. Hecker A, Schellnegger M, Hofmann E, Luze H, Nischwitz SP, Kamolz LP, et al. The impact of resveratrol on skin wound healing, scarring, and aging. Vol. 19, International Wound Journal. 2022.
20. Hu C, Li L. The application of resveratrol to mesenchymal stromal cell-based regenerative medicine. Vol. 10, Stem Cell Research and Therapy. 2019.
21. Huang DD, Shi G, Jiang Y, Yao C, Zhu C. A review on the potential of Resveratrol in prevention and therapy of diabetes and diabetic complications. Vol. 125, Biomedicine and Pharmacotherapy. 2020.

22. In: International Conference on Harmonization. Guideline S1: Rodent carcinogenicity studies for human pharmaceuticals; 2012.
23. International Society for Stem Cell Research (ISSCR). (2008). Guidelines for the clinical translation of stem cells.
24. Jarosova V, Vesely O, Doskocil I, Tomisova K, Marsik P, Jaimes JD, et al. Metabolism of cis-and trans-resveratrol and dihydroresveratrol in an intestinal epithelial model. Nutrients. 2020;12(3).
25. Jayaraman S, Fathima SJH, Veeraraghavan VP, Raj AT, Patil S. Resveratrol and miR-200c: insights into the prevention of oral squamous cell carcinoma. Vol. 18, Future Oncology. 2022.
26. Jia Y, Shao JH, Zhang KW, Zou ML, Teng YY, Tian F, et al. Emerging Effects of Resveratrol on Wound Healing: A Comprehensive Review. Vol. 27, Molecules. 2022.
27. Jung Y, Bauer G, Nolta JA. Concise review: Induced pluripotent stem cell-derived mesenchymal stem cells: Progress toward safe clinical products. Vol. 30, Stem Cells. 2012.
28. Kim JA, Im KO, Park SN, Kwon JS, Kim SY, Oh K, et al. Cytogenetic heterogeneity and their serial dynamic changes during acquisition of cytogenetic aberrations in cultured mesenchymal stem cells. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis. 2015;777.
29. Kornienko JS, Smirnova IS, Pugovkina NA, Ivanova JS, Shilina MA, Grinchuk TM, et al. High doses of synthetic antioxidants induce premature senescence in cultivated mesenchymal stem cells. Scientific Reports. 2019;9(1).
30. Lan T, Luo M, Wei X. Mesenchymal stem/stromal cells in cancer therapy. Vol. 14, Journal of Hematology and Oncology. 2021.
31. Li B, Ouchi T, Cao Y, Zhao Z, Men Y. Dental-Derived Mesenchymal Stem Cells: State of the Art. Vol. 9, Frontiers in Cell and Developmental Biology. 2021.
32. Liehr T. “Classical cytogenetics” is not equal to “banding cytogenetics.” Molecular Cytogenetics. 2017;10(1).
33. Luzak B, Siarkiewicz P, Boncler M. An evaluation of a new high-sensitivity PrestoBlue assay for measuring cell viability and drug cytotoxicity using EA.hy926 endothelial cells. Toxicology in Vitro. 2022;83.

34. Lv FJ, Tuan RS, Cheung KMC, Leung VYL. Concise review: The surface markers and identity of human mesenchymal stem cells. Vol. 32, Stem Cells. 2014.
35. Macrin D, Alghadeer A, Zhao YT, Miklas JW, Hussein AM, Detraux D, et al. Metabolism as an early predictor of DPSCs aging. Scientific Reports. 2019;9(1).
36. Magrone T, Magrone M, Russo MA, Jirillo E. Recent advances on the anti-inflammatory and antioxidant properties of red grape polyphenols: In vitro and in vivo studies. Vol. 9, Antioxidants. 2020.
37. Malaguarnera L. Influence of resveratrol on the immune response. Vol. 11, Nutrients. 2019.
38. Marchionni C, Bonsi L, Alviano F, Lanzoni G, di Tullio A, Costa R, et al. Angiogenic potential of human Dental Pulp Stromal (STEM) Cells. International Journal of Immunopathology and Pharmacology. 2009;22(3).
39. Martins ICF, Raposo NRB, Mockdeci HR, Polonini HC, de Oliveira Ferreira A, Fabri GMC, et al. Delivering Resveratrol on the Buccal Mucosa Using Mucoadhesive Tablets: A Potential Treatment Strategy for Inflammatory Oral Lesions. Current Drug Delivery. 2017;15(2).
40. Matsuno Y, Atsumi Y, Alauddin M, Rana MM, Fujimori H, Hyodo M, et al. Resveratrol and its Related Polyphenols Contribute to the Maintenance of Genome Stability. Scientific Reports. 2020;10(1).
41. Mcgowan-Jordan J, Hastings RJ, Moore S, Standing I, S. Karger Gmbh. ISCN 2020 an International System for Human Cytogenomic Nomenclature (2020): recommendations of the International Standing Committee on Human Cytogenomic Nomenclature including revised sequence-based cytogenomic nomenclature developed in collaboration with the Human Genome Variation Society (HGVS) Sequence Variant Description Working Group.
42. Miyashiro CA, Bernegossi J, Bonifácio B v., de Toledo LG, Ramos MAS, Bauab TM, et al. Development and characterization of a novel liquid crystalline system containing sodium alginate for incorporation of trans-resveratrol intended for treatment of buccal candidiasis. Pharmazie. 2020;75(5).

43. Neri S. Genetic Stability of Mesenchymal Stromal Cells for Regenerative Medicine Applications: A Fundamental Biosafety Aspect. Vol. 20, International journal of molecular sciences. 2019.
44. Olivares-Marin IK, González-Hernández JC, Madrigal-Perez LA. Resveratrol cytotoxicity is energy-dependent. Vol. 43, Journal of Food Biochemistry. 2019.
45. Paczkowska-Walendowska M, Szymańska E, Winnicka K, Szwajgier D, Baranowska-Wójcik E, Ruchała MA, et al. Cyclodextrin as functional carrier in development of mucoadhesive tablets containing polygoni cuspidati extract with potential for dental applications. Pharmaceutics. 2021;13(11).
46. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284(5411).
47. Pupo YM, Leite LMB, Senegaglia AC, Antunes L, Nadal JM, de Lara EL, et al. Effect of hydroxyapatite microspheres, amoxicillin–hydroxyapatite and collagen–hydroxyapatite composites on human dental pulp-derived mesenchymal stem cells. Materials. 2021;14(24).
48. Rao T, Tan Z, Peng J, Guo Y, Chen Y, Zhou H, et al. The pharmacogenetics of natural products: A pharmacokinetic and pharmacodynamic perspective. Vol. 146, Pharmacological Research. 2019.
49. Rebelatto CLK, Aguiar AM, Moretão MP, Senegaglia AC, Hansen P, Barchiki F, et al. Dissimilar differentiation of mesenchymal stem cells from bone marrow, umbilical cord blood, and adipose tissue. Experimental Biology and Medicine. 2008;233(7).
50. Rebelatto CLK, Boldrini-Leite LM, Daga DR, Marsaro DB, Vaz IM, Jamur VR, et al. Quality Control Optimization for Minimizing Security Risks Associated with Mesenchymal Stromal Cell-Based Product Development. International Journal of Molecular Sciences. 2023;24(16).
51. Saqib U, Kelley TT, Panguluri SK, Liu D, Savai R, Baig MS, et al. Polypharmacology or promiscuity? Structural interactions of resveratrol with its bandwagon of targets. Frontiers in Pharmacology. 2018;9(OCT).
52. Sato Y, Bando H, di Piazza M, Gowing G, Herberts C, Jackman S, et al. Tumorigenicity assessment of cell therapy products: The need for global consensus and points to consider. Vol. 21, Cytotherapy. 2019.

53. Seifrtová M, Havelek R, Ćmielová J, Jiroutová A, Soukup T, Brůčková L, et al. The response of human ectomesenchymal dental pulp stem cells to cisplatin treatment. International Endodontic Journal. 2012;45(5).
54. Shevelev AB, la Porta N, Isakova EP, Martens S, Biryukova YK, Belous AS, et al. In vivo antimicrobial and wound-healing activity of resveratrol, dihydroquercetin, and dihydromyricetin against Staphylococcus aureus, Pseudomonas aeruginosa, and Candida albicans. Pathogens. 2020;9(4).
55. Silva M, de Leeuw N, Mann K, Schuring-Blom H, Morgan S, Giardino D, et al. European guidelines for constitutional cytogenomic analysis. Vol. 27, European Journal of Human Genetics. 2019.
56. Silva MC, Haggarty SJ. Human pluripotent stem cell–derived models and drug screening in CNS precision medicine. Vol. 1471, Annals of the New York Academy of Sciences. 2020.
57. Staniowski T, Zawadzka-Knefel A, Skośkiewicz-Malinowska K. Therapeutic potential of dental pulp stem cells according to different transplant types. Vol. 26, Molecules. 2021.
58. Subhan I, Siddique YH. Resveratrol: Protective Agent Against Alzheimer's Disease. Central Nervous System Agents in Medicinal Chemistry. 2024;24(3).
59. Suchanek J, Soukup T, Visek B, Ivancakova R, Kucerova L, Mokryb J. Dental pulp stem cells and their characterization. Biomedical Papers. 2009;153(1).
60. Sun Z, Guo X, Li C, Ling J, Chang A, Zhao H, et al. Exploring the therapeutic mechanisms of resveratrol for treating arecoline-induced malignant transformation in oral epithelial cells: insights into hub targets. Journal of the Science of Food and Agriculture. 2024 Oct 1;
61. Timmers S, Konings E, Bilet L, Houtkooper RH, van de Weijer T, Goossens GH, et al. Calorie restriction-like effects of 30 days of resveratrol supplementation on energy metabolism and metabolic profile in obese humans. Cell Metabolism. 2011;14(5).
62. Tomé-Carneiro J, Gonzálvez M, Larrosa M, Yáñez-Gascón MJ, García-Almagro FJ, Ruiz-Ros JA, et al. One-year consumption of a grape nutraceutical containing resveratrol improves the inflammatory and fibrinolytic status of patients in primary prevention of cardiovascular disease. American Journal of Cardiology. 2012;110(3).

63. Torre ML, Lucarelli E, Guidi S, Ferrari M, Alessandri G, de Girolamo L, et al. Ex vivo expanded mesenchymal stromal cell minimal quality requirements for clinical application. Vol. 24, Stem Cells and Development. 2015.
64. Tou JC. Resveratrol supplementation affects bone acquisition and osteoporosis: Pre-clinical evidence toward translational diet therapy. Vol. 1852, Biochimica et Biophysica Acta - Molecular Basis of Disease. 2014.
65. U.S. Food and Drug Administration. Chemistry, manufacturing, and control (CMC) information for human gene therapy investigational new drug applications: guidance for industry. Silver Spring, MD: U.S. Food and Drug Administration; 2020.
66. Vaz IM, Borgonovo T, Kasai-Brunswick TH, dos Santos DS, Mesquita FCP, Vasques JF, et al. Chromosomal aberrations after induced pluripotent stem cells reprogramming. Genetics and Molecular Biology. 2021;44(3).
67. World Health Organization. World Health Organization Technical Report Series No. 987 Annex 3. 2013. In: Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell bank; 2013.
68. Yang G, Zhang Z, Liu K, Ji X, Fatehi P, Chen J. A cellulose nanofibril-reinforced hydrogel with robust mechanical, self-healing, pH-responsive and antibacterial characteristics for wound dressing applications. Journal of Nanobiotechnology. 2022;20(1).
69. Yang HW, Chun-Yu Ho D, Liao HY, Liao YW, Fang CY, Ng MY, et al. Resveratrol inhibits arecoline-induced fibrotic properties of buccal mucosal fibroblasts via miR-200a activation. Journal of Dental Sciences. 2024;19(2).
70. You JB, Choi AY, Baek J, Oh MS, Im SG, Lee KE, et al. Application of Monodirectional Janus Patch to Oromucosal Delivery System. Advanced Healthcare Materials. 2015;4(15).
71. Zhang J, Li R, Man K, Yang XB. Enhancing osteogenic potential of hDPSCs by resveratrol through reducing oxidative stress via the Sirt1/Nrf2 pathway. Pharmaceutical Biology. 2022;60(1).

ARTIGO 1 - VERSÃO EM INGLÊS

Title page

Title: Evaluation of the effect of different concentrations of resveratrol on human dental pulp stem cells: study of cytotoxicity and genetic stability

Authors: Bruno Eduardo Sant'Anna Falce de Macedo, Patrícia Tolentino da Rosa de Souza, Luciana Reis Azevedo Alanis

Pontifícia Universidade Católica do Paraná, School of Medicine and Life Sciences, Graduate Program in Dentistry

Abstract

Objective: To evaluate the genetic stability of human dental pulp stem cells (DPSCs) and quantify the cytotoxicity of resveratrol at various concentrations. Materials and methods: Dental pulp tissue was obtained from three patients after third molar extraction. The cells were isolated and cultured with different concentrations of resveratrol. Cell viability was measured by flow cytometry after 24 and 96 h in the control group (without resveratrol) and the test groups (with resveratrol at concentrations of 12.5 µM, 25 µM, 50 µM, and 75 µM). G-banding was used to determine genetic stability. Nonparametric Kruskal–Wallis and Dunn's multiple comparison tests were used to compare cell viability between groups. *Results*: DPSC samples were characterized according to the adopted criteria. After 96 h of cultivation, resveratrol at concentrations of 50 µM and 75 µM was found to be cytotoxic (<70% cell viability) for the evaluated samples. Cell viability at a concentration of 50 µM was significantly lower than that at 12.5 µM ($p=0.045$) and the control group ($p=0.018$). Resveratrol at 75 µM concentration showed significantly lower cell viability compared with the control group ($p=0.022$). No significant differences ($p>0.05$) were observed in cell viability of the groups evaluated at different time points of 24 h and 96 h. No clonal changes were observed in the control group at concentrations of 12.5 µM and 25 µM. *Conclusion*: Resveratrol showed cytotoxicity in DPSCs at concentrations of 50 µM and 75 µM; however, DPSCs cultured with resveratrol at concentrations of 12.5 µM and 25 µM showed high cell viability and genetic stability and are promising for *in vivo* studies evaluating the therapeutic use of resveratrol.

Key-words: trans Resveratrol, Dental Pulp, Pluripotent Stem Cells, Mesenchymal Stem Cells, Karyotyping.

Introduction

Resveratrol is a natural polyphenol obtained from foods such as red wine, nuts, peanuts, cocoa, fruits, and herbs (TOU., 2014). It has been widely used in the management of chronic inflammatory diseases (TIMMERS et al., 2011; TOMÉ-CARNEIRO et al., 2012; MALAGUARNERA, 2019; HUANG et al., 2019; FANTACUZZI et al., 2022; SUBHAN & SIDDIQUE., 2024). Resveratrol can regulate the inflammatory response through different signaling pathways such as arachidonic acid metabolism (MAGRONE et al., 2019). Furthermore, resveratrol activates sirtuin 1 (SIRT1), a deacetylase involved in several molecular events, which inhibits the production of inflammatory factors (SAQIB et al., 2018; MALAGUARNERA, 2019). However, the use of resveratrol as an anti-inflammatory agent or facilitator in the repair process through topical application either skin (CHAN., 2002; SHEVELEV et al., 2020; HECKER et al., 2021; YANG et al., 2022; JIA et al., 2022) or mucosa (MARTINS et al., 2018; PACZKOWSKA-WALENDOWSKA et al., 2021), as well as its action against potentially malignant lesions and squamous cell carcinoma (JAYARAMAN et al., 2022; YANG et al., 2023) have still been the subject of discussion.

Before marketing cosmetics or medicines containing resveratrol, it is necessary to investigate their potential to cause cell damage, either through toxicity or by triggering oncogenesis (ICH, 2012; WHO, 2013). *In vitro* toxicological evaluation of substances using human cells, an emerging approach, has replaced the use of animals, and therefore has demonstrated wide acceptance (FRACARO et al., 2023). During cell cultivation, the accumulation of genetic and epigenetic mutations and the occurrence of cellular senescence, which impair cell biology and therapeutic properties, are risk factors for cell transformation (KIM et al., 2015; NERI., 2019). It is necessary to identify the therapeutic responses to natural medicines and their bioactive compounds to ensure their therapeutic efficacy and safety (RAO et al., 2019).

Mesenchymal stromal cells (MSCs) are non-hematopoietic pluripotent stem cells derived from the mesoderm and have self-renewal capacity (BARYAWNO et al., 2019; PITTENGER et al., 1999). MSCs are isolated from various sources such as dental tissues, including dental pulp, periodontal ligament, dental follicle, apical papilla, gingiva, tooth germ, exfoliated deciduous

teeth, and alveolar bone (GRONTOS et al., 2000; DAVE, TOMAR., 2018; LI et al., 2021). Resveratrol can optimize the therapeutic effects of cultured MSCs to ensure their survival, self-renewal, lineage commitment, and anti-aging effects (HU & LI, 2019).

Cytogenetic tests should be performed to ensure that no cell transformation occurs during culture. Karyotyping using G-banding is the gold standard for detecting genetic stability (LIEHR, 2017; ISSCR, 2008). The tumorigenic potential of cultured MSCs is a critical issue that is strictly correlated with genomic instability (NERI, 2019). MSCs have lower tumorigenic potential than that of induced pluripotent stem cells and embryonic stem cells (JUNG, BAUER, NOLTA., 2012).

Cell viability assays allow the evaluation of the potency or toxicity of DRUGS, the effects on cell proliferation and direct cytotoxic effects, in addition to being an important tool for medicine research and development (LUZAK, SIARKIEWICZ, BONCLER., 2022; ADAN, KIRAZ, BARAN., 2016). Generally, non-clinical data on toxicity are necessary to support clinical trials; depending on the intended use, additional studies on cytotoxicity may be necessary, such as genotoxicity, tumorigenicity, reproductive and developmental toxicity, and immunotoxicity studies (EMA, 2024). Toxicity studies should be designed to generate clinically meaningful and relevant data that support the safe use of the product in the intended clinical indication, such as estimation of a safe initial dose and dosing regimen (EMA, 2024).

Given the limitations of investigations that evaluated the cytotoxicity of resveratrol in the culture of dental pulp stem cells (DPSCs) and the genetic stability of cultured DPSCs, the present study aimed to quantify the cytotoxicity of different concentrations of resveratrol through cell viability by flow cytometry and to evaluate the genetic stability of cultured DPSCs through the G-banding karyotyping technique.

Material and Methods

This research project was approved by the Research Ethics Committee of Pontifícia Universidade Católica do Paraná (PUCPR), with opinion number 38458920.4.0000.0020).

Three female patients, aged between 20 and 23 years and treated at the PUCPR Dentistry Clinic, provided their extracted third molars for the present study after signing a Free and Informed Consent Form (FICF).

Healthy erupted or impacted third molars with indications for extraction were used in this study. The impacted third molars were extracted without odontosection. Cell culture was performed from the pulp tissue of three third molars, each tooth coming from a patient, of which two teeth were upper right molars and one tooth upper left molar.

Before tooth extraction, patients rinsed their mouths with 0.12% chlorhexidine digluconate to remove possible contaminants (PUPO et al., 2021). Immediately after surgery, the teeth were cleaned with saline solution in a sterile field and sectioned transversely along the long axis at the cemento-enamel junction (GRONTHOS et al., 2000) using a sterile Zekrya drill. During odontosection, the teeth were irrigated with saline solution and then placed in a collection medium, Iscove's Modified Dulbecco Medium (IMDM) (Gibco, Thermo Fisher Scientific, Grand Island, NY), supplemented with 20% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Grand Island, NY), penicillin (100 units/mL; Sigma-Aldrich, Saint Louis, MO), streptomycin (100 µg/mL; Sigma-Aldrich, Saint Louis, MO), fluconazole (2 mg/mL; Sanobiol, Pouso Alegre, MG), and sodium heparin (5,000 IU/mL; Cristália, Pouso Alegre, MG), and taken to the Cell Technology Center of PUCPR.

Isolation and culture of dental pulp tissue cells

The pulp tissue was removed with a type H endodontic file, mechanically dissociated with a scalpel blade in a petri dish and subjected to enzymatic digestion by 4 mg/mL collagenase type II (Gibco, Thermo Fisher Scientific, Grand Island, NY) for 1 h at 37 °C under continuous agitation (CORRÊA et al., 2018). The sample was then filtered through 100-µm cell filters to obtain a cell

suspension and centrifuged at 400 × *g* for 10 min. The cell suspension was seeded in 25 cm^2 flasks and cultured in IMDM supplemented with 20% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin (CORRÊA et al., 2018).

The cultures were maintained in an incubator at 37 °C in a 5% CO_2 atmosphere, and the medium changed every three days. After achieving confluence (80%), the cells were dissociated by trypsin/0.25% EDTA (Gibco, Thermo Fisher Scientific, Grand Island, NY) and reseeded in 75 cm^2 flasks (CORRÊA et al., 2018).

Cellular characterization by immunophenotyping

For cell characterization by immunophenotyping, daily monitoring of the sample and analysis of cell morphology and adhesion to plastic were performed, in addition to differentiation into adipocytes, chondroblasts, and osteoblasts, according to the ISCT guidelines (DOMINICI et al., 2006). Characterization was conducted after the cells reached P3.

The labeling step consisted of washing the cells with PBS (Gibco, Thermo Fisher Scientific, Grand Island, NY) and incubation in the dark for 30 min (REBELATTO et al., 2008; REBELATTO et al., 2023) with antibodies anti CD29-APC (1:20), CD14-FITC (1:20), CD45-FITC (1:20), CD19-FITC (1:20), CD34-APC (1:20), CD105-APC (1:20), CD73-APC (1:33), and CD90-PE (1:100), (1:20) (Becton Dickinson, San Diego, CA, USA). The cells were then washed with a buffer solution and resuspended in 500 µL of 1% paraformaldehyde solution (Sigma-Aldrich, MO, USA) (PUPO et al., 2021). Mouse IgG1 isotypic antibodies were used as controls (FRACARO et al., 2023).

Approximately 100,000 labeled cells were acquired using FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA) and analyzed using the FlowJo software (FlowJo, Ashland, OR, USA; version 8.1) (PUPO et al., 2021).

Cultivation of DPSCs with resveratrol

After the third passage (P3), DPSCs were placed in 75 cm^2 flasks and incubated with resveratrol (Trans-Resveratrol - Pharmaceutical Secondary Standard; Certified Reference Material, Sigma-Aldrich, Saint Louis, MO) at 37 °C

in a 5% CO_2 atmosphere for 24 and 96 h. Resveratrol was diluted with dimethyl sulfoxide to obtain concentrations of 12.5 µM, 25 µM, 50 µM, and 75 µM.

Cell viability test by flow cytometry

Cytotoxicity was investigated by measuring the cell viability by flow cytometry after 24 and 96 h in the test group (DPSCs cultured in different concentrations of resveratrol: 12.5 µM, 25 µM, 50 µM, and 75 µM) and the control group (DPSCs cultured without resveratrol).

The cells were incubated with Annexin PE (a marker of cell apoptosis) and 7-AAD (a vital dye) at room temperature for 30 min. Isotypic antibodies conjugated with the fluorochromes PE and PE-CY5 were used as controls. After incubation, the cells were washed with PBS and fixed with PBS containing 1% paraformaldehyde. The samples were acquired on FACSCalibur flow cytometer and analyzed using the FlowJo software. The tests were performed in triplicate for each group of samples.

Resveratrol concentrations were considered cytotoxic when cell viability was less than 70% (FDA, 2020).

Cytogenetic evaluation through G-banding

The test group was evaluated at concentrations of 12.5 µM and 25 µM of resveratrol, and compared with the control group.

G-banding was used to detect genetic stability and performed after cultivation (P3). Upon reaching 80% confluence, the cell culture was supplemented with 0.1 µg/mL of colchicine, and the flasks were then incubated at 37 °C for 2–6 h. Changes in cell morphology were monitored using an inverted microscope (BORGONOVO et al., 2014).

DPSCs were dissociated from the vial using 2 mL of heated 0.25% trypsin-EDTA. After 3 min of monitoring, two drops of FBS were added, and the cells were transferred to a centrifuge tube containing the medium. The samples were centrifuged at 400 × *g* for 10 min (BORGONOVO et al., 2014).

For hypotonic treatment, 5 mL of 0.075 M KCl with HEPES was slowly and carefully added, followed by incubation at 37 °C for 30 min and fixation with

methanol: acetic acid (3:1) solution. To improve visualization of metaphases, the samples were washed twice in cold (5 °C) methanol: fresh acetic acid (2:1) solution. Prior to slide preparation, clean slides were steamed, while cells were resuspended in fresh fixative solution and dripped onto the slide (BORGONOVO et al., 2014).

To obtain G bands, the slides were subjected to 60 °C for at least 16 h. They were then immersed in trypsin solution (0.002 g/mL) for 5 s, washed with saline solution, and immediately rinsed with distilled water. Staining was performed using Giemsa (1:20) or Wright's solution (1:6) to produce trypsin and Giemsa (GTG) or trypsin and Wright (GTW) bands, respectively. The quality of the chromosomal bands was evaluated under a microscope at 1000× magnification, and trypsin activity and staining times were adjusted to produce clear and well-stained bands (BORGONOVO et al., 2014).

In each DPSC sample, 20 metaphases were observed under an optical microscope, recorded, and interpreted with the aid of a karyotyping system (MCGOWAN-JORDAN et al., 2020).

Statistical analysis

The mean, median, and standard deviation were calculated for the cell viability measurement of DPSC samples (n=3) for each resveratrol concentration evaluated in the test and control groups. The nonparametric Kruskal–Wallis test for independent samples was used to compare cell viability between the control group and the four test group concentrations at 24 and 96 h. Dunn's multiple comparisons test was used for two-by-two comparisons between resveratrol concentrations, with significant differences in cell viability (96-h time point). The nonparametric Wilcoxon test for paired samples was used to compare the cell viability between the two time points (24 and 96 h) within each group. The power of test for variable cell viability at 96 h according to the groups was 0.938, indicating high reliability of the results. For all tests, a significance level of 5% was adopted ($p < 0.05$).

Results

Cell characterization by immunophenotyping

Cellular characterization of DPSC samples from the three patients was performed according to the criteria suggested by the ISCT (Table 1).

Table 1: Cellular characterization of DPSC samples obtained from the third molars of three patients.

Patient	CD14	CD34	CD45	HLA-DR	CD19	CD73	CD90	CD105	CD29
I	1.60%	0.37%	0.22%	1.50%	0.27%	98.40%	99.00%	98.30%	98.20%
II	1.15%	0.25%	0.23%	1.78%	0.17%	98.70%	98.90%	98.20%	99.20%
III	0.62%	0.08%	0.44%	0.38%	0.19%	99.70%	99.70%	99.50%	99.00%

Cell viability test by flow cytometry

Tables 2 and 3 show the results of the cell viability test of DPSC culture using flow cytometry in the test and control groups after 24 and 96 h, respectively. The viability of DPSC culture of sample III after 24 h was 66.20%, indicating cytotoxicity (Table 2). After 96 h of culture, resveratrol at concentrations of 50 µM and 75 µM showed cytotoxicity against the three samples evaluated (Table 3).

Table 2: Cell viability of DPSCs by flow cytometry after 24 h in the test groups (at resveratrol concentrations of 12.5 μM, 25 μM, 50 μM, and 75 μM) and the control group (without resveratrol).

Patient	Time	Variables	Control	12.5 μM	25 μM	50 μM	75 μM
		Annexin (%)	0.36	0.29	0.43	0.29	0.39
		7-AAD (%)	5.23	14.10	5.08	9.88	20.20
I	24 h	Cell viability (%)	94.77	85.90	94.92	90.12	79.80
		Annexin (%)	0.39	0.27	0.20	0.09	0.14
		7-AAD (%)	4.92	5.86	7.15	9.48	12.40
II	24 h	Cell viability (%)	95.08	94.14	92.85	90.52	87.60
		Annexin (%)	0.89	1.27	0.98	0.60	0.72
		7-AAD (%)	8.71	12.50	7.91	21.20	33.80
III	24 h	Cell viability (%)	91.29	87.50	92.09	78.80	66.20

Table 3: Cell viability of DPSCs by flow cytometry after 96 h in the test groups (at resveratrol concentrations of 12.5 μM, 25 μM, 50 μM, and 75 μM) and the control group (without resveratrol).

Patient	**Time**	**Variables**	**Control**	**12.5 μM**	**25 μM**	**50 μM**	**75 μM**
		Annexin (%)	0.18	0.35	0.28	0.18	0.47
		7-AAD (%)	6.22	6.34	6.67	37.00	45.20
I	96 h	Cell viability (%)	93.78	93.66	93.33	63.00	54.80
		Annexin (%)	0.35	0.61	0.39	0.48	0.42
		7-AAD (%)	2.71	5.17	6.84	58.40	83.40
II	96 h	Cell viability (%)	97.29	94.83	93.16	41.60	16.60
		Annexin (%)	0.88	0.94	0.37	1.07	0.31
		7-AAD (%)	9.69	8.55	5.57	56.10	28.70
III	96 h	Cell viability (%)	93.31	91.45	94.43	43.90	71.30

Table 4 shows mean values of percentages, standard deviations and p-values of cell viability at 24 and 96 h in the test and control groups.

Table 4: Mean and standard deviation of cell viability (%) of DPSC culture samples evaluated by flow cytometry after 24 and 96 h in the test groups (resveratrol concentrations of 12.5 µM, 25 µM, 50 µM, and 75 µM) and the control group (without resveratrol).

Cultivation time	**Control X(%)±SD**	**12,5 µM X(%)±SD**	**25 µM X(%)±SD**	**50 µM X(%)±SD**	**75 µM X(%)±SD**	**p Value**
24 hours	93.713±2.104^{A}	89.180±4.369^{A}	93.287±1.465^{A}	86.480 ± 6.654^{A}	77.867±10.830^{A}	0.595
96 hours	94.793±2.175^{a,A}	93.313±1.716^{a,c,A}	93.640 ± 0.689^{b,A}	49.500 ± 11.748^{b,A}	47.567±28.058^{b,c,A}	0.035*

Same superscript capital letters in columns indicate no significant differences between groups (p>0.05). Wilcoxon test.

* Different superscript lowercase letters in rows indicate significant differences between groups (p<0.05). Same superscript lowercase letters in rows indicate no significant differences between groups (p>0.05). Kruskal–Wallis test and Dunn's test.

There were no significant differences in the cell viability of the DPSC cultures evaluated by flow cytometry after 24 h between the control group and test groups (p=0.595). Significant differences were observed between the control and test groups at 96 h (p=0.035). Cell viability at 50 µM resveratrol was significantly lower than that at 12.5 µM (p=0.045) and compared with the control group (p=0.018). Cell viability at 75 µM resveratrol was significantly lower than that of the control group (p=0.022) (Table 4).

When comparing the cell viability in the groups between the two time points (24 and 96 h), there were no statistically significant differences (p>0.05, Table 4).

The median values of cell viability of the groups at time points of 24 and 96 h are shown in Figures 1 and 2, respectively.

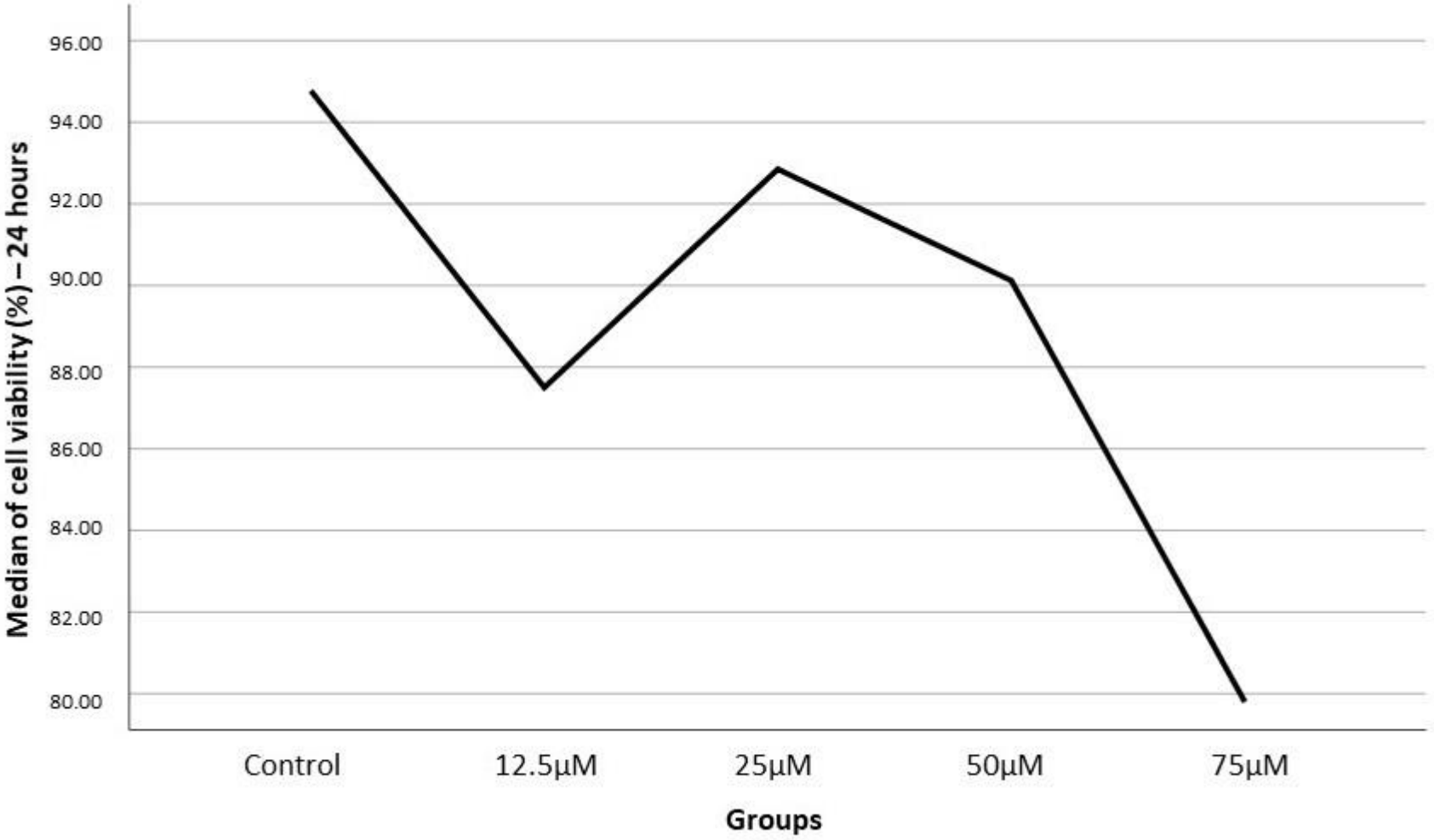

Figure 1: Median cell viability values in the control group and in different concentrations of resveratrol in 24 hours.

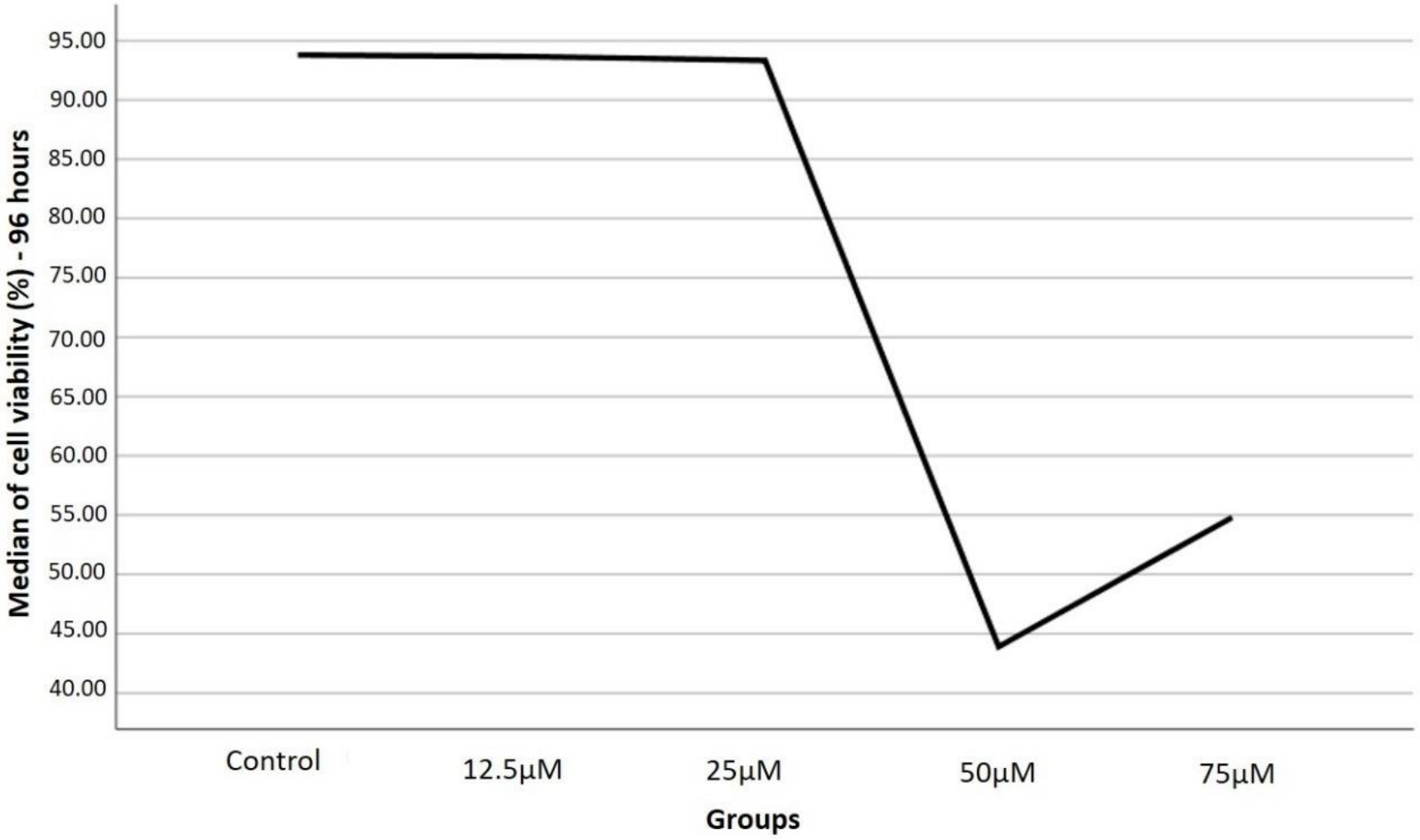

Figure 2: Median cell viability values in the control group and in different concentrations of resveratrol in 96 hours.

Cytogenetic evaluation through G-banding

After 96 hours, no clonal alterations were found in the control group and at concentrations of 12.5 µM and 25 µM resveratrol. At 50 µM and 75 µM resveratrol, G-banding was not possible (Table 5).

Table 5: Karyotypes of DPSCs cultured without resveratrol (control) and with resveratrol at concentrations of 12.5 µM and 25 µM after 96 h.

Patient	Control	12.5 µM	25 µM
I	46,XX[20]	46,XX[20]	46,XX[20]
II	46,XX[20]	46,XX[20]	46,XX[20]
III	46,XX[20]	46,XX[20]	46,XX[20]

The number 46 indicates the number of chromosomes in each cell, XX indicates sex chromosomes, and [20] is the number of metaphases analyzed.

Figure 3 shows karyograms of DPSC samples.

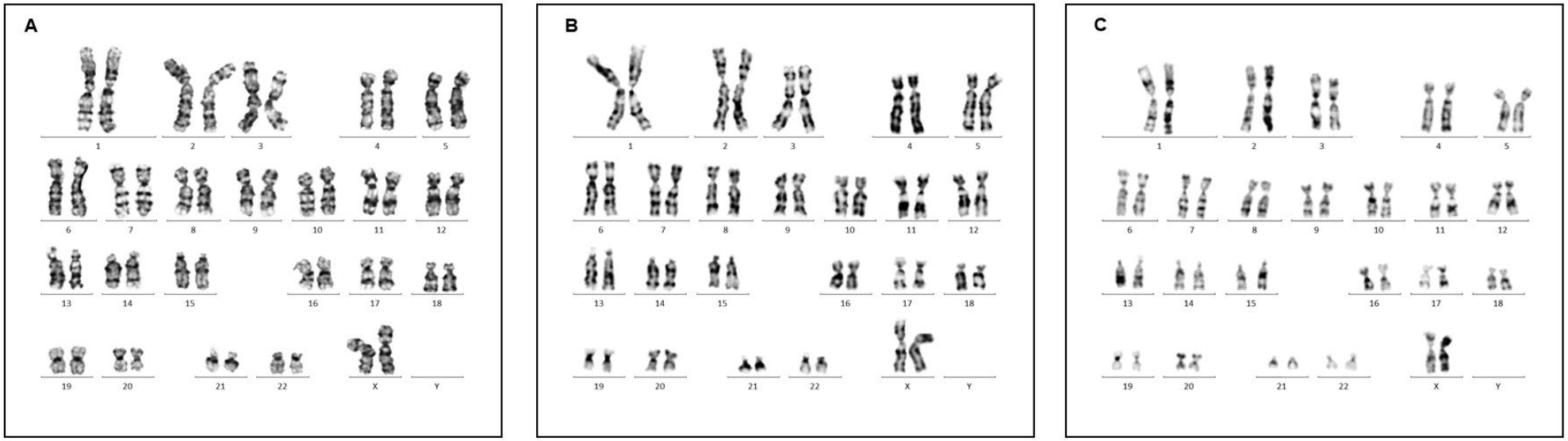

Figure 3: Karyograms of DPSCs samples from patient II. A) cultured without resveratrol (control group), B) cultured at a concentration of 12.5 µM resveratrol, C) cultured at a concentration of 25 µM resveratrol.

Discussion

Considering the limited number of studies that evaluated the genetic stability of DPSCs and the indication of these cells for the study of promising properties of resveratrol as an anti-inflammatory agent and/or facilitator in the repair process, the results of the present study showed cytotoxicity in DPSCs cultured with resveratrol at concentrations of 50 µM and 75 µM after 96 h. In addition, DPSCs cultured with resveratrol at concentrations of 12.5 µM and 25 µM presented high cell viability and genetic stability after 96 h of culture.

The cytotoxicity parameters adopted in this study were based on the criteria suggested by the FDA, according to which the minimum acceptable cell viability is 70%. Thus, concentrations of 50 and 75 µM of resveratrol were cytotoxic to DPSCs after 96 h of culturing. Similar results were found in a study that evaluated the process of DPSC differentiation to neuronal; resveratrol at concentrations of 1, 5, 10, 15, 20, 25, and 50 µM was not cytotoxic during DPSC cultivation, while the concentration of 100 µM was cytotoxic. Furthermore, the concentration of 15 µM for 12 h was considered optimal for the differentiation process (GENG et al., 2017). In another study, the concentration of 10 µM showed significant increase in cell viability and reduction in oxidative stress in DPSCs (ZHANG et al., 2022). In endometrial MSCs cultured in concentrations of 10, 20, 40, and 60 µM of resveratrol, the antioxidant effect was evaluated; a reduction in reactive oxygen species levels was observed at lower concentrations, whereas DNA damage and induction of premature senescence were observed at 40 and 60 µM resveratrol (KORNIENKO et al., 2019). Resveratrol can interact specifically with mitochondrial membranes, depriving cells of adenosine triphosphate (ATP) and increasing levels of reactive oxygen species. Depending on the degree of ATP deprivation caused by resveratrol, the cells may undergo apoptosis or necrosis. These mechanisms appear to be the molecular basis of resveratrol toxicity (OLIVEIRA et al., 2016; OLIVARES-MARIN et al., 2019).

Depending on the concentration of resveratrol, its action is either antioxidant at the physiological level or pro-oxidative cytotoxic, which causes mild oxidative stress and activates antioxidant system as a cellular defense mechanism. Furthermore, the energy state of the cell also influences the cytotoxic effect of resveratrol; by altering the energy state of the cell, it is possible to enhance the

cytotoxic effect of resveratrol. It has also been suggested that the antioxidant effect of resveratrol can be attributed to the adaptive phenotype of cells capable of neutralizing its pro-oxidant effect (OLIVARES-MARIN et al., 2019).

In the present study, no clonal alterations were detected in DPSCs cultured with resveratrol at concentrations of 12.5 µM and 25 µM and without resveratrol. In proposing possible guidelines with minimum quality criteria for the clinical use of cultured MSCs, karyotype analysis should be performed to demonstrate that genomic stability is maintained during expansion (TORRE et al., 2015). Karyotype analysis allows genotoxicity evaluation of chemicals (SATO et al., 2019). Possible clonal alterations in stem cells include aneuploidy, mutations in oncogenes, tumor suppressor genes, and epigenetic mutations (ISSCR, 2008).

Dental pulp stands out as a tissue with remarkable resistance to oncogenesis, although healthy DPSCs share characteristics in common with cancer stem cell phenotypes identified in malignant neoplasms (OLATZ et al., 2020). The high expression of tumor suppressor genes and their ability to interrupt cell proliferation in response to DNA damage (OLATZ et al., 2020; BADIOLA et al., 2015) may partially explain the resistance to oncogenesis in DPSCs. In a study on cultured mouse embryonic fibroblasts, the use of resveratrol contributed to the maintenance of genomic stability, and the cells were protected against mutations in the ARF/p53 pathway (MATSUNO et al., 2020).

G-banding allows the detection of numerical and structural anomalies, such as translocations, inversions, and low levels of mosaicism (VAZ et al., 2021; BEN-DAVID & BENVENISTY, 2012). The detection threshold of this technique is >5% (BEN-DAVID & BENVENISTY, 2012). A limitation of G-banding is its inability to detect small alterations, which may require the use of complementary methods (VAZ et al., 2021), such as fluorescence in situ hybridization (FISH), which can be applied to both interphase and metaphase (SILVA et al., 2018; GRANADA et al., 2020). Chromosome banding analysis remains the most comprehensive method for evaluating chromosomal abnormalities (GRANADA et al., 2020).

In this study, concentrations of 12.5 µM and 25 µM resveratrol did not show cytotoxicity to DPSCs and maintained their genetic stability, which suggests that resveratrol is safe for DPSCs from an oncogenic point of view. These results are

promising for future studies evaluating the anti-inflammatory and repair processes facilitated by resveratrol, mainly in tissues of the stomatognathic system.

The action of resveratrol in the prevention and healing of inflammatory lesions in the oral mucosa was evaluated using mucoadhesives, which appear to be an alternative (MARTINS et al., 2018). A study on the malignant transformation of oral epithelial cells demonstrated that resveratrol can significantly promote apoptosis in cancer cells (SUN et al., 2024). Resveratrol supplementation inhibits myofibroblast activity and suppresses the expression of fibrogenic genes in patients with oral submucosal fibrosis, a potentially malignant lesion (CHANG et al., 2016).

Both the rapid metabolism and excretion of resveratrol are the main factors that imply its clinical use, and improvement in bioavailability has been investigated (HECKER et al., 2021). When analyzing the metabolism of resveratrol isomers in a cellular model, resveratrol dihydrate showed better absorption (99%) than that of trans- or cis-resveratrol (20% and 62%, respectively) (JAROSOVA et al., 2020).

The application of drugs to the mucosa has received great attention in the field of research, as it presents advantages such as precise dose control and prevention of rapid metabolism through oral administration. Despite these advantages, one difficulty with this route of administration is the presence of saliva, which can dissolve the drug and release it via unwanted routes (YOU et al., 2015). A study evaluating the antifungal properties of resveratrol against oral candidiasis demonstrated that the incorporation of sodium alginate into a liquid crystalline system significantly increased mucoadhesion and improved the permanence of resveratrol in the oral cavity (MIYASHIRO et al., 2020).

In this study, cell characterization of DPSCs was performed and evaluated in accordance with the parameters suggested by the ISCT. The results obtained for CD 29 (98.2%, 99.2%, and 99%) were similar to the average values reported in a previous study (98.8 %) (SUCHANEK et al., 2009). The surface marker CD 29, from the β1 integrin family (STANIOWSKI et al., 2021), is commonly used in MSCs and, when evaluated in DPSCs, presents a positive profile (SUCHANEK et al., 2009; MARCHIONNI et al., 2009). In DPSCs, the presence of CD 29 characterizes odonto-osteogenic properties (SEIFRTOVÁ et al., 2012).

Surface markers allow the distinction between stem cell types; they are specific proteins that selectively bind to or adhere to another molecule (STANIOWSKI et al., 2021). Stem cells from different tissues have slightly different surface markers (LAN et al., 2021). However, there are no specific markers for DPSCs (MACRIN et al., 2019), as there is no consensus on the only surface marker capable of identifying MSCs from different tissues (LAN et al., 2021; JUAN et al., 2014). The ISCT (DOMINICI et al., 2006) provides parameters for the characterization of MSCs in different organs and tissues. Establishing guidelines with specific parameters for the cellular characterization of DPSCs would allow researchers to make accurate, non-subjective comparisons. Establishing a standard guarantees that the cultured cells belong to the cell line in question and exhibit the expected properties and characteristics.

The potential limitations of the study are the absence of other concentrations of resveratrol (>12.5 µM, different from 25 µM, and <50 µM), and the non execution of different techniques to quantification of cytotoxicity and the absence of realization of a functional assay. Furthermore, it was not possible to perform G-banding at concentrations of 50 µM and 75 µM, due to the cytotoxicity there was generalized apoptosis, which consequently, compromised the quality of the material to be analyzed, since there was not the minimum number of 20 metaphases available to be recorded and interpreted. Human *ex vivo* systems have potential in the field of translational pharmacology because they intensify the overall medicine development process and can be an important step in determining efficacy prior to clinical trials (SILVA, HAGGARTY., 2020). The use of animal models is essential for understanding medicine administration, passage through the blood-brain barrier, pharmacokinetics, and pharmacodynamics in whole organisms (SILVA, HAGGARTY., 2020).

Conclusion

There was cytotoxicity in DPSCs cultured at concentrations of 50 µM and 75 µM of resveratrol after 96 hours. Resveratrol, at concentrations of 12.5 µM and 25 µM, was not cytotoxic and maintained the genetic stability of DPSCs both at 24 hours and 96 hours of culture. These concentrations proved to be safe and promising for future trials evaluating their therapeutic use *in vivo*.

References

1. Adan A, Kiraz Y, Baran Y. Cell Proliferation and Cytotoxicity Assays. Current Pharmaceutical Biotechnology. 2016;17(14).
2. Badiola I, Santaolalla F, Garcia-Gallastegui P, Ana S del R, Unda F, Ibarretxe G. Biomolecular bases of the senescence process and cancer. A new approach to oncological treatment linked to ageing. Vol. 23, Ageing Research Reviews. 2015.
3. Baryawno N, Przybylski D, Kowalczyk MS, Kfoury Y, Severe N, Gustafsson K, et al. A Cellular Taxonomy of the Bone Marrow Stroma in Homeostasis and Leukemia. Cell. 2019;177(7).
4. Ben-David U and Benvenisty N (2012) Analyzing the genomic integrity of stem cells. In: StemBook. Harvard Stem Cell Instite, Cambridge, MA.
5. Borgonovo T, Vaz IM, Senegaglia AC, Rebelatto CLK, Brofman PRS. Genetic evaluation of mesenchymal stem cells by G-banded karyotyping in a Cell Technology Center. Revista Brasileira de Hematologia e Hemoterapia. 2014;36(3).
6. Chan MMY. Antimicrobial effect of resveratrol on dermatophytes and bacterial pathogens of the skin. Biochemical Pharmacology. 2002;63(2).
7. Chang YC, Lin CW, Yu CC, Wang BY, Huang YH, Hsieh YC, et al. Resveratrol suppresses myofibroblast activity of human buccal mucosal fibroblasts through the epigenetic inhibition of ZEB1 expression. Oncotarget. 2016;7(11).
8. Corrêa NCR, Kuligovski C, Paschoal ACC, Abud APR, Rebelatto CLK, Leite LMB, et al. Human adipose-derived stem cells (ADSC) and human periodontal ligament stem cells (PDLSC) as cellular substrates of a toxicity prediction assay. Regulatory Toxicology and Pharmacology. 2018;92.
9. Crende O, García-Gallastegui P, Luzuriaga J, Badiola I, de la Hoz C, Unda F, et al. Is there such a thing as a genuine cancer stem cell marker? Perspectives from the gut, the brain and the dental pulp. Vol. 9, Biology. 2020.
10. Dave JR, Tomar GB. Dental tissue–derived mesenchymal stem cells: Applications in tissue engineering. Critical Reviews in Biomedical Engineering. 2018;46(5).
11. de Oliveira MR, Nabavi SF, Manayi A, Daglia M, Hajheydari Z, Nabavi SM. Resveratrol and the mitochondria: From triggering the intrinsic apoptotic

pathway to inducing mitochondrial biogenesis, a mechanistic view. Vol. 1860, Biochimica et Biophysica Acta - General Subjects. 2016.

12. Dominici M, le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8(4).
13. European Medicines Agency. Draft guideline on the quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials (second version). Amsterdam: European Medicines Agency; 2024.
14. Fantacuzzi M, Amoroso R, Carradori S, de Filippis B. Resveratrol-based compounds and neurodegeneration: Recent insight in multitarget therapy. Vol. 233, European Journal of Medicinal Chemistry. 2022.
15. Fracaro L, Hochuli AHD, Selenko AH, Capriglione LGA, Brofman PRS, Senegaglia AC. Mesenchymal stromal cells derived from exfoliated deciduous teeth express neuronal markers before differentiation induction. Journal of Applied Oral Science. 2023;31.
16. Geng YW, Zhang Z, Liu MY, Hu WP. Differentiation of human dental pulp stem cells into neuronal by resveratrol. Cell Biology International. 2017;41(12).
17. Granada I, Palomo L, Ruiz-Xivillé N, Mallo M, Solé F. Cytogenetics in the genomic era. 2020 [cited 2024 May 27]; Available from: https://doi.org/10.1016/j.beha.2020.101196
18. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proceedings of the National Academy of Sciences of the United States of America. 2000;97(25).
19. Hecker A, Schellnegger M, Hofmann E, Luze H, Nischwitz SP, Kamolz LP, et al. The impact of resveratrol on skin wound healing, scarring, and aging. Vol. 19, International Wound Journal. 2022.
20. Hu C, Li L. The application of resveratrol to mesenchymal stromal cell-based regenerative medicine. Vol. 10, Stem Cell Research and Therapy. 2019.
21. Huang DD, Shi G, Jiang Y, Yao C, Zhu C. A review on the potential of Resveratrol in prevention and therapy of diabetes and diabetic complications. Vol. 125, Biomedicine and Pharmacotherapy. 2020.
22. In: International Conference on Harmonization. Guideline S1: Rodent carcinogenicity studies for human pharmaceuticals; 2012.

23. International Society for Stem Cell Research (ISSCR). (2008). Guidelines for the clinical translation of stem cells.
24. Jarosova V, Vesely O, Doskocil I, Tomisova K, Marsik P, Jaimes JD, et al. Metabolism of cis-and trans-resveratrol and dihydroresveratrol in an intestinal epithelial model. Nutrients. 2020;12(3).
25. Jayaraman S, Fathima SJH, Veeraraghavan VP, Raj AT, Patil S. Resveratrol and miR-200c: insights into the prevention of oral squamous cell carcinoma. Vol. 18, Future Oncology. 2022.
26. Jia Y, Shao JH, Zhang KW, Zou ML, Teng YY, Tian F, et al. Emerging Effects of Resveratrol on Wound Healing: A Comprehensive Review. Vol. 27, Molecules. 2022.
27. Jung Y, Bauer G, Nolta JA. Concise review: Induced pluripotent stem cell-derived mesenchymal stem cells: Progress toward safe clinical products. Vol. 30, Stem Cells. 2012.
28. Kim JA, Im KO, Park SN, Kwon JS, Kim SY, Oh K, et al. Cytogenetic heterogeneity and their serial dynamic changes during acquisition of cytogenetic aberrations in cultured mesenchymal stem cells. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis. 2015;777.
29. Kornienko JS, Smirnova IS, Pugovkina NA, Ivanova JS, Shilina MA, Grinchuk TM, et al. High doses of synthetic antioxidants induce premature senescence in cultivated mesenchymal stem cells. Scientific Reports. 2019;9(1).
30. Lan T, Luo M, Wei X. Mesenchymal stem/stromal cells in cancer therapy. Vol. 14, Journal of Hematology and Oncology. 2021.
31. Li B, Ouchi T, Cao Y, Zhao Z, Men Y. Dental-Derived Mesenchymal Stem Cells: State of the Art. Vol. 9, Frontiers in Cell and Developmental Biology. 2021.
32. Liehr T. “Classical cytogenetics” is not equal to “banding cytogenetics.” Molecular Cytogenetics. 2017;10(1).
33. Luzak B, Siarkiewicz P, Boncler M. An evaluation of a new high-sensitivity PrestoBlue assay for measuring cell viability and drug cytotoxicity using EA.hy926 endothelial cells. Toxicology in Vitro. 2022;83.
34. Lv FJ, Tuan RS, Cheung KMC, Leung VYL. Concise review: The surface markers and identity of human mesenchymal stem cells. Vol. 32, Stem Cells. 2014.

35. Macrin D, Alghadeer A, Zhao YT, Miklas JW, Hussein AM, Detraux D, et al. Metabolism as an early predictor of DPSCs aging. Scientific Reports. 2019;9(1).
36. Magrone T, Magrone M, Russo MA, Jirillo E. Recent advances on the anti-inflammatory and antioxidant properties of red grape polyphenols: In vitro and in vivo studies. Vol. 9, Antioxidants. 2020.
37. Malaguarnera L. Influence of resveratrol on the immune response. Vol. 11, Nutrients. 2019.
38. Marchionni C, Bonsi L, Alviano F, Lanzoni G, di Tullio A, Costa R, et al. Angiogenic potential of human Dental Pulp Stromal (STEM) Cells. International Journal of Immunopathology and Pharmacology. 2009;22(3).
39. Martins ICF, Raposo NRB, Mockdeci HR, Polonini HC, de Oliveira Ferreira A, Fabri GMC, et al. Delivering Resveratrol on the Buccal Mucosa Using Mucoadhesive Tablets: A Potential Treatment Strategy for Inflammatory Oral Lesions. Current Drug Delivery. 2017;15(2).
40. Matsuno Y, Atsumi Y, Alauddin M, Rana MM, Fujimori H, Hyodo M, et al. Resveratrol and its Related Polyphenols Contribute to the Maintenance of Genome Stability. Scientific Reports. 2020;10(1).
41. Mcgowan-Jordan J, Hastings RJ, Moore S, Standing I, S. Karger Gmbh. ISCN 2020 an International System for Human Cytogenomic Nomenclature (2020): recommendations of the International Standing Committee on Human Cytogenomic Nomenclature including revised sequence-based cytogenomic nomenclature developed in collaboration with the Human Genome Variation Society (HGVS) Sequence Variant Description Working Group.
42. Miyashiro CA, Bernegossi J, Bonifácio B v., de Toledo LG, Ramos MAS, Bauab TM, et al. Development and characterization of a novel liquid crystalline system containing sodium alginate for incorporation of trans-resveratrol intended for treatment of buccal candidiasis. Pharmazie. 2020;75(5).
43. Neri S. Genetic Stability of Mesenchymal Stromal Cells for Regenerative Medicine Applications: A Fundamental Biosafety Aspect. Vol. 20, International journal of molecular sciences. 2019.
44. Olivares-Marin IK, González-Hernández JC, Madrigal-Perez LA. Resveratrol cytotoxicity is energy-dependent. Vol. 43, Journal of Food Biochemistry. 2019.
45. Paczkowska-Walendowska M, Szymańska E, Winnicka K, Szwajgier D, Baranowska-Wójcik E, Ruchała MA, et al. Cyclodextrin as functional carrier in

development of mucoadhesive tablets containing polygoni cuspidati extract with potential for dental applications. Pharmaceutics. 2021;13(11).

46. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284(5411).
47. Pupo YM, Leite LMB, Senegaglia AC, Antunes L, Nadal JM, de Lara EL, et al. Effect of hydroxyapatite microspheres, amoxicillin–hydroxyapatite and collagen–hydroxyapatite composites on human dental pulp-derived mesenchymal stem cells. Materials. 2021;14(24).
48. Rao T, Tan Z, Peng J, Guo Y, Chen Y, Zhou H, et al. The pharmacogenetics of natural products: A pharmacokinetic and pharmacodynamic perspective. Vol. 146, Pharmacological Research. 2019.
49. Rebelatto CLK, Aguiar AM, Moretão MP, Senegaglia AC, Hansen P, Barchiki F, et al. Dissimilar differentiation of mesenchymal stem cells from bone marrow, umbilical cord blood, and adipose tissue. Experimental Biology and Medicine. 2008;233(7).
50. Rebelatto CLK, Boldrini-Leite LM, Daga DR, Marsaro DB, Vaz IM, Jamur VR, et al. Quality Control Optimization for Minimizing Security Risks Associated with Mesenchymal Stromal Cell-Based Product Development. International Journal of Molecular Sciences. 2023;24(16).
51. Saqib U, Kelley TT, Panguluri SK, Liu D, Savai R, Baig MS, et al. Polypharmacology or promiscuity? Structural interactions of resveratrol with its bandwagon of targets. Frontiers in Pharmacology. 2018;9(OCT).
52. Sato Y, Bando H, di Piazza M, Gowing G, Herberts C, Jackman S, et al. Tumorigenicity assessment of cell therapy products: The need for global consensus and points to consider. Vol. 21, Cytotherapy. 2019.
53. Seifrtová M, Havelek R, Ćmielová J, Jiroutová A, Soukup T, Brůčková L, et al. The response of human ectomesenchymal dental pulp stem cells to cisplatin treatment. International Endodontic Journal. 2012;45(5).
54. Shevelev AB, la Porta N, Isakova EP, Martens S, Biryukova YK, Belous AS, et al. In vivo antimicrobial and wound-healing activity of resveratrol, dihydroquercetin, and dihydromyricetin against Staphylococcus aureus, Pseudomonas aeruginosa, and Candida albicans. Pathogens. 2020;9(4).

55. Silva M, de Leeuw N, Mann K, Schuring-Blom H, Morgan S, Giardino D, et al. European guidelines for constitutional cytogenomic analysis. Vol. 27, European Journal of Human Genetics. 2019.
56. Silva MC, Haggarty SJ. Human pluripotent stem cell–derived models and drug screening in CNS precision medicine. Vol. 1471, Annals of the New York Academy of Sciences. 2020.
57. Staniowski T, Zawadzka-Knefel A, Skośkiewicz-Malinowska K. Therapeutic potential of dental pulp stem cells according to different transplant types. Vol. 26, Molecules. 2021.
58. Subhan I, Siddique YH. Resveratrol: Protective Agent Against Alzheimer's Disease. Central Nervous System Agents in Medicinal Chemistry. 2024;24(3).
59. Suchanek J, Soukup T, Visek B, Ivancakova R, Kucerova L, Mokryb J. Dental pulp stem cells and their characterization. Biomedical Papers. 2009;153(1).
60. Sun Z, Guo X, Li C, Ling J, Chang A, Zhao H, et al. Exploring the therapeutic mechanisms of resveratrol for treating arecoline-induced malignant transformation in oral epithelial cells: insights into hub targets. Journal of the Science of Food and Agriculture. 2024 Oct 1;
61. Timmers S, Konings E, Bilet L, Houtkooper RH, van de Weijer T, Goossens GH, et al. Calorie restriction-like effects of 30 days of resveratrol supplementation on energy metabolism and metabolic profile in obese humans. Cell Metabolism. 2011;14(5).
62. Tomé-Carneiro J, Gonzálvez M, Larrosa M, Yáñez-Gascón MJ, García-Almagro FJ, Ruiz-Ros JA, et al. One-year consumption of a grape nutraceutical containing resveratrol improves the inflammatory and fibrinolytic status of patients in primary prevention of cardiovascular disease. American Journal of Cardiology. 2012;110(3).
63. Torre ML, Lucarelli E, Guidi S, Ferrari M, Alessandri G, de Girolamo L, et al. Ex vivo expanded mesenchymal stromal cell minimal quality requirements for clinical application. Vol. 24, Stem Cells and Development. 2015.
64. Tou JC. Resveratrol supplementation affects bone acquisition and osteoporosis: Pre-clinical evidence toward translational diet therapy. Vol. 1852, Biochimica et Biophysica Acta - Molecular Basis of Disease. 2014.
65. U.S. Food and Drug Administration. Chemistry, manufacturing, and control (CMC) information for human gene therapy investigational new drug

applications: guidance for industry. Silver Spring, MD: U.S. Food and Drug Administration; 2020.

66. Vaz IM, Borgonovo T, Kasai-Brunswick TH, dos Santos DS, Mesquita FCP, Vasques JF, et al. Chromosomal aberrations after induced pluripotent stem cells reprogramming. Genetics and Molecular Biology. 2021;44(3).
67. World Health Organization. World Health Organization Technical Report Series No. 987 Annex 3. 2013. In: Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell bank; 2013.
68. Yang G, Zhang Z, Liu K, Ji X, Fatehi P, Chen J. A cellulose nanofibril-reinforced hydrogel with robust mechanical, self-healing, pH-responsive and antibacterial characteristics for wound dressing applications. Journal of Nanobiotechnology. 2022;20(1).
69. Yang HW, Chun-Yu Ho D, Liao HY, Liao YW, Fang CY, Ng MY, et al. Resveratrol inhibits arecoline-induced fibrotic properties of buccal mucosal fibroblasts via miR-200a activation. Journal of Dental Sciences. 2024;19(2).
70. You JB, Choi AY, Baek J, Oh MS, Im SG, Lee KE, et al. Application of Monodirectional Janus Patch to Oromucosal Delivery System. Advanced Healthcare Materials. 2015;4(15).
71. Zhang J, Li R, Man K, Yang XB. Enhancing osteogenic potential of hDPSCs by resveratrol through reducing oxidative stress via the Sirt1/Nrf2 pathway. Pharmaceutical Biology. 2022;60(1).

ANEXOS

Parecer do Comitê de Ética em Pesquisa

Comitê de Ética em Pesquisa da PUCPR

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO PARANÁ - PUC/PR

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: AÇÃO DO RESVERATROL NO CULTIVO DE CÉLULAS-TRONCO DA POLPA DENTAL HUMANA NA LIBERAÇÃO DE MEDIADORES INFLAMATÓRIOS E FATORES DE CRESCIMENTO: ESTUDO IN VITRO.

Pesquisador: PATRICIA TOLENTINO DA ROSA DE SOUZA

Área Temática:

Versão: 1

CAAE: 38458920.4.0000.0020

Instituição Proponente: Associação Paranaense de Cultura - PUCPR

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 4.333.325

Apresentação do Projeto:

Introdução: O resveratrol é um tipo de polifenol natural obtido de alguns alimentos, com potente ação anti-oxidante e anti-inflamatória. Essa substância tem sido estudada no reparo, diminuindo os danos causados por agressões aos tecidos, tanto em desordens cardiovasculares e metabólicas como em doenças respiratórias, neuroinflamação, câncer e no processo de reparo ósseo. O presente estudo tem como objetivo, avaliar in vitro a ação do resveratrol em diferentes concentrações, em células-tronco da polpa dental humana, a partir da quantificação de interleucinas, TNF -alpha e fatores de crescimento.Materiais e métodos: O estudo irá quantificar interleucinas IL-1beta, IL-6, IL-8, TNF-alpha, FGF-2, EGF, VEGF-A sobre células tronco de três pacientes submetidas a aplicação de diferentes concentrações de resveratrol (0,6, 12, 25, 50, 75, 100 e 200 micro molar de resveratrol), em sistema miltiplex (MAGPIX). Além disso, as amostras de células-tronco da polpa dental humana sobre diferentes concentrações de resveratrol, serão submetidas a teste de viabilidade celular por citometria de fluxo, com incubação Anexina PE e 7-AAD, e a utilização de anticorpos isotípicos conjugados com os fluorocromos PE e PE-CY5 como controle.

Objetivo da Pesquisa:

Avaliar in vitro a ação do resveratrol em diferentes concentrações, em células-tronco da polpa dental humana (Dental Pulp Stem Cell - DPSCs), a partir da quantificação de interleucinas IL-1beta,

Endereço: Rua Imaculada Conceição 1155
Bairro: Prado Velho **CEP:** 80.215-901
UF: PR **Município:** CURITIBA
Telefone: (41)3271-2103 **Fax:** (41)3271-2103 **E-mail:** nep@pucpr.br

IL-6, IL-8, TNF-alpha, FGF-2, EGF e VEGF-A, a fim de verificar a concentração ideal de resveratrol para diminuição de citocinas e aumento de fatores de crescimento.

Avaliação dos Riscos e Benefícios:

Riscos:

Não será esperado nenhum malefício ou risco ao paciente pois ao invés de realizarmos o descarte do dente extraído, utilizaremos as células-tronco da polpa para testar a ação do resveratrol na quantificação de mediadores da inflamação e regeneração tecidual.

Benefícios:

O benefício que a pesquisa oferecerá, será futuro para a comunidade como um todo, se a substância teste obtiver bons resultados. O estudo das diferentes concentrações de resveratrol em ação nas células-tronco da polpa dental humana, será importante para o desenvolvimento de um futuro estudo em animais, na aplicação terapêutica da substância testada, em mucosa intra-oral lesionada ou pós-cirúrgico.

Comentários e Considerações sobre a Pesquisa:

Ver Conclusões ou Pendências e Lista de Inadequações

Considerações sobre os Termos de apresentação obrigatória:

Termos de apresentação obrigatória adequados.

Recomendações:

Atualizar o cronograma e corrigir os Campo "Dispensa de TCLE" e "Haverá uso de fontes secundárias de dados (prontuários, dados demográficos, etc)? Foram anexados corretamente o TCUD e o TCLE.

Conclusões ou Pendências e Lista de Inadequações:

Projeto adequado dentro dos preceitos éticos determinados nas resoluções presentes 466/12 e 510/16.

Considerações Finais a critério do CEP:

Lembramos aos senhores pesquisadores que, no cumprimento da Resolução 466/12, o Comitê de Ética em Pesquisa (CEP) deverá receber relatórios anuais sobre o andamento do estudo, bem como a qualquer tempo e a critério do pesquisador nos casos de relevância, além do envio dos relatos de eventos adversos, para conhecimento deste Comitê.

Salientamos ainda, a necessidade de relatório completo ao final do estudo. Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP-PUCPR de forma clara e sucinta,

identificando a parte do protocolo a ser modificado e as suas justificativas.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1633958.pdf	24/09/2020 09:54:39		Aceito
Folha de Rosto	folhaDeRosto_Patricia_assinado_decano.pdf	24/09/2020 09:53:45	PATRICIA TOLENTINO DA ROSA DE SOUZA	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCUD_assinado.pdf	19/09/2020 14:13:50	PATRICIA TOLENTINO DA ROSA DE SOUZA	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_pesquisa_resveratrol.docx	19/09/2020 14:13:14	PATRICIA TOLENTINO DA ROSA DE SOUZA	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_resveratrol.docx	19/09/2020 14:12:23	PATRICIA TOLENTINO DA ROSA DE SOUZA	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

CURITIBA, 12 de Outubro de 2020

Assinado por:
Ana Carla Efing
(Coordenador(a))

TCLE - Termo de Consentimento Livre e Esclarecido

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Você está sendo convidado(a) como voluntário(a) a participar do estudo **AÇÃO DO RESVERATROL NO CULTIVO DE CÉLULAS-TRONCO DA POLPA DENTAL HUMANA NA LIBERAÇÃO DE MEDIADORES INFLAMATÓRIOS E FATORES DE CRESCIMENTO: ESTUDO IN VITRO**, que tem como objetivo avaliar *in vitro,* a ação do resveratrol (substância antioxidante presente em alguns alimentos e bebidas) em diferentes concentrações, em células tronco da polpa dental humana, a partir da quantificação algumas interleucinas e fatores de crescimento (moléculas que atuam no processo inflamatório e recuperação dos tecidos lesionados), a fim de verificar a concentração ideal de resveratrol para diminuição de citocinas inflamatórias e aumento de fatores de crescimento. Acreditamos que esta pesquisa seja importante pois futuramente essa substância poderá ser utilizada sobre lesões em mucosa oral, para auxiliar na sua cicatrização e regeneração.

PARTICIPAÇÃO NO ESTUDO

A sua participação no referido estudo será de ceder o dente extraído, no ato da cirurgia, para que nossa equipe possa fazer a coleta das células da polpa dental que serão utilizadas em nossa pesquisa. No momento da cirurgia, faremos um breve questionário com perguntas referentes aos seus dados pessoais, endereço e telefone para contato, e dados referentes a suas condições de saúde.

RISCOS E BENEFÍCIOS

Através deste Termo de Consentimento Livre e Esclarecido você está sendo alertado de que, da pesquisa a se realizar, para o seu tratamento no momento não será esperado nenhum benefício ou malefício pois ao invés de fazermos o descarte de seu dente extraído, utilizaremos as células da polpa para testar a ação da substância teste (resveratrol) na quantificação de mediadores da inflamação e regeneração tecidual. Portanto, o único benefício que a pesquisa oferecerá, será futuro para a comunidade como um todo, se a substância teste obtiver bons resultados. Mesmo assim, novas pesquisas com essa substância deverão ser realizadas para comprovação de seu benefício. O procedimento de exodontia será realizado de forma habitual, pela disciplina responsável pela cirurgia e você será acompanhado pelos alunos e/ou professores que executarão a cirurgia de exodontia e acompanharão você no pós-operatório.

SIGILO E PRIVACIDADE

Nós pesquisadores garantiremos a você que sua privacidade será respeitada, ou seja, seu nome ou qualquer outro dado ou elemento que possa, de qualquer forma, lhe identificar, será mantido em sigilo. Nós pesquisadores nos responsabilizaremos pela guarda e confidencialidade dos dados, bem como a não exposição dos dados de pesquisa.

AUTONOMIA

Nós lhe asseguramos assistência durante toda pesquisa, bem como garantiremos seu livre acesso a todas as informações e esclarecimentos adicionais sobre o estudo e suas consequências, enfim, tudo o que você queira saber antes, durante e depois de sua participação. Também informamos que você pode se recusar a participar do estudo, ou retirar seu consentimento a qualquer momento, sem precisar justificar, e de, por desejar sair da pesquisa, não sofrerá qualquer prejuízo à assistência que vem recebendo.

RESSARCIMENTO E INDENIZAÇÃO

RÚBRICA DO PARTICIPANTE DA PESQUISA

RÚBRICA DO PESQUISADOR

No entanto, caso tenha qualquer despesa decorrente da participação nesta pesquisa, tais como transporte, alimentação entre outros, bem como de seu acompanhante (se for o caso), haverá ressarcimento dos valores gastos.

De igual maneira, caso ocorra algum dano decorrente de sua participação no estudo, você será devidamente indenizado, conforme determina a lei.

CONTATO

Os pesquisadores envolvidos com o referido projeto são: Patrícia Tolentino da Rosa de Souza, Alexandra Cristina Senegaglia, Edvaldo Antônia Ribeiro Rosa e Luciana Reis Azevedo Alanis. Você poderá manter contato com a pesquisadora principal (Patrícia) no telefone 41 99802-4696.

O Comitê de Ética em Pesquisa em Seres Humanos (CEP) é composto por um grupo de pessoas que estão trabalhando para garantir que seus direitos como participante de pesquisa sejam respeitados. Ele tem a obrigação de avaliar se a pesquisa foi planejada e se está sendo executada de forma ética. Se você achar que a pesquisa não está sendo realizada da forma como você imaginou ou que está sendo prejudicado de alguma forma, você pode entrar em contato com o Comitê de Ética em Pesquisa da PUCPR (CEP) pelo telefone (41) 3271-2103 entre segunda e sexta-feira das 08h00 às 17h30 ou pelo e-mail nep@pucpr.br.

DECLARAÇÂO

Declaro que li e entendi todas as informações presentes neste Termo de Consentimento Livre e Esclarecido e tive a oportunidade de discutir as informações deste termo. Todas as minhas perguntas foram respondidas e eu estou satisfeito com as respostas. Entendo que receberei uma via assinada e datada deste documento e que outra via assinada e datada será arquivada nos pelo pesquisador responsável do estudo.

Enfim, tendo sido orientado quanto ao teor de todo o aqui mencionado e compreendido a natureza e o objetivo do já referido estudo, manifesto meu livre consentimento em participar, estando totalmente ciente de que não há nenhum valor econômico, a receber ou a pagar, por minha participação.

Dados do participante da pesquisa	
Nome:	
Telefone:	
e-mail:	

Local, _____ de _____ de _____.

Assinatura do participante da pesquisa

Assinatura do Pesquisador

RUBRICA DO PARTICIPANTE DA PESQUISA

RUBRICA DO PESQUISADOR

Normas para publicação – Archives of Oral Biology

- **Ethics and Policies**
- **Ethics in publishing**

Authors must follow ethical guidelines stated in Elsevier's Publishing Ethics Policy.

- **Submission declaration**

When authors submit an article to an Elsevier journal it is implied that:

- the work described has not been published previously except in the form of a preprint, an abstract, a published lecture, academic thesis or registered report. See our policy on multiple, redundant or concurrent publication.
- the article is not under consideration for publication elsewhere.
- the article's publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out.
- if accepted, the article will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

To verify compliance with our journal publishing policies, we may check your manuscript with our screening tools.

- **Authorship**

All authors should have made substantial contributions to all of the following:

1. The conception and design of the study, or acquisition of data, or analysis and interpretation of data.
2. Drafting the article or revising it critically for important intellectual content.
3. Final approval of the version to be submitted.

All authors should agree to be accountable for all aspects of the work to ensure that the questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

- **Changes to authorship**

The editors of this journal generally will not consider changes to authorship once a manuscript has been submitted. It is important that authors carefully consider the authorship list and order of authors and provide a definitive author list at original submission.

The policy of this journal around authorship changes:

- All authors must be listed in the manuscript and their details entered into the submission system.
- Any addition, deletion or rearrangement of author names in the authorship list should only be made prior to acceptance, and only if approved by the journal editor.
- Requests to change authorship should be made by the corresponding author, who must provide the reason for the request to the journal editor with written confirmation from all authors, including any authors being added or removed, that they agree with the addition, removal or rearrangement.
- Only in exceptional circumstances will the journal editor consider the addition, deletion or rearrangement of authors post acceptance.
- Publication of the manuscript may be paused while a change in authorship request is being considered.
- Any authorship change requests approved by the journal editor will result in a corrigendum if the manuscript has already been published.
- Any unauthorised authorship changes may result in the rejection of the article, or retraction, if the article has already been published.

- **Declaration of interests**

All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence or bias their work. Examples of potential competing interests include:

- Employment
- Consultancies
- Stock ownership
- Honoraria
- Paid expert testimony
- Patent applications or registrations
- Grants or any other funding

The Declaration of Interests tool should always be completed.

Authors with no competing interests to declare should select the option, "I have nothing to declare".

The resulting Word document containing your declaration should be uploaded at the "attach/upload files" step in the submission process. It is important that the Word document is saved in the .doc/.docx file format. Author signatures are not required.

We advise you to read our policy on conflict of interest statements, funding source declarations, author agreements/declarations and permission notes.

- **Funding sources**

Authors must disclose any funding sources who provided financial support for the conduct of the research and/or preparation of the article. The role of sponsors, if any, should be declared in relation to the study design, collection, analysis and interpretation of data, writing of the report and decision to submit the article for publication. If funding sources had no such involvement this should be stated in your submission.

List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

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.

If no funding has been provided for the research, it is recommended to include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

- **Declaration of generative AI in scientific writing**

Authors must declare the use of generative AI in scientific writing upon submission of the paper. The following guidance refers only to the writing process, and not to the use of AI tools to analyse and draw insights from data as part of the research process:

- Generative AI and AI-assisted technologies should only be used in the writing process to improve the readability and language of the manuscript.
- The technology must be applied with human oversight and control and authors should carefully review and edit the result, as AI can generate authoritative-sounding output that can be incorrect, incomplete or biased. Authors are ultimately responsible and accountable for the contents of the work.

- Authors must not list or cite AI and AI-assisted technologies as an author or co-author on the manuscript since authorship implies responsibilities and tasks that can only be attributed to and performed by humans.

The use of generative AI and AI-assisted technologies in scientific writing must be declared by adding a statement at the end of the manuscript when the paper is first submitted. The statement will appear in the published work and should be placed in a new section before the references list. An example:

- Title of new section: Declaration of generative AI and AI-assisted technologies in the writing process.
- Statement: During the preparation of this work the author(s) used [NAME TOOL / SERVICE] in order to [REASON]. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

The declaration does not apply to the use of basic tools, such as tools used to check grammar, spelling and references. If you have nothing to disclose, you do not need to add a statement.

We advise you to read our policy for authors on the use of generative AI and AI-assisted technologies for Elsevier.

Please note: to protect authors' rights and the confidentiality of their research, this journal does not currently allow the use of Generative AI or AI-assisted technologies such as ChatGPT or similar services by reviewers or editors in the peer review and manuscript evaluation process. We are actively evaluating compliant AI tools and may revise this policy in the future.

- **Preprints**
- **Preprint sharing**

Authors may share preprints, anywhere and at any time, in line with Elsevier's article sharing policy. Sharing preprints, such as on a preprint server, will not count as prior publication.

We advise you to read our policy on multiple, redundant or concurrent publication.

- **Use of inclusive language**

Inclusive language acknowledges diversity, conveys respect to all people, is sensitive to differences, and promotes equal opportunities. Authors should ensure their work uses inclusive language throughout and contains nothing which might imply one individual is superior to another on the grounds of:

- age
- gender
- race
- ethnicity
- culture
- sexual orientation
- disability or health condition

We recommend avoiding the use of descriptors about personal attributes unless they are relevant and valid. Write for gender neutrality with the use of plural nouns ("clinicians, patients/clients") as default. Wherever possible, avoid using "he, she," or "he/she."

No assumptions should be made about the beliefs of readers and writing should be free from bias, stereotypes, slang, reference to dominant culture and/or cultural assumptions.

These guidelines are meant as a point of reference to help you identify appropriate language but are by no means exhaustive or definitive.

- **Reporting sex- and gender-based analyses**

There is no single, universally agreed-upon set of guidelines for defining sex and gender. We offer the following guidance:

- Sex and gender-based analyses (SGBA) should be integrated into research design when research involves or pertains to humans, animals or eukaryotic cells. This should be done in accordance with any requirements set by funders or sponsors and best practices within a field.
- Sex and/or gender dimensions of the research should be addressed within the article or declared as a limitation to the generalizability of the research.
- Definitions of sex and/or gender applied should be explicitly stated to enhance the precision, rigor and reproducibility of the research and to avoid ambiguity or conflation of terms and the constructs to which they refer.

We advise you to read the Sex and Gender Equity in Research (SAGER) guidelines and the SAGER checklist (PDF) on the EASE website, which offer systematic approaches to the use of sex and gender information in study design, data analysis, outcome reporting and research interpretation.

For further information we suggest reading the rationale behind and recommended use of the SAGER guidelines.

Definitions of sex and/or gender

We ask authors to define how sex and gender have been used in their research and publication. Some guidance:

- Sex generally refers to a set of biological attributes that are associated with physical and physiological features such as chromosomal genotype, hormonal levels, internal and external anatomy. A binary sex categorization (male/female) is usually designated at birth ("sex assigned at birth") and is in most cases based solely on the visible external anatomy of a newborn. In reality, sex categorizations include people who are intersex/have differences of sex development (DSD).
- Gender generally refers to socially constructed roles, behaviors and identities of women, men and gender-diverse people that occur in a historical and cultural context and may vary across societies and over time. Gender influences how people view themselves and each other, how they behave and interact and how power is distributed in society.

- **Image manipulation**

We accept that authors sometimes need to manipulate images for clarity but any manipulation of images for the purpose of deception or fraud will be seen as scientific ethical abuse and will be dealt with accordingly.

Authors must adhere to this journal's policy for graphical images:

- No specific feature within an image may be enhanced, obscured, moved, removed or introduced.
- Adjustments of brightness, contrast, or color balance are acceptable if, and only as long as, they do not obscure or eliminate any information present in the original image.
- Nonlinear adjustments such as changes to gamma settings must be disclosed in the figure legend.
- We do not permit the use of generative AI or AI-assisted tools to create or alter images in submitted manuscripts. Please read our policy on the use of generative AI and AI-assisted tools in figures, images and artwork.

To verify compliance with the above, this journal may send your images to a third-party service who screen for image irregularities. Our editors may ask you to provide original data or images if any questions arise as a result of the screening. The final decision as to whether images are acceptable will be taken by our editors.

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

- **Jurisdictional claims**

Elsevier respects the decisions taken by its authors as to how they choose to designate territories and identify their affiliations in their published content. Elsevier's policy is to take a neutral position with respect to territorial

disputes or jurisdictional claims, including, but not limited to, maps and institutional affiliations. For journals that Elsevier publishes on behalf of a third party owner, the owner may set its own policy on these issues.

- Maps: Readers should be able to locate any study areas shown within maps using common mapping platforms. Maps should only show the area actually studied and authors should not include a location map which displays a larger area than the bounding box of the study area. Authors should add a note clearly stating that "*map lines delineate study areas and do not necessarily depict accepted national boundaries*". During the review process, Elsevier's editors may request authors to change maps if these guidelines are not followed.
- 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 and animals**

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

Studies 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. 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.

Studies in animals

All animal experiments should comply with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

Studies should be carried out in accordance with Guidance on the operation of the Animals (Scientific Procedures) Act 1986 and associated guidelines, EU Directive 2010/63 for the protection of animals used for scientific purposes or the NIH (National Research Council) Guide for the Care and Use of Laboratory Animals (PDF).

The sex of animals, and where appropriate, the influence (or association) of sex on the results of the study must be indicated and a statement included in your manuscript that such guidelines as listed above have been followed.

- **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.
- Use spell-check and grammar-check functions to avoid errors.

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

- **Title page**

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.
- Affiliations. Add affiliation addresses, referring to where the work was carried out, below the author names. Indicate affiliations using a lower-case superscript letter immediately after the author's name and in front of the corresponding address. Ensure that you provide the full postal address of each affiliation, including the country name and, if available, the email address of each author.
- Corresponding author. Clearly indicate who will handle correspondence for your article at all stages of the refereeing and publication process and also post-publication. This responsibility includes answering any future queries about your results, data, methodology and materials. It is important that the email address and contact details of your corresponding author are kept up to date during the submission and publication process.
- Present/permanent address. If an author has moved since the work described in your article was carried out, or the author was visiting during that time, a "present address" (or "permanent address") can be indicated by a footnote to the author's name. The address where the author carried out the work must be retained as their main affiliation address. Use superscript Arabic numerals for such footnotes.

- **Abstract**

You are required to provide a concise and factual abstract. 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.

- **Structured abstract**

A structured abstract, by means of appropriate headings, should provide the context or background for your research. Some guidelines:

- State the purpose of your research.
- Outline basic procedures followed such as the selection of study subjects or laboratory animals and observational and analytical methods.
- Include your main findings, providing specific effect sizes and their statistical significance, if possible, and your principal conclusions.
- Emphasize new and important aspects of your study or observations.

- **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.

- **Tables**

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:

When submitting artwork:

- 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 along with the 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).
- Bitmapped line drawings: Save as TIFF, JPG or PNG files using a minimum of 1000 dpi (for single column: min. 3543 pixels, full page width: 7480 pixels).

- 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:

- files that are too low in resolution (for example, files optimized for screen use such as GIF, BMP, PICT or WPG files).
- disproportionally large images compared to font size, as text may become unreadable.

- **Figure captions**

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.

Provide captions in a separate file.

- **Color artwork**

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.

For articles appearing in print, you will be sent information on costs to reproduce color in the printed version, after your accepted article has been sent to production. At this stage, please indicate if your preference is to have color only in the online version of your article or also in the printed version.

- **Generative AI and Figures, images and artwork**

Please read our policy on the use of generative AI and AI-assisted tools in figures, images and artwork, which states:

- We do not permit the use of Generative AI or AI-assisted tools to create or alter images in submitted manuscripts.
- The only exception is if the use of AI or AI-assisted tools is part of the research design or methods (for example, in the field of biomedical imaging). If this is the case, such use must be described in a reproducible manner in the methods section, including the name of the model or tool, version and extension numbers, and manufacturer.
- 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.

- **Supplementary material**

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

- 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.

We recommend you upload research data to a suitable specialist or generalist repository. Please read our guidelines on sharing research data for more information on depositing, sharing and using research data and other relevant research materials.

- **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.

- **Research data**

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 B: Research data deposit, citation and linking

You are **encouraged** to:

- Deposit your research data in a relevant data repository.
- Cite and link to this dataset in your article.
- If this is not possible, make a statement explaining why research data cannot be shared.

- **Data statement**

To foster transparency, you are encouraged 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.

Read more about the importance and benefits of providing a data statement.

- **Data linking**

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.

If your research data has been made available in a data repository there are a number of ways your article can be linked directly to the dataset:

- Provide a link to your dataset when prompted during the online submission process.
- 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).

Learn more about linking research data and research articles in ScienceDirect.

- **Mendeley data**

This journal supports Mendeley Data, enabling you to deposit any research data (including raw and processed data, video, code, software, algorithms, protocols and methods) associated with your manuscript in a free-to-use, open access repository.

Before or during the submission process, you can deposit the relevant datasets to Mendeley Data. Please include the DOI of the deposited dataset(s) in your main manuscript file.

The datasets will be listed and directly accessible to readers next to your published article online.

Learn more about Mendeley Data for journals.

- **Research Elements**

This journal enables the publication of research objects (e.g. data, methods, protocols, software and hardware) related to original research in Elsevier's Research Elements journals.

Research Elements are peer-reviewed, open access journals which make research objects findable, accessible and reusable. By providing detailed descriptions of objects and their application with links to the original research article, your research objects can be placed into context within your article.

You will be alerted during submission to the opportunity to submit a manuscript to one of the Research Elements journals. Your Research Elements article can be prepared by you, or by one of your collaborators.

- **Article structure**
- **Article sections**

Divide your manuscript into clearly defined sections covering all essential elements. For example:

- Abstract
- Keywords
- Introduction
- Materials and methods
- Results
- Conclusions
- Artwork
- Tables with captions

- **Introduction**

The introduction should clearly state the objectives of your work. We recommend that you provide an adequate background to your work but avoid writing a detailed literature overview or summary of your results.

- **Material and methods**

The materials and methods section should provide sufficient details about your materials and methods to allow your work to be reproduced by an independent researcher. Some guidelines:

- If the method you used has already been published, provide a summary and reference the originally published method.
- If you are quoting directly from a previously published method, use quotation marks and cite the source.
- Describe any modifications that you have made to existing methods.

- **Results**

Results should be clear and concise. We advise you to read the sections in this guide on supplying tables, artwork, supplementary material and sharing research data.

- **Discussion**

The discussion section should explore the significance of your results but not repeat them. You may combine your results and discussion sections into one section, if appropriate. We recommend that you avoid the use of extensive citations and discussion of published literature in the discussion section.

- **Conclusion**

The conclusion section should present the main conclusions of your study. You may have a stand-alone conclusions section or include your conclusions in a subsection of your discussion or results and discussion section.

- **Glossary**

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

- **Abbreviations**

Abbreviations which are not standard in the field should be defined in a footnote on the first page of your article.

Abbreviations which are essential to include in your abstract should be defined at first mention in your abstract, as well as in a footnote on the first page of your article.

Before submission we recommend that you review your use of abbreviations throughout your article to ensure that it is consistent.

- **Acknowledgements**

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.

- **Funding sources**

Authors must disclose any funding sources who provided financial support for the conduct of the research and/or preparation of the article. The role of sponsors, if any, should be declared in relation to the study design, collection, analysis and interpretation of data, writing of the report and decision to submit the article for publication. If funding sources had no such involvement this should be stated in your submission.

List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

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.

If no funding has been provided for the research, it is recommended to include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

- **Appendices**

We ask you to use the following format for appendices:

- Identify individual appendices within your article using the format: A, B, etc.
- 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:

- References cited in your abstract must be given in full.
- 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."
- References cited as "in press" imply that the item has been accepted for publication.

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. See the example below, though be aware that the format of such citations should be adapted to follow the style of other references in your paper.

DOI link example (for an article not yet in an issue): VanDecar J.C., Russo R.M., James D.E., Ambeh W.B., Franke M. (2003). Aseismic continuation of the Lesser Antilles slab beneath northeastern Venezuela. Journal of Geophysical Research, https://doi.org/10.1029/2001JB000884.

- **Reference style**

Citations in the text should follow the referencing style used by the American Psychological Association. You are referred to the *Publication Manual of the American Psychological Association,* Seventh Edition (2020) ISBN 978-1-4338-3215-4.

The reference list should be arranged alphabetically and then chronologically. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Examples:

Reference to a journal publication:

Van der Geer, J., Handgraaf T., & Lupton, R. A. (2020). The art of writing a scientific article. Journal of Scientific Communications, 163, 51–59. https://doi.org/10.1016/j.sc.2020.00372.

Reference to a journal publication with an article number:

Van der Geer, J., Handgraaf, T., & Lupton, R. A. (2022). The art of writing a scientific article. Heliyon, 19, Article e00205. https://doi.org/10.1016/j.heliyon.2022.e00205.

Reference to a book:

Strunk, W., Jr., & White, E. B. (2000). The elements of style (4th ed.). Longman (Chapter 4).

Reference to a chapter in a book:

Mettam, G. R., & Adams, L. B. (2020). How to prepare an electronic version of your article. In B. S. Jones, & R. Z. Smith (Eds.), Introduction to the electronic age (pp. 281–304). E-Publishing Inc.

Reference to a website:

Powertech Systems. (2022). Lithium-ion vs lead-acid cost analysis. Retrieved from http://www.powertechsystems.eu/home/tech-corner/lithium-ion-vs-lead-acid-cost-analysis/. Accessed January 6, 2022.

Reference to a dataset:

Oguro, M., Imahiro, S., Saito, S., & Nakashizuka, T. (2015). Mortality data for Japanese oak wilt disease and surrounding forest compositions [dataset]. Mendeley Data, v1. https://doi.org/10.17632/xwj98nb39r.1.

Reference to a conference paper or poster presentation:

Engle, E.K., Cash, T.F., & Jarry, J.L. (2019, November). The Body Image Behaviours Inventory-3: Development and validation of the Body Image Compulsive Actions and Body Image Avoidance Scales. Poster session presentation at the meeting of the Association for Behavioural and Cognitive Therapies, New York, NY.

Reference to software:

Coon, E., Berndt, M., Jan, A., Svyatsky, D., Atchley, A., Kikinzon, E., Harp, D., Manzini, G., Shelef, E., Lipnikov, K., Garimella, R., Xu, C., Moulton, D., Karra, S., Painter, S., Jafarov, E., & Molins, S. (2020, March 25). Advanced Terrestrial Simulator (ATS) v0.88 (Version 0.88) [computer software]. Zenodo. https://doi.org/10.5281/zenodo.3727209.

- **Journal abbreviations**

We ask you to abbreviate journal names according to the List of Title Word Abbreviations (LTWA).

- **Web references**

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 names, dates or reference to a source publication) should also be provided, if known.

You can list web references separately under a new heading directly after your reference list or include them in your reference list.

- **Data references**

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

When citing data references, you should include:

- author name(s)
- dataset title
- data repository
- version (where available)
- year
- global persistent identifier

Add [dataset] immediately before your reference. This will help us to properly identify the dataset. The [dataset] identifier will not appear in your published article.

- **Preprint references**

We ask you to mark preprints clearly. You should include the word "preprint" or the name of the preprint server as part of your reference and provide the preprint DOI.

Where a preprint has subsequently become available as a peer-reviewed publication, use the formal publication as your reference.

If there are preprints that are central to your work or that cover crucial developments in the topic, but they are not yet formally published, you may reference the preprint.

- **Reference management software**

Most Elsevier journals have their reference template available in popular reference management software products. These include products that support Citation Style Language (CSL) such as Mendeley Reference Manager.

If you use a citation plug-in from these products, select the relevant journal template and all your citations and bibliographies will automatically be formatted in the journal style. We advise you to remove all field codes before submitting your manuscript to any reference management software product.

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:

- One author has been designated as the corresponding author and their full contact details (email address, full postal address and phone numbers) have been provided.
- All files have been uploaded, including keywords, figure captions and tables (including a title, description and footnotes) included.
- 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.

- For gold open access articles, all authors understand that they are responsible for payment of the article publishing charge (APC) if the manuscript is accepted. Payment of the APC may be covered by the corresponding author's institution, or the research funder.

- **Submit online**

Our online submission system guides you through the process steps of entering your manuscript details and uploading your files. The system converts your article files to a single PDF file used in the peer-review process.

Editable files (e.g., Word, LaTeX) are required to typeset your article for final publication. All correspondence, including notification of the editor's decision and requests for revision, is sent by email.

Please follow this link to submit your paper.