UNIVERSIDADE FEDERAL DO PAMPA – CAMPUS URUGUAIANA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

LUIZ EDUARDO BEN PILISSÃO

A EXPOSIÇÃO CRÔNICA A NANOPARTÍCULAS DE QUITOSANA CONTENDO ÁCIDO GIBERÉLICO (GA_3) CAUSA REPROTOXICIDADE E DANOS NEURONAIS EM *Caenorhabditis elegans*

DISSERTAÇÃO DE MESTRADO

URUGUAIANA
2023

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Dissertação apresentada ao Programa de Pós-graduação Stricto Sensu em Bioquímica da Universidade Federal do Pampa, como requisito parcial para obtenção do título de mestre em Bioquímica.

Orientadora: Daiana Silva de Ávila.

URUGUAIANA
2023

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P638e Pilissão, Luiz Eduardo Ben
A EXPOSIÇÃO CRÔNICA A NANOPARTÍCULAS DE QUITOSANA CONTENDO ÁCIDO GIBERÉLICO (GA3) CAUSA REPROTOXICIDADE E DANOS NEURONAIS EM Caenorhabditis elegans / Luiz Eduardo Ben Pilissão.
94 p.

Dissertação(Mestrado)-- Universidade Federal do Pampa, MESTRADO EM BIOQUÍMICA, 2023.
"Orientação: Daiana Silva Avila".

1. Toxicologia. 2. pesticidas. 3. nanotecnologia. I. Título.

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Dissertação/Tese apresentada ao Programa de Bioquímica da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Mestre/Doutor em Bioquímica.

Dissertação defendida e aprovada em: 29 de março de 2023.

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Agradecimentos

Agradeço primeiramente a Deus, pela minha vida, pela minha família, por ter saúde para correr atras dos meus objetivos e dos meus sonhos.

Agradeço ao meus pais, Iolanda e Luiz Valmir, por estarem sempre presentes e me apoiando nas minhas decisões. Pela compreensão, amor e educação. Por sempre me proporcionarem segurança e suporte para eu seguir em frente nas minhas escolhas. Muito Obrigado!

Ao meu irmão Bruno, pela convivência, palavras, conversas e por saber que sempre terei alguém pra confiar.

A minha orientadora, Daiana Silva de Ávila, pela oportunidade de ingressar em seu laboratório e permitir que eu me envolvesse em alguns projetos e assim me apresentar o mundo da pesquisa, se tornando uma referência de pesquisadora. Agradeço pela amizade, confiança depositada em mim e por todo aprendizado ao longo desses anos de pareceria que tivemos. Muito Obrigado!

A todos os colegas do Grupo de Pesquisa em Bioquímica e Toxicologia em *Caernohabditis elegans* (GBToxCe), pela parceria, pelos momentos de risadas, de músicas, e principalmente por compartilhar conhecimento e ajuda para o desenvolvimento deste trabalho. Agradeço a esse ser de luz, chamado, Daniela, pela amizade e ajuda durante o período de realização deste trabalho. Você foi fundamental. Agradeço a Nari e Aline pela amizade e pareceria na realização dos experimentos deste trabalho.

À UNIPAMPA e ao programa de pós-graduação pela oportunidade.

Enfim, obrigado a todos que de alguma maneira auxiliaram na realização deste trabalho, seja ela, profissional ou pessoal .**Muito Obrigado!**

“Vitoriosos são aqueles que, mesmo cansados, nunca desistem de lutar.
Quando você acredita, o universo conspira a seu favor.
Seja otimista, mantenha-se firme, em seus propósitos e a vitória será sua!”

Resumo

Muitos produtos químicos são aplicados atualmente na agricultura, e os reguladores de crescimento de plantas (PGRs) estão entre os amplamente empregados. O ácido giberélico (GA_3) é um regulador endógeno do crescimento vegetal usado mundialmente na agricultura; entretanto, pouco se sabe sobre seus efeitos bioquímicos e fisiológicos em mamíferos e seu impacto nos organismos que vivem no meio ambiente. Uma estratégia para direcionar a ação deste regulador para a planta-alvo e reduzir o impacto no meio ambiente é a aplicação de nanotecnologia, cujos efeitos no micro e macroorganismos ainda são pouco conhecidos. Este estudo teve como finalidade avaliar os possíveis efeitos neurotóxicos e reprotóxicos do GA_3 e de nanopartículas (NPs) contendo este PGR no modelo experimental *Caenorhabditis elegans*, que é um modelo considerado como não-alvo dos PGRs e pode ser encontrado no solo. Os vermes foram expostos de forma crônica (48 h) a diferentes concentrações do composto regulador do crescimento de plantas e a dois tipos de nanopartículas de quitosana contendo o ácido giberélico, além dos controles de NPs brancas. Após os tratamentos, os ensaios de toxicidade como sobrevivência, desenvolvimento, reprodução, marcadores de estresses oxidativo e efeitos neuronais foram realizados. Observamos aumento na mortalidade dos vermes expostos. Foi demonstrado um aumento da incidência de eventos apoptóticos em células germinativas, o que foi consistente com as reduções observadas na capacidade reprodutiva. Além disso, a exposição promoveu mudanças comportamentais significativas afetando os movimentos de natação e locomoção radial, que foram associadas a mudanças na intensidade de fluorescência e morfologia dos neurônios dopaminérgicos e colinérgicos. Notavelmente, nossos resultados demostraram a toxicidade induzida pelo GA_3 através da mortalidade apresentada, alterações do perfil reprodutivo e alterações neuronais que refletem no comportamento do verme. Além disso os resultados indicam que os efeitos observados nos parâmetros avaliados foram causados pelas nanopartículas *per se*. Esses resultados sugerem que o desenvolvimento de nanopartículas visando aplicações agrícolas necessita de mais estudos para otimizar a composição e assim reduzir sua toxicidade para organismos não-alvo.

Palavras chaves: nanotecnologia; acido giberélico; especies reativas; disfunção mitochondrial; reprodução; neurotoxicidade.

Chronic exposure to chitosan nanoparticles containing Gibberellic Acid (GA3) cause reprotoxicity and neuronal damage in Caenorhabditis elegans

Abstract

Many chemicals are currently applied in agriculture, and plant growth regulators (PGRs) are among the most widely used. Gibberellic acid (GA3) is an endogenous plant growth regulator used worldwide in agriculture; however, little is known about its biochemical and physiological effects on mammals and its impact on organisms living in the environment. A strategy to direct the action of this regulator to the target plant and reduce the impact on the environment is the application of nanotechnology, which effects on micro and macro-organisms are still little known. This study aimed to evaluate the possible neurotoxic and reprotoxic effects of GA3 and nanoparticles (NPs) containing this PGR in the experimental model *Caenorhabditis elegans*, which is a model considered as a non-target of PGRs and can be found in the soil. The worms were chronically exposed (48 h) to different concentrations of the PGR compound and to two types of chitosan nanoparticles containing GA3, in addition to blank NPs as control. After treatments, toxicity assays such as survival, development, reproduction, oxidative stress markers and neuronal effects were performed. We observed an increase in the mortality of exposed worms. An increased incidence of apoptotic events in germ cells was demonstrated, which was consistent with the observed reductions in reproductive capacity. Furthermore, the exposure promoted significant behavioral changes affecting swimming movements and radial locomotion, which were associated with changes in fluorescence intensity and in the morphology of dopaminergic and cholinergic neurons. Notably, our results demonstrated the toxicity induced by GA3 through the presented mortality, alterations in the reproductive profile and neuronal alterations that reflect in the behavior of the worm. Furthermore, the results indicate that the observed effects on the evaluated parameters were caused by the nanoparticles per se. These results suggest that the development of nanoparticles targeting agricultural applications needs further studies to optimize the composition and thus reduce its toxicity to non-target organisms.

Keywords: nanotechnology; gibberelic acid; reactive species; mitochondrial dysfunction; reproduction; neurotoxicity.

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1.Introdução

A população global tem crescido rapidamente e está projetada para mais de 9 bilhões até 2050, com a maior parte do crescimento nos países em desenvolvimento (Boddula et al., 2019; Grillo et al., 2021; Pandey et al., 2018). Atualmente, mais de 800 milhões de pessoas em todo o mundo estão subnutridas e esse número aumentará para mais de 2 bilhões, levando em consideração a produção agrícola atual e a taxa de crescimento das culturas até 2050 (Usman et al., 2020). Há uma necessidade urgente de aumentar a produção agrícola mundial em mais de 70% para acompanhar essa demanda crescente nas próximas décadas (Grillo et al., 2021; Pandey et al., 2018). A produção e uso de agroquímicos, incluindo fertilizantes e pesticidas, têm aumentado rapidamente para aumentar a produção agrícola e atender à demanda global (Grillo et al., 2021). O mercado global de agroquímicos diretamente relacionados à proteção de cultivos foi estimado em mais de 55 bilhões de dólares em 2017 e foi estimado um aumento de pelo menos 2,5% a cada ano (Phillips, 2020).

Alguns entraves para o atendimento a esta demanda global por alimentos se devem aos solos que se tornam pobres a cada plantio e às doenças nas plantações (Chhipa, 2019). Insetos, ervas daninhas, fungos, bactérias e parasitas combinados respondem por cerca de 40% das perdas nas colheitas, o que equivale a mais de US$ 220 bilhões anualmente (Chhipa, 2019; Pandey et al., 2018; Wang et al., 2022). Mais de 3 milhões de toneladas de pesticidas (equivalentes a aproximadamente US$ 55 bilhões) são usados a cada ano para controlar doenças nas lavouras (Pandey et al., 2018; Slattery et al., 2019). Grandes proporções de pesticidas acabam no meio ambiente e afetam organismos não-alvo. Estima-se que menos de 25% dos pesticidas convencionais realmente atinjam a cultura-alvo e menos de 1% cheguem à praga-alvo eventualmente (Kumar et al., 2019; Wang et al., 2022). Os fertilizantes desempenham um papel importante no aumento do rendimento das culturas e na manutenção da fertilidade do solo (Pandey et al., 2018; Pramanik et al., 2020). As culturas requerem micronutrientes, incluindo cobre (Cu), ferro (Fe), zinco (Zn), manganês (Mn), níquel (Ni), sódio (Na), boro (B), molibdênio (Mo) e

cloreto (Cl) em baixa quantidades, necessárias para função enzimática, respiração celular e metabolismo (Zulfiqar et al., 2019). Além disso, as culturas requerem um grande número de macronutrientes como blocos de construção essenciais para o crescimento, especialmente os macronutrientes primários, incluindo nitrogênio (N), fósforo (P), potássio (K). Macronutrientes secundários, como cálcio (Ca), enxofre (S) e magnésio (Mg), também desempenham um papel crítico no crescimento das culturas (Solanki et al., 2015). Os fertilizantes contribuem com mais de 30% da produção agrícola anualmente. Estima-se que aproximadamente 199,4 toneladas/m^2 de fertilizantes foram aplicadas globalmente em 2017, com um aumento de 2% esperado a cada ano (Adisa et al., 2019). Aproximadamente 50-70% de N, 80% de P e 60% de K aplicados por fertilizantes convencionais são perdidos no ambiente antes de atingirem as culturas-alvo. Mais da metade desses nutrientes perdidos entra nas águas superficiais e subterrâneas por meio de lixiviação e escoamento (Pourzahedi et al., 2018). Semelhante aos pesticidas, a baixa eficiência requer aplicação repetida pesada, especialmente fertilizantes de fósforo e nitrogênio (Adisa et al., 2019; Chhipa and Joshi, 2016; Guha et al., 2020).

Em geral, as formulações convencionais geralmente têm eficiência de uso muito baixa e alta porcentagem de produtos químicos aplicados não atingem os organismos-alvo devido ao rápido escoamento, taxa de aplicação desigual ou baixas taxas de absorção/adsorção (Guha et al., 2020; Usman et al., 2020). Como consequência, os agroquímicos convencionais geralmente requerem aplicações múltiplas com quantidades maiores de produtos químicos para fornecer nutrientes suficientes e controlar pragas (Guha et al., 2020; Kumar et al., 2019; Pourzahedi et al., 2018; Usman et al., 2020). O uso intenso de pesticidas e fertilizantes convencionais não apenas aumenta os custos de insumos, mas também leva a consequências adversas, incluindo o aumento da resistência de patógenos a pesticidas, efeitos adversos em organismos não-alvo, contaminação da água e do solo e aumento da exposição humana através da cadeia alimentar (Chhipa and Joshi, 2016; Kumar et al., 2019; Usman et al., 2020). Portanto, novas tecnologias e práticas inovadoras são inevitavelmente necessárias para aumentar o rendimento das culturas e, ao mesmo tempo, reduzir os impactos ambientais (Kah et al., 2013; Zhang et al., 2020). Ou seja, uma agricultura ecologicamente amigável e sustentável é necessária.

O maior desafio enfrentado pelos pesquisadores agrícolas é produzir alimentos em quantidade e qualidade suficientes para alimentar a população global cada vez maior sem

degradar a saúde do solo e o agroecossistema. Para administrar essas perdas e aumentar a produtividade, os agricultores estão fazendo uso excessivo e indiscriminado de agroquímicos, o que leva à deterioração da saúde do solo, degradação de agroecossistemas, problemas de resíduos, poluição ambiental e resistência a pesticidas em insetos e patógenos. Portanto, há uma necessidade urgente de mudar a forma como usamos os agrotóxicos. As mudanças podem incluir (i) implantação criteriosa de pesticidas e fertilizantes, (ii) detecção rápida e precisa de patógenos e pragas, bem como pesticidas e níveis de nutrientes, e (iii) promoção da saúde do solo.

Considerando o fato de que é necessário produzir mais e com melhor qualidade, muitos produtos químicos são usados atualmente na agricultura, e os reguladores de crescimento de plantas estão entre os que são amplamente empregados. Estes reguladores são hormônios vegetais (fitormônios), que são pequenas moléculas orgânicas comumente usadas para aumentar a produção agrícola (Kaneko et al., 2003). Esses hormônios vegetais promovem o alongamento do caule, superam a dormência em sementes, estão envolvidos no desenvolvimento de frutos, floração, mobilização de reservas alimentares na germinação de sementes, juvenilidade e expressão sexual das plantas (Xiong and Zhu, 2003). A Organização Mundial da Saúde considera as giberelinas como PGR, sendo o ácido giberélico (GA_3) um dos hormônios mais ativos dentro da família das giberelinas (Arous et al., 2001; El-sayyad et al., 2012). As plantas produzem GA_3 naturalmente para promover o crescimento normal, mas atualmente o fitormônio tem sido aplicado de maneira exógena para aumentar o rendimento agrícola (Silverstone and Sun, 2000a).

Embora o GA_3 seja amplamente utilizado na agricultura, seus efeitos na saúde humana são pouco explorados. Apenas alguns experimentos examinaram os possíveis efeitos tóxicos de PGRs, incluindo GA_3 em mamíferos. Muitos produtos químicos como reguladores de crescimento de plantas podem induzir estresse oxidativo e provocar várias doenças. Os humanos são expostos principalmente ao GA_3, bem como a outros PGRs, por meio da ingestão de produtos alimentícios que contêm esses compostos, particularmente frutas frescas, como tâmara, palma, melão, morango, uvas e alguns vegetais, como tomate, pimenta, repolho, couve-flor e oliva, bem como através da água potável (Hussein et al., 2011). Portanto, formulações alternativas que possam reduzir o impacto ambiental e na saúde destes reguladores de crescimento devem ser buscadas, com objetivo de minimizar a contaminação ambiental e assim a exposição humana. Nesse

contexto, a nanotecnologia emergiu como um avanço tecnológico que pode transformar a agricultura e setores aliados, fornecendo novas ferramentas para o gerenciamento molecular de estresses bióticos e abióticos, detecção rápida de doenças e aumento da capacidade das plantas de absorver nutrientes ou pesticidas (Kashyap et al., 2015a).

A nanotecnologia está sendo desenvolvida e aplicada a fim de minimizar tais problemas; por exemplo, nanopartículas carregadas com herbicidas permitem a redução de ingredientes ativos e remoção dos ingredientes inertes por meio de formulações derivadas de constituintes como lipídios, polissacarídeos e polímeros biodegradáveis. Esses nanossistemas possibilitam a redução da concentração dos princípios ativos, pois conferem maior estabilidade contra a degradação do composto bioativo, maior solubilidade e mecanismo de liberação controlada, além de protegerem compostos fotossensíveis, permitindo a utilização de pequenas doses (Silva et al., 2011).

Apesar do potencial de aplicação, alguns estudos sugerem que as NPs podem ser tóxicas. Esses estudos demonstraram a capacidade dos NPs de se acumularem nas células e induzirem toxicidade órgão-específica. Esses estudos, combinados com a exposição humana cada vez maior, demonstram uma necessidade urgente para o desenho de NPs seguras e o desenvolvimento de diretrizes rígidas para o seu desenvolvimento no que diz respeito aos testes de toxicidade (Najahi-Missaoui et al., 2021). Um estudo mostrou uma redução maior na viabilidade celular induzida por NPs de quitosana quando testados *in vitro* usando monócitos de sangue periférico humano (PBMCs) ou linha celular RAW 264.7. Além disso, NPs de quitosana foram mais citotóxicas para PBMCs, aumentou a produção de espécies reativas de oxigênio (ROS) (acima de 156 μg / mL) na linha celular RAW 264,7 e interferiu com a via intrínseca de coagulação(Jesus et al., 2020). Sendo assim estudos sobre a toxicidade desses nanossistemas em diferentes organismos são necessários para verificar a segurança dessas formulações, atestando assim a qualidade do solo onde são aplicadas. Para tanto, faz-se necessária a utilização de um modelo experimental viável que seja sensível e possa sofrer possíveis efeitos decorrentes da nanotecnologia.

Para determinar a segurança destas NPs, modelos que ofereçam ensaios rápidos, de baixo custo e que ajudem a prever os impactos no meio ambiente e os efeitos em mamíferos devem ser empregados, sendo um destes o nematoide invertebrado *Caenorhabditis elegans*. O *C. elegans* é um modelo *in vivo* que vem sendo utilizado em

ensaios de triagens farmacológicas e toxicológicas. Esse modelo apresenta diversas vantagens, entre elas o baixo custo de manutenção, rápida reprodução, possibilidade de monitorar o desenvolvimento larval até a morte, e também foi o primeiro ser vivo a ter o seu genoma e transcriptoma completamente sequenciado e caracterizado, respectivamente. O *C. elegans* é um pequeno nematóide (vermes adultos têm comprimento de ~ 1 mm) que pode ser mantido a baixo custo e manuseado usando técnicas similares às *in vitro*. Ao contrário dos testes de toxicidade usando culturas de células, os testes de toxicidade de *C. elegans* fornecem dados de um animal inteiro com sistemas digestivos, reprodutivos, endócrinos, sensoriais e neuromusculares intactos e metabolicamente ativos (Hunt, 2017a). O fato de o solo ser um dos habitats de *C. elegans* torna este modelo viável para testes de qualidade do solo e toxicidade de seus contaminantes (Höss et al., 2009; Sochová et al., 2007a)

Considerando todo este cenário, da complexidade que envolvem as etapas de produção agrícola, com todos os desafios mencionados acima, como aumento da população mundial problemas com pragas e com o solo, baixa produtividade, uma prática que ganhou bastante espaço ultimamente é o uso de reguladores de crescimento vegetal de forma endógena e o uso da nanotecnologia. Levando-se em conta que o uso contínuo de PGR na agricultura contribui para o aumento dos níveis de contaminantes no solo e nos rios e sabendo o impacto que isso gera no meio ambiente, este trabalho visou avaliar a segurança de um PGR, o GA_3 em relação a efeitos nos sistemas nervoso e reprodutivo, bem como se a nanotecnologia poderia reduzir esta toxicidade, utilizando o nematoide *Caenorhabditis elegans* como modelo experimental.

2. Revisão Bibliográfica

2.1 Reguladores do Crescimento Vegetal

A Revolução Verde surgiu com a adoção de abordagens modernas, como variedades de alto rendimento, agroquímicos e agricultura intensificada (Akhilesh and Sooda, 2020). Embora variedades de alto rendimento estejam sendo promovidas, a maior parte da terra tropical não oferece condições de crescimento adequadas para essas culturas de alto rendimento; e, consequentemente, obtém-se baixo rendimento mesmo após a aplicação dos agroquímicos necessários. O uso indiscriminado desses produtos químicos

causa poluição ambiental, degradação do solo e carga econômica para os agricultores. Diferentes fatores, como o rápido crescimento populacional, cultivo de terras inadequadas para a agricultura, práticas agrícolas inadequadas e erradicação de árvores para combustível e abrigo, podem tornar os solos tropicais frágeis. Além disso, os centros agrícolas regionais sob condições tropicais são altamente afetados por eventos extremos de mudança climática, por exemplo, chuvas fortes, inundações e secas (González-Orozco et al., 2020; Sarkar et al., 2020).

Estas práticas agrícolas modernas com uma abordagem intensiva (por exemplo, cultivo pesado, agroquímicos sintéticos, variedades híbridas e monoculturas) podem deteriorar ainda mais os solos mais frágeis, acelerando a perda de matéria orgânica e degradação do solo, causando danos ambientais. Aspectos vitais da melhoria da cultura através da manutenção da sustentabilidade podem ser alcançados pela modulação do metabolismo da semente que seria alcançado pela técnica de condicionamento de sementes. O condicionamento de sementes é um tratamento de sementes pré-semeadura que permite a hidratação controlada das sementes para absorver água e passar pelo primeiro estágio de germinação, mas não permite a protrusão radical através do tegumento (Devika et al., 2021).O condicionamento das sementes facilita a germinação mesmo em condições adversas, aumenta o desempenho da cultura e aumenta o potencial de rendimento. Para aliviar os efeitos da agricultura moderna, essa técnica surgiu como uma estratégia viável que protege as plantas contra estresses bióticos e abióticos (Sarkar et al., 2020).

O ‘Priming’ é um tratamento bem estabelecido para melhorar a qualidade da semente. As sementes submetidas ao condicionamento fisiológico apresentam taxas de germinação aumentadas, o que resulta em altos níveis de resistência ao estresse bióticos/abióticos e produtividade das culturas. Todas essas características que melhoram a competitividade do produto estão diretamente relacionadas ao vigor da semente, uma característica agronômica complexa controlada por múltiplos fatores genéticos e ambientais (Jisha et al., 2013; Rajjou et al., 2012). O sucesso do ‘priming’ de sementes está fortemente correlacionado com a espécie/genótipo e fisiologia da planta, lote de sementes e vigor, bem como com o método de ‘priming’ aplicado. O ‘priming’ é rotineiramente utilizado no tratamento de sementes de hortaliças, principalmente cenoura (*Daucus carota L.*), alho-poró e cebola (Gênero *Allium*) (DEARMAN et al., 1987), aipo

(*Apium graveolens L.),* alface (*Lactuca sativa L.*), escarola (*Cichorium endivia L.),* pimenta (*Genus Capsicum*) e tomate (*Solanum lycopersicum L.*)(Di Girolamo and Barbanti, 2012; Parera and Cantliffe, 2010). O condicionamento de sementes é categorizado em diferentes tipos: hidropriming, osmopriming, halopriming, priming hormonal e biopriming, e oferece amplos benefícios para as culturas. Por exemplo, a embebição de sementes usando PGR para ativar o metabolismo das sementes é conhecida como 'priming' hormonal.

Neste sentido, os reguladores de crescimento vegetal vem sendo utilizados neste condicionamento e desempenham um papel cada vez mais importante na produção agrícola global. A taxa média de crescimento das vendas globais de reguladores de crescimento vegetal foi superior a 14% na última década. Por muitos anos, a maioria dos reguladores de crescimento vegetal foram considerados pesticidas de baixa ou leve toxicidade.

Os reguladores de crescimento vegetal são definidos como compostos orgânicos sintéticos ou naturais que influenciam o processo biológico em plantas superiores em concentrações muito baixas (Rademacher, 2015). Os PGR podem aumentar ou diminuir o crescimento e desenvolvimento alterando seus processos biológicos normais (Lopez-Lauri, 2016; Rademacher, 2015; Rosen et al., 1960). Quando estes compostos ocorrem naturalmente no interior da planta, são denominados como fitormônios, mas quando aplicados exogenamente são chamados de PGR (Avery Jr., 1937). Os PGR atuam como agentes de sinalização, permitindo que as plantas mantenham a plasticidade durante o crescimento e desenvolvimento, e são, portanto, considerados os principais fatores nas respostas das plantas a estresses bióticos e abióticos (Bücker-Neto et al., 2017; Pál et al., 2018). Em termos gerais, os PGR estão envolvidos no aumento da disponibilidade fisiológica de água e nutrientes essenciais, enquanto ajudam as plantas a reduzir a carga tóxica de sal (Iqbal and Ashraf, 2007). Eles também têm um grande impacto nas atividades das enzimas antioxidantes nas plantas (Faghih et al., 2019; Iqbal and Ashraf, 2013; Qiu et al., 2014; Rhaman et al., 2021).

Sendo assim os reguladores de crescimento de plantas são conhecidos por aumentar o rendimento e trazer o crescimento desejado por unidade de terra e tempo. Os PGR também são essenciais para a germinação, desenvolvimento de flores e raízes,

maturação de sementes, armazenamento e outros processos funcionais nas plantas (Cai et al., 2015; Wu et al., 2017). Os PGR são classificados em várias classes distintas, como auxinas (Aux), citocininas (CKs), óxido nítrico (NO), brassinosteróides (BRs), giberelinas (GAs), ácido salicílico (SA), ácido abscísico (ABA), jasmonatos (JAs) e etileno. Na produção de soja, PGRs como auxina, citocinina, ácido abscísico, etileno, ácido jasmônico, ácido salicílico, giberelinas e brassinosteróides, entre outros, têm sido relatados como promotores de crescimento e produtividade (Basuchaudhuri, 2016; Behr et al., 2018; Giri et al., 2018; Roy Choudhury et al., 2019). Os PGR promovem de forma sustentável o crescimento da raiz e da parte aérea (Kim et al., 2018), aumentam a eficiência do uso da água (Giri et al., 2018), promovem a floração e a formação de vagens (Nagel et al., 2001), aumentam o teor de clorofila (Wang et al., 2018), melhoram a taxa fotossintética (Qi et al., 2013; Travaglia et al., 2009), aumentam a translocação de fotoassimiladores (Liu et al., 2019), aumentam o acúmulo de biomassa (Liu et al., 2019; Mohamed and Latif, 2017) e induzem tolerância a vários estresses abióticos (George et al., 2008; Hamayun et al., 2010) resultando em maior crescimento e rendimento.

Além disso, já foi evidenciado que os PGR foram capazes de melhorar os efeitos tóxicos causados pela metribuzina em *V. radiata*, o que foi ilustrado pelas atividades significativamente aumentadas das enzimas de desintoxicação, bem como pela redução da peroxidação lipídica, o que provavelmente garantiu a estabilidade da membrana e, portanto, induziu a tolerância da planta. Isso também foi evidenciado pelo aumento no rendimento médio das plantas. Entre os PGR (IAA, GA_3 e SA) utilizados neste estudo, o SA foi o que apresentou o melhor efeito. No entanto, o efeito combinado de todos os PGR foi melhor do que qualquer PGR individual. Portanto, podemos concluir que os PGR neutralizam os danos induzidos por herbicidas, estimulando sua resposta antioxidante quando atingem as células vegetais. Além disso, esses resultados fornecem uma base para estudos subsequentes para investigar o papel potencial dos PGR na mitigação da toxicidade de herbicidas nas lavouras. Além disso, foi estabelecido neste estudo que o co-tratamento de PGR com herbicidas leva a um aumento na produção da cultura, o que pode auxiliar na formulação de estratégias para melhorar o rendimento (Kumar et al., 2022).

No entanto, estudos recentes descobriram que muitos reguladores de crescimento de plantas e seus produtos de degradação no meio ambiente são potencialmente prejudiciais a humanos, animais e plantas. Como os principais fatores para controlar a

entrada de reguladores de crescimento de plantas no meio ambiente, os comportamentos ambientais dos reguladores de crescimento de plantas no solo podem exercer uma influência significativa sobre o risco dos reguladores de crescimento de plantas para a segurança ambiental (Chen et al., 2022).

2.1.1 Giberelinas

As giberelinas, comumente conhecidas como ácidos giberélicos, chamaram a atenção de cientistas ocidentais na década de 1950, pois haviam sido descobertas muito antes no Japão. Os produtores de arroz do Japão há muito sabiam de uma doença fúngica chamada muda tola ou doença *bakanae,* em japonês, que faz com que as plantas de arroz cresçam mais altas e eliminem a produção de sementes. Patologistas de plantas descobriram que esses sintomas na planta de arroz foram induzidos por uma substância química secretada por um fungo patogênico, *Gibberella fujikuroi*. Cultivar esse fungo em laboratório e analisar o filtrado da cultura permitiu que cientistas japoneses na década de 1930 obtivessem cristais impuros de dois "compostos" fúngicos que possuíam atividade promotora do crescimento vegetal. Uma delas, por ter sido isolada do fungo *Gibberella*, foi nomeado giberelina A. Em 1950, cientistas da Universidade de Tóquio separaram e caracterizaram 3 giberelinas diferentes da amostra de giberelina A e as nomearam giberelina A_1, giberelina A_2 e giberelina A_3 . O sistema de numeração para giberelinas usado nos últimos 50 anos baseia-se nesta nomenclatura inicial de giberelinas A_1 (GA_1), GA_2 e GA_3 (Gupta and Chakrabarty, 2013a).

No mesmo ano, dois grupos de pesquisa, um da Imperial Chemical Industries na Grã-Bretanha e outro do Departamento de Agricultura dos EUA (USDA) em Illinois, elucidaram a estrutura química do composto que purificaram da filtração da cultura de *Gibberella* e batizaram de ácido giberélico. Posteriormente, esse composto se mostrou idêntico à giberelina isolada pelo cientista japonês. Para este GA_3 também é referido como ácido giberélico. GA_3 é o principal componente na cultura de *Gibberella.* O GA_3 é o GA produzido com mais frequência em fermentações comerciais em escala industrial de *Gibberella* para usos agronômicos, horticultura outros usos científicos. A identificação de um GA a partir de um extrato vegetal foi feita pela primeira vez em 1958 com a descoberta de GA_1 a partir de sementes imaturas de feijão vuner (*Phaseolus cocineus*). À medida que mais e mais ácidos giberélicos foram sendo descobertos e diferentes fontes

vegetais foram caracterizados, um esquema foi adotado em 1968 para numerá-los (GA_1 – GA_4), em ordem cronológica de sua descoberta (Gupta and Chakrabarty, 2013a).

2.1.2 Biossíntese e ação das GAs

As giberelinas são reguladores endógenos do crescimento vegetal, possuindo compostos diterpenóides tetracíclicos. Após esforços valiosos para entender a biossíntese e os movimentos do GAs, o local apropriado do GA bioativo em plantas ou tecidos direcionados por GAs bioativos para iniciar sua ação ainda não foi confirmado. O bioensaio de plantas anãs e sua análise quantitativa revelaram a presença de GA em tecidos ativos em crescimento, ou seja, ápices caulinares, folhas jovens e flores (Jones and Phillips, 1966; Kobayashi et al., 1988). Em contraste, há alguns relatos da presença de GAs em exsudatos do xilema e do floema, indicando um transporte de gás a longa distância (Lang, 1970). As giberelinas são sintetizadas através da via terpenoide e requerem 3 enzimas, a saber, terpeno sintase (TPSs), citocromo P450 monooxigenase (P450s) e desidrogenase dependente de 2-oxoglutarato (2 ODDs), para a biossíntese de GA bioativo a partir de GGDP em plantas (Figura 1). Duas terpeno sintase, *ent* -copalil difosfato sintase (CPS) e *ent* - caureno sintase (KS), localizadas em plastídeos, envolvidas na conversão de GGDP em hidrocarboneto tetracíclico intermediário *ent* - caureno(Helliwell et al., 2001) *ent* -Kaurene é então convertido para GA12 por 2 P450s. Primeiro, *ent* - Kaurene oxidase (KO) presente na membrana externa do plastídio catalisa a oxidação sequencial em C-19 para produzir ácido *ent* -caurenóico (Helliwell et al., 2001). Em segundo lugar, a oxidase do ácido *ent* caurenóico (KAO) presente no retículo endoplasmático é posteriormente convertida em GA_{12} (Helliwell et al., 2001). O GA_4 bioativo é convertido de GA_{12} por meio de oxidações em C-20 e C-3 pela GA 20-oxidase (GA20ox) e GA 3-oxidase (GA_3ox), respectivamente.

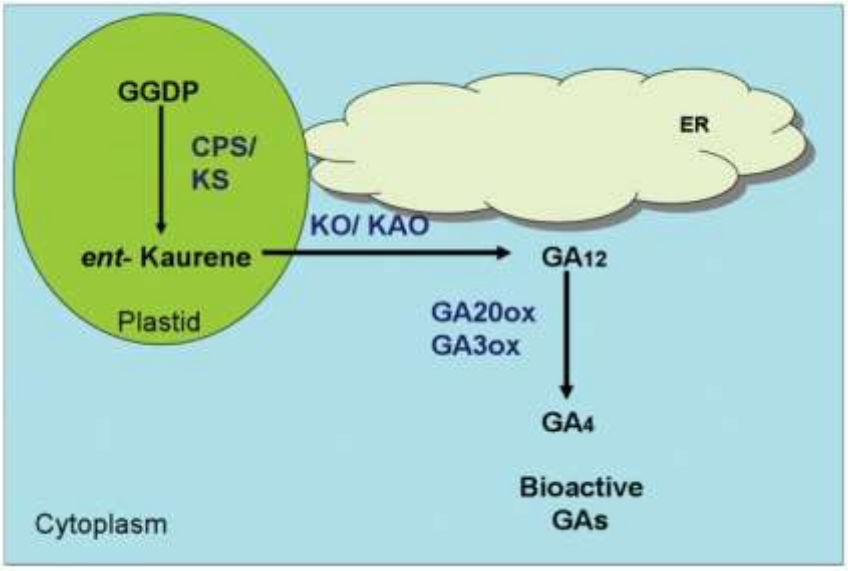


Figura 1. Via de biossíntese de giberelinas; residindo em 3 compartimentos celulares diferentes (plastídio, retículo endoplasmático e citoplasma). GGDP, difosfato de geranilgeranilo; *ent* -CDP, *ent* -copalil difosfato; CPS, *ent* -copalil difosfato sintase; KS, *ent* -caureno sintase; KO, *ent* - caureno oxidase; KAO, oxidase do ácido *ent* - caurenoico. Fonte:(Gupta and Chakrabarty, 2013b).

A quebra da dormência das sementes para a germinação é controlada por alguns fatores físicos (luz, temperatura e umidade) e pelos hormônios endógenos reguladores do crescimento (GA e ABA). O GA estimula a germinação das sementes, enquanto o ABA está envolvido no estabelecimento e manutenção da dormência (Debeaujon and Koornneef, 2000). GA exerce sua influência de duas maneiras, primeiro aumentando o potencial de crescimento do embrião e segundo induzindo enzimas hidrolíticas (Finch-Savage and Leubner-Metzger, 2006; Ogawa et al., 2003). Durante a germinação da semente, o GA embrionário é liberado, o que desencadeia a fraqueza da cobertura da semente, estimulando a expressão gênica envolvida na expansão e modificação celular, conforme relatado em (Finkelstein et al., 2008). A germinação das sementes, o alongamento do caule, o desenvolvimento do tecido meristmético e a diferenciação dos órgãos florais são altamente dependentes do sistema e mecanismo de sinalização do AG. Sendo assim o GA é necessário para quebrar a dormência das sementes levando à sua germinação.

Os reguladores de crescimento vegetal, em especial o ácido giberélico, são amplamente utilizados na agricultura em muitos países. No entanto, pouco se sabe sobre seus riscos para a saúde humana ou suas vias fisiológicas e bioquímicas. Alguns

experimentos examinaram os possíveis efeitos tóxicos de PGRs, incluindo GA 3 em mamíferos. Foi demonstrado que GA_3 causa anormalidades cromossômicas em células humanas e de camundongos (El-Mofty et al., 1994a; Zalinian et al., 1990). Em camundongos de laboratório, GA_3 em uma dose de 75 mg/L causou degeneração do fígado, inflamação do rim e diferenciação sexual prejudicada (Maciej Serda et al., 1995a). Camundongos suíços albinos adultos que receberam GA_3 por gavagem por 22 meses desenvolveram lesões cancerígenas(El-Mofty et al., 1994b). GA_3 também pode causar estresse oxidativo, resultando em radicais livres e danos celulares (El-Mofty et al., 1994b). A principal defesa enzimática são os antioxidantes, que demonstraram caçar os radicais livres; estes incluem superóxido dismutase (SOD), catalase e glutationa peroxidase (GPx), que trabalham em conjunto com uma variedade de antioxidantes não enzimáticos, incluindo a glutationa, que é a segunda linha de defesa (Troudi et al., 2011b). GA_3 também demonstrou causar estresse oxidativo em ratos, resultando no acúmulo de produtos de peroxidação lipídica e aumento dos níveis de malondialdeído (MDA) em órgãos, como fígado, rim e cérebro (Celik and Tuluce, 2007). Já foi demonstrado também neurotoxicidade e estresse oxidativo induzido por ácido giberélico em ratas durante o final da gestação e início do período pós-natal, através de alterações bioquímicas e histológicas (Troudi et al., 2012a).(Alsemeh et al., 2019), demonstrou alterações histológicas e bioquímicas induzidas pelo ácido giberélico no fígado de ratas albinas grávidas e seus descendentes. Além disso , estresse oxidativo e comprometimento da tireoide após tratamento com ácido giberélico em ratas gestantes e lactantes e seus descendentes já foram descritos (Troudi et al., 2011a). Desregulação metabólica e inibição da espermatogênese em células testiculares de ratos tratados com GA_3 são relatados (Ravikumar and Srikumar, 2005). Recentemente foi demonstrado que a exposição de ratos ao GA_3 causa toxicidade hepatorrenal, conforme demonstrado pelo aumento da atividade dos marcadores oxidativos hepáticos e renais, aumento da peroxidação lipídica da membrana e diminuição da atividade das enzimas antioxidantes hepatorrenais, com regulação positiva de citocinas inflamatórias e genes apoptóticos (Soliman et al., 2022a).

Considerando a possível toxicidade do GA_3 e levando-se em conta que os PGR podem se degradar prontamente quando expostos a fatores ambientais como luz e temperatura, resultando em perda de atividade, a aplicação da nanotecnologia na

agricultura poderá aumentar a estabilidade e a atividade deste e outros agentes ativos, ao mesmo tempo em que poderá reduzir os impactos ambientais.

2.2 Nanotecnologia

A nanotecnologia é amplamente aplicada em nossa vida cotidiana e está mudando toda a sociedade. A pesquisa sobre esse tópico disparou na última década. Vem sendo aplicada em vários aspectos da indústria de alimentos e agricultura, incluindo agricultura, irrigação/filtração de água, processamento e embalagem de alimentos, ração animal e aquicultura (Dasgupta and Ranjan, 2018; Sozer and Kokini, 2009). O setor de alimentos e bebidas é uma indústria global de vários trilhões de dólares (Cushen et al., 2012). Uma estimativa recente do impacto econômico global da nanotecnologia é projetada em pelo menos US$ 3 trilhões até 2020, o que pode empregar 6 milhões de trabalhadores nas crescentes indústrias de nanotecnologia em todo o mundo (Roco et al., 2011). Isso é muito atraente e tem impulsionado muitas empresas de alimentos envolvidas no desenvolvimento e comercialização de novos produtos baseados em nanomateriais e na melhoria da eficiência da produção, características, sabor e segurança dos alimentos. Incrivelmente, existem centenas de produtos que já foram comercializados e utilizados no ramo alimentício na última década. A maioria desses produtos são projetados “fora de alimentos”, mas “dentro” da indústria de alimentos, ou seja, materiais que entram em contato com alimentos, mas não são consumidos diretamente pelas pessoas. Nenhum novo produto contendo nanomateriais foi colocado diretamente na alimentação humana, exceto o dióxido de titânio e o óxido de ferro que já foram usados como pigmento e corante alimentar, respectivamente. A razão fundamental é que a regulamentação e a legislação são muito limitadas em relação a presença de nanopartículas em alimentos (Hussain, 2020).

Uma causa mais profunda para a regulamentação limitada é o pouco conhecimento da toxicidade e do risco que novos nanomateriais podem trazer (Bryksa and Yada, 2012; Ray, 2014). Muitos estudos se concentram na toxicidade *in vitro* de nanomateriais, enquanto muito poucos dados de toxicidade *in vivo* estão disponíveis, sem mencionar o efeito crônico de nanomateriais (especialmente nanopartículas metálicas) (Duncan, 2011; Najahi-Missaoui et al., 2020). Pelo menos várias lacunas precisam ser preenchidas: toxicidade do nanomaterial para células, tecidos/órgãos de mamíferos e efeito crônico para o corpo humano; migração de nanomateriais para

alimentos; degradação ou destino ambiental de nanomateriais; bioacumulação de nanomateriais e seu impacto nos ecossistemas.

Outro aspecto importante é a aceitação pública que muitas vezes é ignorada por pesquisadores, fabricantes, bem como autoridades (Arnaldi and Muratorio, 2013; Cormick, 2009). Os nanomateriais podem e têm sido amplamente aplicados em todos os aspectos da indústria de alimentos, desde a agricultura de alimentos, processamento, armazenamento e transporte até o nosso prato. Independentemente da população que aceita esses novos nanoalimentos, os resíduos acabarão sendo descartados no meio ambiente e provocarão impactos específicos na flora, fauna e ecossistemas. Para piorar, o método de descarte adequado ainda não foi mencionado por nenhum pesquisador, empresa alimentícia ou órgão governamental. Além disso os dados de toxicidade in vivo de nanomateriais são muito escassos, especialmente o potencial efeito crônico em órgãos humanos.

A nanotecnologia lida com nanomateriais que possuem pelo menos uma dimensão variando de 1 a 100 nm. Os nanomateriais testados na indústria alimentícia incluem os inorgânicos (NPs de metal e óxido de metal), orgânicos (principalmente NPs de produtos naturais) e combinados (argila). Dentre todas as NPs metálicas, a NP de prata é a mais produzida e aplicada comercialmente devido à sua atividade antimicrobiana, enquanto a NP de ouro é amplamente estudada como sensor/detector. As NPs de dióxido de titânio também são bem estudadas como agente desinfetante, bem como aditivo alimentar (pigmento de cor branca) e intensificador de sabor. NPs de produtos naturais são normalmente concebidos como um sistema de entrega, e também envolvidos como ingredientes ou suplementos na indústria de alimentos. Na agricultura, a nanotecnologia é empregada para aumentar a produção de alimentos, com valor nutricional equivalente ou até superior, qualidade e segurança. O uso eficiente de fertilizantes, pesticidas, herbicidas e fatores/reguladores de crescimento vegetal são as formas mais importantes de melhorar a produção agrícola. A liberação controlada de pesticidas, herbicidas e reguladores de crescimento vegetal pode ser obtida por meio do uso de nanocarreadores. Por exemplo, nanocápsulas de poli (épsilon-caprolactona) foram recentemente desenvolvidas como carreadores de herbicida para atrazina (H. C. Oliveira et al., 2015). O tratamento de plantas de mostarda (*Brassica juncea*) com nanocápsulas de poli (épsilon-caprolactona) carregadas com atrazina aumentaram a atividade herbicida

em comparação com a atrazina comercial, mostrando uma diminuição drástica nas taxas fotossintéticas líquidas e na condutância estomática, um aumento significativo do estresse oxidativo e, finalmente, perda de peso e redução do crescimento das plantas testadas (H. C. Oliveira et al., 2015). Da mesma forma, outros nanocarreadores como NPs de sílica (Cao et al., 2018) e NPs poliméricas (Kumar et al., 2017) também foram desenvolvidos como sistema de liberação modificada para fornecer pesticidas de maneira controlada. Carreadores em nanoescala podem ser utilizados para alcançar perfeitamente a entrega e liberação lenta dessas espécies. Tais estratégias são conhecidas como "agricultura de precisão" que melhora o rendimento das culturas, mas não danifica o solo e a água (Duhan et al., 2017). Além disso, certos nanomateriais per se podem atuar como pesticidas com maior toxicidade e sensibilidade nanomateriais de óxido de metal como ZnO, TiO 2 e CuO são amplamente estudados para proteger plantas de infecções por patógenos devido à sua toxicidade intrínseca. Tomamos ZnO NPs como exemplo. Tem sido demonstrado que ZnO NPs podem efetivamente inibir o crescimento de micróbios como *Fusarium graminearum* (Dimkpa et al., 2013), *Aspergillus niger*, *Aspergillus fumigatus*, *F. culmorum* e *F. oxysporium*, mostrando forte atividade antifúngica e antibacteriana (Rajiv et al., 2013).

A saúde humana após a exposição a nanomateriais como aditivos alimentares e outros ingredientes funcionais de alimentos e produtos agrícolas também é uma grande preocupação. O contato direto de nanomateriais usados como aditivos alimentares/ingredientes funcionais/nutricionais pode representar ameaças à saúde humana. A produção de espécies reativas oxidativas (ROS) atua como um dos principais mecanismos toxicológicos causadores de dano e morte celular (He et al., 2014). A superprodução de ROS pode levar à autofagia (He et al., 2014) danos aos neurônios(Long et al., 2007) e danos graves ao DNA (Singh et al., 2009) e potencialmente mutagênese, carcinogênese e doenças relacionadas ao envelhecimento em humanos. Reações alérgicas e danos causados pela liberação de íons metálicos de nanomateriais também são possíveis resultados adversos da exposição a nanoprodutos alimentares (He and Hwang, 2016). Além disso, o acúmulo de nanomateriais em partes comestíveis (sementes) de plantas (Tripathi et al., 2017) e corpo humano (Chen et al., 2013) pode causar problemas mais graves em concentrações mais altas e interações de longo prazo.

Considerando que os PGR podem se degradar prontamente quando expostos a fatores ambientais, como luz e temperatura, resultando em perda de atividade. Nesse contexto, um dos usos da nanotecnologia na agricultura é fornecer materiais em nanoescala capazes de aumentar a estabilidade e a atividade de vários agentes ativos, ao mesmo tempo em que reduzem os impactos ambientais(Campos et al., 2014; Grillo et al., 2016; Kah and Hofmann, 2014; Khot et al., 2012; Rossi et al., 2014). Por exemplo, o encapsulamento de agroquímicos em nanopartículas pode melhorar suas propriedades físico-químicas e reduzir problemas como sorção e lixiviação, aumentando assim a biodisponibilidade e eficiência, reduzindo as concentrações necessárias e tornando os produtos mais seguros no meio ambiente (Campos et al., 2015; Grillo et al., 2014a).

Sistemas de nanocarreadores baseados em quitosana (CS) têm uma gama de aplicações para proteção de cultivos. As nanopartículas produzidas usando várias técnicas podem ser empregadas como sistemas de entrega de pesticidas e fertilizantes, bem como para entrega de genes (Kashyap et al., 2015b). A própria nanopartícula pode apresentar atividade fungicida, dependendo do peso molecular do CS, do tamanho da nanopartícula e do potencial zeta (Ing et al., 2012). O uso de nanopartículas de CS carregadas com paraquat resultou na redução da toxicidade do herbicida, permitindo o controle de ervas daninhas com menos danos ao meio ambiente (Grillo et al., 2014b). Nanopartículas de CS preparadas com adição de proteínas aniônicas isoladas de *Penicillium oxalicum* pode ser usado no tratamento de sementes para controle de fungos, ao mesmo tempo em que melhora o desenvolvimento da planta (Sathiyabama and Parthasarathy, 2016). Outra aplicação de nanopartículas compostas de CS é aumentar a solubilidade, estabilidade e absorção celular de compostos antioxidantes , aumentando suas aplicações nas indústrias alimentícia e farmacêutica (Jeong et al., 2005). Sistemas de nanocarreadores foram desenvolvidos para reguladores de crescimento de plantas, como brassinosteróides (Quiñones et al., 2010) e *O* -naftilacetil (Tao et al., 2012).(Liu et al., 2013) mostraram que a associação de ácido giberélico e CS gerou um sistema de liberação sustentada , aumentando a solubilidade do princípio ativo e protegendo contra a degradação térmica e fotolítica em diferentes valores de pH.

Pereira et al (2017) avaliaram nanopartículas de CS como sistema carreador para GA. As nanopartículas permaneceram estáveis por 60 dias e os dois sistemas diferiram

quanto ao mecanismo de liberação, sendo a liberação dependente de fatores como pH e temperatura. Ensaios de bioatividade usando *Phaseolus vulgaris* mostraram que as nanopartículas de quitosana revestidas com alginato e preenchidas com acido giberélico foram mais efetivas em aumentar a área foliar e os teores de clorofilas e carotenoides. Os sistemas desenvolvidos apresentaram bom potencial, proporcionando maior estabilidade e eficiência deste hormônio vegetal em aplicações agrícolas (Pereira et al., 2017) .

Nanopartículas de quitosana revestidas com alginato (ALG.CS) e quitosana revestidas com tripolifosfato (TPP.CS) têm sido utilizadas como carreadores de fármacos e agroquímicos em sistemas de liberação sustentada que podem prolongar a duração da ação do ativo químico e melhorar sua estabilidade (Sarmento et al., 2006; Silva et al., 2010). O polímero CS é um polissacarídeo catiônico derivado da desacetilação da quitina (Jiang et al., 2014). Tem muitas aplicações na agricultura, com propriedades que incluem atividade fungicida e melhoria dos mecanismos de defesa das plantas (Kashyap et al., 2015a). O polímero de alginato (ALG), extraído de algas marrons, é atóxico, biocompatível, biodegradável e possui uma carga negativa característica, devido à presença de grupos de ácido urônico (Azevedo et al., 2014). Entre os vários métodos desenvolvidos para preparar nanopartículas de quitosana, a gelificação iônica usando tripolifosfato de sódio (TPP) é atraente porque o processo é relativamente fácil de controlar e não requer o uso de solventes orgânicos (Fan et al., 2012). Nanopartículas de alginato/quitosana (ALG/CS) e quitosana/tripolifosfato (CS/TPP) têm sido utilizadas como carreadores de fármacos e agroquímicos em sistemas de liberação sustentada que podem prolongar a duração da ação do ativo químico e melhorar sua estabilidade(Rampino et al., 2013; Woitiski et al., 2009).Entretanto, estes nanossistemas foram pouco avaliados *in vivo*, em especial em organismos não-alvo, os quais poderão ser afetados pela aplicação destas formulações nas sementes no momento do plantio.

2.3 *Caenorhabditis elegans*

C. *elegans* é um nematoide que se alimenta de fungos e bactérias do solo e de frutas em decomposição. Com pouco mais de 1 mm de comprimento, os adultos podem ser vistos a olho nu. Desde a caracterização inicial do modelo por Sydney Brenner na década de 1960, a pesquisa de *C. elegans* tem sido essencial na elucidação de vários aspectos básicos da biologia (JG et al., 1986). A capacidade de cultivar centenas de

animais em uma única placa de Petri alimentando-se de bactéria, sua transparência, um ciclo de vida rápido (3 dias) uma vida útil curta (2-3 semanas) e crescimento fácil e barato no laboratório tornam estes vermes um modelo ideal para avaliação de efeitos biológicos(Ploeger et al., 2016). Avanços na genética e biologia molecular permitiram a identificação de todas as suas 959 células. O *C. elegans* foi o primeiro organismo multicelular com o genoma totalmente sequenciado (Ruszkiewicz et al., 2018). O tamanho do genoma é aproximadamente 100 milhões de pares de bases de comprimento e, embora seja notavelmente menor que o genoma humano, ambos os genomas têm um número semelhante de genes (vermes 20.000 genes; humanos 23.000). Há uma sobreposição substancial entre *C. elegans* e humanos com relação a genes e vias bioquímicas. As análises de bioinformática sugerem que 60 – 80% dos genes do *C. elegans* são homólogos aos genes humanos(Hulme and Whitesides, 2011). O número reduzido de células, junto com o número de células invariantes e o destino dentro dos tecidos somáticos, permitiram a identificação de cada célula (Wood WB, 1988).

C. elegans são predominantemente hermafroditas, e os machos surgem espontaneamente com uma frequência de aproximadamente 0,1%. Seu corpo é essencialmente um tubo, o trato digestivo dentro de outro tubo, a cutícula e a epiderme. Entre os dois tubos há uma cavidade cheio de fluídos, o pseudoceloma. A anatomia do *C. elegans* inclui os seguintes sistemas: um sistema digestivo, um sistema reprodutivo, sistema nervoso e sistema excretor, todos protegidos pela cutícula. A cutícula do verme, pode ser utilizada como modelo de pele, visto que sua função e composição são semelhantes a da pele humana (Chisholm and Hsiao, 2012). Digno de nota, o intestino de *C. elegans* apresenta uma arquitetura celular semelhante em comparação com animais superiores em termos de polaridade celular das células intestinais (enterócitos), incluindo a presença de domínios apicais e basolaterais, junções celulares e microvilosidades (Fraser et al., 2000; Honnen et al., 2012). Finalmente, os mecanismos de transporte de biomoléculas através das barreiras biológicas são altamente conservadas (Balla and Troemel, 2013; Sato et al., 2014). O *C. elegans* não tem sistema circulatório e depende da difusão passiva no fluído pseudocelômico para o transporte de O_2, CO_2, nutrientes e moléculas exógenas (Wood WB, 1988).

O *C. elegans* tem um ciclo de vida rápido: a 25ºC, a embriogênese dura 14 horas e o desenvolvimento pós-embrionário para adulto dura 36 horas. O desenvolvimento pós-

embrionário progride através de quatro estágios larvais – conhecidos como L1, L2, L3 e L4 – antes de se tornar adulto produtor de ovos. Os estoques de *C. elegans* podem ser mantidos melhor a 16-25ºC, normalmente a 20 ºC. Mesmo com escassez de alimento, o verme pode se manter por meses, pois é capaz de entrar em um estágio larval alternativo, denominado dauer, que é adaptado para sobreviver em condições ambientais adversas. O sistema reprodutivo produz gametas maduros (gametogênese) e fornece a estrutura e o ambiente para fertilização e postura de ovos. A espermatogênese começa durante o estágio larval L4 e se completa no adulto jovem logo após a muda (Hunt, 2017b). Hermafroditas produzem ~ 300 embriões por autofecundação. Os ovos fertilizados no útero são expelidos através da vulva. A postura dos ovos é facilitada pela contração dos músculos sexuais, que são regulados pelo sistema nervoso (Wood WB, 1988). Vários parâmetros podem ser estudados em *C. elegans*, incluindo número de ovos ou progênie, efeitos no desenvolvimento da progênie ou efeitos na reprodução de diferentes gerações filiais. Assim, o *C. elegans* facilita a experimentação e permite resultados iniciais rápidos no campo da reprotoxicidade (Moon et al., 2019).

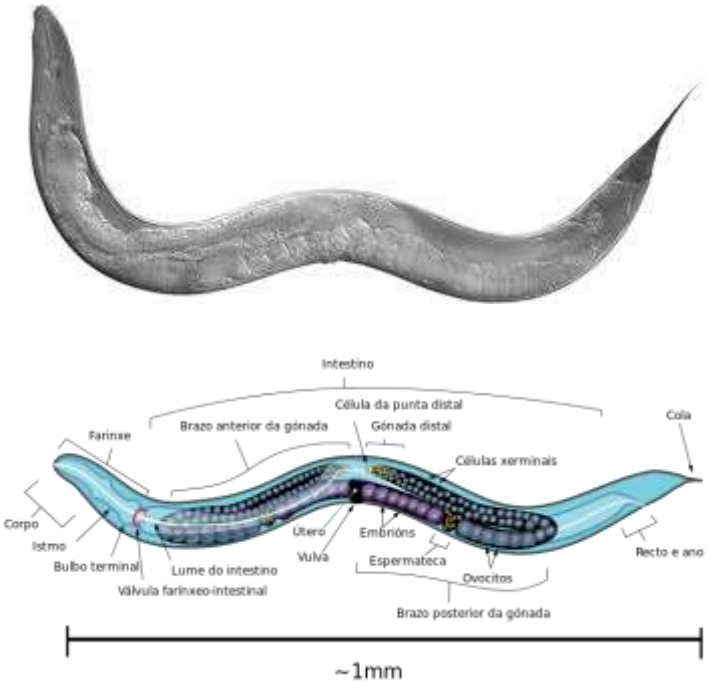


Figura 2: Anatomia e morfologia de *Caenorhabditis elegans*: Na parte superior imagem em microscopia de um exemplar adulto hermafrodita. Abaixo, um esquema representativo da anatomia de *C. elegans* (Fonte: https://www.wormatlas.org/).

O *C. elegans* provou ser um organismo modelo útil para a investigação de aspectos moleculares e celulares de várias doenças humanas complexas, incluindo doença de Alzheimer , doença de Parkinson, diabetes, distrofia muscular e câncer (Braungart et al., 2004; Levitan et al., 1996; Ogg et al., 1997; Sternberg and Han, 1998; Zhou and Chen, 2011). Na última década, este nematoide surgiu como uma ferramenta para a descoberta de medicamentos, pois suas propriedades o tornam um excelente candidato para a triagem e alto rendimento baseada em todo o organismo (Sternberg and Han, 1998). As principais vantagens do uso de *C. elegans* na triagem de alto rendimento incluem: 1) a capacidade de modelo de doenças humanas complexas que não podem ser facilmente reproduzidas in vitro ou em modelos unicelulares, 2) a capacidade de avaliar simultaneamente a eficácia e absorção da droga, distribuição, metabolismo, excreção ou características de toxicidade, 3) um grande repertório de fenótipos classificáveis, 4) a complexidade do sistema multicelular e de múltiplos órgãos existente em um organismo inteiro aumenta as chances de identificar drogas que serão mais eficazes em organismos multicelulares mais complexos, como humanos, 5) a disponibilidade de ferramentas genéticas e recursos genômicos (por exemplo RNAi) que simplificam a identificação de alvos de drogas (Giacomotto and Ségalat, 2010).

Recentemente, há uma tendência crescente de uso de *C. elegans* na avaliação da biossegurança de nanopartículas. Pesquisadores conseguem investigar os efeitos biológicos de NPs em estágios iniciais do verme. A caracterização in vivo imediata de NPs após a síntese abre oportunidades para a sua rápida otimização, eliminando a necessidade de ensaios demorados baseados em cultura de células e supera as dificuldades envolvidas no uso de modelos de mamíferos. É importante notar que o uso de *C. elegans* está em conformidade com os princípios ético amplamente aceitos, conhecidos como os 3 Rs para experimentos com animais que são: Redução do uso de animais superiores, Refinamento das técnicas atuais e, por fim, substituição de animais por modelos alternativos (Gonzalez-Moragas et al., 2015a).

Neste sentido, nosso grupo vem estudando os efeitos de nanossistemas poliméricos em *C. elegans* e já demonstramos que há redução de toxicidade da clozapina, quinina associada à curcumina, eugenol e dos óleos de Neem e de cravo neste modelo animal (Pascoli et al., 2019; Salgueiro et al., 2015; Saraiva et al., 2021; Velasques et al., 2018). Nanocápsulas de poli-ε-caprolactona (PCL) contendo melatonina apresentaram

melhor efeito antioxidante do que a sua forma livre neste nematoide. Por outro lado, nossos estudos indicaram a toxicidade oral dos polímeros PCL e da quitosana (mesmo quando neutralizada com tripolifosfato de sódio (TPP), mas também que a zeína é bastante biocompatível com este nematoide (Salgueiro et al., 2015; Velasques et al., 2018)

3. Justificativa

Considerando que o uso contínuo de PRG na agricultura contribui para o aumento dos níveis de contaminantes no solo e nos rios e sabendo o impacto que isso pode gerar no meio ambiente, este trabalho visa avaliar a segurança de um PGR, o GA_3, e de nanoformulações de quitosana carregadas com GA_3 em relação a efeitos nos sistemas nervoso e reprodutivo em *Caenorhabditis elegans* (Schulenburg and Félix, 2017)(Schulenburg and Félix, 2017)(Schulenburg and Félix, 2017)(Schulenburg and Félix, 2017)(Schulenburg and Félix, 2017)(Schulenburg and Félix, 2017). Nossa hipótese é que as nanopartículas contendo o ácido giberélico e o ácido giberélico livre apresentem perfis de toxicidade diferentes, sendo o composto livre mais tóxico. E com base na literatura e nos estudos prévios em modelos vertebrados, esperamos encontrar alterações no sistema antioxidante, um aumento de espécies reativas e alterações no sistema reprodutor causadas pelo PGR.

4. Objetivos

4.1 Objetivo Geral

Comparar a toxicidade reprodutiva e neuronal causada por formulações contendo ácido giberélico livre e nanoencapsulado em *Caenorhabditis elegans*.

4.2 Objetivos Específicos

-Determinar a segurança das nanopartículas de GA_3 em *Caenorhabiditis elegans* através da avaliação da sobrevivência e longevidade;

- Avaliar se os tratamentos irão causar alterações no nível de espécies reativas e no sistema antioxidante do verme;

-Avaliar os efeitos dos diferentes tratamentos com GA_3 livre e nano encapsulados no sistema neuronal dos vermes;

-Comparar os efeitos dos diferentes tratamentos com GA_3 livre e nanoencapsulada na reprodução dos animais;

- Determinar qual das nanoformulações de GA_3 nanoencapsulado apresenta menor risco ambiental e se este é menor do que o GA_3 livre.

5 ARTIGO CIENTÍFICO

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de artigo científico. As seções Materiais, Resultados e Discussão, Conclusão e Referências Bibliográficas encontram-se no próprio manuscrito. O manuscrito ainda não está formatado pois a revista para a qual será submetido será definida após as considerações da banca.

Chronic exposure to chitosan nanoparticles containing Gibberellic Acid (GA_3) cause reprotoxicity and neuronal damage in *Caenorhabditis elegans*

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Graphical Abstract



Abstract

Many chemicals are currently applied in agriculture, and plant growth regulators (PGR) are among the most widely used. Gibberellic acid (GA_3) is an endogenous plant growth regulator used worldwide in agriculture; however, little is known about its biochemical and physiological effects on mammals and its impact on organisms living in the environment. A strategy to direct the action of this phytohormone to the target plant and reduce the impact on the environment is the application of nanotechnology, whose effects on micro and macroorganisms are still little known. This study aims to evaluate the possible neurotoxic and reprotoxic effects of GA_3 and nanoparticles (NPs) containing this PGR in the experimental model *Caenorhabditis elegans*, which is a model considered as a non-target of PGRs and can be found in the soil. The worms were chronically exposed (48h) to different concentrations of plant growth regulator compound and chitosan nanoparticles containing gibberellic acid, in addition to white NPs controls. After treatments, toxicity assays such as survival, development, reproduction, oxidative stress markers and neuronal effects were performed. We observed an increase in the mortality of exposed worms. An increased incidence of apoptotic events in germ cells was demonstrated, which was consistent with the observed reductions in reproductive capacity. Furthermore, exposure promoted significant behavioral changes affecting swimming movements and radial locomotion, which were associated with changes in fluorescence intensity and morphology of dopaminergic and cholinergic neurons. Notably, our results demonstrated the toxicity induced by GA_3 through the presented mortality, alterations in the reproductive profile and neuronal alterations that reflect in the behavior of the worm. Furthermore, the results indicate that the observed effects on the evaluated parameters were caused by the nanoparticles per se. These results suggest that the development of nanoparticles targeting agricultural applications needs further studies to optimize the composition and thus reduce its toxicity to non-target organisms.

Keywords: nanotoxicology; reactive species; mitochondrial dysfunction; plant growth regulators

Introduction

Many chemicals are currently used in agriculture, and plant growth regulators (PGRs) are among those. The amounts of these substances applied to the environment may soon exceed those of insecticides (NICKELL, 1979). These regulators are plant hormones (phytormones), which are small organic molecules commonly used to increase crops production (Ashikari et al., 2005; Silverstone and Sun, 2000). These plant hormones promote stem elongation, overcome seed dormancy, are involved in fruit development, flowering, mobilization of food reserves in seed germination, youthfulness and plant sexual expression (Xiong and Zhu, 2003). Therefore, PGR are commonly used in agriculture to promote plant growth and development (Azim, 2017; Troudi et al., 2011b). One example is gibberellic acid (GA_3), a naturally occurring plant growth regulator of the gibberellin class, being one of the most active hormones within the gibberellin family (Arous et al., 2001; El-sayyad et al., 2012). GA_3 is a hormone that accelerates and increases plant yield by stimulating cell division, increasing DNA synthesis and causing cell elongation, leaf expansion and flower development (De Oliveira et al., 2017; Silverstone and Sun, 2000). Plants naturally produce GA_3 to promote normal growth, but currently the phytohormone has been applied exogenously to increase agricultural yields (Silverstone and Sun, 2000). In fact, GA_3 or one of its metabolites, when applied to dwarf varieties of peas, broad beans or corn, greatly accelerates their growth (Jones, 1973).

Although GA_3 is widely used in agriculture, its effects on human health are poorly explored. Some studies have examined the possible toxic effects of PGR including GA_3 in mammals. Many chemicals like plant growth regulators can induce oxidative stress, lipid peroxidation and cause various diseases. Previous studies have shown that regulators were present in the diet of herbivorous and omnivorous animals, where hormone treatments caused increased serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), amylase and creatine phosphokinase (CPK) compared to control mice. Studies with animal models have shown that the subacute administration of GA_3 caused an increase in lipid oxidation and alteration of the antioxidant system in the testes of rats. Chronic administration of GA_3 has also been reported to increase the risk of tumor formation and oxidative stress in vertebrates (Sabry, 2017). GA_3 has also been shown to cause alarming toxicity to mammalian tissues, particularly in the breast and lung (El-Mofty et al., 2009) kidney and liver, and GA_3 was shown to induce liver neoplasia in frogs, suggesting that the tumors

could be misdiagnosed as hepatocellular carcinomas (El-Mofty and Sakr, 1988; H. Ustun, 1992). In addition, studies have shown GA_3- induced neurotoxicity in rat pups which mothers were treated with GA_3, since there was a significant increase in malondialdehyde levels and a decrease in the activities of antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase in their brain and cerebellum.

Humans are primarily exposed to GA_3, as well as other PGRs, through ingestion of food products that contain these compounds, particularly fresh fruits such as dates, palms, melons, strawberries, grapes and some vegetables such as tomatoes, peppers, cabbage, cauliflower and olive, as well as through drinking water (Hussein et al., 2011; Troudi et al., 2011b). Because GA_3 is widely used and because animals and humans depend on plants to feed, the general population is at risk of exposure when consuming plant food products (Vikramathithan et al., 2009). In addition, occupational exposure of workers to GA_3 in agricultural areas can occur through dermal contact or dust inhalation in the workplace, which may cause acute toxicity (Arteca, 1996). In addition, PGRs application may contribute to increased levels of contaminants in soil and rivers (Hermosin et al., 2013; Moreno-González et al., 2013; Palma et al., 2014). Therefore, alternative formulations that can reduce the environmental and health impact of these growth regulators must be sought, in order to minimize environmental contamination and thus human exposure. Thus, alternative formulations that can reduce the environmental impact of these PRGs should be sought. Nanotechnology is being developed and applied in order to minimize such problems; for example, nanoparticles loaded with herbicides allow the reduction of active ingredients and removal of inert ingredients through formulations derived from constituents such as lipids, polysaccharides and biodegradable polymers (Silva et al., 2011). These nanosystems make it possible to reduce the concentration of active principles, as they provide greater stability against degradation of the bioactive compound, greater solubility and a controlled release mechanism, in addition to protecting photosensitive compounds, allowing the use of small doses. Efficient use of fertilizers, pesticides, herbicides and plant growth regulators are the most important ways to improve agricultural production. The controlled release of pesticides, herbicides and plant growth regulators can be achieved through the use of nanocarriers. All these features reduce the need for continuous applications and therefore reduce pollution and the risk of human poisoning (Chen and Yada, 2011; Nair et al., 2010; J. L. de Oliveira et al., 2015).

To determine the safety of these NPs, models that offer quick, low-cost tests that help predict impacts on the environment and effects on mammals should be used, one of which is the invertebrate nematode *Caenorhabditis elegans*. *C. elegans* is an in vivo model that has been used in pharmacological and toxicological screening tests. This model has several advantages, including low maintenance costs, rapid reproduction, the possibility of monitoring larval development until death, and it was also the first living being to have its genome and transcriptome completely sequenced and characterized, respectively. *C. elegans* is a small nematode (adult worms are ~1 mm long) that can be maintained at low cost and handled using similar techniques as in vitro. Unlike toxicity tests using cell cultures, *C. elegans* toxicity tests provide data from an entire animal with intact, metabolically active digestive, reproductive, endocrine, sensory, and neuromuscular systems (Hunt, 2017a). The fact that the soil is one of the habitats of *C. elegans* makes this model viable for tests of soil quality and toxicity of its contaminants (Höss et al., 2009; Sochová et al., 2007b). *C. elegans* is often used in nanoparticle studies, as it allows the assessment of nanotoxicity endpoints, such as reductions in body length, progeny and locomotion behavior, in addition to effects on survival and lifespan (Gonzalez-Moragas et al., 2015b; Li et al., 2012; Mohan et al., 2010; Scharf et al., 2013).

One of the biggest advantages of the model is its nervous system, which is well characterized and considered to be structurally and functionally similar to that of mammals. The nematode nervous system contains 302 neurons in adult hermaphrodites (383 in males), divided into 118 morphologically distinct classes, and 56 glial cells, which together form more than 7600 synapses (Ruszkiewicz et al., 2018). It has dopaminergic, cholinergic, glutamatergic and GABAergic systems, for example. *C. elegans* is the only organism which neuronal connections diagram has been determined - all neurons and the main interactions between them are well characterized and mapped. The biochemistry of the nervous system is also highly conserved in mammals - as worms express ion channels, receptors, vesicular transporters and other similar synaptic components (Bargmann, 1998). Its transparent bodies also provide researchers with the ability to visualize morphological changes in the nervous system via fluorescent proteins, making it suitable for observing changes due to toxic insults in vivo. Other advantages include the rapid life

cycle, which are relevant for breeding investigations and the possibility of predicting the findings for mammals due to the high genetic homology. After the embryonic period, they develop into four larval stages, from L1 to L4. Hermaphrodite adults can produce up to 300 offspring during their short reproduction period (4 days). Reproduction is a very sensitive process and many parameters are used to assess reprotoxicity in this animal. Several studies provide evidence that this worm responds after exposure to various natural compounds with increased resistance to stress and/or even extending lifespan (Possik and Pause, 2015).

Considering that the continuous use of regulators in agriculture contributes to the increased levels of contaminants in the soil and in the rivers and knowing the impact that this generates in the environment, this work aims to evaluate the safety of a PGR, the GA_3, in relation to the effects in the nervous and reproductive systems, as well as whether nanotechnology can reduce this toxicity, using the nematode *Caenorhabditis elegans* as an experimental model.

Material and methods

Chemicals

GA_3 (Figure 1), cholesterol, dopamine and 5(6)-carboxy-2',7'-dichlorofluoroscein diacetate were obtained from Sigma. CMXRos Mitotracker was obtained from Thermo Fisher. All other reagents were obtained from local suppliers and were of analytical grade.

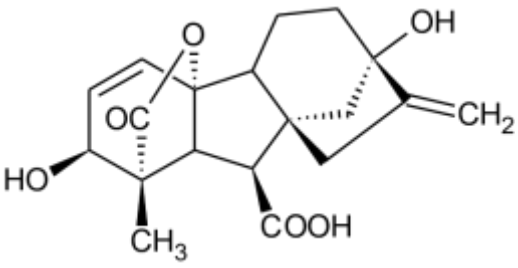


Figure 1: Chemical structure of gibberellic acid (GA_3).

Preparation and Characterization of Nanoparticles

The nanoparticles of CS.TPP.GA_3 (chitosan NP/sodium tripolyphosphate/GA_3) and ALG.CS.GA_3 (chitosan NP/alginate-GA_3) were synthesized and characterized by the Environmental Nanotechnology Laboratory of UNESP/SOROCABA. Parameters such as

polydispersion, encapsulation efficiency, stability and zeta potential, as well as microscopy to observe the morphology of NPs were used as parameters during characterization.

Strains, Maintenance and Synchronization of *Caenorhabditis elegans*

The strains used during the study were N2 (wild type); BY200 (*dat-1::GFP(vtIs1*); LX929 *vsIs48* [*unc-17p :: GFP*]; MD701 (*bcls39 [lim-7p :: ced-1 :: gfp + lin-15 (+)]* V. All strains were obtained from the *Caenorhabditis* Genetic Center (Minesota, USA), as well as the strain of *Escherichia coli* OP50 used as food for the worms. During this study, the nematodes were kept in NGM medium (nematode growth media) with bacteria E. coli OP50, preserved in an incubator at 20ºC. Worms at stage L1, used in all exposures, were obtained by a synchronization process, which consists of exposing pregnant worms to a lysis solution (0.45 N NaOH, 2% HOCl w/v) in order to separate the eggs from the worms. After that, the eggs are placed in plates with liquid medium. After 14 h, the eggs hatch and release the L1 larvae.

Chronic exposure to the nanoparticles

Worms at the first larval stage (L1) were exposed to different concentrations of the formulations (active ingredient GA_3, empty TPP.CS NPs, empty ALG.CS NPs, GA_3 loaded TPP.CS NPs and GA_3 loaded ALG.CS NPs) for 30 min in liquid medium (0.5% saline solution), then transferred with the treatment for NGM plates with *E. coli* OP50. After 48h of exposure, the worms were analyzed. This treatment method was used to simulate a chronic agricultural application. For this reason, the worms were not washed after exposure to the treatments. Initially, we performed a dose-response curve to obtain a lethal concentration (LC_{50}) using the following concentrations: 1, 5, 10, 50, 100, 125 and 150 μg/mL).

Survival test

Worms at the L1 larval stage (2,000 worms), treated as previously described, were placed on NGM plates with *E. coli* OP50 bacteria. After 48h, the number of surviving worms on each plate was counted. A transparent grid was placed below the NGM plate and 25 quadrants were analyzed under a dissecting microscope, where live worms were differentiated from dead worms (immobile and without body or pharyngeal reaction to touch). At least 5 independent experiments were done.

Body size

To assess developmental delays, wildtype treated worms were transferred to microscope slides (containing 1 mM Levamisole) and then images were acquired using an inverted microscope (MEDILUX MDL-INV-1) connected to a digital camera (SAMSUNG ST64). The length and body area of 10 worms per group/per assay was measured using ImageJ software. At least 5 independent experiments were done.

Reproduction rate

Reproduction was assessed by counting the progeny size after exposures. One worm at the L4 stage from each treatment (in triplicates) was transferred individually daily to NGM plates with *E. coli* OP50 and that worm was allowed to lay eggs. The hatched larvae (L2 larvae) were counted until the end of the reproductive period of each worm. At least 5 independent experiments were done.

Assessment of apoptotic bodies

To verify apoptosis, strain MD701 (*bcls39 [lim-7p :: ced-1 :: gfp + lin-15 (+)*] V, which has a CED-1 protein tagged with green fluorescent protein (GFP) was used. CED-1 is a caspase protein that binds to the surface of germ cells that detach and degrade during apoptosis. Worms at stage L1 were exposed as described above. After treatment, 10 worms from each group, on the first day of adulthood, were separated for be evaluated and photographed using the fluorescent microscope EVOS FLoid Cell Imaging Station (Thermo Fisher Scientific) to perform apoptotic cell counts and obtain representative images. At least 5 independent experiments were done.

Swimming-induced paralysis assay (SWIP)

This behavioral assay was performed using the N2 strain, which was treated as described above. 48 hours after treatment, 5 worms per treatment group were placed in a well of a 24-well plate, containing 300µl of M9 buffer and exposed to dopamine (5mM) in the well. The paralysis rate was counted for 5 min after the addition of dopamine.

Control worms were only exposed to dopamine. A negative control group that did not receive dopamine was also evaluated and these worms continued to swim during the five minutes of observation. At least 5 independent experiments were done.

Swimming test

Ten wildtype worms of each tested concentration were transferred to plates containing 2 mL of M9 buffer (0.02 M KH_2PO_4, 0.04 M Na_2HPO_4 and 0.085 M NaCl) and habituated to the new environment for 1 min. Subsequently, the swimming movements were recorded and the videos analyzed using Image J software with Worm Tracker plugin. We measured the speed and distance traveled by the worms in liquid medium for 1 minute. At least 5 independent experiments were done.

Assessment of cholinergic and dopaminergic neurons

LX929 and BY200 worms exposed as described above were collected 48 hours after exposure with saline 0.5% in microtubes and washed 3 times with the buffer after natural sedimentation. Then, the worms were transferred to glass slides containing Levamisole (1 mM) and covered with cover slips to obtain images in a fluorescent microscope Floid Cell Imaging Station® (Thermo Fisher Scientific). To verify the morphology of the neurons, 10 worms were evaluated per concentration and the number of worms that presented neurons with abnormal morphology in the axons and dendrites was counted. In addition, we evaluated the fluorescence intensity of these neurons using ImageJ software (National Institutes of Health, USA). At least 5 independent experiments were done.

Reactive oxygen species levels determination

To detect the levels of reactive oxygen species (ROS), 48h after exposure to the GA_3 and the nanoformulations, N2 strain worms were transferred to a solution containing 50 mM of 5(6)-carboxy-2',7'-dichlorofluoroscein diacetate (carboxy-DCFH-DA, Sigma) a well-established method to detect and quantify ROS (Eruslanov and Kusmartsev, 2010). Worms were incubated for 1 hour at 20°C and protected from light. Excess dye was removed by washing the worms 3 times with phosphate bufferby natural sedimentation. For imaging, worms were placed on glass slides containing Levamisole (1 mM) for anesthesia and images of 10 worms per group were acquired. Fluorescence was observed

using a Floid Cell Imaging Station® microscope (Thermo Fisher Scientific) and intensity was measured using ImageJ software (National Institutes of Health, USA). The results were normalized by dividing the observed fluorescence by the size of the worm. At least 5 independent experiments were done.

SOD-3 expression

To verify the antioxidant system of *C. elegans* after exposure, the expression of SOD-3 (superoxide dismutase-3) was evaluated using a strain which the promoter gene for this protein is marked with green fluorescent protein (GFP). To evaluate SOD-3, CF1553 worms were exposed as described above. In the analysis, the worms were transferred to glass slides containing Levamisole (1 mM) and covered with coverslips to obtain images in a Floid Cell Imaging Station® microscope (Thermo Fisher Scientific). The fluorescence intensity of 10 worms was further evaluated using ImageJ software (National Institutes of Health, USA). At least 5 independent experiments were done.

Mitotracker Assay

Mitotracker assay was performed to observe alterations in the mitochondrial integrity according to (Exil et al., 2010). To assess membrane integrity, N2 worms were exposed as described above. First, a stock solution was prepared with 50 µg of MitoTracker Red + 650 µL of DMSO, protected from light. Then the worms exposed to the treatment were washed and suspended in an M9 lid after the washes. Then 45µL of the Mitotracker stock solution was added to the eppendorf with the worms and homogenized for 2 hours in the dark. In the sequence, the animals were transferred to NGM plates, previously seeded with bacteria, to carry out the intestinal cleaning of the dye for 2 hours. In the analysis, the worms were transferred to glass slides containing Levamisole (1mM) and covered with coverslips to obtain images in a Floid Cell Imaging Station® microscope (Thermo Fisher Scientific). The fluorescence intensity of 10 worms was further evaluated using ImageJ software (National Institutes of Health, USA). The results were normalized by dividing the observed fluorescence by the size of the worm At least 5 independent experiments were done.

Statistical analysis

Statistical analyzes were performed using GraphPad Prism 8.0 software. Data were submitted to the Shapiro-Wilk normality test to verify normal and non-normal distribution. For behavioral tests (non-parametric data) post hoc Kruskal-Wallis and Dunn tests were used. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test as post hoc were performed for analysis of survival, body size, neuronal abnormality score, mitochondrial function and behavior analysis (parametric data). A $p < 0.05$ was considered statistically significant.

Results

Worms survival after exposure to the formulations

To find the lethal concentration of the NPs, a concentration-response curve was performed. The results demonstrated that the empty and GA_3-loaded TPP.CS and ALG.CS nanoparticles reduced worms survival rate (Figures 2A-D). However, mortality rate of worms exposed to free GA_3 was similar to that observed in worms exposed to empty NP, which may indicate that the observed toxicity is caused by the formulation itself. Through the survival curve, we chosen two concentrations to be used in the next experiments: 50 μg/mL, as it is a lower concentration than the LC_{50} for all NPs, and 150 μg/mL, which was the concentration that showed mortality in the group exposed to GA_3.

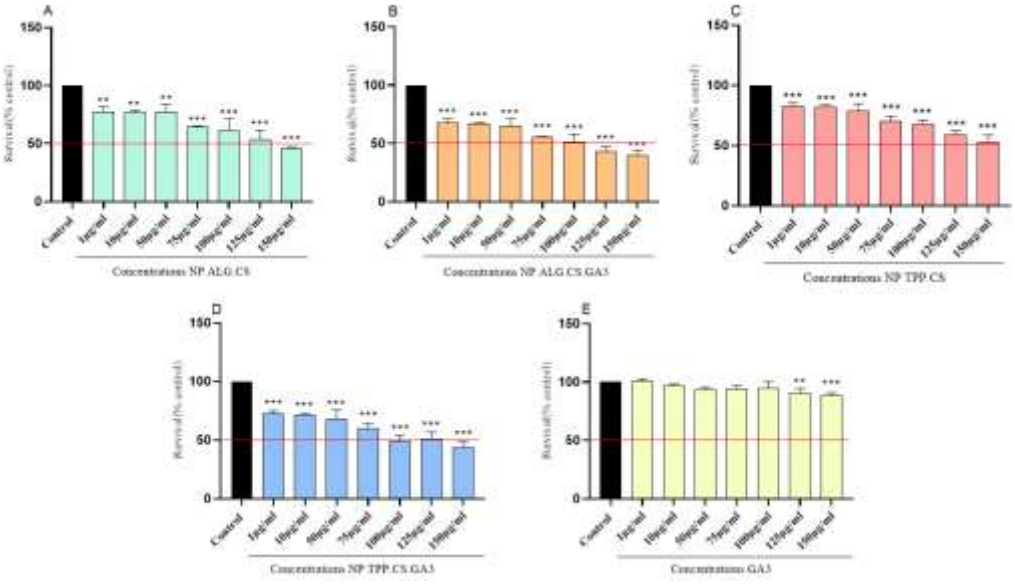
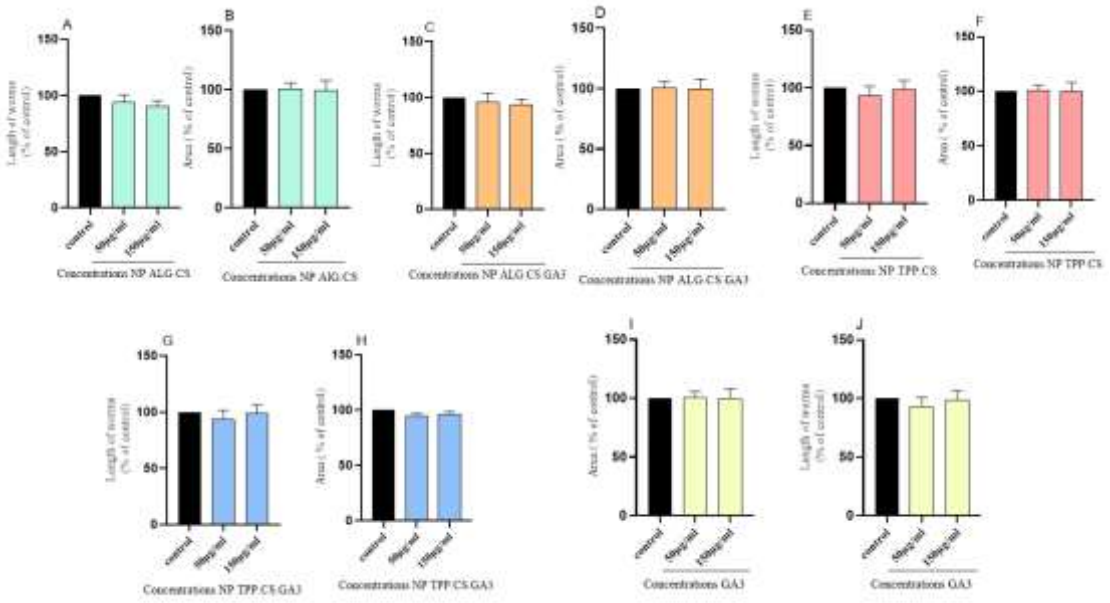


Figure 2: Survival rate after exposure to loaded and unloaded NPs and GA_3. Worms exposed to different concentrations of (A) unloaded ALG.CS NPs; (B) GA_3 loaded ALG.CS NPs; (C) unloaded TPP.CS NPs; (D) GA_3 loaded TPP.CS NPs and (E) GA_3. The red line represents 50% of worm survival. Statistical

significance was determined using the One-way ANOVA test, followed by Tukey's post hoc test. Symbols indicate significant differences compared to the control group: ** p <0.01 *** p < 0.001. (n=5 independent experiments).

NPs do not alter worm development

The effects of acute exposure to nanoparticles on worm development were evaluated 48 hours after exposure. Results showed that exposure did not cause a significant delay in this parameter. None of the tested NPs caused any change in body length (Figure 3A-E) and area (Figure 3 F-J) of *C. elegans* when compared to the control. Representative images are presented in Figure 3 K-P).



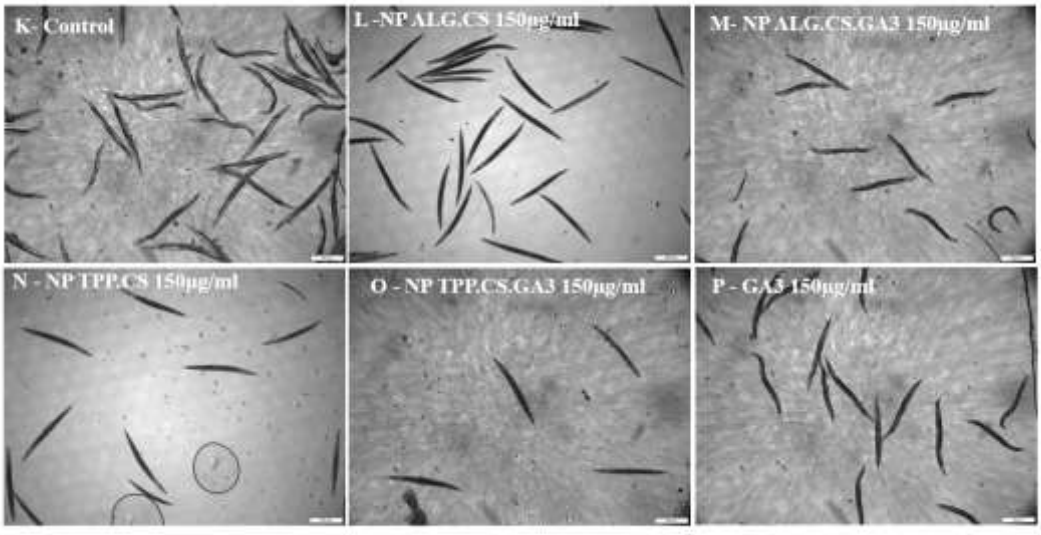


Figure 3: *C. elegans* development after exposure to NPs. Size and body area of worms exposed to different concentrations of (A) unloaded ALG.CS NPs; (B) GA_3 loaded ALG.CS NPs; (C) unloaded TPP.CS NPs;

(D) GA_3 loaded TPP.CS NPs and (E) GA_3. Results show that exposure did not cause any significant delay in these parameters. In K,L,M,N,O and P representative images of worms exposed to the highest concentration tested. Statistical significance was determined using the One-way ANOVA test, followed by Tukey's post hoc test. (n=5 independent experiments).

NPs cause alterations in the reproductive profile independent of GA_3

The reproductive assays showed that the tested concentrations of all treatments reduced the number of eggs laid by the worms at 150µg/ml (Figures 4, 5 and 6). Figure 4 shows the effects of NPs ALG.CS and ALG.CS.GA_3 on the reproductive parameter. Figures 4A, 4B and 4C show the effects of ALG.CS NP, where figure 4A shows the number of worm descendants, where we did not observe significant change in the number of laid eggs. Figure 4B shows the number of eggs produced by a single worm. In this parameter, we observed that NP without gibberellic acid caused a significant reduction in egg production by the worm. Figure 4C shows the posture of the worms per day, during the reproductive period of the animals. Therefore, we can again observe an effect of free NP, significantly decreasing the number of eggs laid on day 1 and 2, at the highest concentration. When we analyzed these same parameters, in relation to the NP of ALG.CS filled with GA_3, (figure 4D, 4E and 4F) we noticed an aggravation of these effects of changing the number of eggs laid by the animals. When we observe the effects of the NP of ALG.CS.GA_3 (figure 4D), compared to the number of descendants, we see a significant reducing effect in the reduction of the number of descendants. Figure 4E shows the number of eggs produced by a single worm. In this parameter, we observed that the NP filled with GA_3 caused a significantly greater reduction in egg production by the worm. Figure 4Fshows the posture of the worms per day during the reproductive period. We can observe that the empty NP by itself already causes a change in the reproductive profile of the earthworms (figure 4C) and these effects are exacerbated when we encapsulate GA_3, further reducing posture on days 1, 2 and 3 (figure 4F), showing that GA_3 participates in the reprotoxic effect.

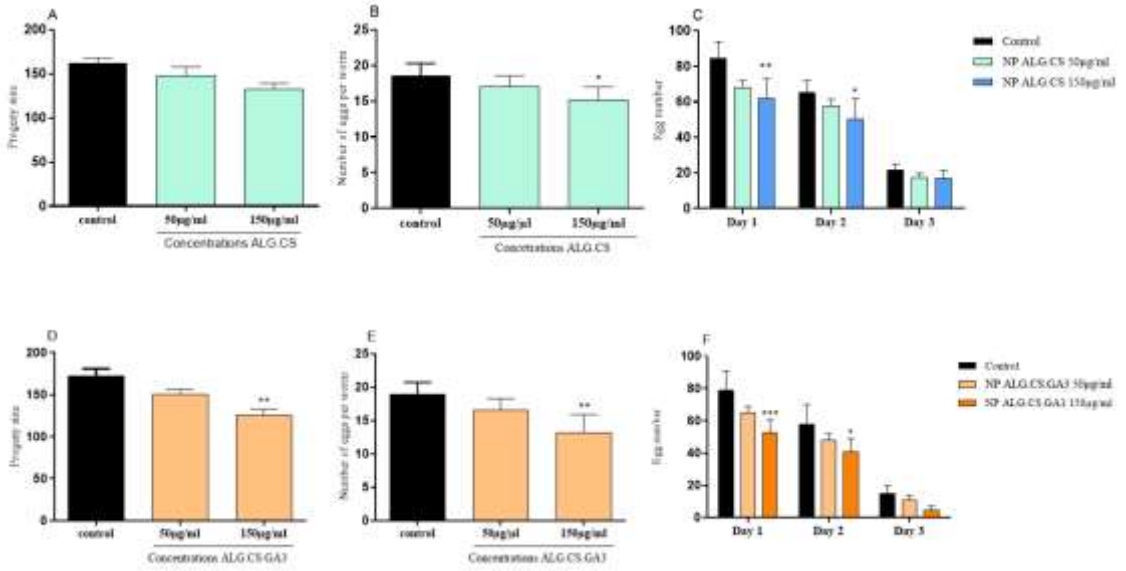


Figure 4: Brood size of worms exposed to NP.ALG.CS. In A, B and C we have the reproductive profile of the ALG.CS NP, showing the total progeny, the number of eggs per worm and the number of eggs per day, respectively. In D, E and F we have the reproductive profile of the AlG.CS.GA_3 NP, showing the total progeny, the number of eggs per worm and the number of eggs per day, respectively. Statistical significance was determined using the One-way ANOVA test, followed by Tukey's post hoc test. The symbols indicate significant differences compared to the control group:* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$. (n=5 independent experiments).

Figure 5 demonstrates the same effect evaluated above, however with the NP of TPP.ALG and TPP.ALG.GA_3. And here the reproductive toxicity profile was very similar. Empty TPP.CS NPs cause a greater reduction in the number of eggs laid per worm (figure 5B). When we evaluate these parameters with the PN filled with GA_3, this effect is aggravated, reducing the progeny (figure 5D) maintaining the toxicity in relation to the number of eggs laid per worm and further altering the laying of eggs per day, on the day 1, and significantly reducing on day 2 at the highest concentration.

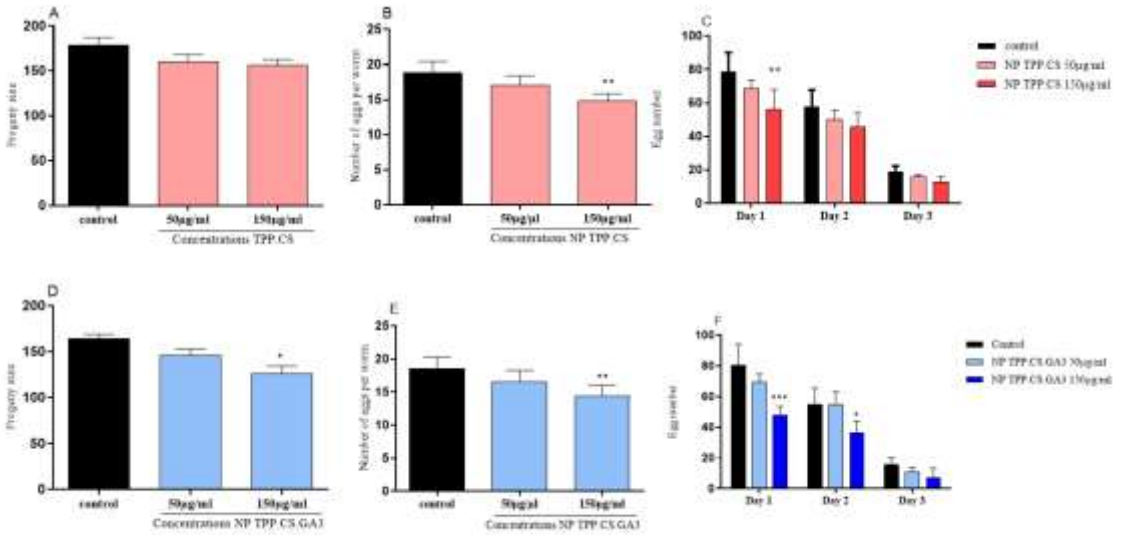


Figure 5: Brood size of worms exposed to NP.TPP.CS. In A, B and C we have the reproductive profile of the ALG.CS NP, showing the total progeny, the number of eggs per worm and the number of eggs per day, respectively. In D, E and F we have the reproductive profile of the AlG.CS.GA_3 NP, showing the total progeny, the number of eggs per worm and the number of eggs per day, respectively. Statistical significance was determined using the One-way ANOVA test, followed by Tukey's post hoc test. The symbols indicate significant differences compared to the control group:* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$. (n=5 independent experiments).

Finally, we evaluated the effects of acute exposure to GA_3, which also interfered with egg production. In figure 5 we can observe that after treatment the number of eggs was significantly reduced at the highest concentration (figure 6A). Likewise, the number of eggs produced per worm decreased when compared to the control group (Figure 6B). In Figure 6C the number of eggs laid is distributed per day and it is possible to observe a decrease in egg laying on day 1 in the two concentrations tested, on day 2 in the highest concentration and we also observed an effect on day 3 at the highest concentration.

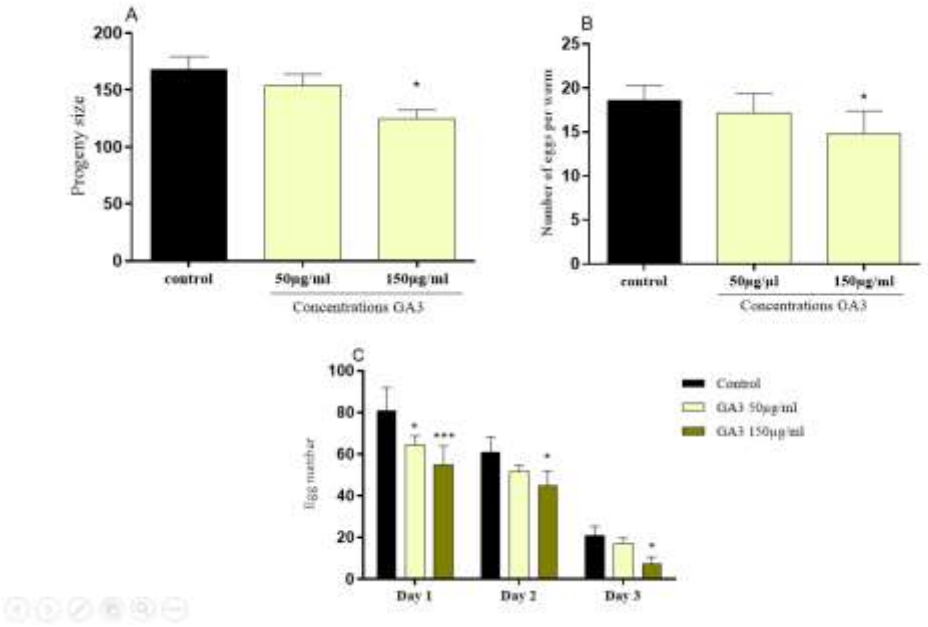


Figure 6: Brood size of worms exposed to GA_3. In A, B and C we have the reproductive profile of GA_3, showing the total progeny, the number of eggs per worm and the number of eggs per day, respectively. In D, E and F we have the reproductive profile of the AlG.CS.GA_3 NP, showing the total progeny, the number of eggs per worm and the number of eggs per day, respectively. Statistical significance was determined using the One-way ANOVA test, followed by Tukey's post hoc test. The symbols indicate significant differences compared to the control group:* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$. (n=5 independent experiments).

GA_3 increased the number of apoptotic cells

Next, we addressed the different mechanisms associated with the decrease in egg production induced by the regulator. Thus, using the MD701 strain (which has apoptotic cells marked with GFP in the gonadal region) we demonstrated a significant increase in the number of apoptotic cells in this region of the animals treated with (7A) unloaded ALG.CS NPs; (7B) GA_3 loaded ALG.CS NPs; (7C) unloaded TPP.CS NPs; (7D) GA_3 loaded TPP.CS NPs and (7E) GA_3 at the highest concentration of 150µg/mL, suggesting that GA_3 induces apoptosis and these NPs did not prevent this effect.

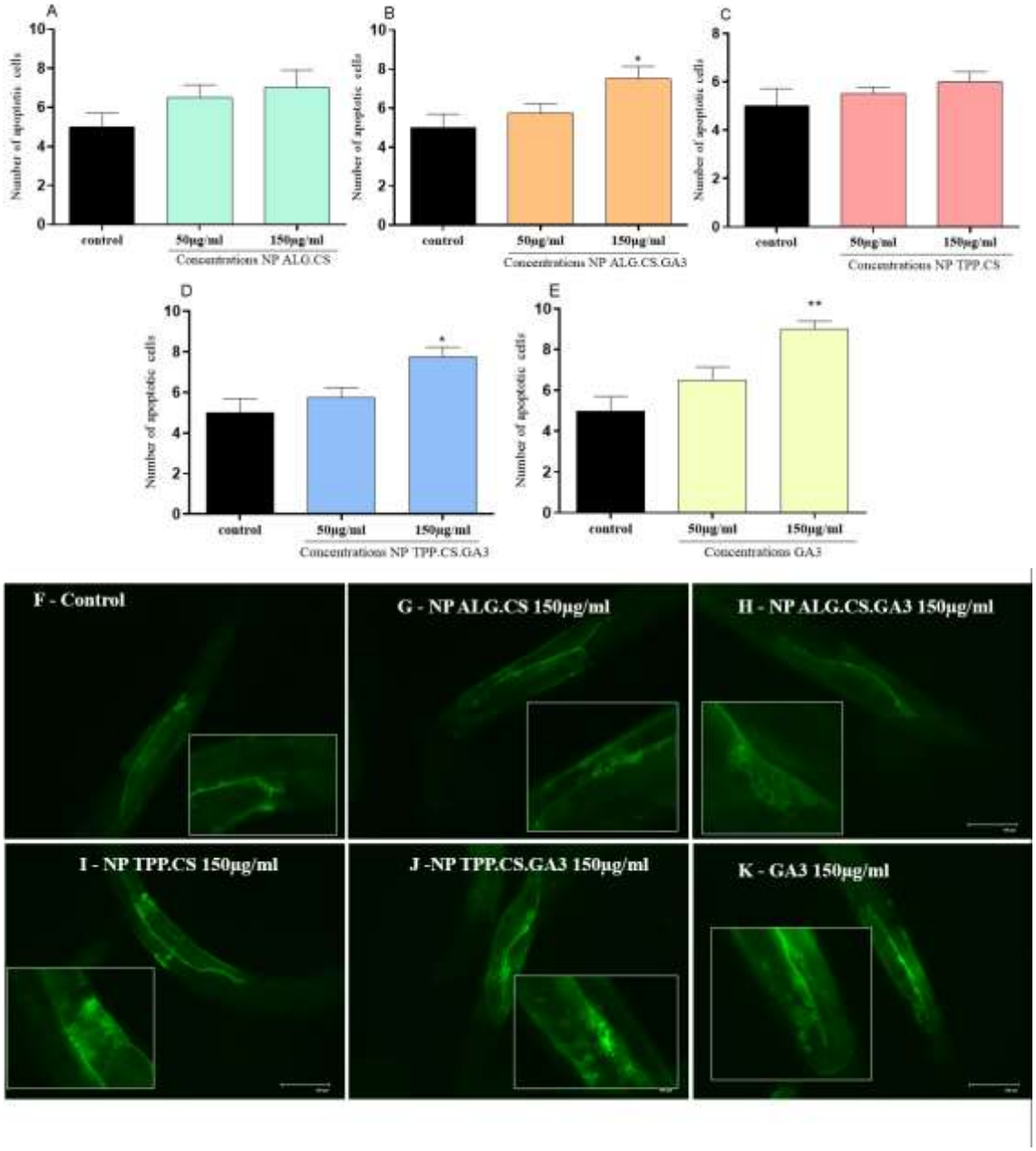


Figure 7: Effect of exposure to GA_3 and NPs on the number of apoptotic cells in wild-type worms. Worms exposed to different concentrations of (A) unloaded ALG.CS NPs; (B) GA_3 loaded ALG.CS NPs; (C) unloaded TPP.CS NPs; (D) GA_3 loaded TPP.CS NPs and (E) GA_3;. in F, G, H, I, J and K are representative images of the control group versus worms exposed to NPs and plant hormone at the highest concentration. (n=50). Statistical significance was determined using the One-way ANOVA test, followed by Tukey's post hoc test. The symbols indicate significant differences in relation to the control group:* $p < 0.05$ ** $p < 0.01$ (n= 5 independent experiments).

NPs increases the time required to paralyze in an isolated and GA_3- independent manner

Then, we performed behavioral tests based on the locomotion assessments. In this first test, paralysis induced by swimming, the objective is to evaluate the dopaminergic

system of the animals. We observed that the worms treated with GA_3 at the highest concentration, presented a longer time (in seconds) necessary to paralyze when compared to the control group (figure 8A-E). Likewise, when evaluating the empty NPs, the paralysis time was longer when compared to the control, and when these NPs are loaded with the hormone, this time to respond to dopamine was impaired in relation to GA_3 and empty NPs.

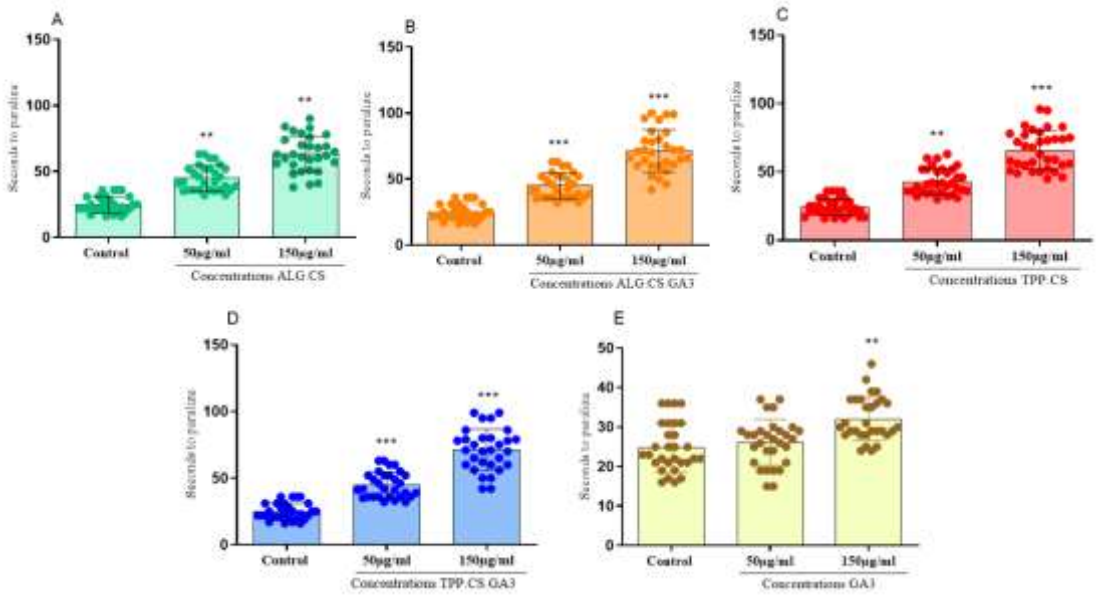


Figure 8: Effect of GA_3 and NPs exposure on swimming-induced paralysis. The graphics show the time required for the worms to paralyze after the addition of dopamine. Worms exposed to different concentrations of (A) unloaded ALG.CS NPs; (B) GA_3 loaded ALG.CS NPs; (C) unloaded TPP.CS NPs; (D) GA_3 loaded TPP.CS NPs and (E) GA_3. Statistical significance was determined using the One-way ANOVA test, followed by Tukey's post hoc test. The symbols indicate significant differences compared to the control group:* $p < 0.05$ ** $p < 0.01$ ***$p<0.001$ (n= 5 independent experiments).

Chitosan NP exposure decreases swimming movement rate independent on GA_3

The next behavioral test performed was the swimming movement, which evaluates whether the treatment with GA_3 loaded or unloaded NPs affect the movement of *C. elegans*. Both the speed and the distance traveled by the worms have changed noticeably in all treatment groups. In figures 9A, 9C, 9E, 9G and 9I it is possible to observe an increase in the swimming speed caused by all treatments, whereas Figures 9b, 9D, 9F, 9H and 9J depict a reduced travelled distance, suggesting that the movement of

these animals is being inefficient, they move faster, but the movement may be weak and they cannot leave the place.

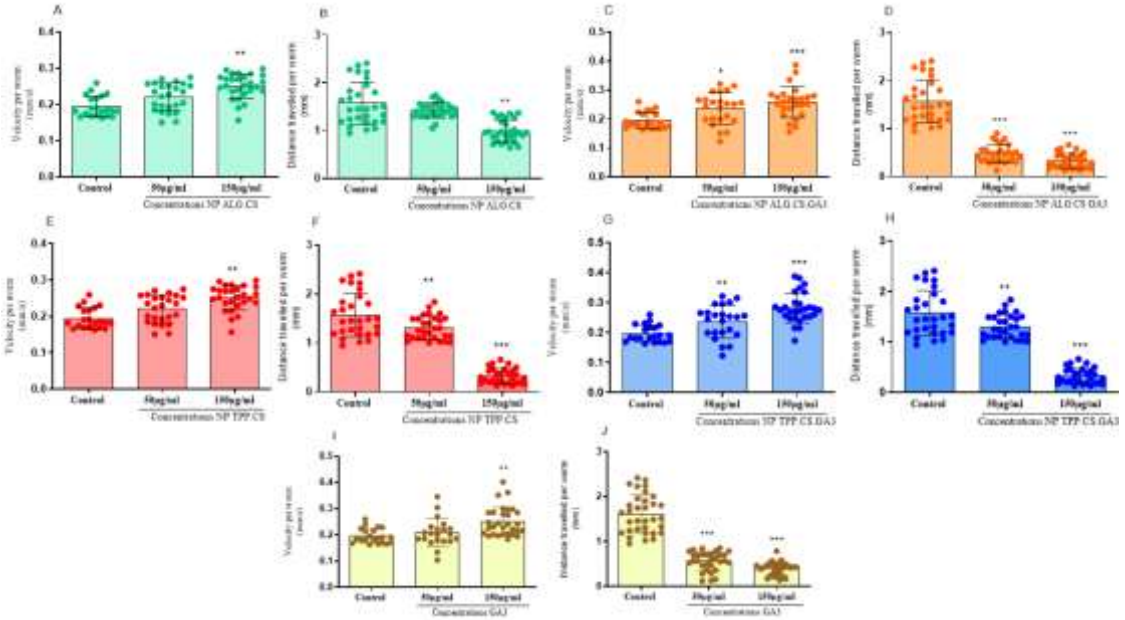


Figure 9: evaluation of swimming movement rate after exposure to loaded and unloaded NPs and GA_3.Worms exposed to different concentrations of (A) unloaded ALG.CS NPs; (B) GA_3 loaded ALG.CS NPs; (C) unloaded TPP.CS NPs; (D) GA_3 loaded TPP.CS NPs and (E) GA_3. Statistical significance was determined using the One-way ANOVA test, followed by Tukey's post hoc test. The symbols indicate significant differences compared to the control group:* p <0.05 ** p <0.01 ***p<0.001 (n= 5 independent experiments).

In Figure 10 we demonstrate the dopaminergic effects of L1 worms exposed to loaded and unloaded NPs. We observed that damage to dopaminergic neurons was more evident when compared to worms exposed to free GA_3, once treatments reduced GFP fluorescence and altered the neuronal morphology. This demonstrates that empty nanoparticles damaged these neurons.

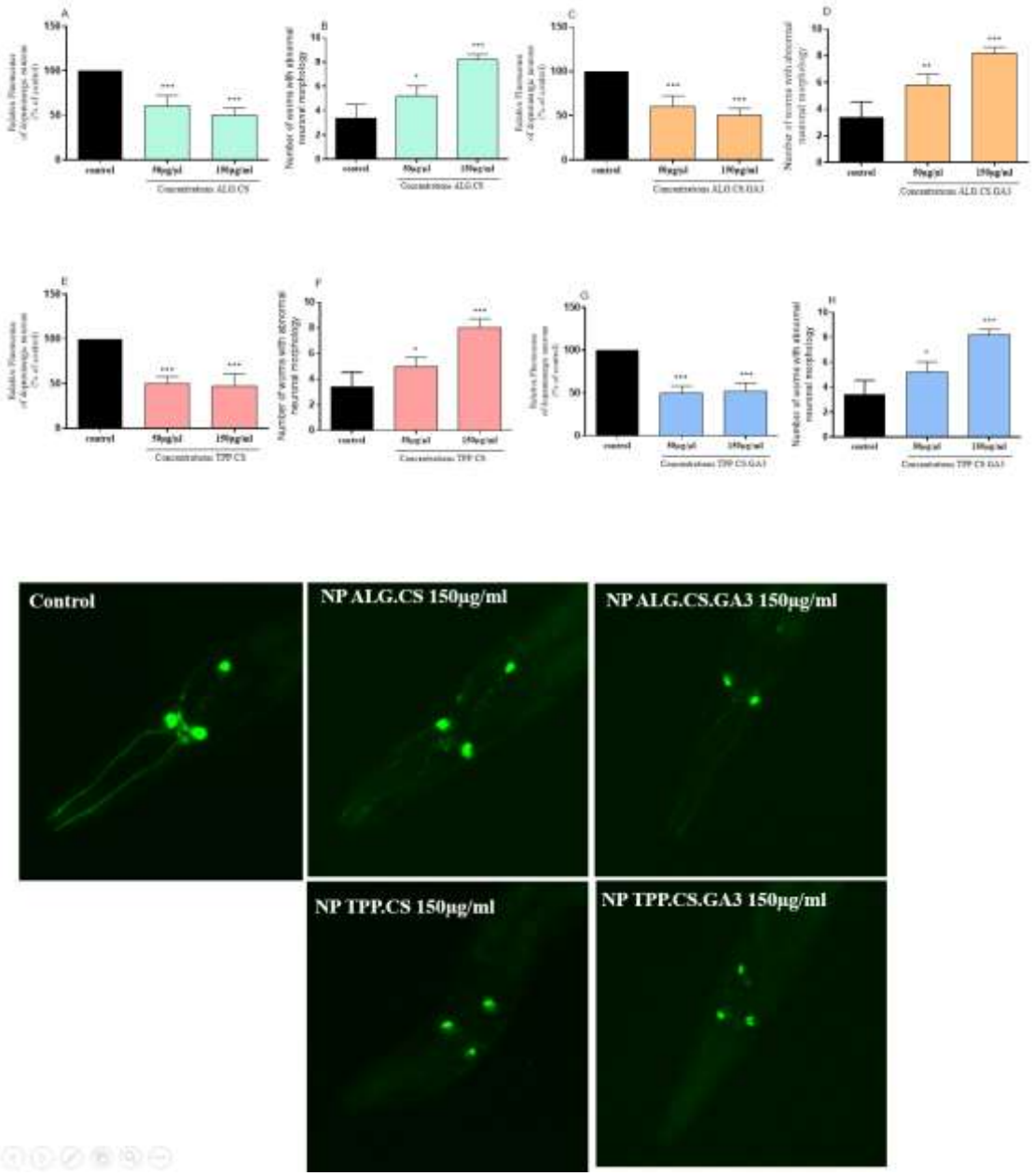


Figure 10: Effect of exposure to NPs on the BY200 strain. A) fluorescence of dopaminergic neurons from worms exposed to ALG.CS and B) the number of worms showing any sign of morphological neurodegeneration from worms exposed to ALG.CS. In C and D corresponds to the fluorescence of dopaminergic neurons and the number of worms that show any sign of morphological neurodegeneration corresponding to worms exposed to ALG.CS.GA_3. E and F correspond to these effects on exposure to TPP.CS NP and G and H correspond to effects on TPP.CS.GA_3 NP. below are representative images indicating reduced fluorescence (neuronal degeneration) and morphological changes.

The dopaminergic damage induced by NP is more pronounced than the cholinergic

Based on the behavioral results, we decided to visualize in vivo the neurons responsible for SWIP and swimming behaviors, which were changed by the treatments. In figure 10 and 12, we demonstrate that L1 worms exposed to GA_3 for 48h had reductions in the fluorescence of dopaminergic neurons compared to those unexposed. Furthermore, we observed changes in cholinergic morphology and even degeneration. The alterations analyzed in the animals' neurons were subtle alterations (small dots without spaces and interruptions in the neuronal processes), large dots in the dendrites, notorious morphological alterations, larger, abundant and evident dots, with possible interruptions or spaces in the extensions and marked spaces in the cell, even with loss of soma or fluorescent marker.

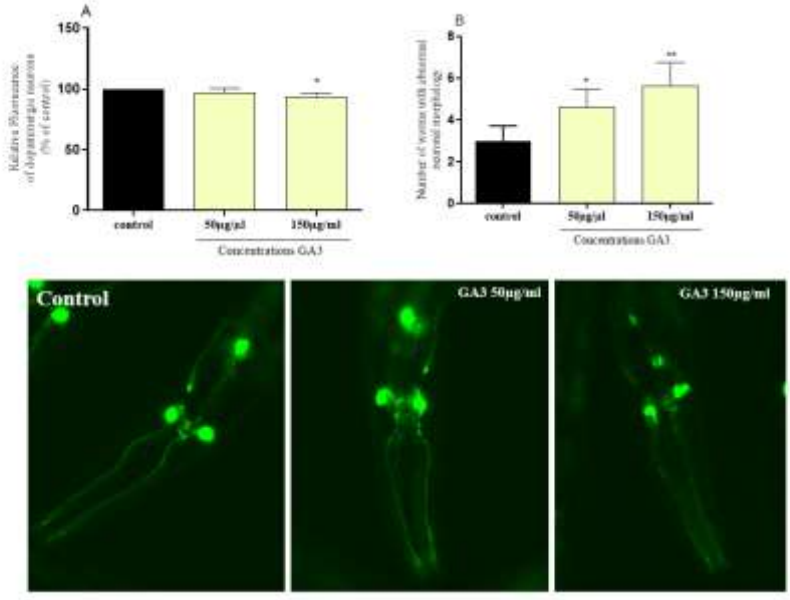
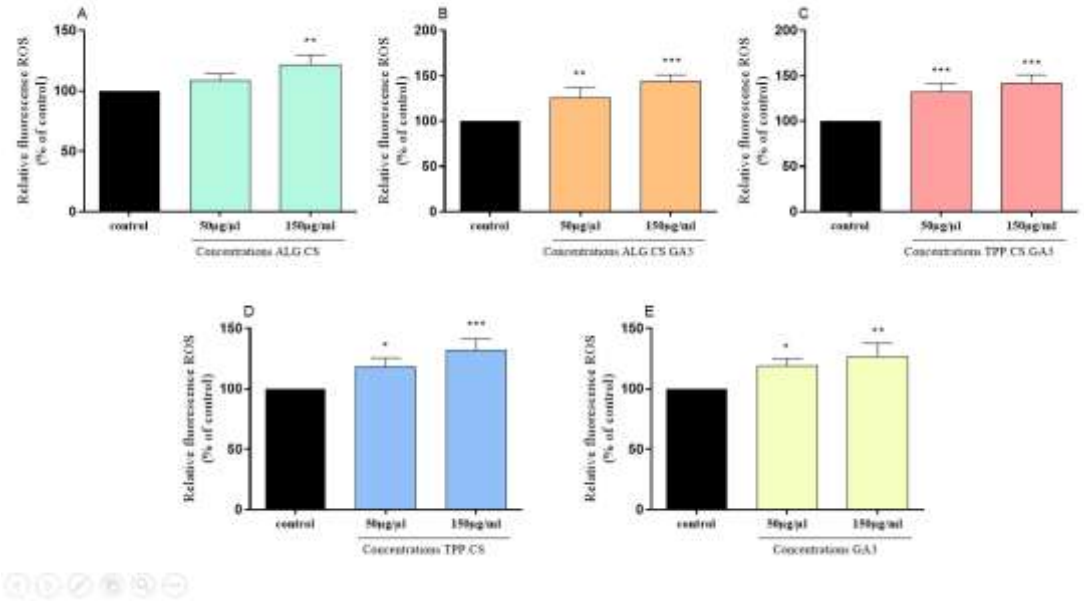


Figure 10: Effect of GA_3 exposure on the BY200 strain. A) fluorescence of dopaminergic neurons. B) the number of worms showing any sign of morphological neurodegeneration. Statistical significance was determined using the One-way ANOVA test, followed by Tukey's post hoc test. Symbols indicate significant differences compared to the control group:* $p < 0.05$ ** $p < 0.01$. (n=5 independent experiments).

After evaluating dopaminergic neurons, we used the LX929 strain to evaluate the effects on cholinergic neurons. In relation to its fluorescence, we did not observe differences, however in relation to the presence of morphological alterations, we observed that expose worms to all treatments presented neurons with abnormal morphology when compared to the control group (Figure 11).



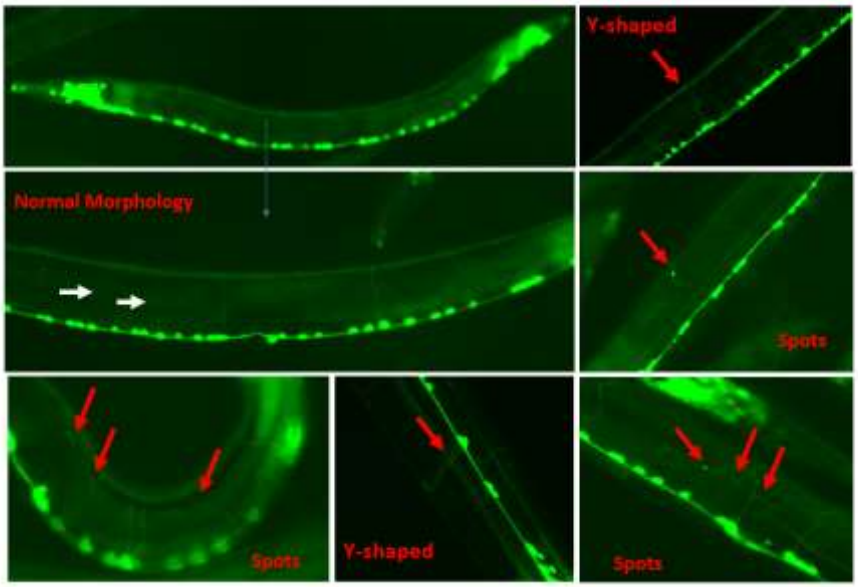


Figure 11: Cholinergic neurons evaluation using LX929. Worms exposed to different concentrations of (A) unloaded ALG.CS NPs; (B) GA_3 loaded ALG.CS NPs; (C) unloaded TPP.CS NPs; (D) GA_3 loaded TPP.CS NPs and (E) GA_3. Below are representative images of the abnormal morphology of neurons. Statistical significance was determined using the One-way ANOVA test, followed by Tukey's post hoc test. The symbols indicate significant differences compared to the control group:* $p < 0.05$ ** $p < 0.01$ ***$p < 0.001$. (n=5 independent experiments).

Chitosan NP and GA_3 increase ROS production

Considering the observed neurobehavioral effects, we evaluated the levels of reactive species, as a way to elucidate the presented toxicity mechanisms. In all analyses, after

exposure to NPs and GA_3 there was an increase in ROS production when compared to the control group (Figures 13A – E). In figure A, we observe that the growth regulator caused an increase in ROS at the two tested concentrations. Regarding free ALG.CS NP, a significant increase was observed only in the highest concentration tested.

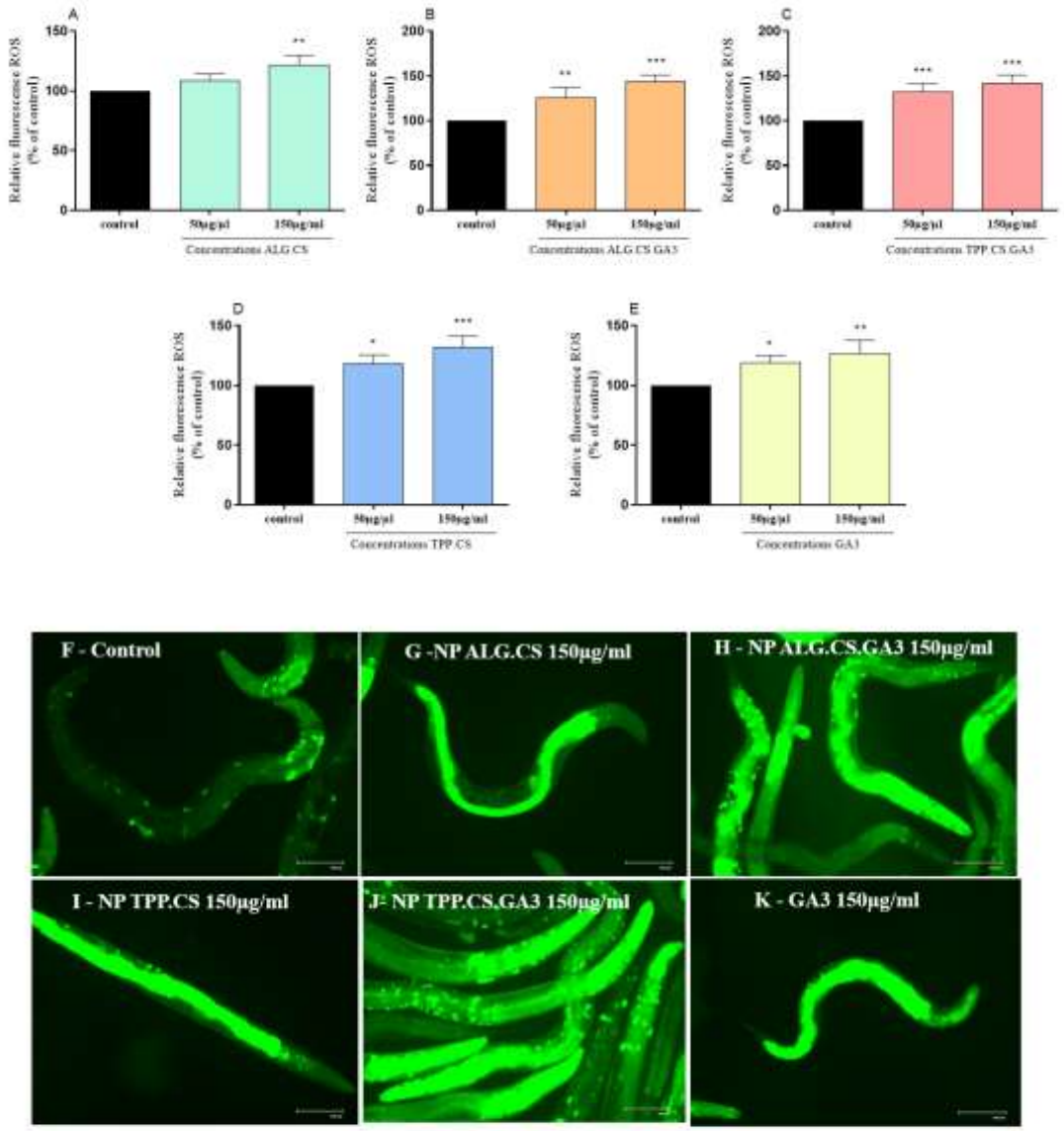


Figure 12: ROS levels in *C. elegans* after treatment with NP and GA_3. Worms exposed to different concentrations of (A) unloaded ALG.CS NPs; (B) GA_3 loaded ALG.CS NPs; (C) unloaded TPP.CS NPs; (D) GA_3 loaded TPP.CS NPs and (E) GA_3. In F, G, H, I, J and K are representative images of the control group versus worms exposed to NPs and plant hormone at the highest concentration Statistical significance was determined using the One-way ANOVA test, followed by Tukey's post hoc test. The symbols indicate significant differences compared to the control group:* $p < 0.05$ ** $p < 0.01$ ***$p < 0.001$. (n=5 independent experiments).

Increased SOD-3 expression

Considered the increased ROS levels, we next verified the expression of SOD-3 (Figure 13). We observed a significant increase in GFP expression of this enzyme after exposure to 150 µg/mL GA_3 (Figure 13A). In relation to empty NPs, an increase in enzyme expression was also observed at the highest concentration tested. When these same nanoparticles loaded with GA_3 were evaluated, the increase in expression was greater when compared to empty nanoparticles, thus showing a summative effect of GA_3.

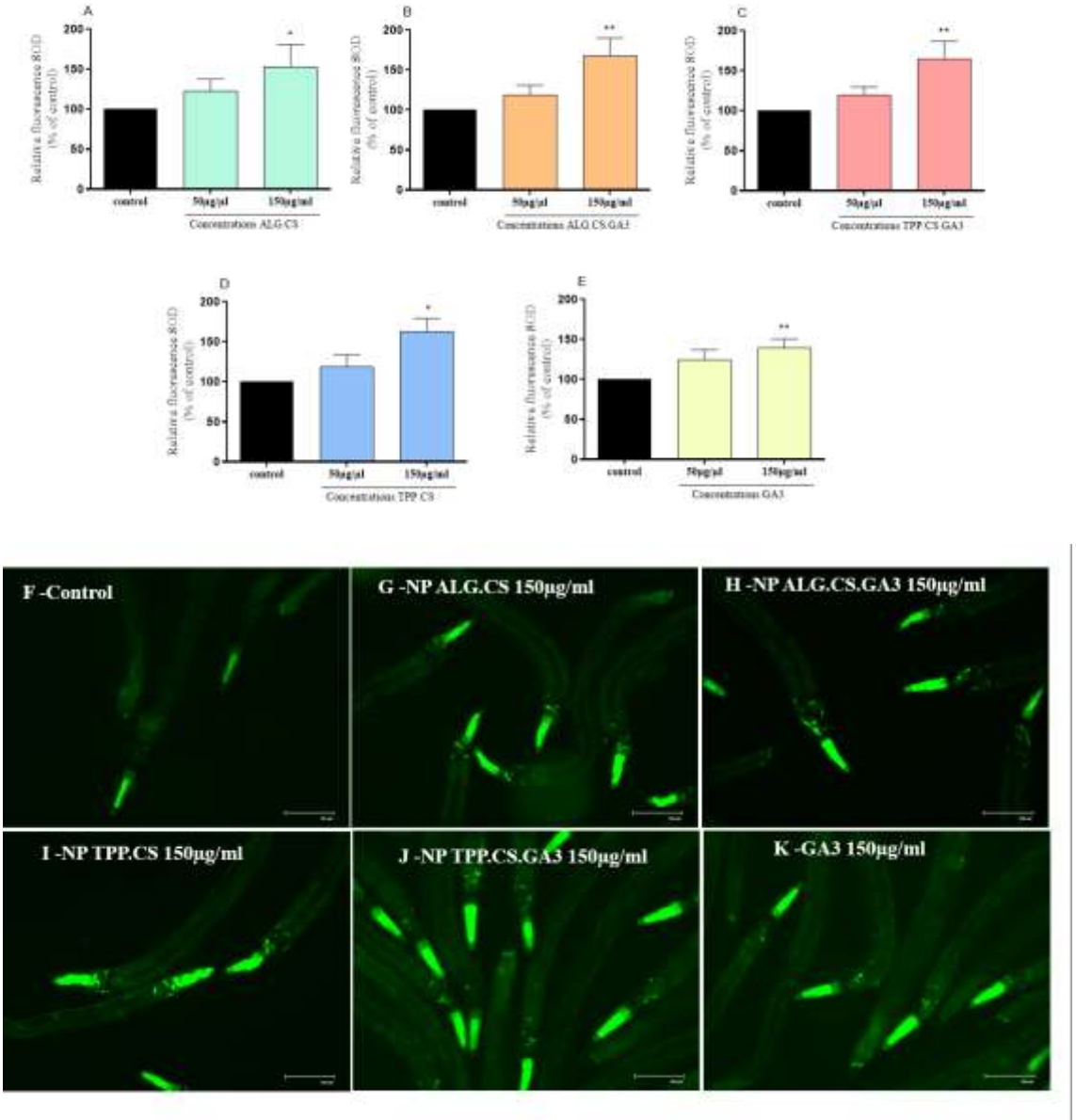
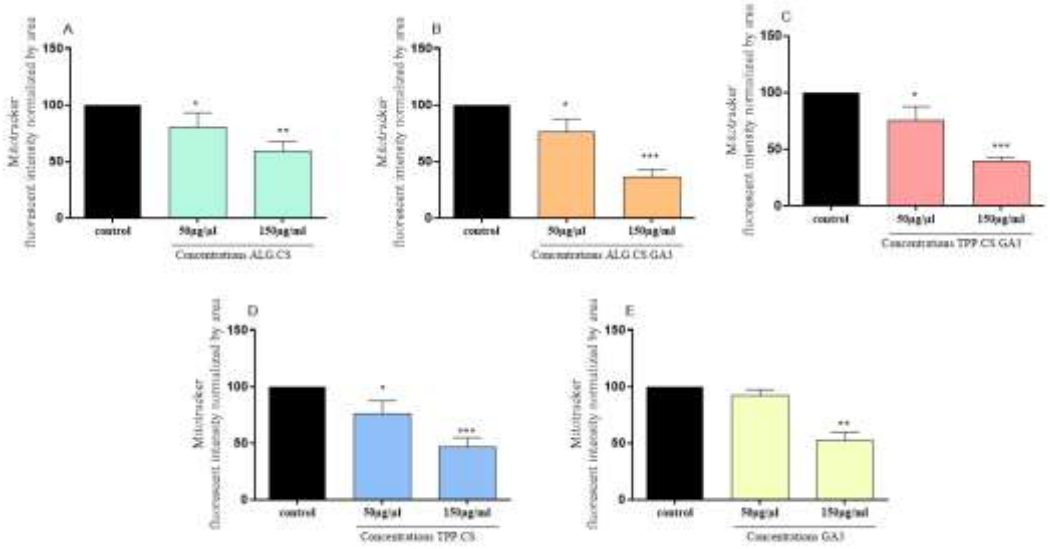


Figure 13: Relative levels of SOD in *C. elegans* after treatment with NP and GA_3. Worms exposed to different concentrations of (A) unloaded ALG.CS NPs; (B) GA_3 loaded ALG.CS NPs; (C) unloaded TPP.CS NPs; (D) GA_3 loaded TPP.CS NPs and (E) GA_3. In F, G, H, I, J and K are representative images of the control group versus worms exposed to NPs and plant hormone at the highest concentration..

Statistical significance was determined using the One-way ANOVA test, followed by Tukey's post hoc test. The symbols indicate significant differences compared to the control group:* p <0.05 ** p <0.01 ***p<0.001 . (n=5 independent experiments).

GA_3 and chitosan NPs alter mitochondrial viability

To search for an association among reproductive and neuronal damage and oxidative stress with mitochondrial impairment, we used the MitoTracker Red marker, which stains mitochondria that maintain membrane potential integrity. We observed a reduction in fluorescence in exposed nematodes, especially in the head area where most of the neurons are located (Figure 14).



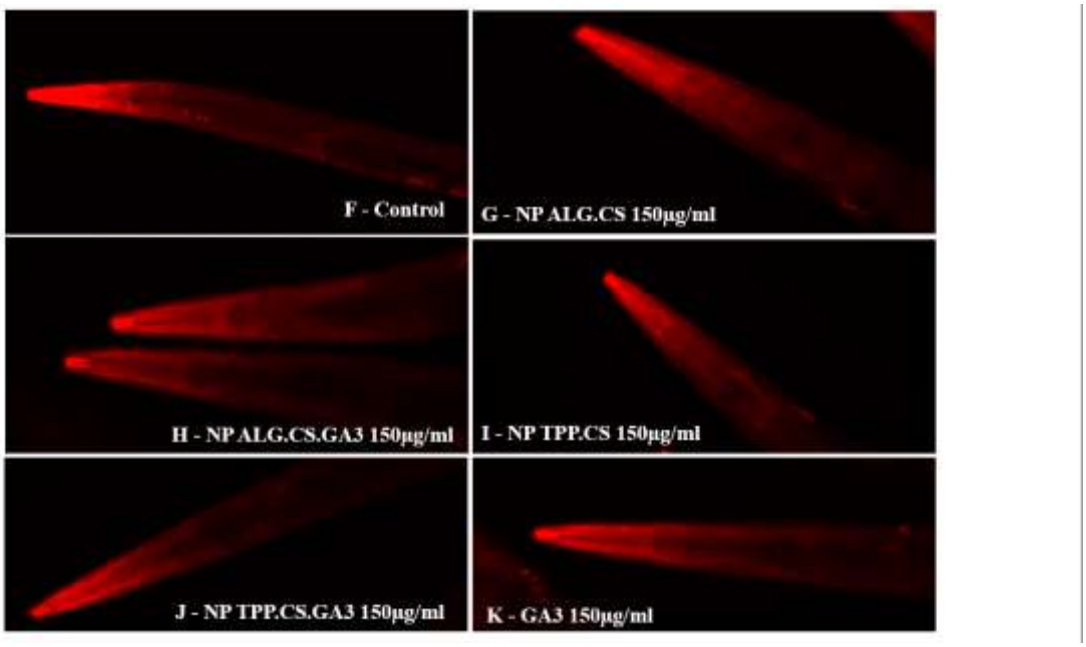


Figure 14: Mitochondrial dysfunction associated with exposure to NP. Worms exposed to different concentrations of (A) unloaded ALG.CS NPs; (B) GA_3 loaded ALG.CS NPs; (C) unloaded TPP.CS NPs; (D) GA_3 loaded TPP.CS NPs and (E) GA_3 . In F, G, H, I, J and K are representative images of the control group versus worms exposed to NPs and plant hormone at the highest concentration. Statistical significance was determined using the One-way ANOVA test, followed by Tukey's post hoc test. The symbols indicate significant differences compared to the control group:* $p < 0.05$ ** $p < 0.01$ ***$p < 0.001$. (n=5 independent experiments).

Representative images of worms stained with the fluorescent marker Mitotracker CMXRos and analyzed after 48h of exposure to GA_3 and NP. A reduction in fluorescence was observed in exposed worms, indicating a reduction in viable mitochondria, specifically in the head area.

Discussion

To better understand the impact of the findings of this work, we must first recognize that agriculture keeps pace with global food needs and obviously must increase its production due to population growth. Faced with challenges such as climate change, pest control and the very high demand resulting from population growth, agriculture must adapt with a focus on high productivity, relying on the use of growth regulators. GA_3, an endogenous hormone, applied to dwarf varieties of peas, broad beans or maize greatly accelerates growth (Jones, 1973). This PGR was therefore the focus of our interest due to the paucity of data on its effects on reproductive and neurological impairment in *C.*

elegans, a non-target organism that lives in the soil. To improve the efficacy and reduce its possible toxicity, chitosan NPs were developed and tested as well.

The investigation of toxicity began with the effects of nanoparticles and GA_3 on the mortality rate of worms, in order to plot dose-response curves to obtain the LC_{50}. Figure 2 shows that exposure to GA_3-loaded nanoparticles caused increased mortality rate compared to untreated worms. However, mortality was similar to that observed in worms exposed to empty NPs, which may indicate that the observed toxicity is caused by the nanoformulations. Another fact that goes in the direction of this is the low toxicity showed by GA_3, which caused mortality only at the highest tested concentrations. This lack of toxicity observed with GA_3 indicates that the mortality observed in Fig. 2 is caused by the nanoparticles themselves. If we observe in the graphs, all LC_{50} were similar for all formulations, that is, there was no significant difference in the mortality rate between worms treated with empty nanoparticles versus loaded NPs. Some studies have reported the dependency relationship between the composition and size of nanoparticles in *C. elegans* (Contreras et al., 2014; Höss et al., 2015).

Campos et al., 2017 evaluated three nanoparticle formulations: solid lipid nanoparticles loaded or not with atrazine and simazine, SLN; polymeric nanoparticles, NC_PCL loaded with atrazine; and chitosan/tripolyphosphate, TPP.CS, whether or not loaded with paraquat. All formulations, whether or not loaded with pesticides, increased lethality in a dose-dependent manner with similar LC_{50} in *C. elegans.* However, at the two highest concentrations used there was a mortality rate of 12%. Previous studies demonstrate that PGRs produced substantial organ toxicity in the lung and spleen during the 25-day subacute exposure period (Celik and Tuluce, 2007). Death or abortion was not observed in mothers treated with GA_3 during the experimental period (21 days). However, in their nursing pups, clinical signs such as ataxia, reduced activity, tremors, and nasal discharge have been observed (Troudi et al., 2012b). There are few data on the investigation of lethal doses of GA_3 for animals and humans. According to (Alsemeh et al., 2019), the ingestion of food and drinking water containing large amounts of GA_3 can induce liver failure, histological and biochemical changes in the liver of pregnant albino rats and their offspring. Hepatorenal dysfunction was also already observed following GA_3 exposure in rats (Soliman et al., 2022b).

Reduction in *C. elegans* body size is an important endpoint of nanotoxicity as reported by some studies (Hsu et al., 2012; Wang et al., 2009; Wu et al., 2012). Genetic or environmental factors can promote alterations in the worms size *(Nagamatsu and Ohshima, 2004; So et al., 2011).* Worms exposed to GA_3, unloaded and loaded NPs did not show significant changes in body length. This allows us to suggest that any alterations after treatment are not due to delay in larval development, such as reprotoxicity, since all worms reached the adult stage. In fact, our data demonstrate that acute exposure to GA_3 and chitosan NPs caused a change in the reproductive profile. All treatments decreased the number of eggs per worm and the number of eggs laid during the reproduction period. Notably, GA_3 loaded NPs presented a more pronounced reproductive toxicity in relation to empty NPs. The alterations in *C. elegans* reproduction has been associated with damage in cell division, mainly of the gametes and also with apoptosis (Ruan et al., 2012). Our results demonstrated a significant increase in the number of apoptotic cells in the germline cells, thus interfering with the physiology of the reproductive system. The germline provides the crucial link between generations, and its proper function depends on the precise execution of the process of cell meiosis and chromosome division. Furthermore, it is known that the theory that the environment influences chromosomal segregation and behavior is not new, but it is still misunderstood (Hunt, 2006). It has already been described that worms treated with 0.05% polymeric nanoparticles showed a significant reduction in the number of hatched larvae.(Campos et al., 2017) Of note, GA_3 effects on animals reproduction is not a novelty, since it has been reported to cause oxidative stress and DNA damage in rat testes (Soliman et al., 2010), in addition to estrogen-like actions in mice and induced testosterone secretions (Gawienowski et al., 1977; Gawienowski and Chatterjee, 1980). GA 3 was found to affect sexual differentiation and other physiological traits in mice (Maciej Serda et al., 1995b). Studies are available on the acute and chronic effects of GA_3 on biochemical functions, spermatogenesis and sperm parameters in mice, chicks and rabbits (Maciej Serda et al., 1995b).

In the present study, we evaluated the neural damage of dopaminergic and cholinergic neurons, as well as the behaviors governed by these neuronal subtypes. Besides reproduction, neurons are sensitive to toxicants in worms. Therefore, we subsequently evaluated possible effects behaviors controlled by dopaminergic and cholinergic systems and also observed these neurons morphology. Indeed, we have found

this observed dopaminergic degeneration may also contribute to the observed effects on swimming movements, as shown in figures 8 and 9, as it has been established that morphological changes and death of dopaminergic neurons are strongly associated with locomotor deficits in nematodes, mammals and humans (Duncan et al., 2012; Lakso et al., 2003; Lebowitz and Khoshbouei, 2020). Dopaminergic neurons play an important role in modulating locomotion behavior (Sawin et al., 2000). Locomotor behaviors require control of neural circuits and muscle functions. The functions of dopaminergic neurons are closely related to locomotor and chemotaxis behaviors in *C. elegans* (Omura et al., 2012). Our results demonstrate that chitosan NP causes degeneration of dopaminergic neurons. Previous work has used the level of GFP expression as an indicator of neurodegeneration (Settivari et al., 2009).

Our findings show for the first time that NP-induced functional (behavioral) impairments can occur in the presence of observable damage to these neurons. Considering that *C. elegans* lacks a functional blood-brain barrier, nanoparticles and even GA_3 have more opportunities to interact with neurons. We hypothesize that the underlying mechanism of neurotoxicity may be involved in oxidative damage to neurons. *C. elegans* moves through the topsoil, navigating both liquids using swimming behavior and more solid matrices using crawling, often transitioning from one form of locomotion, or gait, to another. The transition from swimming to crawling is controlled by dopaminergic neurons, particularly the ADE and PDE neurons, whereas that from crawling to swimming is mediated by serotonergic neurons VC-4, VC-5, AIM and RIH(Vidal-Gade et al., 2011). General sinusoidal locomotion is mediated by cholinergic motor neurons. The ability to lay eggs is governed by mixed cholinergic/serotonergic neurons, HSNL/R (Desai et al., 1988).

Because the neurons that govern each of these behaviors are well understood, *C. elegans* is a powerful model for directly exploring behavioral dysfunctions resulting from neuronal damage. Our aim was to examine the possible differential sensitivity of behaviors governed by different subsets of neurons, as well as to elucidate the nature of cellular changes in damaged neurons. Our results indicate that behavioral damage, which can influence the overall behavior of nematodes in their soil environment, is a sensitive early indicator of neuronal damage that precedes overt structural impairment. Yuan et al. showed that Tween-80-modified chitosan nanoparticles (TmCS-NPs) induced

neurobehavioral toxicity, including decreased spontaneous movement and a hyperactive effect in treated zebrafish (*Danio rerio*)embryos. Furthermore, NPs inhibited axonal development of motor neurons and remarkably affected the muscular structure of the embryos (Yuan et al., 2016).

Considering the observed neurobehavioral and reprotoxic effects, we evaluated whether oxidative stress would be involved in these processes. In all analyses, after exposure to unloaded and loaded NPs and GA_3, there was an increase in ROS production when compared to the control group. ROS are one of the main factors involved in the initiation and progression of neurodegenerative diseases. Oxidative stress is a condition produced by an imbalance between oxidants and antioxidants in a biological system. Imbalance occurs because of excessive level of reactive oxygen species (ROS) or malfunctioning antioxidant system. The crucial role of molecular oxygen in biology is unquestionable, as it is essential for the proper cellular functioning and life of all organisms. Although oxygen is crucial for life and is involved in signal transduction, gene transcription and other cellular activities, coincidentally, it also has a deleterious effect on biomolecules in the form of free radicals and ROS(Chiurchiù et al., 2016). The body's defense system in the form of antioxidants has also co-evolved to combat/neutralize the effect of reactive oxygen species. Several antioxidants such as glutathione, taurine, creatine, zinc, vitamin E, vitamin C, vitamin A and polyphenols (tea extract) are produced against ROS. Antioxidant effect is enhanced and supported by antioxidant enzymes such as SOD, catalase, glutathione peroxidase and so on (Fang et al., 2002; Poulsen et al., 1998; Sharma et al., 2012). ROS contribute significantly to the deterioration of neuronal cells by modulating the function of biomolecules. These species target different biomolecules (DNA, RNA, lipids and proteins) and processes (nucleic acid oxidation, lipid peroxidation) in the cell. The brain consumes a large amount of ROS due to the high ATP needs. ROS involved in neurodegeneration include hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and highly reactive hydroxyl radical ($HO^\bullet$). Reactive nitrogen species (RNS), such as nitric oxide (NO), also have a deleterious effect on neurons. The literature is rich in evidence that there is a significant connection between ROS and neurodegenerative diseases (Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Machado-Joseph disease, etc.), as well as aging (El-Bachá et al., 1998; von Arnim et al., 2010; Yu et al., 2009).

As oxidative stress is characterized by an imbalance between total antioxidant defenses and generated reactive species, we evaluated SOD-3 expression. The increase in SOD-3 expression observed in all treatments can be explained due to the increase in reactive ROS generated by exposures, named stress response. SOD-3 is a mitochondrial enzyme, and notably, the main site of ROS production in the cell is the mitochondria because of ATP production process. However, oxidative stress leads to mitochondrial dysfunction. Therefore, we investigated mitochondrial viability in nematodes chronically exposed to the NPs and GA_3. We observed a reduction in fluorescence in nematodes exposed to both unloaded, GA_3-loaded NPs and GA_3 in the head area where dopaminergic and several cholinergic neurons are located. This bioenergetic impairment may be responsible for the damage to these neurons and for the locomotor and reproductive deficits described here. Mitochondrial disruption has been linked to impaired health in worms (Maglioni et al., 2019), an effect that has also been observed in humans (Bornstein et al., 2020).

ATP is essential for cell function, signaling, and general activities. Mitochondria are responsible for the production of ATP through the electron transport chain and oxidative phosphorylation. They are also involved in producing molecules to overcome oxidative stress along with programmed cell death and other respiratory functions in the cell. Enrichment of mitochondria with various redox enzymes and mitochondrial dysfunction is hypothesized to be responsible for the production of ROS in the cellular environment (Chakrabarti et al., 2011; Federico et al., 2012). Therefore, it is hypothesized that high levels of ROS production in mitochondria, which have longer half-lives - like H 2 O 2 ; lipid hydroperoxide; or active aldehydes such as MDA, acrolein, and so on (generated elsewhere but ultimately reach the mitochondria) - can cause mitochondrial dysfunction and ultimately impede biological processes that lead to various diseases(Singh et al., 2019).

Agriculture faces challenges such as climate change, pest management and population growth. As a consequence, there is a need for highly productive and sustainable agriculture(Charles et al., 2014). The use of growth regulators helps to increase crop production, but due to physical and chemical issues that affect the stability and bioavailability of these products, larger amounts are needed to obtain the desired effect, thus increasing environmental contamination and risks to human health. Taking

this into account, nanotechnology offers solutions for optimizing and improving the use of agrochemicals in agriculture. Among the alternatives is the use of nanocapsules that involve these compounds, thus promoting an increase in the bioavailability and distribution of the active principle, reducing the frequency of applications.

Recently, studies have described formulations with chitosan/tripolyphosphate nanoparticles loaded with GA_3, and these formulations have demonstrated positive effects on plants, improving plant development, and also increasing fruit productivity. However, no in vivo toxicological studies with animals, using nanoparticles with GA_3 has been evaluated so far. The observed toxicity was caused by the nanomaterial, thus indicating that the formulations need to be improved. Therefore, we demonstrate for the first time the effects of these nanomaterials and GA_3 against mitochondrial damage. Our study demonstrates that unloaded, GA_3-loaded chitosan NPs and GA_3 exposure affects reproductive and neuronal systems in *C. elegans* by inducing apoptosis, oxidative stress and mitochondrial dysfunction. This opens up perspectives for using these studies as a way of obtaining toxicity data to support the discussion on the regulatory frameworks for nanotechnology and its use in agriculture. Another important point is that this study reports the use of *C. elegans* to investigate toxicological aspects of nanomaterials, especially nanoparticles loaded with GA_3.

Conclusion

Chronic treatment demonstrated low safety in relation to nanoparticles, demonstrating mortality even in empty nanoparticles. These results indicate that these formulations have some toxicity in this organism. In response to the harmful effects caused by reactive species, cellular defense mechanisms are activated, including the antioxidant system. These antioxidants act by protecting or repairing macromolecules that are targets of the action of these reactive species in cells. Our results show that there was an increase in the expression of the antioxidant enzyme SOD-3 in response to the increase in reactive species generated. Associating with this there was neuronal and reproductive toxicity due to exposure of nanos. These toxicity effects were likely triggered by mitochondrial compromise. Taken together, the results of this work show that there is a significant difference between the toxicity of charged and uncharged nanoparticles and GA_3. And that despite the toxicity presented by the empty nano itself, GA_3 has been

shown to contribute to the toxicity of the formulations. However, in some parameters, the observed toxicity was caused by the nanomaterial, thus indicating that the formulations need to be improved. Furthermore, this study demonstrates that C. elegans is an excellent indicator for toxicity studies of nanos filled with plant growth regulators. This opens up perspectives for iusing these studies as a way of obtaining toxicity data to support the discussion on regulatory frameworks for nanotechnology and its use in agriculture. Another important aspect is that this study is the first to report the use of C. elegans to investigate toxicological aspects of nanoparticles loaded with GA_3 reported in the literature.

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5. Conclusões

- A exposição crônica às NP, nas concentrações testadas alteraram de maneira significativa a sobrevivência em *C. elegans*;

- A exposição crônica às NP causou um aumento no nível de espécies reativas e uma ativação do sistema antioxidante do verme;

- A exposição crônica às NP e ao GA3 livre gerou alterações morfológicas nos neurônios dopaminérgicos;

- A exposição crônica às NP altera parâmetros reprodutivos como o tamanho da progênie sem alterar o tamanho corporal dos *C. elegans*;

6. Perspectivas

Com base nos resultados apresentados neste estudo, demonstramos a toxicidade das NPs brancas. Esses resultados sugerem que o desenvolvimento de nanopartículas visando aplicações agrícolas necessita de mais estudos para otimizar a composição e assim reduzir sua toxicidade para organismos não-alvo.

7. Referências

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