UNIVERSIDADE FEDERAL DO PAMPA

GIULIANNA ECHEVERRIA MACEDO

MECANISMOS BIOQUÍMICOS DE TOXICIDADE DO COMPOSTO 1-OCTEN-3-OL EM MACHOS E FÊMEAS DE *Drosophila melanogaster*

São Gabriel
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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Doutora em Ciências Biológicas.

Orientador: Thaís Posser

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Dedico este trabalho a minha mãe Ana Cristina e ao meu pai Zilmar (*in memorian*) pelo amor, carinho e suporte de sempre. Minha eterna gratidão.

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“Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota”.

Madre Teresa de Calcutá

RESUMO

Os poluentes atmosféricos podem afetar tanto a qualidade do ar dos ambientes externos, como internos. A exposição a poluição interna fúngica pode contribuir para o surgimento de sintomas alérgicos e inflamatórios, e tem sido considerada uma grave ameaça à saúde pública. Estudos apontam que a prevalência destes sintomas pode variar de acordo com o sexo, visto que homens e mulheres podem apresentar diferentes níveis de resposta inflamatória. Entre os principais poluentes de ambientes domésticos, estão os compostos orgânicos voláteis fúngicos (COVs), comumente encontrados em locais úmidos e comprometidos pela água. O 1-octen-3-ol (pop. álcool de cogumelo), é o COV mais predominante nestes ambientes e o responsável pelo odor característico do mofo. A exposição ao 1-octen-3-ol tem sido associada ao aumento de marcadores inflamatórios e prevalência de renite alérgica e conjuntivite nos habitantes destes locais. Pesquisas com *Drosophila melanogaster* revelaram a neurotoxicidade e o potencial inflamatório do 1-octen-3-ol. No entanto, estudos a respeito dos efeitos do 1-octen-3-ol sobre a mitocôndria, uma organela envolvida no desenvolvimento de respostas inflamatórias, e como este composto atua nos diferentes sexos são escassos. *D. melanogaster* (pop. mosca da fruta), é um modelo relevante para o estudo de doenças humanas, distúrbios inflamatórios e compreensão de questões relacionadas ao sexo. Neste trabalho investigou-se o efeito da exposição inalatória ao 1-octen-3-ol sobre alvos bioquímicos e inflamatórios, e sobre a morfologia e taxa bioenergética mitocondrial de *D. melanogaster*. Fêmeas e machos de *D. melanogaster* foram expostos separadamente a uma atmosfera contendo 1-octen-3-ol (1, 2,5 e 5 μL/L) por 24 e/ou 48 horas. Constatou-se elevada taxa de mortalidade e comprometimento locomotor em ambos os sexos (5 μL/L em 24 horas). No grupo das fêmeas observaram-se: i) um aumento na atividade da superóxido dismutase (SOD) e diminuição da catalase (CAT); ii) inibição do complexo I e II no sistema de transporte de elétrons, diminuição na taxa bioenergética mitocondrial e disruptura no perfil das cristas mitocondriais; iii) aumento da atividade de caspase 3/7 e fosforilação de ERK; iv) aumento da expressão gênica de $p38^{MAPK}$ e JNK, e diminuição na expressão de NF-κβ e p53; v) e aumento nos níveis de nitrito. Enquanto que os machos exibiram: i) um aumento nas atividades da glutationa-S-transferase (GST) e SOD; ii) inalteração na atividade de caspase 3/7; iii) aumento na geração de espécies reativas de oxigênio (ERO) na menor concentração, e uma diminuição na maior concentração; iv) queda na viabilidade celular e nos níveis de nitrito. É provável que nas fêmeas a mitocôndria, juntamente com a ativação de fatores pró-inflamatórios e a cascata de vias de sinalização apoptóticos possam ser um alvo crucial para a toxicidade do 1-octen-3-ol. À medida que nos

machos, um aumento na geração de ERO poderia estar envolvido com o comprometimento da atividade mitocondrial observado através da queda na viabilidade celular. No entanto, as razões para tais diferenças permanecem indefinidas, e nossos achados apontam que o 1-octen-3-ol possa deflagrar respostas únicas e modular diferentes vias em *D. melanogaster* de ambos os sexos, o que poderia estar relacionado a maior susceptibilidade vista nos machos.

Palavras-Chave: Mosca da fruta; Mofo; COV; Poluição do ar interno; Síndrome do Edifício Doente; Diferenças relacionadas ao sexo.

ABSTRACT

Atmospheric pollutants can affect both outdoor and indoor air quality. Exposure to indoor fungal pollution contributes to the emergence of a set of allergic and inflammatory symptoms, configuring a serious threat to public health. The prevalence of such symptoms between the sexes has been a target of studies, which pointed to different inflammatory response levels in males and females. Fungal volatile organic compounds (VOCs) are among the main pollutants found in damp or water-damaged indoor places. 1-Octen-3-ol (pop. mushroom alcohol) is the most abundant fungal VOC found in these spaces and responsible for the typical musty odor. Exposure to 1-octen-3-ol induces inflammatory markers and episodes of allergic rhinitis and conjunctivitis in the inhabitants of these places. In *Drosophila melanogaster*, 1-octen-3-ol has revealed neurotoxicity and inflammatory potential. However, the effects of 1-octen-3-ol towards mitochondria, an organelle involved in the development of the inflammatory response, and how this compound acts in different sexes are scarce. *D. melanogaster* (pop. fruit fly), is a relevant model for the study of human diseases, inflammatory disorders, as well as in the understanding of issues related to sex. The present work aimed to investigate the effects of 1-octen-3-ol on biochemical, inflammatory targets and on mitochondrial morphology and bioenergetic rate in *D. melanogaster*. Females and males of *D. melanogaster* were exposed separately to an atmosphere containing 1-octen-3-ol (1, 2.5 and 5 µL/L) for 24 and/or 48 hours. Exposure to 1-octen-3-ol (5 µL/L) for 24 hours was able to induce mortality rate and locomotor impairment. In our results from the female group, we observed that 1-octen-3-ol i): increased the activity of Superoxide dismutase (SOD) and decreased the catalase (CAT) activity; ii) induced an inhibition of complex I and II in the electron transport system, a decrease in the bioenergetic rate and disarrangement in mitochondrial cristae profile; iii) increased activity of caspase 3/7 and ERK phosphorylation; iv) increased gene expression of $p38^{MAPK}$ and JNK, and decreased expression of NF-κB and p53; v) and an increase in nitrite levels. While the group of males exhibited: i) an increase in glutathione-S-transferase (GST) and SOD activities; ii) unchanged caspase 3/7 activity; iii) increase in the generation of reactive oxygen species (ROS) at the lowest concentration, in contrast to a decrease in the highest concentration; iv) and a drop in cell viability and in nitrite levels. These findings point to the mitochondria as a crucial target for the toxicity of 1-octen-3-ol in parallel with activation of pro-inflammatory factors and apoptotic signaling pathway cascade in females. However, in male flies, an increase in ROS generation could be involved with the impairment of mitochondrial activity observed through the decrease in cell viability. Nevertheless, the reasons for such differences remain undefined,

and our findings indicate that 1-octen-3-ol can trigger unique responses and modulate different pathways in *D. melanogaster* of both sexes, which could be related to greater susceptibility seen in males.

Keywords: Fruit fly; Mold; VOC; Indoor air pollution; Sick Building Syndrome; Sex-related differences.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

Apaf-1 – Fator de ativação de protease apoptótica-1

ATP – Adenosina trifosfato

CAT – Catalase

Cit C – Citocromo C

Cl^- – Cloro

CO – Monóxido de carbono

CO_2 – Dióxido de carbono

COVs – Compostos orgânicos voláteis

DAMPs – *Danger-associated molecular patterns*, Padrões moleculares associados a danos

DNA – Ácido desoxirribonucleico

eNOS – Óxido nítrico sintase endotelial

ERK – Quinase regulada por sinais extracelulares

ERN – Espécies reativas de nitrogênio

ERO – Espécies reativas de oxigênio

$FADH_2$ – Dinucleotídeo de flavina e adenina

FDA – *Food and Drug Administration*, Agência Federal de Administração de Alimentos e Medicamentos

Fe^{2+} – Íon ferroso

G6PDH – Glicose 6-fosfato desidrogenase

GPCRs – Receptores acoplados à proteína G

GPx – Glutationa peroxidase

GR – Glutationa redutase

GSH – Gluationa reduzida

GSSG – Glutationa oxidada

GST – Glutationa S-transferase

H_2O – Água

H_2O_2 – Peróxido de hidrogênio

HOCl – Ácido hipocloroso

IL1β – Interleucina 1β

IL-6 – Interleucina 6

IL-8 – Interleucina 8

iNOS – Óxido nítrico sintase induzível

JH – Hormônio juvenil

JNK – c-Jun N-terminal quinase

MAPK – Proteínas quinases ativadas por mitógenos

MM – Membrana mitocondrial

MMI – Membrana mitocondrial interna

MOMP – Poro de permeabilização da membrana externa

MPO – Mieloperoxidase

NADH – Dinucleotídeo de nicotinamida e adenina

NADPH – Fosfato de dinucleotídeo de nicotinamida e adenina

NF-κβ – Fator nuclear kappa-β

nNOS – Óxido nítrico sintase neuronal

NO – Óxido nítrico

NOS – Óxido nítrico sintase

NOx – Óxido de azoto

O_2 – Oxigênio molecular

$O_2^{\bullet-}$ – Radical superóxido

O_3 – Ozônio

OH- – Íon hidroxila

$OH^{\cdot}$ – Radical hidroxila

OMS – Organização mundial da saúde

$ONOO^{-\bullet}$ – Peroxinitrito

PAMPs – *Pathogen-associated molecular patterns*, Padrões moleculares associados a patógenos

PM_{10} – Particulate matter, Material particulado (entre 2,5 e 10 micrômetros)

$PM_{2,5}$ – Particulate matter, Material particulado (menor ou igual a 2,5 micrômetros)

PPP – Pentose fosfato

Q – Ubiquinona

RRPs – Receptores de reconhecimento de padrões

RTKs – Receptores de tirosina quinases

rTNF – Receptores de fatores de necrose tumoral

SAPK – Proteínas quinases ativadas por estresse

SBS – *Sick building syndrome*

SED – Síndrome do edifício doente

SNC – Sistema nervoso central

SO_2 – Dióxido de enxofre

SOD – Superóxido dismutase

STE – Sistema de transporte de elétrons

TNF-α – Fator de necrose tumoral-α

TRLs – Receptores do tipo Toll

VOCs – Volatile organic compounds

APRESENTAÇÃO

No item **INTRODUÇÃO**, consta uma breve revisão da literatura sobre os temas trabalhados nesta tese. A metodologia realizada e os resultados obtidos que fazem parte desta tese estão apresentados sob a forma de manuscritos, que se encontram nos itens **MANUSCRITOS**. No mesmo constam as seções: Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas. O item **CONSIDERAÇÕES FINAIS**, encontrado no final desta tese, apresenta interpretações e comentários gerais sobre os resultados dos manuscritos presentes neste trabalho. As **REFERÊNCIAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO** e **CONSIDERAÇÕES FINAIS** desta tese.

SUMÁRIO

1. Introdução

A qualidade do ar é essencial para a saúde e o bem-estar dos seres vivos. Desta forma, a exposição aos poluentes atmosféricos tem se tornado uma problemática ambiental, uma vez que eles podem afetar tanto a qualidade do ar dos ambientes externos como dos internos, além de prejudicar a saúde dos indivíduos expostos (EPA, 2021). Dentre os principais contribuintes para uma baixa qualidade do ar nos ambientes internos, estão o mofo e os compostos orgânicos voláteis fúngicos (COVs), comumente encontrados em locais úmidos e comprometidos pela água (BENNET; INAMDAR, 2015; ZAM et al., 2017).

O COV 1-octen-3-ol é um produto natural derivado da oxidação enzimática e clivagem do ácido linoleico (XIONG et al.,2017). Este composto pode ser produzido através do metabolismo de fungos e plantas, além de ser encontrado em emanações de mamíferos, incluindo humanos (GALLAGHER et al., 2008; KIGATHI et al., 2009; MAJEED et al., 2016; MATSUI et al., 2018; SEO; BAEK, 2009). Nos fungos, o 1-octen-3-ol é liberado principalmente pelos gêneros *Aspergillus*, *Penicillium*, *Alternaria*, *Cladosporium*, *Mucor* e *Ulocladium*, os quais são frequentemente encontrados em espaços fechados (BENNET; INAMDAR, 2015). Este composto é particularmente importante, pois é o responsável pelo odor característico do mofo no interior dos ambientes, sendo considerado um indicador do crescimento de fungos nestes locais (BENNET; INAMDAR, 2015; INAMDAR et al., 2012).

Pesquisas têm relatado episódios de toxicidade causados pelo 1-octen-3-ol em humanos e no modelo de invertebrado *Drosophila melanogaster*. A exposição humana a ambientes úmidos e mofados e com a presença de 1-octen-3-ol tem sido associada ao aumento da incidência de sintomas inflamatórios e alérgicos nos moradores e/ou trabalhadores destes ambientes (MENDELL et al., 2011; WÅLINDER et al., 2008; ZAM et al., 2017). Diferenças relacionadas ao sexo têm sido observadas quanto ao surgimento de tais sintomas, visto que homens e mulheres podem apresentar diferentes níveis de resposta inflamatória. Estudos com a mosca da fruta *D. melanogaster* revelaram a neurotoxicidade e o potencial inflamatório do 1-octen-3-ol.

Drosophila melanogaster é um modelo extremamente vantajoso e largamente utilizado em pesquisas científicas de diversas áreas, como de bioquímica, toxicologia, genética, biologia molecular e para o *screening* de diferentes compostos. Apesar de ser um organismo

distantemente relacionado evolutivamente dos seres humanos, o seu genoma compartilha cerca de 75% de homologia com genes associados a doenças humanas (UGUR; CHEN; BELLEN, 2016; YAMAGUCHI; YOSHIDA, 2018). Além disso, assim como nos vertebrados, fêmeas e machos de *D. melanogaster* podem apresentar diferenças em aspectos como a longevidade e em mecanismos envolvidos em respostas imunológicas e ao estresse (BELMONTE et al., 2020; BUCHON; SILVERMAN; CHERRY, 2014). Tais vantagens permitem que *Drosophila* seja um modelo relevante para a compreensão de mecanismos implicados em uma variedade de doenças humanas, como a asma e distúrbios inflamatórios, além de ser útil para a investigação de diferenças relacionadas ao sexo em diferentes patologias (ROEDER; ISERMANN; KABESCH, 2009).

Os mecanismos celulares e moleculares do 1-octen-3-ol são pouco explorados na literatura, e estudos a respeito dos seus efeitos sobre a mitocôndria, uma organela envolvida no desenvolvimento de respostas inflamatórias, e como este composto atua nos diferentes sexos são escassos. Portanto, tendo em vista a necessidade de informações a respeito da toxicidade deste composto, em conjunto com as associações feitas entre a sua exposição e o desenvolvimento de sintomas inflamatórios nos indivíduos expostos, pesquisas que melhor esclareçam as relações de toxicidade deste composto se fazem necessárias. Além do mais, se torna indispensável a padronização de um modelo experimental que colabore com tais investigações, auxiliando na compreensão dos mecanismos de atuação do 1-octen-3-ol em ambos os sexos, e sobre aspectos fundamentais envolvidos na resposta inflamatória. Deste modo, este trabalho foi desenvolvido com o objetivo de avaliar os mecanismos bioquímicos e moleculares envolvidos na exposição inalatória ao composto orgânico volátil fúngico 1-octen-3-ol em fêmeas e machos de *Drosophila melanogaster*.

2. Revisão de literatura

2.1 Meio ambiente e poluentes atmosféricos

Define-se o meio ambiente como “o conjunto de condições, leis, influências e interações de ordem física, química e biológica, que permite, abriga e rege a vida em todas as suas formas” (BRASIL, lei nº 6.938, Art. 3º inciso I). Assim sendo, o meio ambiente pode ser considerado um bem comum e compartilhado que a todos pertence, uma rede de inter-relações bióticas (seres vivos) e abióticas (hidrosfera litosfera e atmosfera). No entanto, determinados fatores são

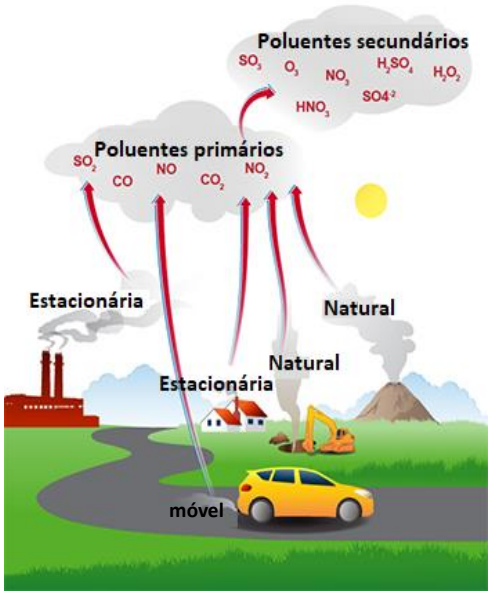
capazes de interferir na qualidade ambiental, entre os quais se destacam os poluentes atmosféricos, que podem ser compreendidos como “qualquer forma de matéria em quantidade, concentração, tempo ou outras características, que tornem ou possam tornar o ar impróprio ou nocivo à saúde” (CONAMA, resolução nº 491/2018). Portanto, os poluentes podem ser gases e partículas sólidas dispersas no ar, capazes de provocar alterações nas propriedades físicas, químicas e biológicas do ar atmosférico, e consequentemente causarem efeitos adversos a todos os seres vivos, desequilibrando os ecossistemas.

Entre os principais tipos de poluentes encontrados no ambiente estão o monóxido de carbono (CO), dióxido de enxofre (SO_2), óxidos de nitrogênio (NOx), ozônio (O_3), materiais particulados (PM_{10} e $PM_{2,5}$) e compostos orgânicos voláteis (COVs) (ALMETWALLY; BIN-JUMAH; ALLAM, 2020; LEE; LEE; BAE, 2014; MANISALIDIS et al., 2020). As fontes emissoras de poluentes podem ser estacionárias, móveis ou naturais. As fontes estacionárias são fixas e vão desde indústrias, fornos, caldeiras, até unidades de aquecimento doméstico como fogões a lenha e chaminés. As fontes móveis incluem carros, caminhões, ônibus, motocicletas, entre outros. Já as fontes naturais compreendem materiais particulados e gases de erupções vulcânicas, além de COVs de vegetações (EPA, 2021; MMA, 2009).

Estes poluentes podem ser primários, isto é, quando contaminam o ambiente diretamente a partir da sua emissão; ou secundários, resultantes de reações entre os poluentes primários com substâncias presentes na camada baixa da atmosfera, ou seja, são subprodutos dos principais poluentes (EPA, 2021; MMA, 2021) (Figura 1).

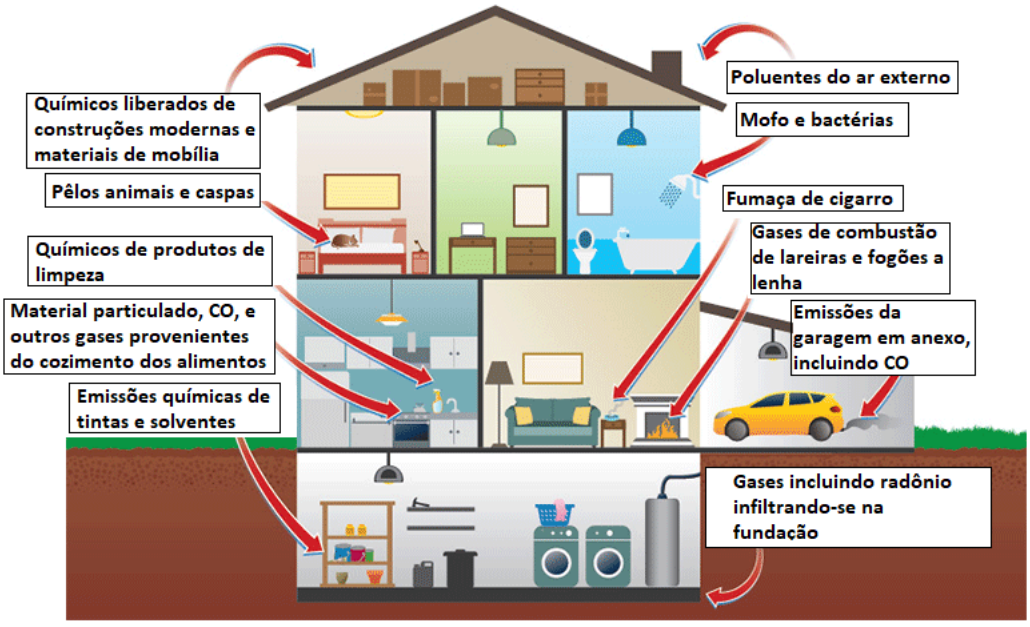
Além dos poluentes afetarem significativamente a qualidade do ar em espaços abertos, eles são capazes de adentrar em ambientes domésticos, industriais e comerciais e alterar a qualidade do ar nestes lugares. Alguns exemplos de poluentes externos que prejudicam estes locais são os materiais particulados e poluentes gasosos advindos do ar exterior; ou ainda contaminantes naturais como o radônio, que pode ser gerado por rochas sob as construções, e desta forma penetrar nestes ambientes diminuindo a qualidade do ar. Além disso, contaminantes liberados no próprio local, como por exemplo gases provenientes de atividades humanas como cozinhar e limpar, ou ainda de materiais de construção e poluentes biológicos como o mofo podem prejudicar consideravelmente a qualidade do ar (EPA, 2021; HARRISON; HESTER, 2019) (Figura 2).

Figura 1 – Principais poluentes do ar externo



Fonte: Adapatado de https://www.epa.gov/expobox/exposure-assessment-tools-media-air#outdoorair

Figura 2 – Principais poluentes presentes nos ambientes internos



Fonte: Adapatado de https://www.epa.gov/expobox/exposure-assessment-tools-media-air#outdoorair

2.1.1 Compostos orgânicos voláteis

Os compostos orgânicos voláteis conhecidos como COVs ou VOCs do inglês "*Volative organic compounds"*, além de estarem entre os poluentes do ar externo mais significativos, são considerados um dos grandes responsáveis por diminuírem a qualidade do ar nos espaços internos. Os COVs são definidos como uma ampla classe de substâncias sólidas e líquidas a base de carbono e que possuem baixa solubilidade em água. São compostos com baixa massa molecular, ponto de ebulição na faixa de 50-100°C (compostos muito voláteis) a 240-260°C (compostos semi-voláteis) e pressões de vapor de saturação superiores a 102 kPa a 25°C. São substâncias altamente voláteis, que em temperatura e pressão ambiente rapidamente entram na fase gasosa (BERENJIAN; CHAN; MALMIRI, 2012; EPA, 2017; ISO16000-6, 1989; KAMAL; RAZZAK; HOSSAIN, 2016; ZHANG et al., 2017).

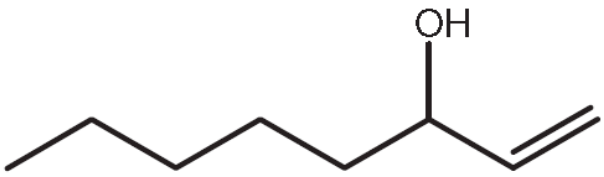
Estes compostos podem ser emitidos de fontes variadas, através de emissões antropogênicas, ou seja, produzidas pelo próprio homem, ou por meio de fontes naturais, denominadas biogênicas. Os COVs antropogênicos compreendem compostos derivados da refinação, combustão e evaporação de produtos à base de petróleo, emissões industriais, gases de escape de veículos, além de serem utilizados como solventes na fabricação de tintas, adesivos, produtos de limpeza e automotivos, *sprays* de aerosol, pesticidas, fluidos de limpeza a seco, entre outros (BENNET; INAMDAR, 2015; EPA, 2017; WANG et al., 2013; ZHANG et al., 2017). Em contraste, os COVs biogênicos são naturalmente gerados principalmente a partir do metabolismo de plantas, bactérias e fungos (BENNET; INAMDAR, 2015; WANG et al., 2013).

Entre as fontes naturais, os fungos são emissores de uma ampla variedade de COVs e são comumente encontrados no interior de locais mofados e comprometidos pela água. Os compostos voláteis liberados pelos fungos possuem tamanhos, tipos, quantidades e classes químicas diferentes, e compreendem hidrocarbonetos, ácidos, aldeídos, compostos aromáticos, cetonas, terpenos, álcoois, tióis e seus derivados (BENNET; INAMDAR, 2015; MORATH; HUNG; BENNETT, 2012). Os COVs fúngicos possuem facilidade de vaporização e difusão, e devido seu baixo peso molecular, podem prontamente serem transportados pelo ar. Portanto, no interior dos ambientes estes compostos podem se acumular e atingir níveis elevados, e consequentemnte prejudicar a qualidade do ar e a saúde dos seus habitantes (MORATH; HUNG; BENNETT, 2012; WÅLINDER et al., 2008; WHO, 2009; WHO, 2010).

2.1.1.1 Composto orgânico volátil fúngico 1-octen-3-ol

O 1-octen-3-ol é o COV fúngico mais abundante no interior de ambientes úmidos e acometidos pelo mofo, podendo atingir concentrações muito elevadas de até 900 μg/m^3 (0,16 ppm) (INAMDAR et al., 2012; MOREY et al., 1997) (Figura 3). Conhecido como "álcool de cogumelo", o 1-octen-3-ol é um composto de baixo peso molecular de 8 carbonos, que é produzido através da oxidação enzimática e clivagem do ácido linoleico (XIONG et al.,2017; XU et al., 2015).

Figura 3 – Fórmula estrutural do composto orgânico volátil 1-octen-3-ol



Fonte: Combet et al (2006, p. 319)

Este COV pode existir na forma de dois isômeros opticamente ativos que ocorrem naturalmente, a forma R, que possui um odor de cogumelos frescos, e a forma S que apresenta um odor típico de grama ou mofo (COMBET et al. 2006; INAMDAR; MORATH; BENNET, 2020). O 1-octen-3-ol é liberado principalmente pelos fungos dos gêneros *Aspergillus*, *Penicillium*, *Alternaria*, *Cladosporium*, *Mucor* e *Ulocladium*, comumente encontrados em espaços fechados (BENNET; INAMDAR, 2015). Este composto é particularmente importante, pois é o responsável pelo odor característico do mofo no interior dos ambientes, sendo considerado um indicador do crescimento de fungos nestes locais (BENNET; INAMDAR, 2015).

Além dos fungos, o 1-octen-3-ol também pode ser encontrado em algas, plantas e em emanações de mamíferos, incluindo humanos (CHEN et al., 2019; GALLAGHER et al., 2008; KIGATHI et al., 2009; MAJEED et al., 2016; MATSUI et al., 2018; SEO; BAEK, 2009). Estudos têm descrito que este COV pode ainda atuar como um agente atrativo para as moscas tsé-tsé *Glossina pallidipes* e *G. morsitans* e para os dípteros da família Tabanidae, além de alterar a postura de ovos e o comportamento de *Phthorimaea operculella*, e possuir um potencial inseticida para *Tribolium castaneum* (Herbst) (ANFORA et al., 2014; CUI et al., 2021;

FRENCH; KLINE, 1989; HALL et al., 1984).

Ainda, o 1-octen-3-ol é utilizado como um aditivo alimentar, aprovado pela Agência Federal de Administração de Alimentos e Medicamentos dos Estados Unidos da América - EUA, a FDA do inglês "*Food and Drug Adminstration*" (ASP 1154, Regnum 172.515), e considerado uma característica desagradável no vinho, além de também ser usado como um agente aromatizante e ingrediente de perfume (EPA, 2003).

2.2 Efeitos adversos dos poluentes do ar sobre os organismos

Ambientes com uma qualidade do ar reduzida são um risco para a saúde e o bem-estar da população e estão associados a uma alta taxa de morbidade e mortalidade (MANISALIDIS et al., 2020). De acordo com a Organização Mundial da Saúde (OMS), estima-se que ocorram anualmente cerca de 3,8 milhões de mortes prematuras em decorrência de uma baixa qualidade do ar nos espaços internos (WHO, 2014). Neste sentido, a qualidade do ar nestes locais é especialmente importante, uma vez que grande parte das pessoas permanecem a maior parte do seu tempo dentro de ambientes fechados (TRAN; PARK; LEE, 2020). Portanto, a poluição no interior dos ambientes tem sido considerada um fator de risco para os seus habitantes e um sério problema de saúde pública, pois pode contribuir para uma série de efeitos nocivos sobre os seres vivos, representando uma maior ameaça à saúde em relação a poluição do ar em espaços abertos (TRAN; PARK; LEE, 2020).

2.2.1 Efeitos da exposição ao mofo e ao 1-octen-3-ol

Particularmente, a presença de umidade e de mofo no interior dos ambientes tem sido correlacionada ao surgimento de sintomas respiratórios e alérgicos nos ocupantes destes locais (MENDELL et al., 2011; ZAM et al., 2017). Estudos anteriores demonstraram uma associação entre exposição ao 1-octen-3-ol e episódios de toxicidade em humanos e no modelo de invertebrado *Drosophila melanogaster*. A exposição humana voluntária a 10 mg/m^3 de 1-octen-3-ol durante 2 horas induziu irritações nos olhos, náuseas leves, dores de cabeça e um aumento em marcadores inflamatórios nas secreções nasais (WÅLINDER et al., 2008). Além disso, foram obtidas correlações positivas entre os níveis de 1-octen-3-ol e a prevalência de rinite alérgica e conjuntivite em pessoas expostas (ARAKI et al., 2012; SMEDJE; NORBÄCK; EDLING 1997). Em *Drosophila melanogaster*, exposição ao 1-octen-3-ol foi capaz de prejudicar a atividade locomotora, causar atraso na metamorfose e efeitos tóxicos nos estádios

de pupa e adulto, induzir peroxidação lipídica, morte celular por apoptose, neurotoxicidade e respostas inflamatórias mediadas pelo óxido nítrico (INAMDAR; BENNETT, 2014; INAMDAR et al, 2013; INAMDAR et al., 2014; INAMDAR; MASUREKAR; BENNET, 2010; YIN et al., 2015).

Embora estudos demonstrem uma correlação positiva entre a presença do mofo e a exposição ao 1-octen-3-ol ao desenvolvimento de doenças respiratórias e inflamatórias, os mecanismos bioquímicos celulares e moleculares deste COV fúngico ainda não são muito explorados na literatura. Além do mais, informações a respeito da toxicocinética deste composto, ou seja, a forma como o 1-octen-3-ol inalado é distribuído e eliminado do corpo humano, são escassas. Neste sentido, alguns estudos têm reportado que quando inalados, os compostos orgânicos voláteis são rapidamente absorvidos pelos pulmões, distribuídos para o sangue arterial e posteriormente para os órgãos-alvo dos seres humanos. Estes COVs podem ser biotransformados em produtos com uma maior ou menor toxicidade, processo este que ocorre principalmente no fígado. Ainda, fatores como a idade e a gordura corporal podem influenciar na toxicocinética destes compostos (EPA, 2005; NRC, 2009).

A exposição a locais com a presença de compostos fúngicos também tem sido relacionada ao desenvolvimento de uma condição denominada Síndrome do Edifício Doente (SED), mais conhecida pela sigla SBS, do inglês "*Sick Building Syndrome*" (BOŽIĆ; ILIĆ; ILIĆ, 2019). Esta síndrome é caracterizada pelo surgimento de um conjunto de sintomas alérgicos apresentados pelos moradores e/ou trabalhadores destes espaços, como dores de cabeça, irritações nos olhos, nariz ou garganta, tosse seca, alergias e aumento da incidência de asma (KHAN; KARUPPAYIL, 2012; TRAN; PARK; LEE, 2020). A asma é uma doença inflamatória crônica das vias aéreas muito comum, e a sua prevalência varia de acordo com o sexo e a idade (JEFFREY et al., 2018; SHAH; NEWCOMB, 2018; ZHANG; ZEIN, 2019). Estudos têm apontado que a prevalência dos sintomas desta síndrome pode variar de acordo com o sexo, visto que homens e mulheres podem apresentar diferentes níveis de resposta inflamatória (LEE; PARK; JEONG, 2016; ZHANG et al., 2014). Além disso, tem sido sugerido que homens podem apresentar um maior risco de morte em respota a exposição a poluição do ar (OLIVEIRA et al., 2011). Diferenças relacionadas ao sexo também têm sido observadas quanto ao risco, incidência e patogênese de doenças pulmonares, e em doenças e respostas imunológicas e inflamatórias (CHAMEKH et al., 2017; GARGAGLIONI; MARQUES; PATRONE, 2019; KLEIN; FLANAGAN, 2016; RATHOD et al., 2017).

De acordo com a literatura, o sexo pode afetar uma gama de questões fisiológicas em várias espécies, de insetos a mamíferos, e possivelmente fatores biológicos, como antecedentes genéticos e hormônios sexuais estão relacionados (BELMONTE et al., 2020; CASIMIR et al., 2018). É provável que a presença de dois cromossomos X no sexo feminino seja um dos principais fatores responsáveis pela diferença na resposta imune observada entre os sexos (ORTONA et al., 2016). Sabe-se que mulheres são mais tolerantes a condições inflamatórias agudas, enquanto os homens a condições inflamatórias crônicas. A presença de um pH mais baixo no sexo feminino também poderia influenciar a intensidade da resposta inflamatória, resultando em um aumento nos níveis de citocinas pró-inflamatórias (CASIMIR et al., 2018). Além disso, tais diferenças poderiam estar associadas aos níveis de óxido nítrico (NO), pois mulheres apresentam níveis mais elevados em comparação aos homens (GOÏTA et al., 2020; KHALIULIN; KARTAWY; AMAL, 2020). Apesar de estudos apontarem esta diferença, as razões para tais variações ainda não são bem compreendidas.

Além dos sintomas da SBS, outros efeitos são observados em indivíduos expostos a um ambiente com uma baixa qualidade do ar, entre os quais se destacam sintomas associados à gripe (febre, calafrios, aperto no peito, dores musculares e tosse), e doenças cardiovasculares e alérgicas como dermatite atópica (TRAN; PARK; LEE, 2020). Neste sentido, as mitocôndrias exercem um papel fundamental, pois alterações na sua função e morfologia podem contribuir para o desenvolvimento de doenças inflamatórias alérgicas, como a asma (CLOONAM et al., 2020; BHATRAJU; AGRAWAL, 2014; IYER; MISHRA; AGRAWAL, 2017; MABALIRAJAN; GHOSH, 2013; REDDY, 2011).

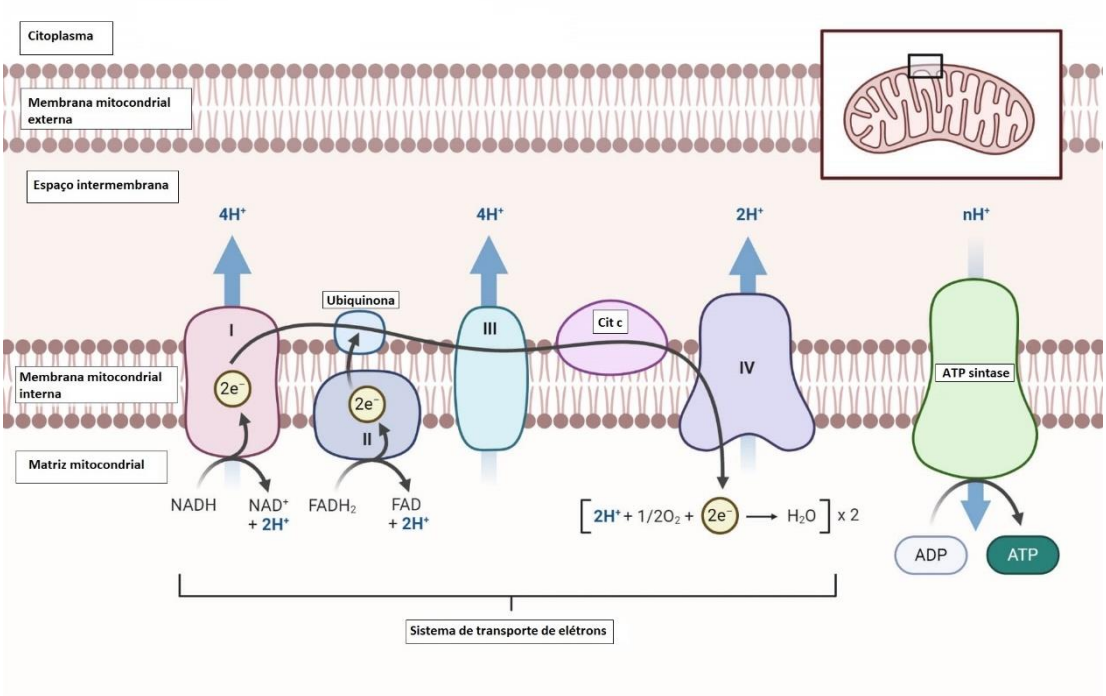
2.2.2 Alterações na função e morfologia das mitocôndrias

Estudos têm reportado que a exposição a polutentes do ar pode acarretar em mudanças na funcionalidade e na morfologia das mitocôndrias, podendo inclusive afetar a produção energética das mesmas (BOOVARAHAN; KURIAN, 2018; BRETON et al., 2019; CHEW et al., 2020). As mitocôndrias são organelas fundamentais para a manutenção da homeostase e produção energética das células, e desempenham papéis cruciais em importantes processos como a sobrevivência, morte celular e respostas inflamatórias (CHABAN; BOEKEMA; DUDKINA, 2014; MISSIROLI et al., 2020; SPINELLI; HAIGIS, 2018).

As mitocôndrias produzem a energia necessária através de um sistema de transporte de

elétrons (STE) acoplado a fosforilação oxidativa, a qual compreende cinco complexos enzimátivos (I, II, III, IV e V) presentes na membrana mitocondrial interna (MMI). De forma geral, os elétrons provenientes dos substratos oxidáveis NADH (dinucleótido de nicotinamida e adenina) e $FADH_2$ (dinucleótido de flavina e adenina) oriundos do Ciclo de Krebs e da oxidação dos ácidos graxos são transferidos para o oxigênio (O_2), o qual age como o aceptor final de elétrons. O transporte de elétrons ocorre em várias etapas ao longo dos complexos enzimáticos, onde o NADH e o $FADH_2$ são reduzidos pelos complexos I (NADH-ubiquinona oxidorredutase) e II (succinato desidrogenase). Subsequentemente seus elétrons são transportados do complexo I ou II para o complexo III (ubiquinona-citocromo c oxidorredutase), e então para o complexo IV (citocromo c oxidase), onde o O_2 é reduzido e ocorre a geração de água (H_2O). Ligado a este processo de transporte de elétrons, há a geração de um gradiente de prótons, que o complexo V (ATP sintase) utiliza para produzir energia através da síntese de ATP (adenosina trifosfato) (CHABAN; BOEKEMA; DUDKINA, 2014; SPINELLI; HAIGIS, 2018) (Figura 4).

Figura 4 – Representação esquemática do sistema de transporte de elétrons acoplado a fosforilação oxidativa

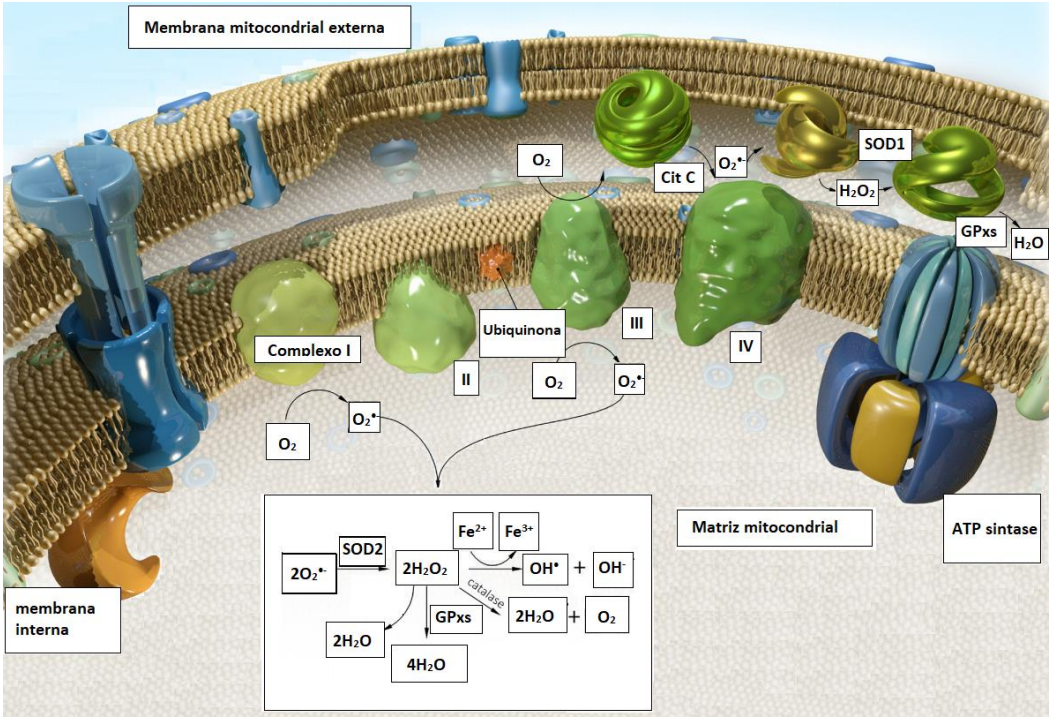


Fonte: Adaptado de https://microbenotes.com/electron-transport-chain/

Durante este processo, naturalmente ocorre a formação endógena de espécies reativas de oxigênio (ERO), como é o caso do radical superóxido ($O_2^{\bullet-}$). A geração do radical $O_2^{\bullet-}$ pode

ocorrer através dos complexos I e III. Essa produção pode se dar pela transferência direta de elétrons do NADH para a ubiquinona (Q), ou por sítios específicos presentes no complexo I. A presença destes sítios facilita o vazamento dos elétrons do STE para a matriz mitocondrial (MM), reduzindo parcialmente o O_2 a $O_2^{\bullet-}$ (KUSSMAUL; HIRST, 2006). O radical $O_2^{\bullet-}$ presente na MM pode ser rapidamente transformado em peróxido de hidrogênio (H_2O_2) espontanemante ou através de uma reação catalisada pela enzima superóxido dismutase mangânes (MnSOD), também conhecida como SOD2 (ONUKWUFOR; BERRY; WOJTOVICH, 2019; SNEZHKINA et al., 2019); ou pela enzima superóxido dismutase cobre-zinco (Cu-ZnSOD), conhecida como SOD1 no espaço intermembrana (KUDRYAVTSEVA et al., 2016). Posteriormente o H_2O_2 pode ser decomposto em O_2 e H_2O pela ação da enzima catalase (CAT), ou removido através da atividade do sistema da glutationa peroxidase (GPx) (SPINELLI; HAIGIS, 2018) (Figura 5).

Figura 5 – Representação esquemática do processo de geração de ERO mitocondriais

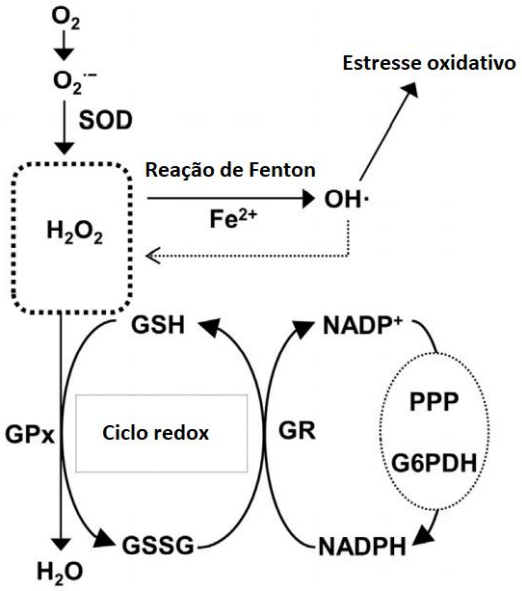


Fonte: Adaptado de Kudryavtseva et al (2016, p. 44882)

O sistema GPx auxilia no processo de eliminação de ERO através de reações redox (oxidação e redução) da glutationa (GSH). GSH é um tripeptídeo e excelente doador de elétrons, amplamente distribuído nas células e detentor do importante papel biológico de eliminar do organismo xenobióticos e compostos endógenos, como o H_2O_2 por exemplo. A enzima GPx

catalisa doação de elétrons da forma reduzida da glutationa (GSH) para o H_2O_2 (ALI et al., 2019; ZHANG et al., 2020). Neste processo, o tripeptídeo GSH é oxidado ao dissulfeto de GSH (GSSG), que é a sua forma oxidada. Posteriormente, esta forma oxidada é novamente reduzida a GSH, através da ação da enzima glutationa redutase (GR), às custas de NADPH (Fosfato de dinucleótido de nicotinamida e adenina), fornecido pela ação da glicose 6-fosfato desidrogenase (G6PDH) na via da pentose fosfato (PPP) (ALI et al., 2019; HIGUCHI, 2014). A enzima glutationa-S-transferase (GST) é particularmente importante neste processo, pois ela pode catalisar a conjugação da GSH com os xenobióticos, facilitando a sua eliminação do organismo (CHATTERJEE; GUPTA, 2018). No entanto, quando há H_2O_2 em excesso, pode ocorrer a reação de Fenton, onde o H_2O_2 existente na mitocôndria reage com o íon Fe^{+2} o qual é então oxidado ao íon Fe^{3+}, e assim gerando o íon hidroxila (OH^-) e o altamente instável e reativo radical hidroxila ($OH^{\bullet}$) (LATUNDE-DADA, 2017) (Figura 6).

Figura 6 –Representação esquemática do ciclo redox da glutationa



Fonte: Adapatado de Higuchi (2014, p. 186)

Portanto, em condições fisiológicas, os níveis de ERO celulares são estáveis, permanecem em um equilíbrio dinâmico, e participam de importantes processos biológicos como respostas imunes, sinalização e morte celular (PIZZINO et al., 2017). Este equilíbrio é modulado pelo sistema de defesa antioxidante, do qual fazem parte o tripeptídeo glutationa (GSH) e as enzimas antioxidantes SOD, CAT, GST e GPx (ALI et al., 2019). Porém, frente a

exposição a certos compostos, como metais pesados e poluentes atmosféricos, pode ocorrer um aumento excessivo na produção das espécies reativas e ocasionar um estado de estresse oxidativo (GANGWAR et al., 2020; GAWDA et al., 2017; LENI; KÜNZI; GEISER, 2020; PIZZINO et al., 2017).

O estresse oxidativo é caracterizado por um desbalanço entre os níveis das espécies reativas e a capacidade antioxidante do organismo de combatê-las e eliminá-las (LIGUORI et al., 2018). Além das espécies reativas derivadas da redução do oxigênio ($O_2^{\bullet-}$, H_2O_2 e $OH^{\bullet}$), destacam-se as espécies reativas de nitrogênio (ERN), principalmente o óxido nítrico (NO) e o radical reativo peroxinitrito ($ONOO^{-\bullet}$), resultante da interação entre o $O_2^{-\bullet}$ e NO (ALI et al., 2019; PIZZINO et al., 2017). Interessantemente, diferenças relacionadas ao sexo também têm sido observadas nestes aspectos, sugerindo-se que homens podem apresentar níveis mais elevados de estresse oxidativo em relação as mulheres, além de diferenças no metabolismo e em respostas dependentes da glutationa (TENKORANG; SYNDER; CUNNINGHAM, 2018; WANG; AHN; ASMIS, 2020).

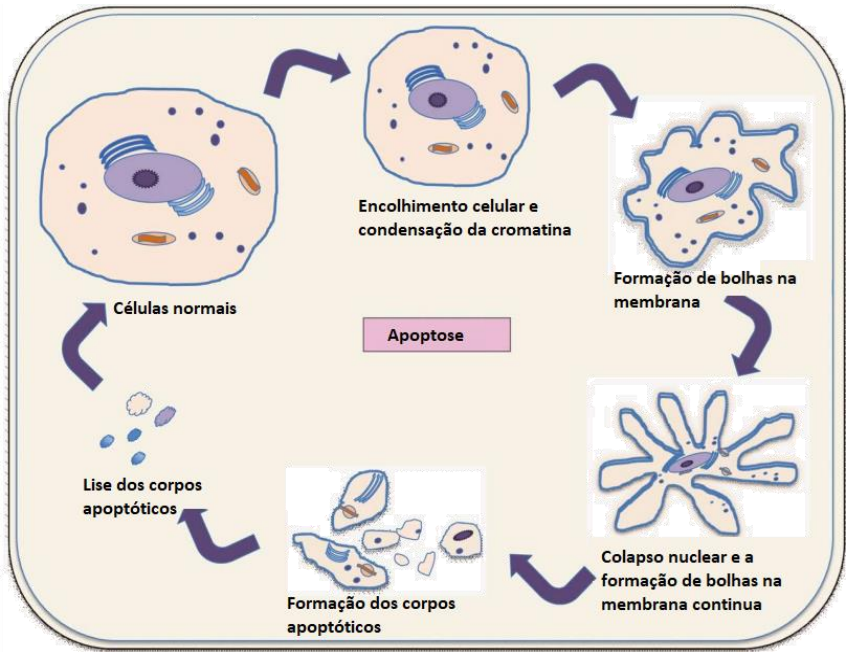
O estresse oxidativo pode causar danos em estruturas essenciais para a sobrevivência das células, como proteínas, lipídeos, ácidos nucléicos e inclusive sobre as próprias mitocôndrias (ISLAM, 2016; PIZZINO et al., 2017). Assim sendo, a exposição aos poluentes ambientais pode aumentar a produção de ERO, causar um dano oxidativo e disfunção mitocondrial (falha na integridade da membrana e na taxa bioenergética), capaz de comprometer biomoléculas essenciais, e levar a um processo de inflamação e morte celular (SUHAILI et al., 2017; WEST, 2017).

2.2.3 Apoptose

A associação entre a exposição a poluentes atmosféricos e a morte celular apoptótica tem sido sugerida (HUANG et al., 2020; PEIXOTO; GALVÃO; DE MEDEIROS, 2017; PIAO et al., 2018; WANG et al., 2019). A apoptose é um processo de morte celular programada altamente regulado e conservado entre as espécies, dependente de sinalização celular específica que controla a proliferação e a sobrevivência celular (D'ARCY, 2019). Este processo ocorre naturalmente no organismo; entretanto, sob condições adversas, pode ser exacerbado, possivelmente, como um mecanismo de defesa. Morfologicamente, a apoptose é caracterizada pelo encolhimento celular, fragmentação do DNA, condensação da cromatina, compactação do

citoplasma, formação de bolhas na membrana celular e colapso nuclear. Posteriormente, ocorre a formação dos corpos apoptóticos que são fagocitados por macrófagos, prevenindo a liberação de componentes intracelulares e um processo inflamatório (NAGATA, 2018; OBENG, 2020) (Figura 7).

Figura 7 – Representação esquemática do processo de apoptose



Fonte: Adaptado de Larrubia et al (2013, p. 1880)

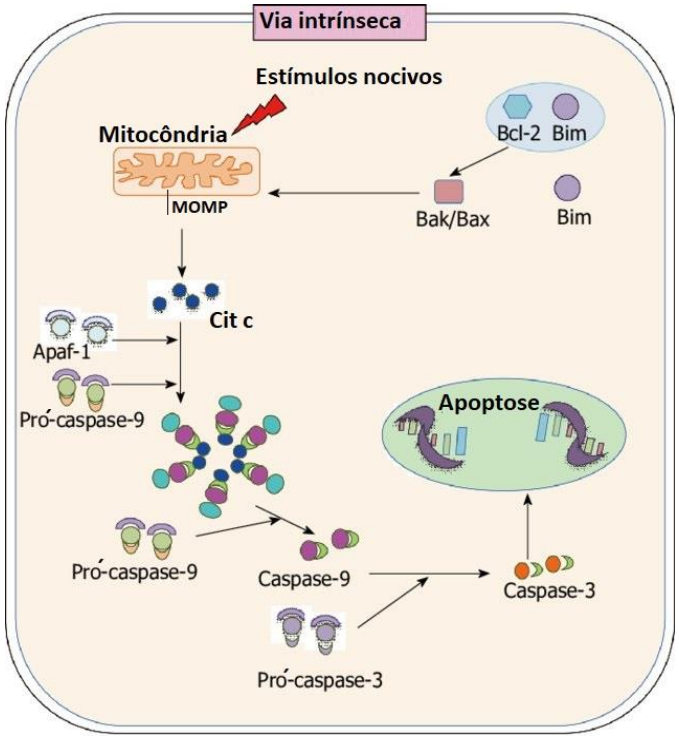
A apoptose pode ser desencadeada por sinais extrínsecos e intrínsecos (D'ARCY, 2019). Na via extrínseca, fatores externos podem reconhecer e se ligar aos receptores de fatores de necrose tumoral (rTNF) presentes na membrana, chamados de receptores de morte celular, iniciando assim uma resposta de sinalização celular e ativando a cascata das caspases (JAN; CHAUDHRY, 2019). Enquanto que a via intrínseca mitocondrial é composta por proteínas antiapoptóticas (Bcl2 e Bclx) e pró-apoptóticas (Bax e Bak, caspases iniciadoras e efetoras), e pode ser ativada frente a estímulos nocivos, como danos ao DNA, estresse metabólico, hipóxia, e presença de ERO (D'ARCY, 2019; REDZA-DUTORDOIR; AVERILL-BATES, 2016; SUHAILI et al., 2017; ZHOU; YANG; XING, 2011).

As caspases são proteases que possuem cisteína em seu sítio ativo, capazes de reconhecer e clivar especificamente resíduos de aspartato em outras proteínas. Estas proteases são sintetizadas na forma de precursores inativos (Pró-caspases), que são ativados (Caspases) após sinais de morte celular, e desempenham um papel fundamental neste processo. Nos

mamíferos, elas podem ser divididas em iniciadoras (2, 8, 9 e 10) e efetoras (3, 6 e 7) (OBENG et al., 2020).

Quando a via mitocondrial é ativada, proteínas pró-apoptóticas são liberadas após consequente formação do poro de permeabilização da membrana externa (MOMP), perda do potencial transmembrana mitocondrial e liberação do citocromo C (Cit c) do espaço intermembrana mitocondrial para o citoplasma. O citocromo c juntamente com Apaf-1 (fator de ativação de protease apoptótica-1) e pró-caspase-9 formam o apoptossoma (complexo apoptótico). Subsequentemente a pró-caspase-9 é clivada dentro deste complexo e libera a caspase 9 ativa, que posteriormente é capaz de ativar a pró-caspase-3, resultando na ativação das caspases efetoras (caspase 3/7) e iniciando apoptose (D'ARCY, 2019; REDZA-DUTORDOIR; AVERILL-BATES, 2016; SUHAILI et al., 2017) (Figura 8).

Figura 8 – Representação esquemática da via intrínseca de morte celular



Fonte: Adaptado de Larrubia et al (2013, p. 1882)

Ademais, a apoptose desempenha um importante papel na regulação da inflamação, pois muitos fatores e vias de sinalização que são ativados na resposta inflamatória estão envolvidos na regulação da apoptose (GREEN; LLAMBI, 2015; OPDENBOSCH; LAMKANFI, 2019; YANG et al., 2015). A participação da apoptose em doenças inflamatórias também tem sido

descrita, e desregulações no processo de morte celular têm sido relacionadas a doenças inflamatórias crônicas (GIRARDOT et al., 2017; REDZA-DUTORDOIR; AVERILL-BATES, 2016).

2.2.4 Inflamação

Como mencionado anteriormente, de acordo com a literatura existe uma relação entre a exposição aos poluentes atmosféricos e o desenvolvimento de respostas inflamatórias nos indivíduos expostos (FITCH et al., 2020; GAO et al., 2020; LEE et al., 2018). A inflamação é uma resposta essencial do sistema imune que visa manter a homeostase e sobrevivência dos organismos (BRASILEIRO FILHO, 2016). É um processo fisiológico que pode ser desencadeado frente a estímulos adversos, como patógenos (bactérias, vírus, fungos e parasitas), danos que alterem a integridade tecidual, como queimaduras, congelamento, radiação, necrose, traumas e cortes, ou decorrente da exposição a poluição por exemplo (AGHASAFARI; GEORGE; PIDAPARTI, 2019; FITCH et al., 2020; GAO et al., 2020; KUMAR; ABBAS; ASTER, 2013; LEE et al., 2018).

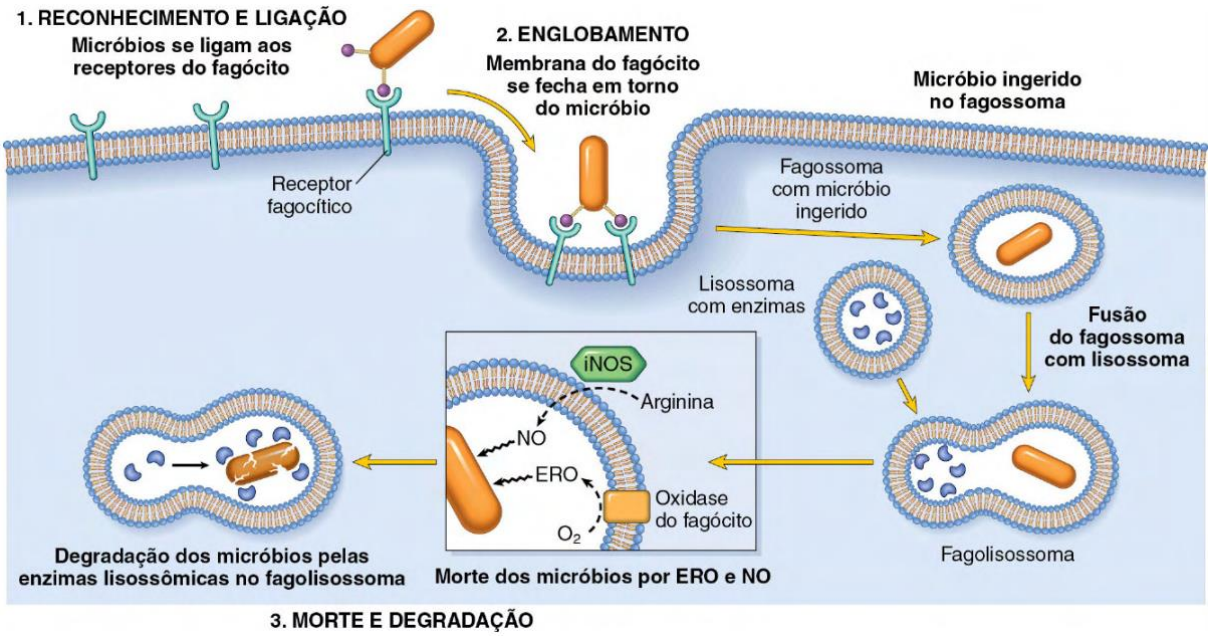
O processo inflamatório compreende uma rede de respostas organizadas e é caracterizada basicamente por eventos vasculares e celulares, que envolvem o recrutamento de células de defesa, plasma e fluidos para o local do dano, os quais atuam para a restauração da homeostase (ABDULKHALEQ et al., 2018). A inflamação pode ser aguda, quando é rapidamente resolvida pelo organismo; ou crônica, quando tem uma duração prolongada, podendo ser resultante de uma inflamação aguda, ou derivada de infecções persistentes, doenças inflamatórias autoimunes, ou ainda pela exposição prolongada a agentes potencialmente tóxicos (KUMAR; ABBAS; ASTER, 2013).

De modo geral, o mecanismo inflamatório envolve primeiramente o reconhecimento da lesão. As células do organismo detectam os estímulos nocivos através de receptores de reconhecimento de padrões (RRPs), principalmente receptores do tipo Toll (TRLs), presentes na membrana plasmática e nos endossomos (KUMAR; ABBAS; ASTER, 2013). Estes receptores identificam moléculas que podem ser provenientes de patógenos, as PAMPs, do inglês "*Pathogen-associated molecular patterns*", ou de células em estresse ou tecido danificado, as DAMPs "*Danger-associated molecular patterns*" (GOULOPOULOU; MCCARTHY; WEBB, 2016; ROH; SOHN, 2018). Deste modo, ocorre um reconhecimento de

ligantes e os TRLs ativam vias de transdução de sinal que levam principalmente à translocação nuclear do fator de transcrição NF-κB (fator nuclear kappa-B). NF-κB induz a produção e liberação de mediadores pró-inflamatórios, como as citocinas interleucina-1-beta (IL1β), IL-6, IL-8, fator de necrose tumoral-alpha (TNF-α), entre outros (KANY; VOLLRATH; RELJA, 2019). Os mediadores inflamatórios estimulam o recrutamento das células de defesa, os leucócitos (monócitos e neutrófilos) para o local da lesão, onde elas então realizam a fagocitose (KUMAR; ABBAS; ASTER, 2013).

A fagocitose é muito importante e durante este processo ocorre o reconhecimento e fixação da partícula (do patógeno ou de restos celulares) ao leucócito; o engolfamento da partícula e a formação de um vacúolo fagocítico (fagossoma); e posteriormente a destruição e degradação do material ingerido. Nesta última etapa, ocorre a fusão do fagossoma com o lisossoma formando assim o fagolisossoma. Quando isto ocorre, as partículas danosas são expostas a importantes mecanismos de destruição presentes no lisossoma (KUMAR; ABBAS; ASTER, 2013) (Figura 9).

Figura 9 – Representação esquemática do processo de fagocitose durante a inflamação



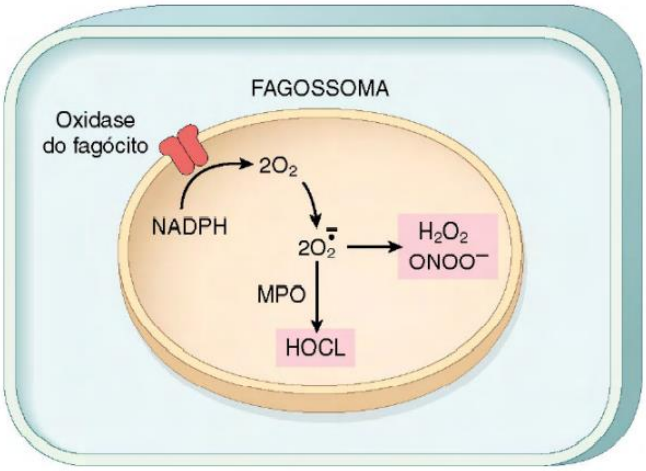
Fonte: KUMAR; ABBAS; ASTER (2013, p. 39).

O processo de fagocitose leva a uma condição denominada surto oxidativo, caracterizada por um súbito aumento do consumo de oxigênio, do catabolismo do glicogênio, aumento da oxidação da glicose e da produção de ERO dentro do fagolisossoma (EL-BENNA

et al., 2016; KUMAR; ABBAS; ASTER, 2013). Essas ERO são geradas a partir da enzima NADPH-oxidase (Oxidase do fagócito), que usa NADPH (fosfato de dinucleotídeo de nicotinamida-adenina reduzido) citosólico para transferir elétrons via FAD para o oxigênio, assim formando o radical $O_2^{\bullet-}$. Este radical $O_2^{\bullet-}$ pode ser dismutado espontaneamente em H_2O_2, o qual pode reagir com o Fe^{2+} e formar o radical $OH^{\bullet}$ através de reção de Fenton (EL-BENNA et al., 2016) (Figura 9). Além disso, o lisossoma contém a enzima Mieloperoxidase (MPO), a qual catalisa reações do H_2O_2 com o Cloro (Cl^-), a fim de formar o oxidante mais tóxico, o ácido hipocloroso (HOCl) (EL-BENNA et al., 2016; KUMAR; ABBAS; ASTER, 2013).

Conforme demonstrado na figura 9, o NO também pode desempenhar um importante papel na resposta inflamatória, pois participa dos processos de vasodilatação, relaxamento do músculo liso e neurotransmissão (CINELLI et al., 2020). O NO é formado por uma família de enzimas conhecidas como NOS (óxido nítrico sintases) através da oxidação do aminoácido L-arginina a L-citrulina, utilizando oxigênio e NADPH como co-fatores necessários. Existem três isoformas de NOS, as quais incluem a nNOS ou NOS1 (NOS neuronal), a iNOS ou NOS2 (NOS induzível), e a eNOS ou NOS3 (NOS endotelial). Todas as isoformas podem ser encontradas em uma variedade de tecidos e de células (CINELLI et al., 2020; PAPI; AHMADIZAR; HASANVAND, 2019). Portanto, durante o processo inflamatório, NO pode ser induzido por citocinas e mediadores inflamatórios nos leucócitos e células endoteliais, podendo reagir com o radical $O_2^{\bullet-}$ e formar o $ONOO^{-\bullet}$, um agente altamente reativo que também participa da lesão celular (Figura 10).

Figura 10 – Representação esquemática da síntese de $O_2^{\bullet-}$, HOCl e $ONOO^{-\bullet}$ no fagossoma durante a inflamação



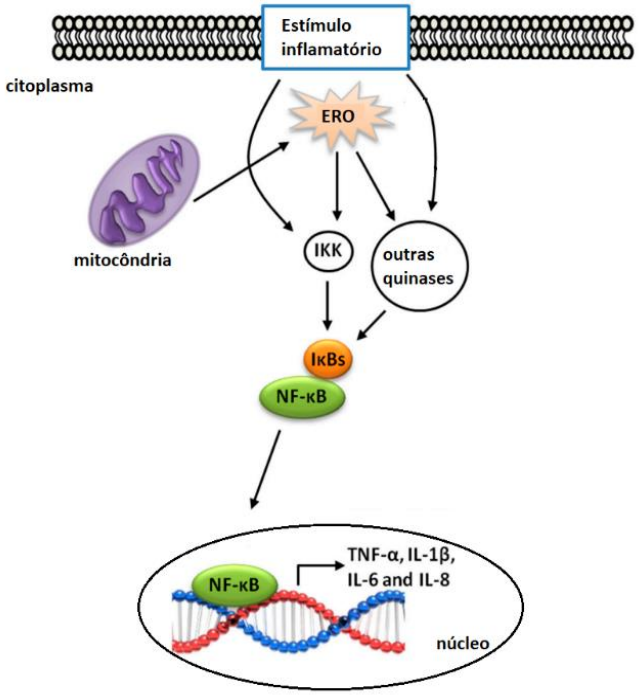
Fonte: KUMAR; ABBAS; ASTER (2013, p. 14).

Normalmente a produção de ERO é controlada na inflamação, pois após o surto oxidativo, mecanismos antioxidantes como CAT, SOD e GSH desempenham seus papéis nos tecidos e no sangue (KUMAR; ABBAS; ASTER, 2013). Em baixos níveis, as espécies reativas atuam na destruição do agente lesivo e podem aumentar a expressão de moléculas de adesão, citocinas e quimiocinas, e amplificar a cascata de mediadores inflamatórios. Porém, em condições patológicas inflamatórias, ERO podem ser geradas em excesso, e podem se difundir para fora das células fagocíticas, e, portanto, induzir estresse oxidativo localizado e lesão tecidual. Por consequência, as ERO geradas pelas respostas inflamatórias também podem estimular vias que levam a amplificação da inflamação. Deste modo, o estresse oxidativo e inflamação são processos que se relacionam, pois as ERO são capazes de induzir um processo inflamatório (BISWAS, 2016; CHATTERJEE, 2016; LUGRIN et al., 2014).

Além disso, fatores de transcrição, como o NF-κB, também pode ser modulado por ERO. Além do NF-κB desempenhar um papel crítico na regulação de genes envolvidos em respostas imunes e inflamatórias, este fator de transcrição também contribui na diferenciação celular, proliferação e apoptose, visto que pode levar a expressão de proteínas caspases através da ativação da proteína p53 (CARRÀ et al., 2020; LIU; CHEN, 2017). Em condições fisiológicas, o NF-κB permance ligado à subunidade inibitória IκB no citoplasma, no entanto, na presença de um estímulo (citocinas, fatores de crescimento, mitógenos, componentes microbianos, contaminantes do ar), a proteína IκB é fosforilada e degradada, permitindo a migração de NF-κB para o núcleo, promovendo a transcrição de genes envolvidos na inflamação (LI et al., 2019; LIU; CHEN, 2017; YOSHIDA et al., 2009) (Figura 11).

As proteínas MAPK (proteínas quinases ativadas por mitógenos) também podem ser moduladas por ERO, e em resposta a exposição a poluentes atmosféricos (MCCULLOUGH et al., 2014; REZATABAR et al., 2018). As MAPK compreendem um grupo de proteínas específicas de Serina/Treonina que requerem fosforilação destes resíduos para se tornarem ativas. Existem três vias principais altamente reguladas, a JNK1/2 (c-Jun N-terminal quinase), a $p38^{MAPK}$ e a ERK1/2 (Quinase Regulada por Sinais Extracelulares) (KIM; CHOI, 2010; MORRISON, 2012; SUN et al., 2015).

Figura 11 – Representação esquemática do mecanismo de NF-κB



Fonte: Adaptado de Minatel et al (2016, p. 10)

Resumidamente, sinais extracelulares ou intracelulares podem ativar as vias de sinalização MAPK através de uma proteína GTPase e/ou da fosforilação de proteínas quinases que se encontram a jusante de receptores de membrana celular, como receptores de tirosina quinases (RTKs) e receptores acoplados à proteína G (GPCRs) (KIM; CHOI, 2010; MELE; JOHNSON, 2020; MORRISON, 2012). As três vias MAPK exercem seus efeitos através da fosforilação e ativação de proteínas sequenciais em suas cascatas de quinases, partindo das MAPK quinase quinases (MKKKs), que fosforilam e ativam MAPK quinases (MKKs), as quais por sua vez fosforilam e ativam MAPKs (CUENDA; ROUSSEAU, 2007). Após ativadas, as três vias MAPK fosforilam substratos específicos no citoplasma ou no núcleo, regulando a proliferação, diferenciação, sobrevivência e morte celular, além de respostas inflamatórias (GUO et al., 2020; KIM; CHOI, 2010).

Cada uma destas cascatas de sinalização responde a diferentes sinais e e contribui para uma série de processos biológicos nos vertebrados. Particularmente, as vias JNK e $p38^{MAPK}$ pertencem a um grupo de proteínas quinases ativadas por estresse (SAPKs). Sendo assim, o estresse oxidativo, danos ao DNA, citocinas inflamatórias e fatores de crescimento podem induzir a ativação da via JNK. Esta via desempenha um papel importante na proliferação,

sobrevivência, migração celular, apoptose, atividade neuronal, sinalização de insulina e inflamação (HAMMOUDA et al., 2020; PLOTNIKOV et al., 2011). Já a via $p38^{MAPK}$ é ativada principalmente em resposta a estresse ambiental e citocinas inflamatórias. A ativação desta via auxilia em processos de apoptose, senescência celular, sobrevivência e inflamação (CUENDA; ROUSSEAU, 2007; PLOTNIKOV et al., 2011). Enquanto que a via ERK é induzida por fatores de crescimento e mitógenos (sinais extracelulares que induzem o crescimento e a diferenciação celular), e participa do desenvolvimento embrionário do sistema nervoso central (SNC) e na regulação da função cerebral de adultos, além de contribuir para a proliferação, diferenciação, sobrevivência celular, inflamação, e, em algumas condições, em resposta ao estresse e apoptose (GUO et al., 2020; MORRISON, 2012; SUN; NAN, 2017; WORTZEL; SEGER, 2011).

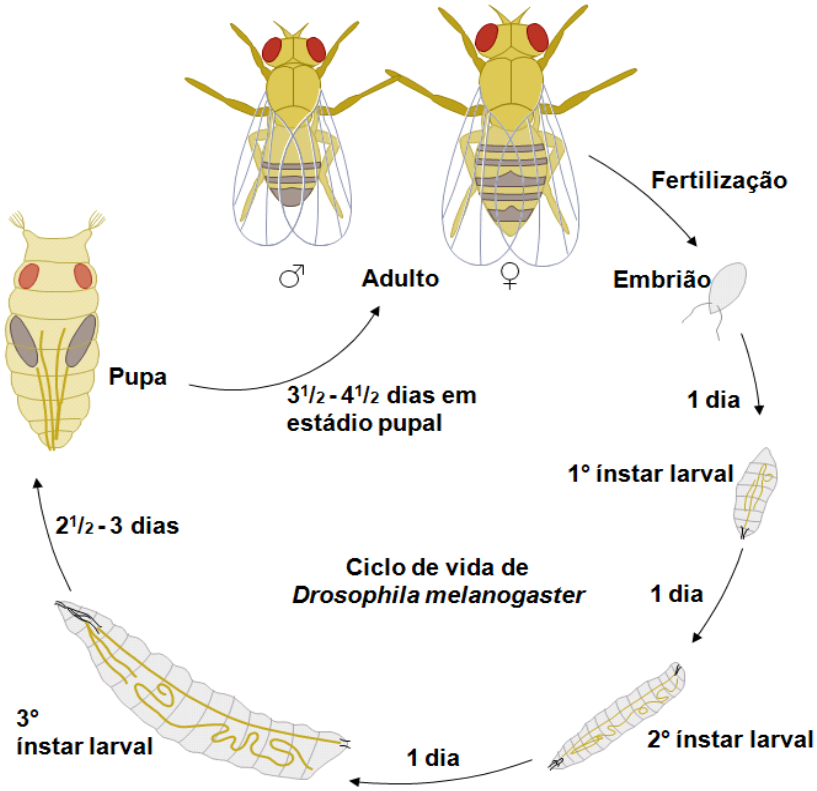
2.3 *Drosophila melanogaster*

Drosophila melanogaster (Figura 12) comumente conhecida como a “mosca da fruta”, é um poderoso organismo modelo que se destaca devido às vantagens que apresenta. É um inseto holometábolo, pertencente à ordem Diptera, família Drosophilidae, e ao gênero *Drosophila*. Seu ciclo de vida é rápido, e em condições ideais de temperatura (25±1ºC) e umidade relativa do ar (60%) necessita de cerca de 10-12 dias para ser completado (Figura 13). É facilmente replicada dentro de espaços pequenos e apresenta uma alta taxa de fecundidade, obtendo-se uma prole abundante em um curto período de tempo, além de possuir um dimorfismo sexual aparente (YAMAGUCHI; YOSHIDA, 2018).

Figura 12 – Exemplar adulto de *Drosophila melanogaster*

Fonte: https://kxci.org/podcast/drosophila-melanogaster/

Figura 13 – Ciclo de vida de *Drosophila melanogaster*



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Embora *D. melanogaster* e os seres humanos sejam organismos que estejam distantemente relacionados evolutivamente, o genoma de *D. melanogaster* compartilha aproximadamente 60% de homologia com o genoma humano, incluindo cerca de 75% de homologia com genes relacionados a doenças humanas (UGUR; CHEN; BELLEN, 2016; YAMAGUCHI; YOSHIDA, 2018). Portanto, *D. melanogaster* possui vias metabólicas e genes homólogos aos mamíferos que estão envolvidos em importantes processos biológicos, como em respostas inflamatórias, imunológicas e ao estresse, além da morte celular apoptótica. Entre os genes homólogos identificados em *Drosophila*, e implicados em tais processos podem-se destacar os que codificam as enzimas antioxidantes SOD (Sod1 e Sod2), GST (GstD1-8, GstS1 e GstE12) e CAT (MACKAY; BEWLEY, 1989; MISHRA, 2019; PAUL et al., 2007; SAISAWANG; WONGSANTICHON; KETTERMAN, 2012; SMITH et al., 2019); as proteínas quinases ativadas por mitógenos, $p38^{MAPK}$ (p38a e p38b), ERK (rolled) e JNK (Basket)

(BITEAU et al., 2011; BORNSTEIN et al., 2015; HAN et al., 1998; OELLERS; HAFEN, 1996; SHILO, 2014; VRAILAS-MORTIMER et al., 2011); o gene da NOS (dNOS1) (STASIV et al., 2001) e os três homólogos do fator de transcrição NF-κB (Dorsal, Dif e Relish) (CHOWDHURY et al., 2019). Já os componentes da morte celular apoptótica incluem as proteínas Dronc, Dredd e Strica (semelhantes às caspases 9 e 8 iniciadoras de mamíferos) e Dcp-1, Drice, Decay e Damm (semelhantes às caspases efetoras de mamíferos 3 e 7) (CLAVIER et al., 2016; KIM et al., 2014; WHITE et al., 2017).

Além disso, da mesma forma que ocorre nos vertebrados, fêmeas e machos de *Drosophila* podem apresentar diferenças em aspectos como a longevidade e em mecanismos envolvidos em respostas imunológicas e ao estresse (BELMONTE et al., 2020; BUCHON; SILVERMAN; CHERRY, 2014). Assim como os humanos, *Drosophila* apresenta uma relação entre problemas imunológicos e hormonais, e é possível que os cromossomos X e Y e o hormônio juvenil (JH) estejam implicados no dimorfismo sexual que é observado nas respostas imunes da mosca (BELMONTE et al., 2020; MILLINGTON; RIDEOUT, 2018). Tais fatos permitem que *Drosophila* seja um modelo interessante para elucidar diferentes mecanismos envolvidos em uma variedade de doenças humanas, como a asma e distúrbios inflamatórios (ROEDER; ISERMANN; KABESCH, 2009), além de ser útil para a investigação de diferenças relacionadas ao sexo em determinadas patologias. Ainda, *D. melanogaster* tem sido utilizada no estudo dos efeitos da exposição a contaminantes ambientais, como o material particulado e compostos orgânicos voláteis, incluindo o 1-octen-3-ol (EOM et al., 2017; INAMDAR; MASUREKAR; BENNET, 2010; WANG et al., 2017).

3. Justificativa

A exposição aos poluentes atmosféricos tem se tornado uma problemática ambiental, pois eles afetam tanto a qualidade do ar dos ambientes externos como dos internos, além de prejudicar a saúde dos indivíduos expostos. Os compostos orgânicos voláteis fúngicos, sobretudo o 1-octen-3-ol, estão entre os principais poluentes do ar interno, e pesquisas têm correlacionado à sua presença ao desenvolvimento de sintomas alérgicos e inflamatórios. Além disso, diferenças relacionadas ao sexo têm sido observadas quanto ao surgimento de tais sintomas nos habitantes destes espaços. O 1-octen-3-ol em particular, é responsável pelo odor característico do mofo e comumente encontrado em locais mofados e infiltrados. Devido as associações entre a presença do 1-octen-3-ol e aos sintomas inflamatórios observados nos

habitantes, há necessidade de estudos que melhor esclareçam as relações de toxicidade deste composto. Neste sentido, a padronização de um modelo experimental, a fim de compreender os mecanismos de atuação do composto 1-octen-3-ol em ambos os sexos e sobre aspectos fundamentais envolvidos na resposta inflamatória se torna indispensável. Desta forma, nosso estudo corrobora para este fim, pois busca contribuir com a elucidação de importantes alvos bioquímicos e moleculares em fêmeas e machos de *Drosophila melanogaster* frente a exposição inalatória ao 1-octen-3-ol.

4. Objetivos

4.1 Objetivo geral

Avaliar os mecanismos bioquímicos e moleculares envolvidos na exposição inalatória ao composto orgânico volátil fúngico 1-octen-3-ol em fêmeas e machos de *Drosophila melanogaster*.

4.2 Objetivos específicos

- Comparar o efeito da exposição inalatória ao 1-octen-3-ol sobre a sobrevivência e desempenho locomotor entre fêmeas e machos de *D. melanogaster*;

- Investigar o efeito da exposição inalatória ao 1-octen-3-ol sobre a atividade das enzimas antioxidantes em *D. melanogaster* fêmeas e machos;

- Investigar o efeito da exposição inalatória ao 1-octen-3-ol sobre a atividade de caspases 3/7 em *D. melanogaster* fêmeas e machos;

- Investigar o efeito da exposição inalatória ao 1-octen-3-ol sobre o nível de fosforilação da proteína quinase ERK em *D. melanogaster* fêmeas;

- Investigar os níveis de NO em *D. melanogaster* fêmeas e machos após exposição inalatória ao 1-octen-3-ol;

- Verificar a presença ou ausência de alterações a nível transcricional dos genes $p38^{MAPK}$, JNK, NF-κB e p53 em *D. melanogaster* fêmeas após exposição inalatória ao 1-octen-3-ol.

- Analisar se há alterações morfológicas e sobre a taxa bioenergética mitocondrial de *D. melanogaster* fêmeas após exposição inalatória ao 1-octen-3-ol;

- Investigar o efeito da exposição inalatória ao 1-octen-3-ol sobre a viabilidade celular de *D. melanogaster* machos;

- Investigar o efeito da exposição inalatória ao 1-octen-3-ol sobre a produção de espécies reativas de oxigênio de *D. melanogaster* machos.

5. Resultados

Os resultados que fazem parte desta tese estão apresentados sob a forma de manuscritos. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas, encontram-se nos manuscritos:

5.1 Capítulo I - Fungal compound 1-octen-3-ol induces mitochondrial morphological alterations and respiration dysfunctions in *Drosophila melanogaster*.

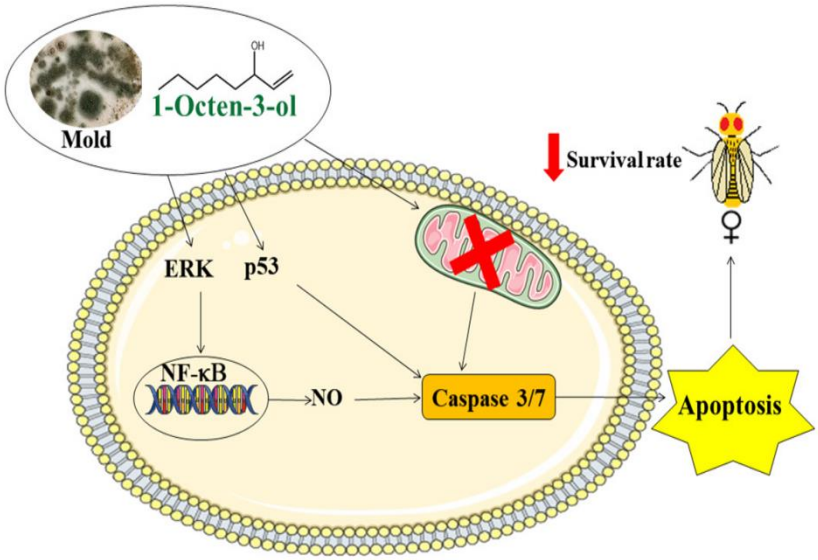
Artigo publicado na revista Ecotoxicology and Environmental Safety.

5.2 Capítulo II - The fungal indoor air pollutant 1-octen-3-ol induces ROS and inhibits nitric oxide and cell viability in males of *Drosophila melanogaster.*

Os resultados do capítulo II estão dispostos em um manuscrito na forma em que foram submetidos para publicação na revista científica internacional Experimental and Clinical sciences eISSN 1611-2156.

5.1 Capítulo I - Fungal compound 1-octen-3-ol induces mitochondrial morphological alterations and respiration dysfunctions in *Drosophila melanogaster.*

Graphical abstract:



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Fungal compound 1-octen-3-ol induces mitochondrial morphological alterations and respiration dysfunctions in *Drosophila melanogaster*

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ABSTRACT

Fungal volatile organic compounds (VOCs) comprise a group of compounds commonly found in damp or water-damaged indoor places affecting air quality. Indoor fungal pollution is a severe threat to human health, contributing to the onset of allergic diseases. The compound 1-octen-3-ol, known as "mushroom alcohol", is the most abundant VOC and confers the characteristic mold odor. Exposure to 1-octen-3-ol induces inflammatory markers and episodes of allergic rhinitis and conjunctivitis; however, the effects of this compound towards mitochondria are fairly known. The present study aimed to evaluate the effects of 1-octen-3-ol on inflammatory targets and on mitochondrial morphology and bioenergetic rate in *D. melanogaster*. Drosophilas were exposed by inhalation to 2.5 µL/L and 5 µL/L of 1-octen-3-ol for 24 h. Observation showed a decreasing in the survival and locomotor ability of flies. Superoxide dismutase (SOD) activity was induced whereas Catalase (CAT) activity was inhibited. Analysis of the mitochondria respiration, detected inhibition of complex I and II in the electron transport chain and a decreased bioenergetic rate. Electronic microscopy provided morphological insights of the mitochondrial status in which a disarrangement in mitochondrial cristae profile was observed. 1-Octen-3-ol induced increased activity of caspase 3/7 and ERK phosphorylation. The mRNA relative steady-state levels of $p38^{MAPK}$ and JNK were down-regulated, whereas NF-κB and p53 were up-regulated. In parallel, nitrite levels were induced in relation to the non-exposed group. These findings point to the mitochondria as a crucial target for the toxicity of 1-octen-3-ol in parallel with activation of pro-inflammatory factors and apoptotic signaling pathway cascade.

1. Introduction

Volatile organic compounds (VOCs) are carbon-based substances that are highly evaporative, with low molecular weight and low water solubility. These compounds dissipate rapidly at room temperature and pressure and are capable of damaging human health and the environment (Kamal et al., 2016). VOCs are released from different sources, including anthropogenic and biogenic emissions. Anthropogenic VOCs comprise industrial activities, such as combustion and evaporation of petroleum-based products, paint thinners, cleaning agents, wall covering among others. In contrast, biogenic VOCs are naturally generated by the metabolism of living organisms such as plants, bacteria, and fungi (Bennett and Inamdar, 2015).

Fungi comprise a diverse group of organisms able to grow in almost any environment. These organisms produce different chemical classes of VOCs, including hydrocarbons, acids, aldehydes, ketones, terpenes and alcohols. These fungal VOCs can quickly diffuse and accumulate, especially in damp or water-damaged indoor places, impoverishing the air quality (Bennett and Inamdar, 2015). Indoor fungal pollution has been considered a severe threat to public health, contributing to the emergence of the "sick building syndrome" (SBS). This condition is characterized by a series of symptoms, such as headache, eye, nose or throat irritation, dry cough, allergies and increased incidence of asthma in the inhabitants

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of these spaces (AL-Maliki et al., 2017). Asthma is a prevalent chronic inflammatory airway disease and several factors including mitochondrial dysfunction and oxidative stress may contribute to its onset (Liu and Chen, 2017).

The most abundant VOC within these water-damaged environments is the C-8 compound 1-octen-3-ol, known as "mushroom alcohol", released by many fungal genera such as *Aspergillus*, *Penicillium*, *Alternaria*, *Cladosporium*, *Mucor* and *Ulocladium* (Bennett and Inamdar, 2015). 1-Octen-3-ol is responsible for the characteristic odor of mold and can be considered an indicator of fungal growth indoors (Inamdar et al., 2012). Exposure to this compound has been associated with toxicity both in humans and in the invertebrate model *Drosophila melanogaster*. Voluntary human exposure to 1-octen-3-ol for 2 h at (10 mg/m^3) induced eye irritation, mild nausea, headache and increased inflammatory markers in nasal secretions (Wålinder et al., 2008). Also, a study demonstrated positive correlations between 1-octen-3-ol levels and the prevalence of allergic rhinitis and conjunctivitis in exposed people (Araki et al., 2012). In *D. melanogaster*, 1-octen-3-ol was able to induce locomotor damage, lipid peroxidation, developmental delay, apoptosis cellular death, neurotoxicity, and nitric oxide-mediated inflammatory response (Inamdar et al., 2014, 2013; 2010; Inamdar and Bennett, 2014; Yin et al., 2015).

D. melanogaster, commonly known as the "fruit fly", is a powerful model organism that has been successfully used to understand mechanisms involved in human disease, inclusive asthma, and inflammatory disorders, as well as to explore the toxicity of various compounds such as atmospheric pollutants (Eom et al., 2017; Hammer et al., 2018; Wang et al., 2017).

Studies have suggested that exposure to air pollutants contributes to changes in mitochondrial function and morphology (Boovarahan and Kurian, 2018). Mitochondria play a critical role in survival, apoptosis as well as inflammatory responses. Also, functional and morphological alterations in its structure may contribute to the development of allergic inflammatory diseases such as asthma (Liu and Chen, 2017). However, as far as we know, the mechanisms of action of VOCs towards mitochondrial morphological and functional aspects are scarce.

Despite that, the cellular biochemical mechanisms involved in the action of 1-octen-3-ol toxicity remain unclear. Considering that indoor fungal pollution has been strongly associated with negative effects to human health, here we investigated the action of 1-octen-3-ol on inflammatory targets, and mitochondrial bioenergetics and morphology using *Drosophila melanogaster* as a model organism.

2. Materials and methods

2.1 Chemicals reagents

The racemic form of 1-octen-3-ol (≥98.0%) (68225) was purchased from Sigma-Aldrich® (Sao Paulo, SP). Apo-ONE˜ ® Homogeneous Caspase 3/7 Assay (G7790) and Griess Reagent System (G2930) were obtained from Promega (Madison, WI). DNAse I Amplification Grade (18068015) was purchased from Invitrogen™ by Thermo Fisher Scientific (Waltham, MA) and iScript™ cDNA Synthesis kit (1708891) was obtained from Biorad (Hercules, CA). All other reagents were commercial products purchased with highest purity available. Other materials, including food ingredients, were from standard commercial suppliers.

2.2 Drosophila melanogaster stock and exposure to 1-octen-3-ol

The Harwich strain of *Drosophila melanogaster* was obtained from our own breeding stock. The culture of flies was maintained at 25 ± 1 °C, 12 h dark-light photoperiod and 60–70% relative humidity, and reared on food as previously described by Gomes et al. (2020). Flies were exposed through inhalation to the compound 1-octen-3-ol. For this purpose, glass flasks (100 cm^3, 50 mm × 85 mm) were cover with parafilm (Parafilm® M) and in the inner side of parafilm cap was fixed a layer of filter paper. 1-Octen-3-ol diluted in DMSO (Dimethyl sulfoxide) in a final concentration of 0.0020% was applied on filter paper. Flasks were kept on an orbital shaker for 5 min for 1-octen-3-ol vaporization. Along all exposure period, a piece of cotton soaked with 1.5 mL of 1% sucrose solution was present on the bottom glass flasks as a food supply. The final concentration values of 1-octen-3-ol and DMSO were calculated based on the volume of the vial. Groups of thirty female and male adult flies (1–5 days old) were separately exposed by inhalation up to 48 h to 1-octen-3-ol (1 µL/L, 2.5 µL/L and 5 µL/L), vehicle (DMSO 0.0020%) or distilled water (control), pipetted on a filter paper layer. The concentrations of 1-octen-3-ol used in this work were based on a concentration curve where the concentrations able to cause less than 50% of mortality (2,5 µL/L and 5 µL/L) were chosen for further studies. LC_{50} found by PROBIT analysis was 12.49 µL/L for thirty female flies after 24 h of exposure. A total of twelve biological replicates were used per group (n = 12).

2.3 Survival and locomotor performance assays

For survival curve, the number of live and dead flies was registered each 24 h and LC_{50} of the flies were determined by PROBIT analysis. The behavioral test was determined using the Negative Geotaxis as previously described by Gomes et al. (2020). Groups of 10 flies (male and female separately) were exposed to 1-octen-3-ol (2.5 µL/L and 5 µL/L) for 24 h and 48 h, then the ability of flies to reach the top (5 cm) of the flask was recorded after 6 s. A total of three biological replicates were used per group (n = 3). Taking into account similar results obtained for female and male groups in behavioral and survivorship tests, only female and the period of 24 h were used for further experiments.

2.4 Assays of antioxidants enzymes

The activity of catalase (CAT), glutathione-S-transferase (GST) and superoxide dismutase (SOD) enzymes were measured as previously described by Costa-Silva et al. (2018). Groups of twenty flies (control, DMSO, 2.5 µL/L and 5 µL/L) were homogenized in 1000 µL of 20 mM HEPES buffer pH 7.0 and centrifuged at 20,000×*g* for 30 min at 4 °C. All assays were performed at room temperature using the Agilent Cary 60 UV/VIS® spectrophotometer (Santa Clara, CA). A total of nine biological replicates were used per group (n = 9). Results were normalized according to the protein concentration of the samples and expressed as percentage of control.

2.5 Caspase assay

The cell death by apoptosis was evaluated following to the manufacturer's suggested protocol (Apo-ONE® Homogeneous Caspase 3/7 Assay) according Saraiva et al. (2018). The fluorescence emission of supernatants from groups of twenty flies (control, DMSO, 2.5 µL/L and 5 µL/L) were monitored at regular intervals of 20 min at 485nm$_{ex}$/530nm$_{em}$. The assay was read in an IS 400 MM Pro Bruker Imaging System® (Billerica, MA). The wells density was quantified using the Scion Image® Software. Results were normalized according to the protein concentration and expressed as percentage of control. A total of six biological replicates were used per group (n = 6).

2.6 Nitric oxide levels

Nitric oxide (NO) was determined by measuring nitrite (NO^{2-}) following the indications of the Griess reagent system manufacturer (Promega® Madison, WI) according to Saraiva et al. (2018) with slight modification. Groups of twenty flies (control, DMSO, 2.5 µL/L and 5 µL/L) were homogenized in 500 µL KPi pH 7.4 buffer and centrifuged at 10,000×*g* for 10 min at 4 °C. The absorbance was read at 535 nm using an EnsPire® multimode plate reader PerkinElmer (Waltham, MA). Results were expressed as a percentage of the control group. A total of three biological replicates were used per group (n = 3).

2.7 Gene expression analysis

The effect of 1-octen-3-ol on gene expression of *D. melanogaster* was analyzed by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). The relative steady-state level of mRNA of $p38^{MAPK}$, JNK, NF-κB and p53 genes (described in Table 1) was analyzed. Groups of twenty flies (control and 5 µL/L) were homogenized in 500 µL of TRI-Reagent and approximately 1 µg of total RNA of each sample was treated with DNase I (DNAseI Amplification Grade), and the cDNA (iScript™ cDNA Synthesis Kit) of the samples was synthesized. qRT-PCR was performed as previously described by Saraiva et al. (2018) using a thermal cycler BIO RAD CFX 96 Touch (Biorad®). The mRNA expression levels of the genes of interest were normalized with the GPDH (Glyceraldehyde-3-phosphate dehydrogenase) reference endogenous gene. A total of three biological replicates were used per group (n = 3).

2.8 Western blotting analysis

Quantification of the phosphorylation of ERK1/2 and β-actin expression were performed by Western blotting as previously described by Macedo et al. (2017). Groups of forty flies (control, DMSO, 2.5 µL/L and 5 µL/L) were homogenized in 200 µL of specific buffer (pH 7.0) and centrifuged at 3000×*g* for 10 min at 4 °C. Subsequently the membranes were incubated overnight (4 °C) with primary antibody produced in rabbit anti-phosphorylated ERK1/2 and anti-β-actin HRP conjugated. Following incubation, membranes were washed in Tris-buffered saline with Tween and incubated for 1 h in room temperature with secondary specific antibody (peroxidase-linked anti-IgG). The blottings were visualized on the IS 4000 MM Pro Bruker imaging system® (Billerica, MA) using ECL-detection reagent, and the band density was quantified using the Scion Image® software. Results were expressed as a percentage of the control group. A total of five biological replicates were used per group (n = 5).

2.9 High-resolution respirometry and transmission electron microscopy assays

The righ-resolution respirometry (HRR) assay followed the protocol previously described by Carvalho et al. (2017). Changes in the oxygen flux through mitochondrial complexes, mitochondrial bioenergetic patterns and mitochondrial membrane integrity were performed using groups of fifty flies (control and 5 µL/L). All experiments were performed at 24 °C using Oxygraph-2k and DatLab 4.0 software (O2k, Oroboros Instruments, Innsbruck, Austria), with continuous stirring at 750 rpm. A total of eight biological replicates were used per group (n=8). The effect of 1-octen-3-ol on mitochondrial ultrastructure of *D. melanogaster* was evaluated by transmission electron microscopy (TEM). Groups of fifty flies (control and 5 µL/L) were prepared as HRR sample and fixed for 2 h 30 min with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2).

Table 1
Genes tested by quantitative real-time RT-PCR analysis and forward and reverse primers.

Gene		Primer sequences
GPDH	LEFT RIGHT	5' – GCTCCTCAATGGTTTTTCCA 5' – ATGGAGATGATTCGCTTCGT
p38 MPK2b	LEFT RIGHT	5' – GTCCTCGTTTACCGCAATGT 5' – CGCCGATCTGAACAACATAA
JNK	LEFT RIGHT	5' – ATGGATATGGCCACGCTAAG 5' – CTTTCTGTGCCTGGTGAACA
NF-κB	LEFT RIGHT	5' – TGTGCTTTCTCTTGCCCTTT 5' – CCGCAGAAACCAGAGAGTTC
p53	LEFT RIGHT	5' - GCTTGGGGCACGTACATATT 5' - ACATGAGCCGGTCTGTAACC

The samples were post-fixed for 2 h in 1.0% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.2) and dehydrated in acetone. After, the samples were embedded in EmBed1 812-DER736 resin and ultrathin sections were cut and stained with uranyl acetate and lead citrate for TEM visualization in JEOL 1210 microscope.

2.10. Statistical analysis

Lifespan measurement was determined by comparing the survival curves with a log-rank (Mantel–Cox) test. Normality tests (Kolmogorov Smirnov, *D'Agostino-Pearson omnibus* and Shapiro-Wilk) were performed. Parametric data were analyzed by analysis of variance by One- Way or Two-Way ANOVA and Tukey's *post hoc* test, or Unpaired T Test. LC_{50} was calculated using PROBIT analysis. Results were expressed as mean ± standard error (S.E.M). Results were considered statistically significant when $p < 0.05$.

3. Results

3.1. Exposure to 1-octen-3-ol induces mortality and locomotor deficits in D. melanogaster

After 24 h of treatment with 5 µL/L of 1-octen-3-ol it was observed a decreasing of 19% and 45% in the survival of female and male flies respectively. After 48 h, females and males exposed with 2.5 µL/L showed a reduction of 10% and 30% in survival respectively, and exposure to 5 µL/L caused a decreasing in 50% and 76% in fly survival respectively (Fig. 1A and B). The survival curve data was used for calculation of LC_{50} after 24 h of exposure by PROBIT analysis and a value of 12.35 µL/L for females and 5.41 µL/L for males was obtained. 1- Octen-3-ol induced locomotor deficits after exposure to the highest concentration of 1-octen-3-ol from 24 h (control group: 8.7 flies in the top for females and 8.8 flies for males; group 5 µL/L: 6.3 flies in females and 5.4 in males) (Fig. 1C), this effect was maintained after 48 h of exposure (Fig. 1D) (control group: 9 flies in the top for female and 8.6 flies for male, group 5 µL/L: 5.9 flies in female and 5.3 in male group).

3.2. 1-Octen-3-ol modulates activity of antioxidant enzymes of D. melanogaster

Only the concentration of 2.5 µL/L of 1-octen-3-ol caused a reduction of 37% in CAT activity (Fig. 2A). SOD activity was stimulated in 92% and 97% at 2.5 µL/L and 5 µL/L groups, respectively (Fig. 2B). No changes in GST enzyme activity was observed (Fig. 2C).

3.3. 1-Octen-3-ol induces apoptosis cell death and ERK phosphorylation in D. melanogaster

Exposure to 1-octen-3-ol for 24 h was able to induce a significant increase of 70% (2.5 µL/L) and 68% (5 µL/L) in caspase 3/7 activity in female flies compared to the control group (Fig. 3A). Phosphorylation of ERK was stimulated by 1-octen-3-ol in 78% at 2.5 µL/L and 160% at 5 µL/L (Fig. 3B). No alteration in B-actin expression was observed (Fig. 3C).

3.4. 1-Octen-3-ol modulates the nitrite levels and relative steady-state levels of mRNA in D. melanogaster

The compound 1-octen-3-ol induced an increasing of 29% in nitrite levels at 5 µL/L (Fig. 4A). qRT-PCR analysis revealed a significant reduction in relative steady-state levels of $p38^{MAPK}$ (0.52 fold) and JNK (0.28 fold), while a stimulation in relative steady-state levels of NF-κB (0.63 fold) and p53 (0.78 fold) was observed (Fig. 4B).

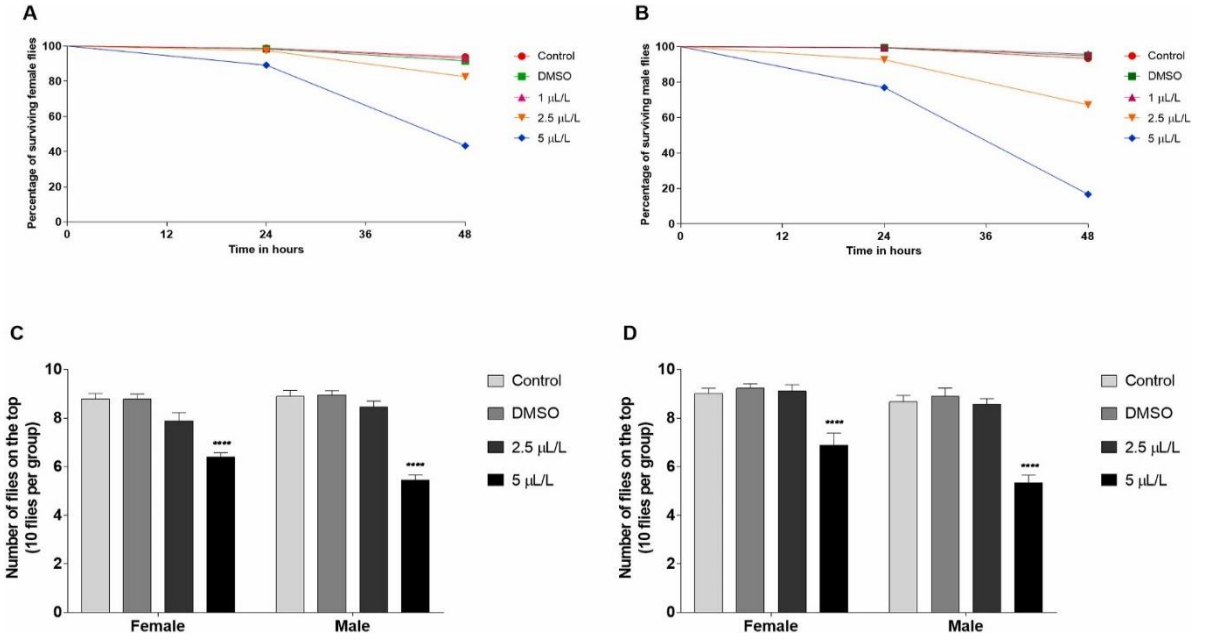

Fig. 1. Survivalship of (A) females and (B) males of *D. melanogaster* after exposure to 1-octen-3-ol (1 µL/L, 2.5 µL/L and 5 µL/L) for 48 h and locomotor performance of females and males of *D. melanogaster* exposed for (C) 24 h and (D) 48 h to 1-octen-3-ol (2.5 µL/L and 5 µL/L). Results are represented as mean ± S.E.M. ****indicates statistical difference from control ($p < 0.0001$).

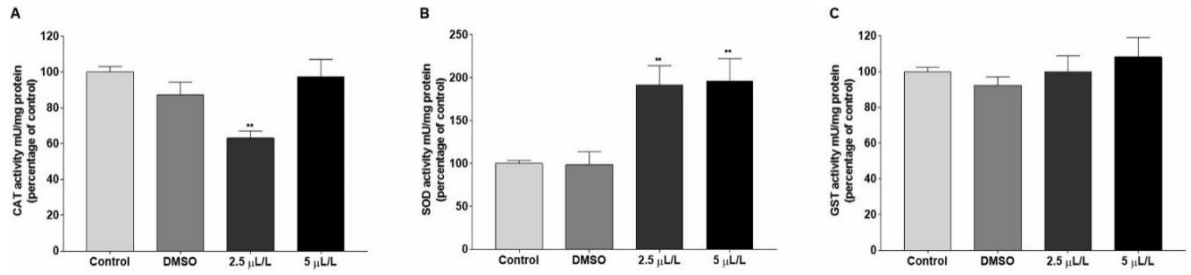

Fig. 2. Antioxidant enzymes activity of (A) CAT, (B) SOD and (C) GST of females of *D. melanogaster* exposed to 1-octen-3-ol (2.5 µL/L and 5 µL/L) for 24 h. Results are represented as mean ± S.E.M. ** indicates statistical difference from control ($p < 0.01$).

3.5. 1-Octen-3-ol induced morphological alterations and respiration dysfunction in mitochondria of D. melanogaster

Female flies treated for 24 h with 5 µL/L 1-octen-3-ol were homogenized for mitochondrial isolation and morphological analysis by electronic microscopy and oxygen consumption by oxidative phosphorylation (Fig. 5). High-resolution respirometry revealed that while the baseline respiration (Routine) and CILeak remained unchanged, the oxidative phosphorylation (OXPHOS) was affected at levels of complexes I (CI-Oxphos) and II (CI & CII-Oxphos) by 1-octen-3- ol (Fig. 5A). In addition, a significant decrease in the bioenergetic capacity of the flies was also observed (Fig. 5B). Regarding mitochondrial morphology, the control group presented normal-shaped mitochondria (elongated and round) with many crests and with the outer and inner membrane intact (Fig. 5C and D). In the group treated with 1-octen-3-ol, a loss of internal morphology was observed, showing a rupture of the inner membrane and mitochondrial cristae (Fig. 5E, H).

4. Discussion

We demonstrated here that exposure of Drosophila to 1-octen-3-ol triggers inflammatory and apoptotic responses associated with a dysregulation of mitochondrial morphological and functional status, indicating induction of an intrinsic pro-apoptotic mechanism.

Fungal VOCs are mainly present in damp or water-damaged places, and the presence of these compounds impair the air quality and affect human health, increasing the risk of acute and chronic respiratory diseases such as asthma and allergies (AL-Maliki et al., 2017; Khan and Karuppayil, 2012). 1-Octen-3-ol belongs to the groups of VOCs and is the main responsible for the characteristic odor of mold (Inamdar et al., 2012). Exposure to 1-octen-3-ol is related to the onset of inflammatory diseases in humans. Exposure for 2 h to 10 mg/L of this compound induced inflammatory markers in nasal secretions (Wålinder et al., 2008). Additionally, a positive correlation between 1-octen-3-ol exposure and allergic rhinitis and conjunctivitis episodes was reported (Araki et al., 2012).

In spite of a relatively simple immune system, studies with *Drosophila melanogaster* have contributed with significant results that impacted our understanding of vertebrate immunity, once many functional and molecular similarities are shared with vertebrates (Shaukat et al., 2015). Drosophilas were reported to be susceptible to VOCs toxicity causing mortality (Inamdar et al., 2010). Specifically, 1-octen-3-ol caused loss of

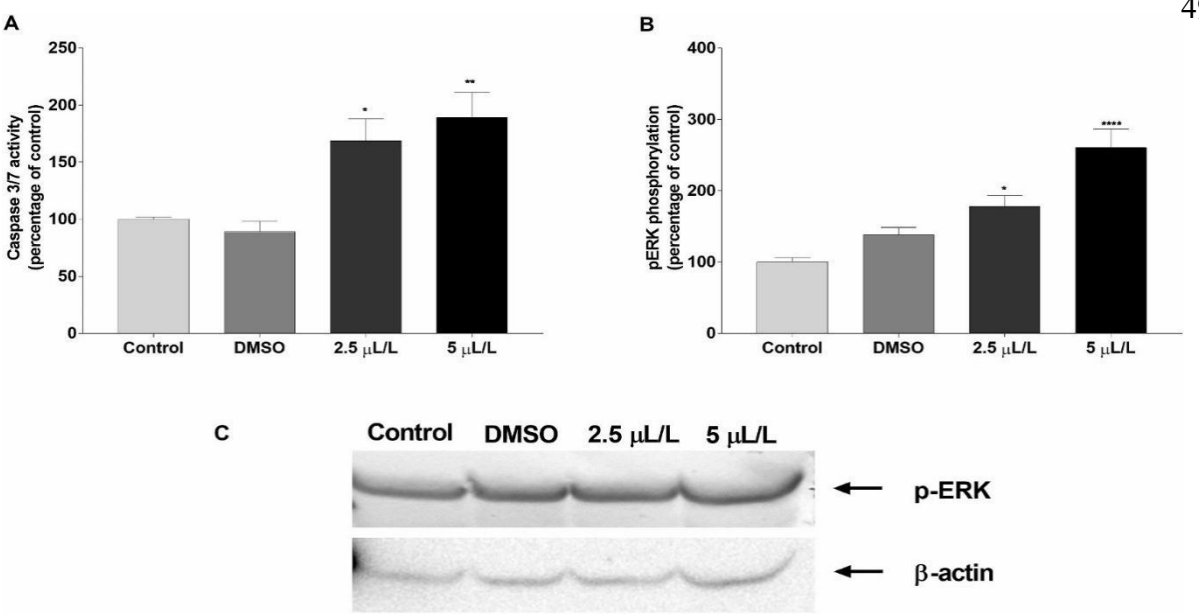

Fig. 3. Effects of 24 h of exposure to 1-octen-3-ol (2.5 µL/L and 5 µL/L) on (A) caspase 3/7 activity and (B–C) ERK phosphorylation of females of *D. melanogaster*. (B) ERK phosphorylation normalized by B-actin expression. (C) Representative Western blotting showing phosphorylated form of ERK and B-actin expression. Results are represented as mean ± S.E.M. *, **, **** indicates statistical difference from control ($p < 0.05$, $p < 0.01$, $p < 0.0001$).

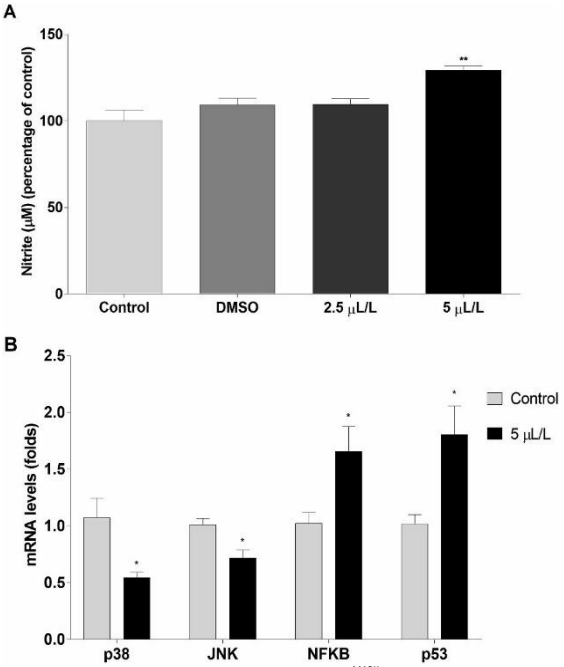

Fig. 4. Nitrite content (A) and gene expression of $p38^{MAPK}$, JNK, NF-κB and p53 (B) of females of *D. melanogaster* exposed for 24 h to 1-octen-3-ol (2.5 µL/L and/or 5 µL/L). Results are represented as mean ± S.E.M. *, ** indicates statistical difference from control ($p < 0.05$, $p < 0.01$).

dopaminergic neurons and Parkinson's disease-like symptoms (Inamdar et al., 2013) and induced NO in flies hemocytes, increasing peroxynitrite levels (Inamdar and Bennett, 2014). A delay in metamorphosis and toxic effects on pupae and adult stages was also reported in flies (Yin et al., 2015).

Herein the compound 1-octen-3-ol reproduced the toxicity already demonstrated for this model where both female and male flies exposed to 2.5 µL/L and 5 µL/L of 1-octen-3-ol up to 48 h presented impairment in locomotor performance and survival rate. *D. melanogaster* have a behavior known as negative geotaxis, which under normal conditions, causes them to rapidly climb to the top. However, when this behavior is affected, flies lose this ability, failing to reach the top, or taking longer than normal (Halmenschelager and da Rocha, 2019)**.** To test the locomotor capacity of the flies, they were challenged to climb up to the 5 cm mark on glass tubes for a limited time of 6 s. The impairment of the behavioral activity induced by 1-octen-3-ol was evidenced by the higher number of flies at the base of the flask in relation to control flies, which reached the top faster. Male flies were more susceptible than females to the toxicity of compound as evidenced by the LC_{50} value. Similarly, was demonstrated higher susceptibility of males to particulate substances (Wang et al., 2017). Gender differences in the inflammatory response were reported for humans in all ages, females are more tolerable to acute inflammatory conditions whereas males to chronic inflammatory conditions. Although the reasons for this gender difference in the response to inflammation are not well described, it may involve multiple aspects such as hormonal and genetic factors (related to X chromosome). Additionally, the lower pH in females could influence the intensity of the inflammatory response, leading to an increase in pro-inflammatory cytokines levels (Casimir et al., 2018).

Environmental pollutants can affect mitochondrial homeostasis, causing dysfunction of ATP production and changes in mitochondrial structure integrity (Boovarahan and Kurian, 2018). Here we demonstrated that exposure to 1-octen-3-ol induced mitochondrial respiration dysfunction by HRR assay and morphological damage in this organelle. *D. melanogaster* exposed to 5 µL/L of 1-octen-3-ol presented a reduction in mitochondrial respiration at levels of complexes I and II, and a general decreasing in bioenergetic capacity. Mitochondria are the energetic center of the cell and are essential organelles for the maintenance of cellular homeostasis and ATP production (Chaban et al., 2014; Spinelli and Haigis, 2018). In general, the energy is produced through the electron transport system (ETS) coupled with oxidative phosphorylation (OXPHOS) that comprises five enzymatic complexes (I, II, III, IV and V) present in the internal mitochondrial membrane (IMM) (Chaban et al., 2014). Damage in this system compromises the bioenergetic capacity of mitochondria (Carvalho et al., 2017). Mitochondrial ETS is the main source of ROS and most of the superoxide radical (O2•-) are originated

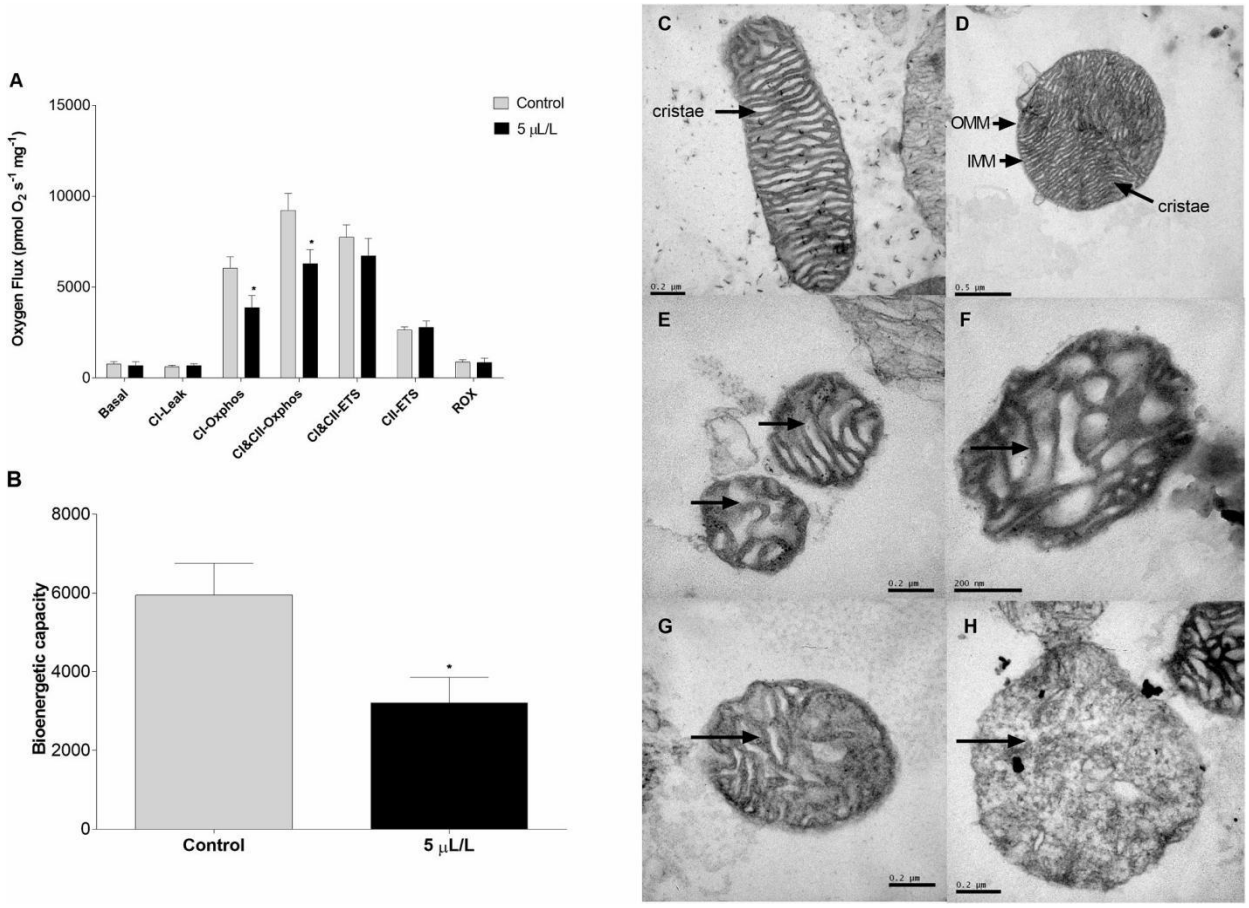

Fig. 5. Effects of 24 h of exposure to 1-octen-3-ol (5 µL/L) on (A–B) mitochondrial function and (C–H) morphology of females of *D. melanogaster*. (A) Representative graphic of O_2 flux and (B) analysis of bioenergetics capacity by HRR. (C–D) Control group mitochondria presented normal shape (elongated and round) and outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM) intact, as demonstrated by TEM. Arrows indicate OMM, IMM and intact mitochondrial ridges. (E–H) 1-Octen-3-ol group mitochondria with inner membrane and cristae rupture. Arrows indicate loss of IMM, disrupted and disconnected mitochondrial ridges. Results are presented as means ± S.E.M. * indicates statistical difference from control ($p < 0.05$).

from NADH-ubiquinone oxidoreductase (complex I) and ubiquinol, but cytochrome c oxidoreductase (complex III) also contribute in a lesser extension for $O_2^{\bullet-}$ production (Kussmaul and Hirst, 2006). Complex I can generate $O_2^{\bullet-}$ through direct electron transfer (NADH to ubiquinone), or due to the presence of sites (FMN group, iron-sulfur clusters, and Q binding site) that facilitate the leakage of electrons from the ETS to mitochondrial matrix, partially reducing oxygen (O_2) to $O_2^{\bullet-}$. In mitochondria, $O_2^{\bullet-}$ anion can be converted to hydrogen peroxide (H_2O_2) by spontaneous or enzymatic dismutation via manganese superoxide dismutase (MnSOD) (Onukwufor et al., 2019). Deficiencies in complex I are linked with enhanced ROS production and disturbed energetic efficiency (Kussmaul and Hirst, 2006). The inhibition of complex I by rotenone had been found to enhance the production of $O_2^{\bullet-}$ anion and reproduces features similar to Parkinson's disease in animal model (Heinz et al., 2017).

Our study provides evidences for an imbalance in oxidative status. It was shown an enhancement in Superoxide dismutase (SOD) activity in this study in both concentrations (2.5 µL/L and 5 µL/L). SOD catalyses the dismutation of $O_2^{\bullet-}$ radicals to the less reactive molecule H_2O_2, which is decomposed into O_2 and H_2O by the Catalase (CAT) (Chaban et al., 2014). In mitochondria, when in excess H_2O_2 can react with Fe^{+2} (via Fenton reaction) and generates hydroxyl radical ($OH^{\bullet}$), a potent oxidant molecule. It is demonstrated in parallel an inhibition in CAT activity, which in conformity with our hypothesis, could contribute to increased levels of H_2O_2 that by Fenton reaction could increase $OH^{\bullet}$ generation, leading to oxidative damage by this radical.

Analysis of mitochondrial morphology by transmission electron microscopy revealed that exposure to 1-octen-3-ol for 24 h causes mitochondrial shape abnormalities such as a reduction in number and form of cristae and a rupture in IMM. Mitochondrial morphological alterations, including failure in membrane integrity and bioenergetic rate, had been found to precede the phenomenon of apoptotic cell death (Suhaili et al., 2017). Apoptosis is a highly regulated programmed cell death process dependent on specific cellular signaling. It occurs naturally in the organism; however, under adverse conditions, it can be exacerbated possibly as a defense mechanism. Apoptosis is regulated by a mitochondrial intrinsic pathway composed of anti-apoptotic (Bcl2 and Bclx) and pro-apoptotic proteins (Bax and Bak, initiation and effectors caspases). When this pathway is activated, pro-apoptotic proteins are associated to each other to compose the outer membrane permeabilization pore (MOMP) leading to a release of cytochrome C. Cytochrome C and Apaf-1 (Apoptotic protease activating factor-1) form the apoptosome (apoptotic complex), subsequently activating effector caspases (caspase 3/7) and initiating apoptosis (Suhaili et al., 2017). Many components of apoptosis are well conserved between *D. melanogaster* and mammals, including the caspases, the main executioners of the apoptotic process. Seven caspases were described in Drosophilas, among them the caspases called Dronc, Dredd and Strica are similar to the mammalian initiator caspases, whereas Dcp-1, Drice, Decay and Damm are resembled to mammalian effector caspases. Particularly the caspases Dcp-1 and Drice that were analyzed in this work are homologous to the mammal caspases 3 and

7 (White et al., 2017). In this study, a significant increase in caspase 3/7 activity in flies exposed to the compound indicates apoptotic cell death. Augmented levels of p53 mRNA (tumor suppressor 53) was also detected. P53 plays a pivotal role in apoptosis, inflammation and immunity. In situations of cell stress, p53 activation can lead to NF-κB transcriptional factor activation which induces caspases expression (Carra et al., 2020`). Our results are similar to previous studies done by Inamdar et al. (2014) which showed an increase in caspase 3 activity after 24 h of exposure to 0.5 μL/L of 1-octen-3-ol in *D. melanogaster*. In addition, exposure for 1 h to different concentrations (0–1000 μL/L) of 1-octen-3-ol displayed cytotoxicity to human embryonic stem cells (Inamdar et al., 2012).

The transcription nuclear factor kappa B (NF-κB) plays a critical role in the regulation of genes involved in immune responses, inflammation, cell differentiation, proliferation and apoptosis (Carra et al., 2020` ; Liu et al., 2017). Under physiological conditions, NF-κB is bound to the IκB inhibitory subunit at the cytoplasm. In the presence of a stimulus (cytokines, growth factors, mitogens, microbial components and stress agents) the IκB protein is phosphorylated and degraded, allowing migration of NF-κB to the nucleus promoting gene transcription (Liu et al., 2017). *D. melanogaster* genome has three homologous of NF-κB genes (Dorsal, Dif and Relish), with immune response functions (Chowdhury et al., 2019). In the present study, 1-octen-3-ol was able to act as a stimulus that increased the relative steady-state levels in NF-κB mRNA. In line with this work, exposure to the indoor air pollutant two-ethyl-one-hexanol (2-EH) and the VOC methylamine activated NF-κB in mice spleen cells and in human bronchial cell line (Li et al., 2019; Yoshida et al., 2009). Another result reported here, is an increase in nitrite levels by 1-octen-3-ol exposure. A previous study reported under hypoxic conditions augmented expression of nNOS mediated by NF-κB leading to an enhanced NO production in developing retina and apoptotic cell death by caspase 3 activation NO-mediated (Rathnasamy et al., 2014). Inamdar and Bennett (2014) showed that *D. melanogaster* hemocytes exposed to 0.5 μL/L of 1-octen-3-ol revealed an inflammatory response through NO activation. NO signaling pathway is conserved in several species, including in *D. melanogaster*, where NO participates in important processes such as immunity (Eleftherianos et al., 2014). Pro-inflammatory stimulus activates the NF-κB/iNOS/NO signaling pathway (Du et al., 2015). According to our results, we may suggest that NF-κB/iNOS/NO could account for the toxicity of the VOC, thus activation of NF-κB factor could lead to a higher expression of NOS contributing to augmented NO formation and resulting in apoptotic cell death and oxidative damage by augmented levels of peroxynitrite. pathways that play critical roles in biological procestes of cell proliferation and differentiation, environmental stresses, apoptosis and response to pro- inflammatory stimulus (Lu et al., 2019). MAPK are functionally conserved cascades present in *D. melanogaster* and involved in important processes, such as normal development, stress response and immunity (Ryan et al., 2020). Environmental pollution can modulate MAPK signaling pathway.

MAPK (Mitogen-Activated Protein Kinases) including ERK1/2 (Extracellular Signal Regulated Kinase), JNK1/2 (c-Jun N-terminal kinase) and $p38^{MAPK}$ comprise three well-known MAPK pathways that play critical roles in biological processes of cell proliferation and differentiation, environmental stresses, apoptosis and response to pro- inflammatory stimulus (Lu et al., 2019). MAPK are functionally conserved cascades present in *D. melanogaster* and involved in important processes, such as normal development, stress response and immunity (Ryan et al., 2020). Environmental pollution can modulate MAPK signaling pathway. MAPKs have been shown to play an essential role in proinflammatory signaling in human cells exposed to ozone pollutant (O_3) (McCullough et al., 2014). Herein 1-octen-3-ol decreased relative steady-state levels of p38 (0.52 fold) and JNK (0.28 fold) in females of *D. melanogaster*. $p38^{MAPK}$ is associated with apoptosis cell death, cell survival and inflammation mediation (Sheller-Miller et al., 2018) whereas JNK signaling pathway played a pro survival role in 1-octen-3-ol-induced loss of dopaminergic neurons in *D. melanogaster* (Inamdar et al., 2014). Taken into account the pro survival role played by these kinases, the reduction in their expression could contribute to the apoptotic cell death evidenced caspase 3/7 activation. In counterpoint, 1-octen-3-ol treatment induced an increase of 78% (2.5 μL/L) and 160% (5 μL/L) in ERK phosphorylation. ERK signaling pathway plays a key role in inflammatory processes providing induction of NF-κB activity contributing for augmented production of NO by overexpression of NOS (Sun et al., 2017).

Here we demonstrated that exposure to fungal VOC 1-octen-3-ol for 24 h induced a series of events leading to decreased survival in *D. melanogaster*. Our hypothesis to explain the loss in flies' survival involves a possible direct action of 1-octen-3-ol on p53 leading to activation of apoptotic signaling pathway evidenced by caspase 3/7 activation. In parallel an inhibition in mitochondrial complex I and II culminating with diminished bioenergetic rate and lower ATP levels, cyt C release and deflagration of apoptotic signaling. A third hypothesis is related to increased activation of ERK, leading to the induction of NF-κB expression and playing a critical role in inflammatory response with the release of nitric oxide synthase and other cytokines, leading to augmented NO levels and culminating in apoptosis cell signaling activation.

5. Conclusions

This study investigated the toxicity of one of the most abundant water-damaged indoor VOCs as a pro-inflammatory compound. In summary, the data reported shows that inhalation for a short period to 1- octen-3-ol induced substantial mitochondrial morphological alterations and impairments of mitochondrial respiration in *Drosophila melanogaster*. Exposure to this compound decreased survival and locomotor ability of flies, which occurred in parallel with alterations in antioxidant enzymes levels, and bioenergetic failure due to morphological disruption of mitochondria and electron transport chain inhibition, culminating in the activation of apoptosis through different routes and, consequently, cell death. Our findings point to the mitochondria as a crucial target for the toxicity of 1-octen-3-ol and may contribute to a possible understanding of the effects of exposure to this VOC on human health. Finally, this work highlighting *D. melanogaster* as a potent model for the study of indoor air pollutants.

Sample credit author statement

Giulianna Echeverria Macedo: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - review & editing, Writing - original draft, Visualization Patrícia de Brum Vieira: Methodology, Conceptualization, Resources, Writing - original draft, - Review & Editing, Visualization, Supervision Nathane Rosa Rodrigues: Methodology Karen Kich Gomes: Methodology Illana Kemmerich Martins: Methodology Jeferson Luis Franco: Funding acquisition, Project administration, Supervision, Resources Thaís Posser Funding acquisition, Project administration, Supervision, Resources, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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5.2 Capítulo II - The fungal indoor air pollutant 1-octen-3-ol induces ROS and inhibits nitric oxide and cell viability in males of *Drosophila melanogaster.*

The fungal indoor air pollutant 1-octen-3-ol induces ROS and inhibits nitric oxide and cell viability in males of *Drosophila melanogaster*

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Abstract

Indoor fungal pollution contributes to the emergence of a set of allergic diseases configuring a public health problem. The prevalence of allergic symptoms between the sexes has been a target of studies, which pointed to different inflammatory response levels in males and females. 1-Octen-3-ol (pop. mushroom alcohol) is the most abundant fungal volatile organic compound (VOC) found in damp indoor spaces and responsible for the typical musty odor. Exposure to 1-octen-3-ol induces inflammatory markers in humans and *Drosophila melanogaster*. We have previously shown that exposure to 1-octen-3-ol induces a sex-specific response in males and females of *D. melanogaster* on survival and locomotor activity. For elucidation of the biochemical mechanism implied in this phenomenon, male flies were exposed to an atmosphere containing 1-octen-3-ol (2.5 μL/L and 5 μL/L) for 24 hours. Our results showed that VOC decreased the cell viability and nitrite levels and induced reactive oxygen species (ROS) measured by DCF-DA assay with stimulation of Glutathione-S-transferase and Superoxide dismutase activity without altering caspase activation. Our study contributes to the comprehension of the reported differential response of males and females to inflammatory conditions, suggesting NO pathway involvement and apoptotic cell death.

Keywords: Mold; Fruit fly; Sex-differences; VOC, asthma.

Introduction

Indoor air quality is critical since most of the population remains indoors for most of their time (Tran et al. 2020). World Health Organization (WHO) estimates a 3.8 million of premature deaths annually attributed to the poor quality of air inside the ambient (WHO 2014). Therefore, household air pollution can be a risk factor for its inhabitants' wellness and is considered a serious public health problem (Tran et al. 2020). Volatile organic compounds (VOCs) are indoor air pollutants, with a high evaporative rate diffusing rapidly in the atmosphere at ambient temperature and pressure. They are low-molecular-weight compounds with potential to causing inflammation in human airways, as demonstrated previously (Kamal et al. 2016; Kwon et al. 2018). The sources of VOCs indoors can be synthetic or natural emissions. Synthetic sources can derive from paints, solvents, cleaning agents, and aerosol sprays (Kamal et al. 2016).

In comparison, natural sources of VOC comprise the metabolism of living organisms, such as plants, bacteria, and fungi (Bennett and Inamdar 2015). Among the natural sources, fungi are emitters of many VOCs and are commonly found widespread in moldy and water-damaged indoor buildings. Their indoor ambient occurrence has been related to an illness known as sick building syndrome (SBS), which is characterized by a set of allergic symptoms presented by these spaces' inhabitants. The main symptoms include irritation in the eyes, nose, throat, headache, and an increase in asthma incidence (Kwon et al. 2018; Tran et al. 2020). Asthma is a frequent chronic inflammatory lung disease that affects both males and females differently (Zhang and Zein 2019). Studies have suggested that men and women respond differently to atmospheric pollution and the appearance of SBS symptoms (Oliveira et al. 2011; Zhang et al. 2014; Lee et al. 2018). Sex-related differences respecting the immunity and inflammatory conditions have been described in several species, from insects to mammals; however, the reasons for such variations are still not well understood (Chamekh et al. 2017; Belmonte et al. 2020).

1-Octen-3-ol is the most common fungal VOC and is found in damp and water-damaged environments and is responsible for mold's characteristic odor (Bennett and Inamdar 2015). It comprises a C-8 compound, commonly called "mushroom alcohol," and mainly released from the metabolism of fungi of the genera *Aspergillus, Penicillium, Alternaria, Cladosporium, Mucor,* and *Ulocladium* (Bennett and Inamdar 2015). Studies demonstrated that exposure to 1-octen-3-ol induces toxicity in humans and other organisms such as the fruit fly model,

Drosophila melanogaster. Human volunteers exposed to 1-octen-3-ol at 10 mg/m^3 for 2 hours presented eye irritation, mild nausea, headache, and induction of inflammatory markers in nasal secretions (Wålinder et al. 2008). Besides, positive correlations between the levels of 1-octen-3-ol and the incidence of allergic rhinitis and conjunctivitis have already been demonstrated in exposed persons (Araki et al. 2012). In *D. melanogaster*, exposure to 1-octen-3-ol resulted in alterations in several parameters, such as impaired locomotor performance, lipid peroxidation, developmental delay, apoptosis cell death, neurotoxicity, and nitric oxide-mediated inflammatory response (Inamdar et al., 2014, 2013, 2010; Inamdar and Bennett, 2014; Yin et al., 2015).

In our previous study, we showed that *D. melanogaster* females exposed to an atmosphere containing 1-octen-3-ol exhibited alterations in inflammatory targets and disruption in mitochondrial morphology and bioenergetic rate. Furthermore, we also demonstrated that male flies are more susceptible to the toxicity of 1-octen-3-ol in relation to females in terms of survival and locomotor capacity (Macedo et al. 2020). *D. melanogaster* is a model of insect that stands out as a powerful tool that shares genetic similarities with humans, including about 75% of homology with genes related to human diseases (Yamaguchi and Yoshida 2018). Additionally, *D. melanogaster* has an apparent sexual dimorphism, in which females and males differ in essential aspects, such as in lifespan and immune and stress responses (Belmonte et al. 2020). Therefore, this model has proven to be a reliable instrument for elucidating different mechanisms involved in human diseases, such as asthma and inflammatory disorders, as well as for studying sex differences in response to various environmental pollutants, as VOCs (Belmonte et al., 2020; Eom et al., 2017; Inamdar et al., 2010; Millington and Rideout, 2018; Roeder et al., 2009; Wang et al., 2017).

In this sense, as far as we know, sexual differences considering the toxicity of 1-octen-3-ol have not been explored. The study of effects of 1-octen-3-ol, in males and females of *D. melanogaster* contributes to understanding sex differences regarding the response to inflammatory conditions in humans. Thus, taking into account the facts that *D. melanogaster* males showed more susceptibility to 1-octen-3-ol (Macedo et al. 2020), in this study the toxicity of VOC on dehydrogenase activity, apoptosis, cell death, nitric oxide (NO) content, ROS, additionally to the activity of antioxidant enzymes were investigated.

2. Materials and Methods

2.1 Chemical Reagents

Racemic form of 1-octen-3-ol (≥98.0%) (68225); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, M2128); 2′,7′-dichloroflfluorescein diacetate (DCF-DA, 35845); and Resazurin sodium salt (R7017) were purchased from Sigma-Aldrich® (São Paulo, SP). Apo-ONE® Homogeneous Caspase 3/7 Assay (G7790) and Griess Reagent System (G2930) were obtained from Promega (Madison, WI). All other reagents were commercial products purchased with the highest purity available. Other materials, including food ingredients, were from standard commercial suppliers.

2.2 Drosophila melanogaster Stock and Culture

For this study, *Drosophila melanogaster* (Harwich strain) was acquired from our breeding stock. The flies were kept in incubators at 25 ± 1°C, 12 h dark-light photoperiod, and 60–70% relative humidity and raised in glass tubes measuring 50 mm × 85 mm containing standard diet. The standard cornmeal diet was constituted of cornflour, salt, wheat germ, powdered milk, sugar, soy flour, rye flour, antifungal agent (Nipagin®), and supplemented with dry yeast, according to Gomes et al., (2020).

2.3 1-Octen-3-ol Exposure Protocol

The 1-octen-3-ol exposure protocol and concentrations used in this study were based on our recently published work (Macedo et al. 2020) where through a mortality curve, it was found the LC_{50} of 5.41 μL/L for this compound. Briefly, groups of thirty male flies (1-5 days old) inhaled the fungal VOC for 24 hours while being fed with 1% sucrose solution. The treatment groups were divided into control group (distilled water), vehicle (Dimethyl sulfoxide – DMSO 0.0020%), and 1-octen-3-ol at concentrations of 2.5 μL/L and 5 μL/L.

2.4 Protein Quantification

The protein concentration of the samples was determined using bovine serum albumin (BSA) by the method of Bradford, (1976).

2.5 Determination of Dehydrogenase Activity and Arbitrary Steady-State ROS Levels

Groups of twenty male flies (control, DMSO, 2.5 μL/L, and 5 μL/L) were homogenized

in 1000 µL of mitochondrial isolation buffer (220 mM mannitol, 68 mM sucrose, 10 mM KCl, 10 mM HEPES and 0.1% BSA) and subsequently centrifugated at 1000 ×*g* for 10 min at 4°C. The supernatant rich in mitochondria was used to measure mitochondrial dehydrogenases activities by Resazurin fluorescence assay at $544nm_{ex}/590nm_{em}$ and colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay. The steady redox state was determined with the fluorescent dye DCF-DA (2,7- dichlorofluorescein diacetate) at 485 nm_{ex}/530 nm_{em}. The assays were performed as described by Macedo et al., (2017). All assays were carried out using an EnsPire® multimode plate reader PerkinElmer (Waltham, MA). A total of four-five biological replicates were used per group (n = 4-5). The results were adjusted based on the protein concentration of the samples and expressed as a percentage of the control group.

2.6 Enzyme Activity Assay

Groups of twenty male flies (control, DMSO, 2.5 µL/L, and 5 µL/L) were homogenized in 1000 µL of 20 mM HEPES buffer pH 7.0 and centrifuged at 20,000 ×*g* for 30 min at 4°C. The supernatant was used to determine the activities of glutathione-S-transferase (GST) and superoxide dismutase (SOD) enzymes, as earlier described by Costa-Silva et al., (2018). All assays were realized at room temperature (25 ± 1°C) utilizing the Agilent Cary 60 UV/VIS® spectrophotometer (Santa Clara, CA). A total of nine biological replicates were used per group (n = 9). The results were standardized based on the protein concentration of the samples and expressed as a percentage of the control group, and the enzyme activity was expressed as mU/mg/protein.

2.7 Caspase 3/7 Activity Assay

Groups of twenty male flies (control, DMSO, 2.5 µL/L, and 5 µL/L) were used to evaluate the cell death by apoptosis, according to Macedo et al., (2017). The flies were homogenized in 500 µL of 20 mM HEPES buffer pH 7.0, and centrifuged at 20,000 ×*g* for 1 min at 4°C. The caspase activity 3/7 was determined as suggested in the manufacturer's protocol (Promega®, WI, USA). The fluorescent emission was supervised at regular intervals of 20 min at $485nm_{ex}/530nm_{em}$. The samples were read in an IS 400 MM Pro Bruker Imaging System® (Billerica, MA). The density of the wells was calculated using Scion Image® Software. The results were standardized based on the samples' protein concentration and expressed as a

percentage of the control group. A total of six biological replicates were used per group (n = 6).

2.8 Determination of Nitric Oxide Content

Nitric oxide was evaluated by nitrite levels (NO_2^-) using the Griess reagent system protocol (Promega® Madison, WI) following Saraiva et al., (2018). Flies exposed to the treatment protocol were homogenized in 500 µL KPi pH 7.4 buffer and centrifuged at 10,000 ×*g* for 10 min at 4ºC. Briefly, the supernatant was distributed in 96 well plates in the presence of Griess reagent. The samples' absorbance was read at 535nm by an EnsPire® multimode plate reader PerkinElmer (Waltham, MA). The NO_2^- concentration was calculated from a nitrite standard curve (0-100 µM). The results were expressed as a percentage of the control group. A total of three biological replicates were used per group (n = 3).

2.9 *Statistical Analysis*

Data were subjected to Kolmogorov Smirnov and *D'Agostino-Pearson omnibus* and Shapiro-Wilk normality tests. Parametric data were analyzed by One Way ANOVA followed by Tukey's *post hoc* test. Results were expressed as mean ± standard error (S.E.M). Results were considered statistically significant when $p < 0.05$ about the control group.

3. Results

3.1 1-Octen-3-ol Alters the Dehydrogenase Activity without altering Caspase 3/7 activity

The MTT assay is a well-defunded tool in estimating living cells' metabolic activity (Grela et al. 2018) since the reduction of the tetrazolium is catalyzed by mitochondrial and non-mitochondrial enzymes (Grela et al. 2018). Male flies exposed to 1-octen-3-ol for 24 hours demonstrated a 30% (2.5 µL/L) and 28% (5 µL/L) decrease in MTT reduction control group (Figure 1A). It was evaluated the cellular viability of cells by Resazurin assay to reinforce this data, and in accordance, it was observed decay of 40% in the fluorescence of this compound at 5 µL/L (Figure 1B). No alteration in caspase 3/7 activity was observed after 1-octen-3-ol exposure in males of *D. melanogaster* (Figure 1C).

3.2 1-Octen-3-ol Modulates the Activity of the Antioxidant Enzymes and the steady-state levels of ROS in D. melanogaster males

The activity of antioxidant enzymes GST and SOD was evaluated; according to results, 1-octen-3-ol (2.5 μL/L) increased these antioxidant enzymes in 40% (GST) and 33% (SOD) (Figure 2 A, B). ROS steady-state levels were evaluated in the homogenate of flies; the compound leads to an increasing (28%) in ROS steady-state by DCF-DA fluorescence at 2.5 μL/L and a decreasing (63%) at 5 μL/L (Figure 2C).

3.3 1-Octen-3-ol Reduces the Nitric Oxide Content of D. melanogaster males

The NO levels evaluated by the content of nitrite in the flies *D. melanogaster* males was significantly decreased comparing the group, with a drop of 17% at 2.5 μL/L and 28% at 5 μL/L (Figure 3).

4. Discussion

The present study investigated biochemical responses to 1-octen-3-ol exposure in *D. melanogaster* males to compare with previous studies where females were submitted to the same exposition protocol (Macedo et al., 2020). 1-Octen-3-ol is a fungal VOC found in damp-contaminated indoor buildings, responsible for the distinctive smell of mold. This compound's presence worsens air and life quality, causing detrimental health effects (Bennett and Inamdar, 2015).

Drosophila has contributed to understanding biochemical and physiological responses to different environmental pollutants, including VOCs (Eom et al., 2017; Inamdar et al., 2010). In this regard, the 1-octen-3-ol caused toxicity in humans as well as in *D. melanogaster*. Exposure to moldy environments induced nasal inflammation biomarkers and the predominance of asthma and allergies (Araki et al., 2012) in humans. In *D. melanogaster*, expose for 48 hours, 1-octen-3-ol caused flies mortality, locomotor damage, lipid peroxidation (Inamdar et al., 2010, 2013). In accordance, our group demonstrated that both female and male flies exposed to 1-octen-3-ol (2.5 μL/L and 5 μL/L) up to 48 hours impaired locomotor performance and survival, and higher susceptibility of males in comparison with females was evidenced (LC_{50} of 12.35 μL/L for females and 5.41 μL/L for males).

The current study evaluated cell viability, enzymes SOD and GST activity, ROS steady-state levels, and NO levels in males after 24 hours of exposition. The fungal compound decreased the flies' cells' viability, as evidenced by MTT and resazurin assay. This effect could

be attributed to a disruption in the mitochondrial function since succinate dehydrogenase reduces MTT to an MTT formazan. Thus, this assay indirectly serves to assess the cellular energy capacity (Chacon et al. 1996). In this respect, Macedo et al. (2020) demonstrate compromised cell respiration, bioenergetics capacity, and disarrangement of mitochondria cristae in female flies exposed to this compound.

In this study, an increase in ROS steady-state levels was observed at a lower concentration with an increase in activity of SOD and GST. Inhibition of mitochondrial respiration by chemicals as rotenone increased superoxide anion generation. Moreover, it was demonstrated stimulation of superoxide anions by the compound Paraquat and parallel induction in SOD activity, possibly by stimulating SOD synthesis (Niwa et al. 1990). The enzyme glutathione S-transferase (GST) participates in redox reactions with oxidation and reduction of the glutathione (GSH), promoting detoxification of ROS and, consequently, protecting the tissues from oxidative damage (Smith et al. 2019). Like mammals, *D. melanogaster* has genes encoding SOD and GST, which protect against oxidative stress and are induced under stress conditions (Mishra 2019; Smith et al. 2019). Herein, ROS steady-state levels, SOD, and GST were induced by 1-octen-3-ol in the males of *D. melanogaster*, similarly to observed in Saraiva et al. (2018). These data were not observed at higher concentrations, possibly due to a failure in the energetic machinery induced by the alcohol at the highest concentration.

It was previously demonstrated (Inamdar and Bennett 2014) increased nitrite levels and NOS activity in hemocytes of *D. melanogaster* exposed for a short period (6 hours) to 1-octen-3-ol, indicating an inflammatory response. A significant induction in NO levels was reported for females (Macedo et al. 2020). The present study follows previous work (Saraiva et al. 2018) that shows that males of *D. melanogaster* exposed to the fungicide Mancozeb demonstrated lower nitrite levels.

The differential response of NO system to chemical stressors between sex is not fully understood. It was demonstrated that the compound bisphenol A (BPA) differently affected the production of NO in cells of both sexes; that study also demonstrated that expression of inducible nitric oxide synthase isoform (iNOS) and NF-κB was higher in females than males' neutrophils (Ratajczak-Wrona et al. 2019). Other analyses indicated that females have higher NO levels than males (Goïta et al. 2020; Khaliulin et al. 2020). The differential response to this

1-octen-3-ol regarding nitrite levels may express a differential tolerance to this agent between sex. It was demonstrated that women are more resistant to acute inflammatory conditions while men to chronic inflammatory conditions; thus, we can not exclude that a more prolonged exposition period could lead to a more pronounced nitrite production.

The absence of induction in executioner caspases 3 and 7 diverged from our earlier work in which their activity was stimulated, indicating apoptotic cell death (Macedo et al. 2020). Sex-based differences in apoptosis markers in response to chemical agents are scarcely explored in the literature. In this regard, previous studies suggested that factors like sex and age influence ionizing radiation-induced apoptosis and the possible existence of sex-specific modifiers of inborn apoptotic pathways (Applebaum et al., 2014; Klein and Flanagan 2016). In this line, it was demonstrated that activity of caspase 3 was more noticeable in females than males in response to exposure to the organophosphate malathion, possible due to a more pronounced inhibition of growth factors (Salama et al. 2019). Moreover, it was demonstrated in a comparative study that female bovine blastocyst is more prone to apoptosis when compared with males in reason of gene expression and metabolism differences (Ghys et al. 2016).

5. Conclusion

The present study reports the harmful effect of 1-octen-3-ol mushroom alcohol in males of *D. melanogaster*. Our initial findings indicated that *Drosophila* males exposed to 1-octen-3-ol inhalation for 24 hours exhibited more susceptibility to the compound than females. In line with this work, here we show that males exposed to the same protocol exhibited a significant drop in cell viability and nitrite levels without altering caspases activities, besides an increase in ROS generation and antioxidant enzymes GST and SOD were observed only in the lower concentration. Our data draw attention to the hazardous effects that this fungal VOC can present in *D. melanogaster* and the differential responses of males and females. The results presented contribute to the comprehension of differential response related to sex and suggest the possible modulation of NO, and apoptotic pathway; however, further studies are necessary to clarify this hypothesis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Acknowledgments

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Figure 1A

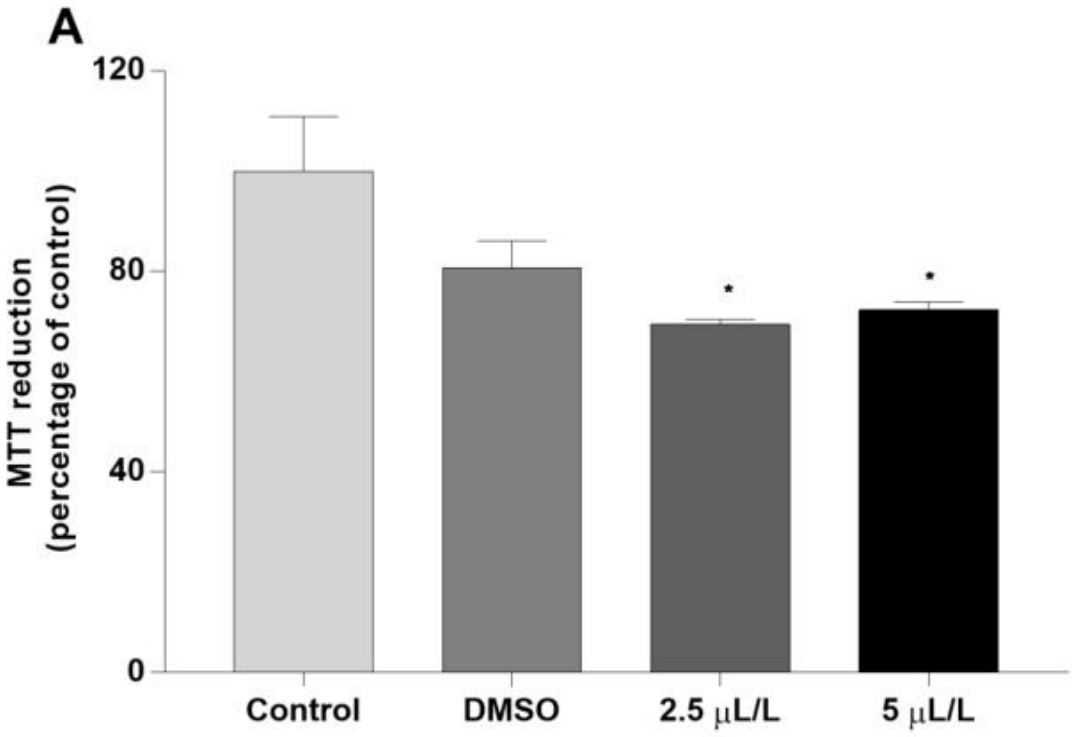
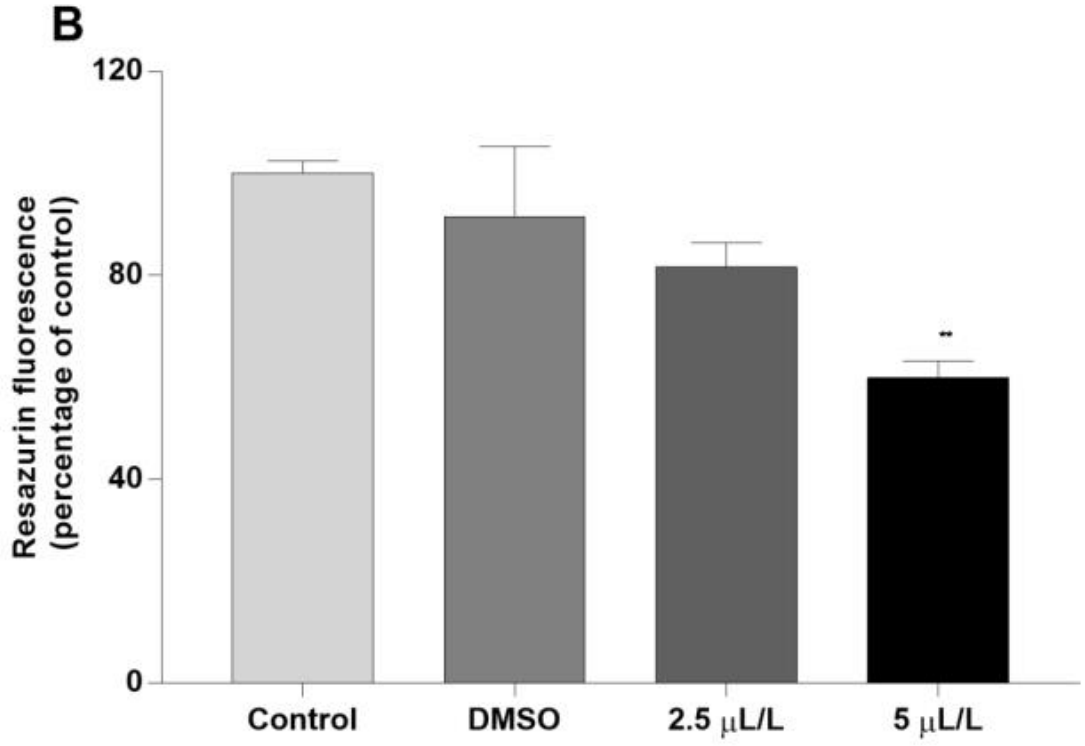

A
120
80
40
0
MTT reduction
(percentage of control)
*
*
Control
DMSO
2.5 μL/L
5 μL/L

Figure 1B


B
120
80
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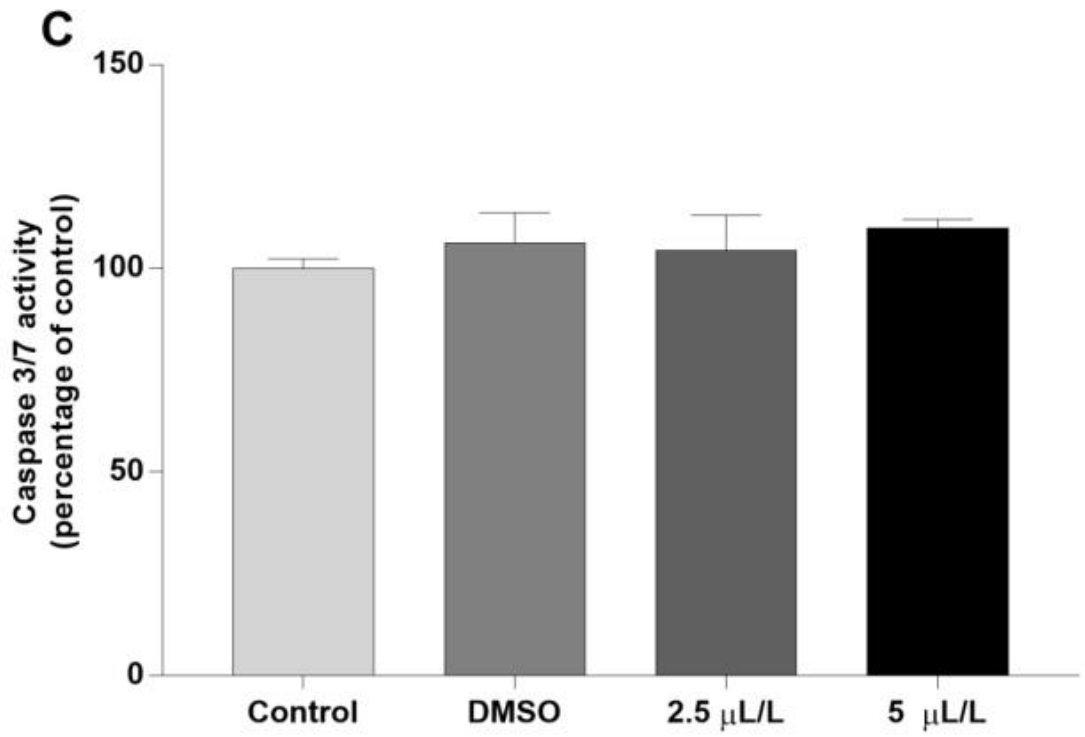
Resazurin fluorescence
(percentage of control)
**
Control
DMSO
2.5 μL/L
5 μL/L

Figure 1C



Effects of exposure to 1-octen-3-ol on cellular viability and caspase 3/7 activity in *D. melanogaster males*. (A) MTT reduction, (B) Resazurin fluorescence and (C) caspase 3/7 activity were performed after 24 hours of exposure to 1-octen-3-ol (2.5 μL/L and 5 μL/L). Results are represented as mean ± S.E.M. *, ** indicates statistical difference from control group ($p < 0.05$, $p < 0.01$).

Figure 2A

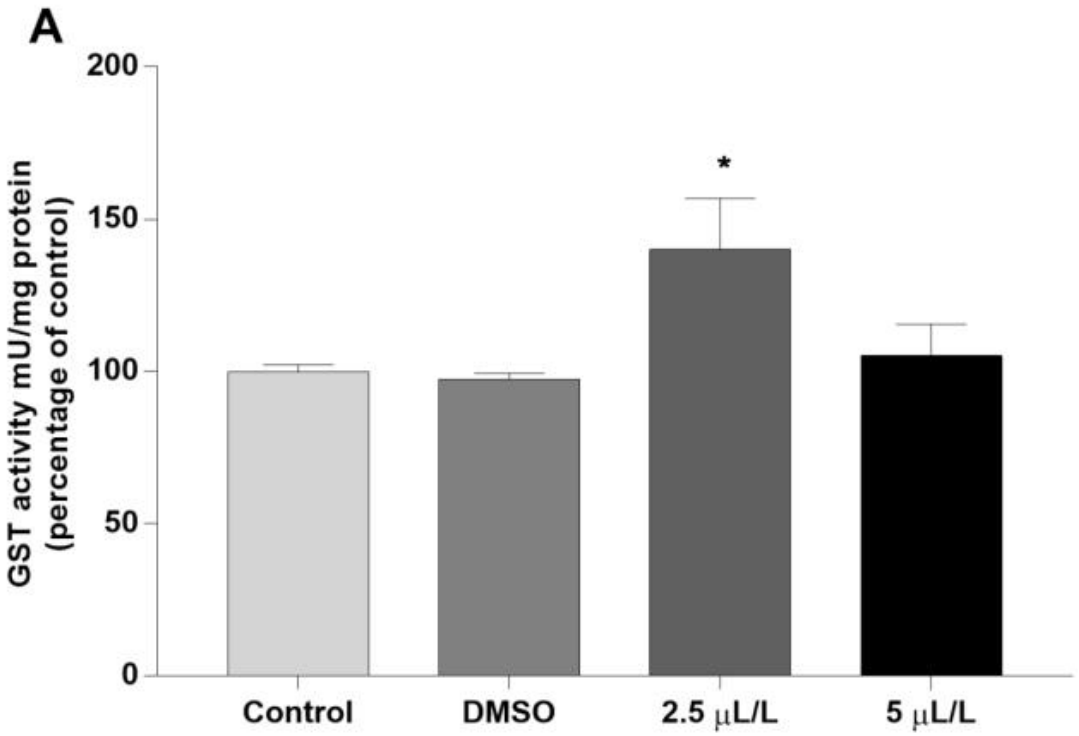

A
200
150
100
50
0
GST activity mU/mg protein
(percentage of control)
*
Control
DMSO
2.5 μL/L
5 μL/L

Figure 2B

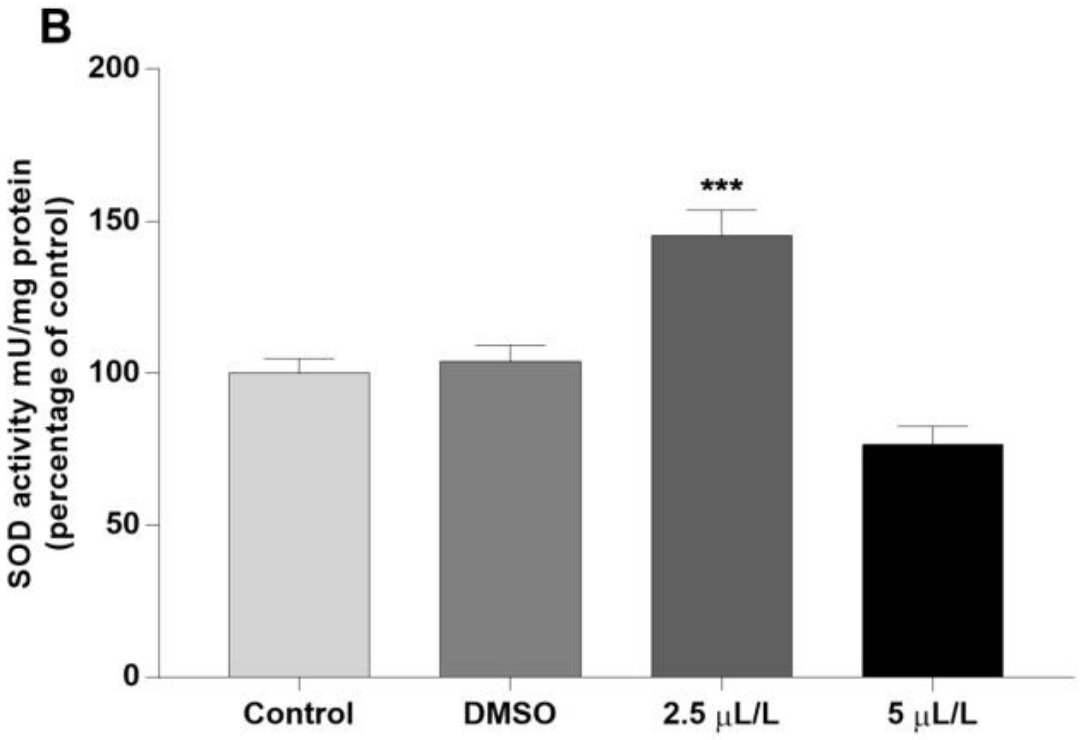
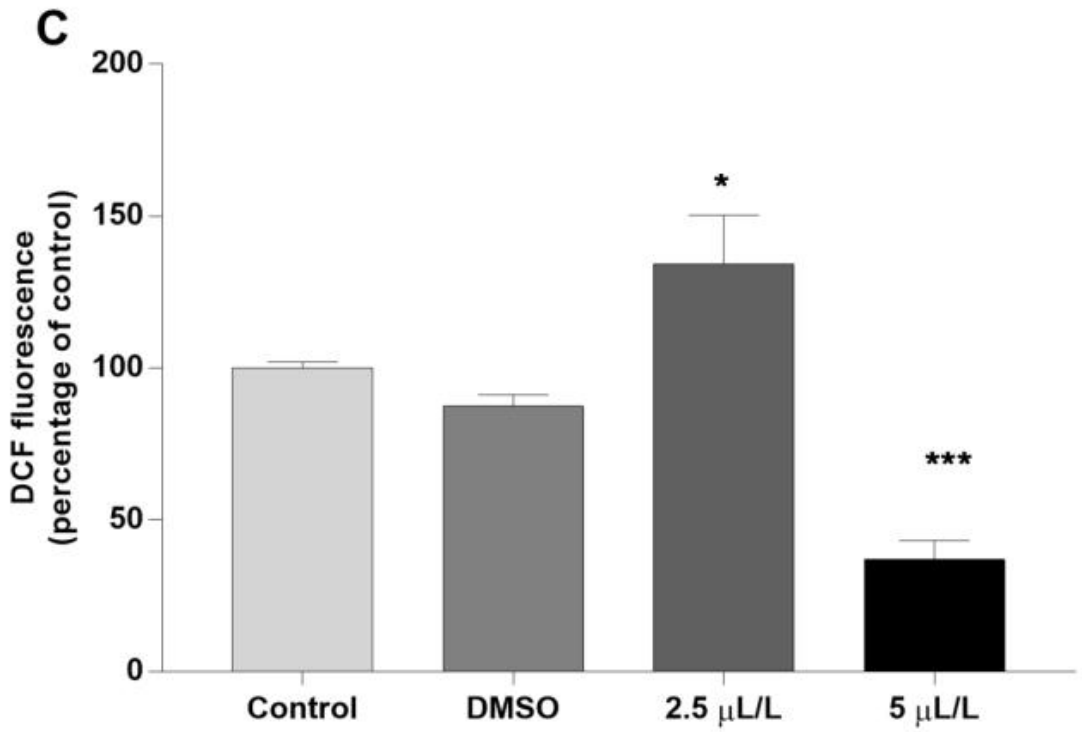
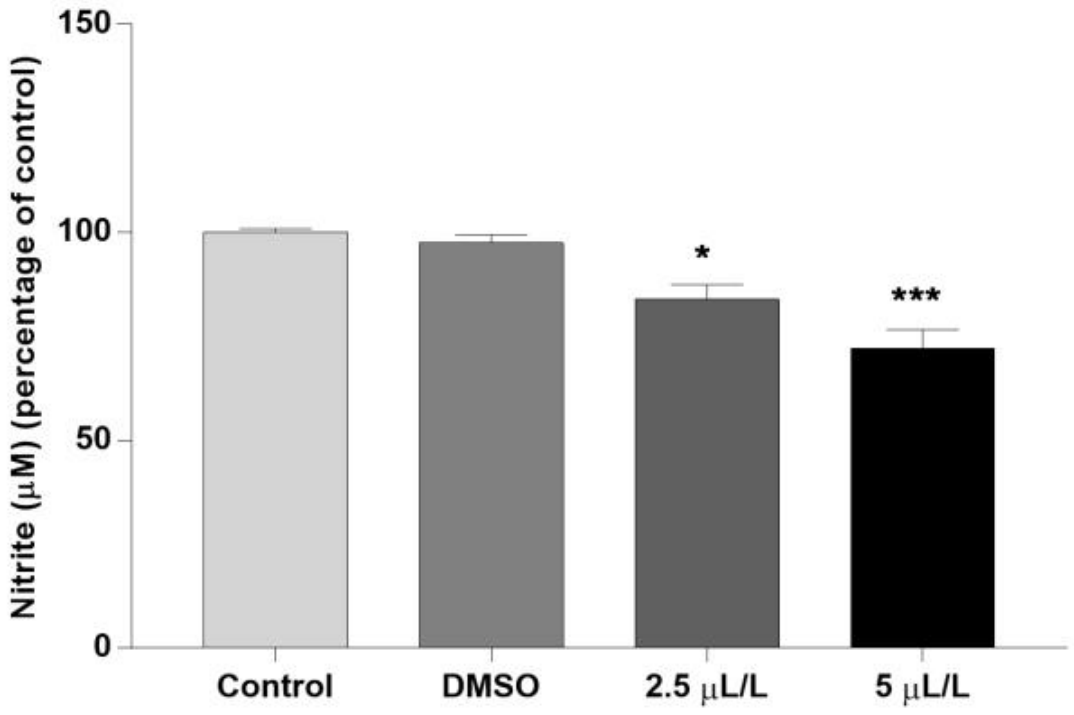

B
200
150
100
50
0
SOD activity mU/mg protein
(percentage of control)
***
Control
DMSO
2.5 μL/L
5 μL/L

Figure 2C



Antioxidant enzymes activity of (A) GST and (B) SOD, and (C) ROS generation of *D. melanogaster* males exposed to 1-octen-3-ol (2.5 μL/L and 5 μL/L) for 24 hours. Results are represented as mean ± S.E.M. *, *** indicates statistical difference from control group ($p < 0.05$, $p < 0.001$).

Figure 3



Nitrite levels of *D. melanogaster* males after exposure to 1-octen-3-ol (2.5 μL/L and 5 μL/L) for 24 hours. Results are represented as mean ± S.E.M. *, *** indicates statistical difference from control group ($p < 0.05$, $p < 0.001$).

6. Considerações finais

O presente estudo investigou a toxicidade do álcool de cogumelo 1-octen-3-ol como um composto pró-inflamatório em *Drosophila melanogaster*. Através deste trabalho pode-se verificar que os machos de *D. melanogaster* são mais susceptíveis a toxicidade do 1-octen-3-ol em relação as fêmeas. Com base nos resultados obtidos, é possível sugerir que este COV fúngico possua diferentes mecanismos de ação nos dois sexos. Enquanto que nas fêmeas é provável que a mitocôndria, juntamente com a ativação de fatores pró-inflamatórios e a cascata de vias de sinalização apoptóticos possam ser um alvo crucial para a toxicidade do 1-octen-3-ol, nos machos, um aumento na geração de ERO poderia estar envolvido com o comprometimento da atividade mitocondrial observado através da queda na viabilidade celular.

No entanto, as razões para tal sensibilidade permanecem indefinidas e muitos mecanismos relacionados à toxicidade do 1-octen-3-ol nos machos de *D. melanogaster* permanecem incompreendidos. Ademais, é possível que a susceptibilidade ao 1-octen-3-ol possa estar relacionada a fatores hormonais e também aos níveis de óxido nítrico, que é naturalmente mais elevado em fêmeas de várias espécies. Portanto, investigar a ação deste composto sobre alvos moleculares, inflamatórios, além da análise da funcionalidade e integridade mitocondrial dos machos poderia ser interessante para uma melhor compreensão acerca das diferenças relacionadas ao sexo.

Em suma, este trabalho atenta para os efeitos perigosos que a inalação por um curto período de tempo ao 1-octen-3-ol pode apresentar sobre *D. melanogaster*, ressaltando a importância deste modelo para o estudo de questões inflamatórias, bem como para a compreensão dos efeitos da poluição em ambientes internos e a toxicidade destes contaminantes em ambos os sexos.

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