PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
MESTRADO EM CIÊNCIA ANIMAL

LARISSA FERREIRA RÓS MARIANO

ANÁLISE DA REMODELAÇÃO CARDÍACA DE RATOS SUBMETIDOS À EXPOSIÇÃO CRÔNICA AO HERBICIDA 2,4-D

Presidente Prudente – SP
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Dissertação apresentada à Pró-Reitoria de Pesquisa e Pós-Graduação, Universidade do Oeste Paulista, como parte dos requisitos para obtenção do título de Mestre em Ciência Animal.
Área de Concentração: Fisiopatologia Animal.

Orientadora:
Prof.ª Drª. Francis Lopes Pacagnelli

Presidente Prudente – SP
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Presidente Prudente, 18 de abril de 2024.

BANCA EXAMINADORA

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Profª. Drª. Francis Lopes Pacagnelli
Universidade do Oeste Paulista – Unoeste
Presidente Prudente-SP

_____
Profº. Dr. Rafael Stuani Floriano
Universidade do Oeste Paulista – Unoeste
Presidente Prudente-SP

_____
Profª Drª. Mariana Janini Gomes
Texas A&M University
College Station, EUA

DEDICATÓRIA

Ao meu marido, Thaoan Bruno Mariano, pessoa com quem compartilho meus dias, que é a inspiração e razão de seguir meus sonhos. Desconheço alguém mais paciente e carinhoso durante minha trajetória acadêmica e de vida.

Aos meus pais, Delcides e Antônio, por me proporcionarem o privilégio de sempre estudar e mostrar o valor da educação dentro e fora de instituições de ensino. É com muito amor que lhes dedico este trabalho, é uma conquista nossa!

Aos meus sogros, que com apoio me mantiveram firme nessa trajetória.

Aos meus amigos de vida e de trabalho que foram pacientes e compreensivos e me distraíram o suficiente para deixar os dias mais alegres.

Á minha querida professora e orientadora dessa pesquisa, Professora Doutora Francis Lopes Pacagnelli, que me companha desde a graduação, que confiou no meu potencial e sabendo das dificuldades dos últimos tempos me tranquilizou e me resgatou para finalizar esse projeto.

E àquela adolescente de 17 anos, no final do ensino médio, que ainda não sabia qual graduação cursar e não sabia nada sobre um programa de mestrado, mas já havia a vontade de compartilhar seus aprendizados e aprender muito mais sobre ciência. Desde sempre tão estudiosa e hoje pode colher os frutos dessa dedicação.

AGRADECIMENTOS

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Agradeço a minha orientadora, Professora Doutora Francis Lopes Pacagnelli, por me direcionar em cada etapa e por me fazer refletir e aprender além da escrita. Obrigada por me manter motivada durante todo o processo.

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O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001

“Não sei o que possa parecer aos olhos do mundo, mas aos meus pareço apenas ter sido como um menino brincando à beira-mar, divertindo-me com o fato de encontrar de vez em quando um seixo mais liso ou uma concha mais bonita que o normal, enquanto o grande oceano da verdade permanece completamente por descobrir à minha frente.”

(Isaac Newton)

RESUMO

Análise da remodelação cardíaca de ratos submetidos à exposição crônica ao herbicida 2,4-D

Introdução: O ácido 2,4-diclorofenoxiacético (2,4-D) é um dos herbicidas clorofenoxi mais amplamente utilizados em todo o mundo devido sua seletividade, eficiência e baixo custo. O 2,4-D é rapidamente absorvido no trato respiratório e pode afetar toxicamente tanto animais quanto seres humanos em uma ampla gama de órgãos, como, fígado, rins, músculos, pulmões, trato gastrointestinal, sistema nervoso (central, periférico), sistema endócrino, sistema reprodutivo e coração. **Objetivo:** O objetivo desse estudo foi avaliar os efeitos crônicos da nebulização com o 2,4-D na remodelação cardíaca de ratos Wistar. **Métodos:** Foi utilizados 30 ratos Wistar adultos machos divididos em 3 grupos: grupo controle exposto à nebulização de 10 ml de solução de cloreto de sódio à 0,9% (GCI, n=10), grupo baixa concentração exposto ao 2,4-D com $3{,}71x10^{-3}$g de ingrediente ativo por hectare (GBI, n=10) e grupo alta concentração exposto ao 2,4-D com $9{,}28x10^{-3}$g de ingrediente ativo por hectare (GAI, n=10). Em um período de exposição de 180 dias, foram utilizadas duas caixas conectadas a nebulizadores ultrassônicos para pulverização de herbicidas. Após esse período, os ratos foram eutanasiados para coleta e estudo do tecido cardíaco. A remodelação cardíaca foi avaliada em relação à hipertrofia, fibrose e vascularização. Para avaliação da hipertrofia as áreas dos cardiomiócitos foram avaliadas após coloração por hematoxilina e eosina, na avaliação do tecido conjuntivo foram analisadas as medidas de fibrose pelas lâminas coradas com Picrosirius Red (PSR). A análise vascular foi feita a análise da espessura arteriolar por meio da coloração por meio da técnica de Verhoeff (VVG). Para todas essas abordagens a dimensão fractal (DF) foi aplicada pelo método de box-counting. A normalidade dos dados foi avaliada pelo teste de Shapiro-Wilk. Os dados paramétricos foram avaliados por ANOVA seguido do teste de Tukey e para dados não paramétricos foi utilizado o teste de Kruskal-Wallis seguido do pós-teste de Dunn ($p<5\%$). **Resultados**: Não houve alteração da área dos cardiomiócitos; houve aumento do colágeno cardíaco nos animais expostos a baixa dose do 2,4-D ($p<0{,}0001$) e diminuição

da dimensão fractal do colágeno no grupo de alta dose (p=0,010); e não houve diferença na quantidade entre os colágenos do tipo I e III; e na análise da espessura das arteríolas não houve modificações. **Conclusão:** Após 180 dias de exposição ao agrotóxico 2,4-D houve alteração na remodelação cardíaca demonstrado pelo aumento do colágeno e da dimensão da fractabilidade.

Palavras chaves: Herbicida, Cardiotoxicidade, Ácido 2,4-Diclorofenoxiacético.

ABSTRACT

Analysis of cardiac remodeling in rats subjected to chronic exposure to the herbicide 2,4-D

Background: 2,4-Dichlorophenoxyacetic acid (2,4-D) is one of the most widely used chlorophenoxy herbicides worldwide due to its selectivity, efficiency and low cost. 2,4-D is rapidly absorbed in the respiratory tract and can toxically affect both animals and humans in a wide range of organs, such as liver, kidneys, muscles, lungs, gastrointestinal tract, nervous system (central, peripheral), endocrine, reproductive system and heart. Workers who use herbicides have a high risk of contamination by residues, which can occur through contact with the skin, ingestion and inhalation. **Objective**: To evaluate the chronic effects on cardiac remodeling of adult male Wistar rats that inhaled 2,4-D by nebulization. **Methods:** 30 adult male Wistar rats were used, divided into 3 groups: a control group exposed to the nebulization of 10 ml of 0.9% sodium chloride solution (GCI, n=10), a low concentration group exposed to 2.4-D with $3.71x10^{-3}$g of active ingredient per hectare (GBI, n=10) and a high concentration group exposed to 2.4-D with $9.28x10^{-3}$g of active ingredient per hectare (GAI, n=10). During an exposure period of 180 days, two boxes connected to ultrasonic nebulizers were used to spray the herbicides. After this period, the rats were euthanized for the collection and study of cardiac tissue. Cardiac remodelling was assessed in terms of hypertrophy, fibrosis and vascularization. To assess hypertrophy, the areas of the cardiomyocytes were evaluated after staining with hematoxylin and eosin. To assess connective tissue, fibrosis measurements were analyzed using slides stained with Picrosirius Red (PSR). Arteriolar thickness was analyzed using the Verhoeff technique (VVG). For all these approaches, the fractal dimension (FD) was applied using the box-counting method. Data normality was assessed using the Shapiro-Wilk test. Parametric data was evaluated by ANOVA followed by Tukey's test and for non-parametric data the Kruskal-Wallis test was used followed by Dunn's post-test ($p<5\%$). **Results:** Analysis of the measurement of cardiomyocyte area showed no change; there was an increase in collagen in the animals exposed to a low dose of 2,4-D ($p<0.0001$) and evaluation of the LV

by means of fractal dimension showed a reduction in fractal dimension in GAI when compared to GCI (p=0.010); and there was no difference in the amount between type I and III collagens; and analysis of the thickness of the arterioles showed no change. **Conclusion**: After 180 days of exposure to the pesticide 2,4-D there was an alteration in cardiac remodeling demonstrated by the increase in collagen and the dimension of fractility.

Keywords: Herbicide, Cardiotoxicity, 2,4-Dichlorophenoxyacetic acid.

LISTA DE ABREVIATURAS

2,4-D: dichlorophenoxyacetic acid

SG Saline group

LCG: Low concentration group

HCG: High concentration group

HE: hematoxylin-eosin method

PSR: Picro Sirius Red

VVG: Verhoeff

DF: fractal dimension

SUMÁRIO

1 ARTIGO CIENTÍFICO

ORIGINAL ARTICLE

CHRONIC INHALATION EXPOSURE TO THE HERBICIDE 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D) ALTERS CARDIAC COLLAGEN IN WISTAR RATS

Larissa Ferreira Rós Mariano[1], Thaoan Bruno Mariano[1], Rafael Stuani Floriano[1], Mariana Janini Gomes[2], Letícia Estevam Engel[1], Gisele Albonguetti Nai[1], Francis Lopes Pacagnelli[1*].

[1] Universidade do Oeste Paulista (UNOESTE), Presidente Prudente, SP, 19.067-175, Brazil

[2] Texas A&M University, College Station, Texas, United States of America

*Correspondence: Francis Lopes Pacagnelli (francispacagnelli@unoeste.br), Universidade do Oeste Paulista (UNOESTE), 19050-680 Presidente Prudente, SP, Brazil

Tel.: +55 18 3229 3264, Fax: +55 18 3229 2080

TOXICOLOGY RESEARCH

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ABSTRACT

2,4-dichlorophenoxyacetic acid (2,4-D) is one of the most used in the world and exposure to herbicides can affect animals and humans, causing toxic effects that include cardiotoxicity. This is the first study to evaluate cardiac remodeling after experimental simulation of environmental exposure by chronic inhalation (6 months) to the herbicide 2,4-D. Thirty male Wistar rats were exposed to two different concentrations of the 2,4-D formulation (low – 187.17 mg/m3; and high – 467.93 mg/m3) and the control group exposed to nebulization of chloride solution 0.9% sodium. Inhalation exposure lasted 6 months. Mice hearts were collected for histology. There was a difference between exposure concentrations in relation to the increase in cardiac collagen ($p<0.0001$). In mice exposed to a low dose of 2,4-D and a decrease in the fractal dimension of cardiac collagen in the high dose of 2, 4-D ($p=0.010$). There was no difference in relation to anatomical parameters, cardiomyocyte area, collagen types I and III and analysis of arteriole thickness. Chronic exposure at different doses to the 2,4D herbicide had the potential to cause damage to cardiac remodeling by altering cardiac collagen in rats.

Keywords: herbicide, cardiotoxicity, 2,4-dichlorophenoxyacetic acid, pesticide exposure, environmental exposure, collagen

INTRODUCTION

2,4-Dichlorophenoxyacetic acid (2,4-D) is one of the most used chlorophenoxy herbicides worldwide since the 1940s to the present day [1]. The mixture of 2,4-D butoxyethanol esters and 2,4,5-trichlorophenoxyacetic acid gave rise to the military herbicide used in the Vietnam War called Agent Orange [2]. The wide use of this pesticide is due to its cost-benefit, selectivity, effectiveness and broad spectrum in controlling weeds and because it is easily soluble in water and other solvents that result in rapid penetration through leaves and roots [3, 4].

In the US, the annual cost of markets for 2,4-D is almost 57 million dollars [5]. In China, production reached 40,000 tons in 2010 and due to this increase in the extensive application of 2,4-D, there is an increase in human exposure due to domestic and agricultural use and the consumption of contaminated food and water [6].

The human population can be exposed to 2,4-D occupationally, in agricultural production through skin absorption and inhalation, paraoccupationally in food, water and air, since during crop spraying the herbicide is carried by the wind which generates contamination of workers and the population [7, 8]. Lack of use of personal protective equipment and chronic exposure can lead to health problems [9]. 2,4-D is rapidly absorbed in the respiratory tract and can toxically affect both animals and humans in a wide range of organs such as the liver, kidneys [10], lungs [11], gastrointestinal tract [12], nervous system (central, peripheral) [13], endocrine system, reproductive system [14] and cardiovascular system [15, 16].

In the cardiovascular system, studies reported that the administration of 2,4-D for 6 weeks promoted an increase in blood pressure and arrhythmias [16] and that the consumption of nebulized feed with a high dose of 2,4 D promoted an increase in apoptotic markers in the rat heart. However, cardiac structural assessment after chronic inhalation exposure to different doses of 2,4-D that simulates occupational exposure has not been investigated.

Cardiac evaluation after chronic exposure to this herbicide is very relevant since its use is worldwide and workers are exposed to its inhalation during spraying of coffee, sugar and other food crops. And, understanding its

mechanisms of action in cardiac tissue can guide public health approaches. The objective of this study was to evaluate the effects of 6 months of low and high dose inhalation exposure of 2,4-D on the heart of rats.

METHODS

Ethical approval

The animals used in the research did not belong to another institution/individual/farm. They were treated humanely in accordance with institutional guidelines and internationally accepted principles for the use and treatment of laboratory animals, as set out in international guidelines, with due consideration for the relief of distress and discomfort. This study was approved by the Animal Use Ethics Committee of the Universidade do Oeste Paulista (Protocol nº 5684) and was carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institute of Health (Guide Update Committee for the care and use of laboratory animals 2011).

Animal protocol

This study was initially described by Mello et al. (2018) [17]. We used 30 healthy adult male Wistar rats weighing between 200 and 250g, with an average age of 60 ± 5 days, provided by the Central Animal Farm of the Universidade do Oeste Paulista (UNOESTE) and they were allocated in collective plastic box (5 rats per box). Which measured 30×16×19 cm. The rats were kept in a temperature-controlled room (22 ± 2°C, 50-60% relative humidity) and with a 12-hour light/dark cycle (7 am to 7 pm, light period; 7 pm to 7 am, dark period). To determine the minimum sample size for comparisons between groups, it was used the article by Parizi et al. (2020) [18] as a basis. From these data it was concluded that at least 5 elements were needed per group.

Throughout the experimental period, rats were exposed to the herbicide for 5 consecutive days per week at the same time, to simulate occupational exposure in crops. The rats received filtered water and commercial rat chow (Supralab®, Alisul, Brazil) ad libitum. All mice in each specific group were exposed to the herbicide at the same time. A commercial formulation of (2,4-

dichlorophenoxy) acetic acid (2,4-D; Nortox SA, Arapongas, Paraná, Brazil, registered with the Ministry of Agriculture, Livestock and Supply nº 03009) was used. The formulation consisted of 806g/L of dimethylamine salt of (2,4-dichlorophenoxy) acetic acid (2,4-D), 670g/L of 2,4-D acid equivalent and 424g/L of inert ingredients. The 2,4-D formulation was diluted in 0.9% sodium chloride. The rats were randomly distributed into three experimental groups (n = 10/group):

- SG, saline group: exposed to nebulization of 10 ml of 0.9% sodium chloride solution.
- LCG (2,4-D low concentration group): exposed to herbicide mist with 3.71×10-3 g of active ingredient per hectare (gia/ha) (4.6 µl of the pesticide was added to the saline), corresponding to 187.17 mg/m3 of 2,4-D.
- HCG (2,4-D high concentration group): exposed to herbicide nebulization at 9.28×10-3 gia/ha (11.5 µl of pesticide was added to saline solution), corresponding to 467.93 mg /m3 of 2,4-D.

The concentrations of 2,4-D herbicide were diluted in 10 ml of 0.9% sodium chloride to perform nebulization. The solutions were prepared at the time of use. The different concentrations of the 2,4-D herbicide were formulated based on the product label, which shows the different concentrations of herbicide for each type of crop to be sprayed, and dose adjustments were made in the area of the mist box to simulate the occupational environmental exposure [18]. Rats from different groups were exposed for five consecutive days per week over a period of 180 days. Rats were exposed with the concentration for their specific experimental group [17].

2,4-D exposure protocol

Exposure occurred through inhalation in plastic boxes connected to an ultrasonic nebulizer (Pulmosonic Star®, Brazil). Daily exposure periods lasted approximately 15 minutes; this is the time required for the entire solution to nebulize [17]. The concentrations of 2,4-D for each experimental group were chosen according to the standard application of the product and agronomic prescription for different crops. For a better interpretation and comparison of results in the literature, the concentrations of 2,4-D used in agriculture (grams of

active ingredient per hectare - g aiha-1) were adjusted to the dimensions of the box and converted to the units of mg/m− 3 and parts per million (ppm) [17].

All rats were exposed to the herbicide for 6 months and euthanized after this period. Anesthesia and euthanasia were performed with sodium thiopental (Syntec, USA) at doses of 100 mg/kg of weight administered into the peritoneal cavity. Indications of death were loss of reflexes and absence of respiratory movements and heartbeat [19]. After euthanasia, the heart was dissected into atria, right ventricle, left ventricle and weighed. Cardiac remodeling of the left ventricle was evaluated by anatomical data with the relationship of left ventricle weight/length of the tibia, and by histological analyses: evaluation of cardiomyocytes in relation to hypertrophy and nuclear organization, the extracellular matrix by analysis of quantity, organization (fractal dimension) and types of collagens and vascular aspects by analyzing arteriolar thickness.

Fig 1. Experimental design

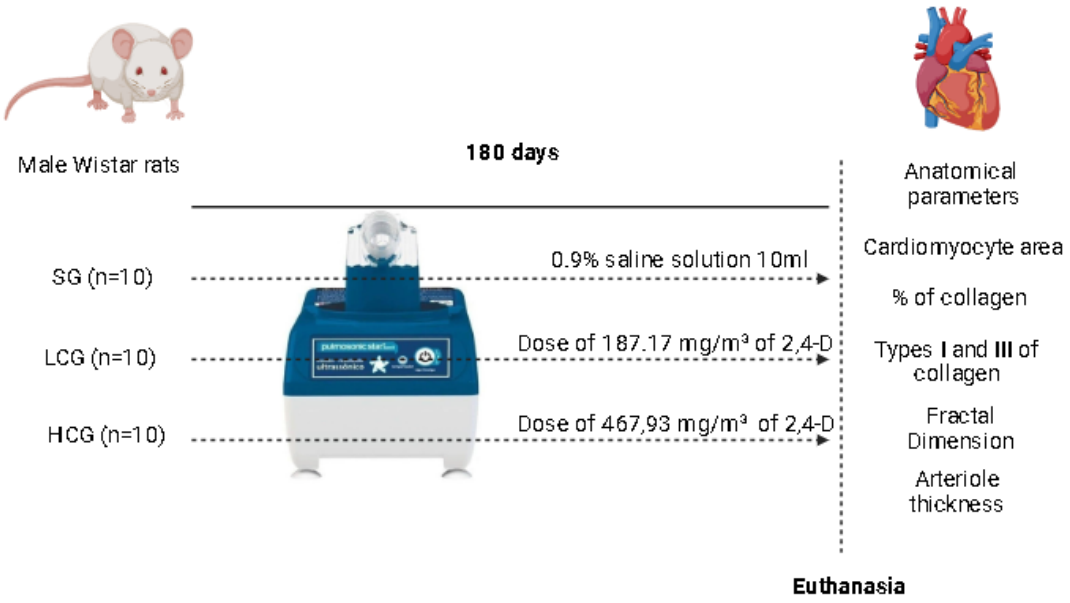

Fig. 1– Experimental design. SG: Saline group, LCG: Low concentration group, HCG: High concentration group, ml: milliliter, mg/m3: milligrams per cubic meter, VE: left ventricle. Created with BioRender.com

Anatomical parameters analysis

The rats were euthanized, and their hearts were dissected into the left ventricle (LV), right ventricle (RV) and atria, and were subsequently weighed.

The weights obtained were normalized by the tibial length, used as an index of ventricular hypertrophy [15].

Cardiomyocyte area analysis

The left ventricle was fixed in 10% buffered formalin for 48 hours. For histological analysis, 4 µm coronal sections were made and the tissues were fixed in paraffin blocks. The slides were stained with Hematoxylin-Eosin (HE) solution, and the analysis carried out consisted of measuring the transversal areas of the cardiomyocytes. We used a LEICA DMLS microscope (DM750, Leica Microsystems, Wetzlar, Germany) with 100x magnification. Digital images of coronal sections were analyzed using the ImagePro-Plus program. From each slide, 15 histological fields were selected. Fifty transverse cardiomyocytes were then selected, they must have a round shape and a visible nucleus in the center of the cell, located in the subendocardial layer of the LV muscular wall. As an indicator of cell size, the average sectional areas of each group were used [20]. A qualitative analysis was obtained to assess cell quantity, necrosis, fibrosis, and inflammation [21, 22, 23, 24].

Arteriolar thickness analysis

For arteriolar assessment, the VVG technique (Verhoeff) was used, and arteriolar thickness was measured at 400 x magnification, with 4 thicknesses for each arteriole, with 4 arterioles per animal [25].

Collagen quantification

LV fixation and preparation of slides were performed in the same way as the previous item (cardiomyocyte area). However, the staining of the samples was in accordance with the laboratory's standard protocol, they were stained with Picrosirius red (PSR). Heart sections stained with PSR were used to quantify fibrosis and 20 captures from each slide were analyzed using ImageJ® following the software instructions for collagen quantification [26].

Fractal dimension

Left ventricular sections stained with Picrosirius red (PSR) were evaluated using fractal dimension. 20 animal images were captured, with a magnification of 400x to evaluate collagen organization. Analysis was performed using ImageJ®, following software instructions for collagen quantification. Fractal dimension calculation is performed in two dimensions in planar binary images. To analyze the fractal dimension, the images were binarized for reading and the DF was estimated using the box-counting method. The software considered the box-counting method in two dimensions, allowing the quantification of the distribution of pixels in this space, without considering the texture of the image. The result is that two images with the same pixel distribution, one binarized and the other in gray levels, will have the same DF. The analysis of fractal histological slides was based on the relationship between the resolution and the evaluated scale, and the result was expressed quantitatively as the DF of the object (Log Nr/Log r-1; Nr which represents the quantity of equal elements necessary to attest the original object as a scale applied to the object.), whose value is between 0 and 2 [27, 28].

Collagen types analysis

The LV sections stained with Picrosirius Red were analyzed with images captured on the LEICA microscope (model DM750, Leica Microsystems, Wetzlar, Germany) using a video camera with a 40x objective with 400x amplification. Images of cardiac tissue were captured by a computer coupled to a video camera to evaluate interstitial collagen. The images were sent to a computer equipped with Image-Pro Plus (Media Cybernetics, Silver Spring, United States). Using polarized light, it was possible to differentiate type I (red) and type III (green) collagen. The measurement of the color of these collagens was carried out using the ImageJ® software, using 20 captures from each slide [29, 30].

Statistical analysis

Data normality was assessed using the Shapiro-Wilk test. Parametric data were evaluated by ANOVA followed by Tukey's test. For non-parametric data, the Kruskal-Wallis test was used followed by Dunn's post-test. Data were expressed as mean ± standard deviation and median, minimum and maximum. GraphPad Prism 10.1.0 software (GraphPad Software, Boston, MA, USA) was used. The significance level for consideration was $p < 0.05$.

RESULTS

Analysis of cardiac anatomical parameters in rats exposed to chronic 2,4-D inhalation

There was no significant difference between the groups for the anatomical variables analyzed, which demonstrates that 2,4-D did not promote hypertrophic changes in the atria and ventricles (Table 1).

Table 1. Analysis of cardiac anatomical parameters of rats exposed to chronic 2,4D inhalation.

VARIABLES	SG (n=10)	LCG (n=10)	HCG (n=10)	*p value*
FBW (g)	**463.5 ±25.88**	**487.5±21.13**	**463.5±29.12**	**0.07**
LV (g)	**1.05±0.08**	**1.012±0.07**	**1.00±0.05**	**0.21**
LV/Tibial length (g/cm)	**0.23±0.02**	**0.22±0.01**	**0.22±0.01**	**0.29**
RV (g)	**0.25±0.05**	**0.24±0.03**	**0.23±0.01**	**0.61**
RV/Tibial length (g/cm)	**0.05±0.01**	**0.05±0.008**	**0.05±0.006**	**0.59**
Atria (g)	**0.08±0.03**	**0.08±0.02**	**0.10±0.02**	**0.37**
Atria/Tibial length (g/cm)	**0.01±0.007**	**0.01±0.005**	**0.02±0.006**	**0.17**

Data as mean ± SD or median and interquartile range. SG: Saline group, LCG: Low concentration group, HGC: High concentration group. FBW: final body weight. LV: Left Ventricle. LV/tibial length: left ventricle to tibial length ratio. RV: Right Ventricle. RV/tibial length: ratio of right ventricle to tibia length. Atria/tibial length: relationship of the atria to the length of the tibial. ANOVA followed by Tukey or Kruskal - Wallis followed by Dunn.

Area of cardiomyocytes on the effect of 2,4-dichlorophenoxyacetic acid

Figure 2 shows the area of cardiomyocytes stained with HE and evaluated for hypertrophy. After 180 days of exposure to 2,4-dichlorophenoxyacetic acid, no LV hypertrophy was observed.

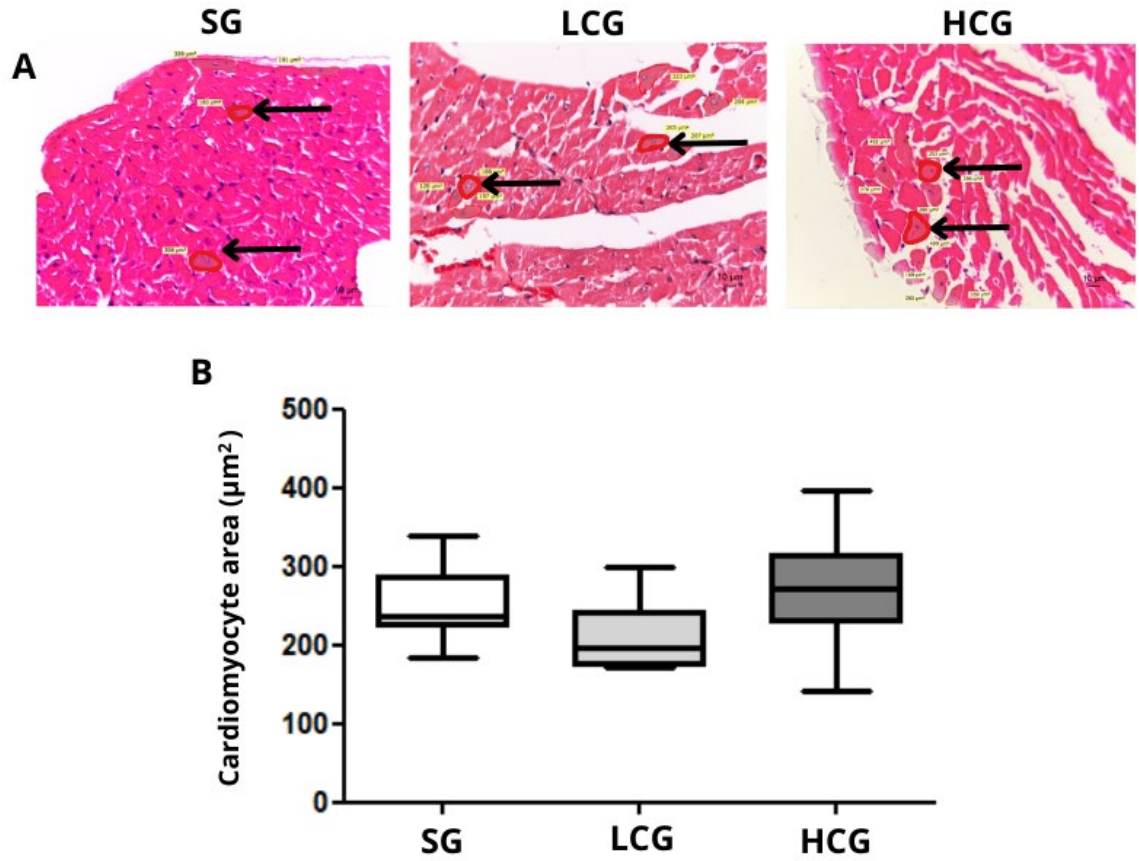

Fig. 2 A Transverse sections of the LV of rats exposed to inhalation of the 2,4-D herbicide were stained using the Hematoxylin and Eosin (HE) technique and visualized with a 40x objective and 400x magnification, the black arrows point to the circled illustrative measurements of the areas of cardiomyocytes. B Quantitative analysis of the cardiomyocyte area. Data expressed as median and interquartile range. Kruskal-Wallis followed by Dunn. SG: Saline group; LCG: Low concentration group; HCG: High concentration group; µm2: square micrometers.

Arteriole thickness induced by 2,4-dichlorophenoxyacetic acid

The analysis of arteriolar thickness analyzed by the method VVG (Verhoeff), is represented in Figure 3. Exposure to the pesticide did not cause an increase in arteriolar thickness.

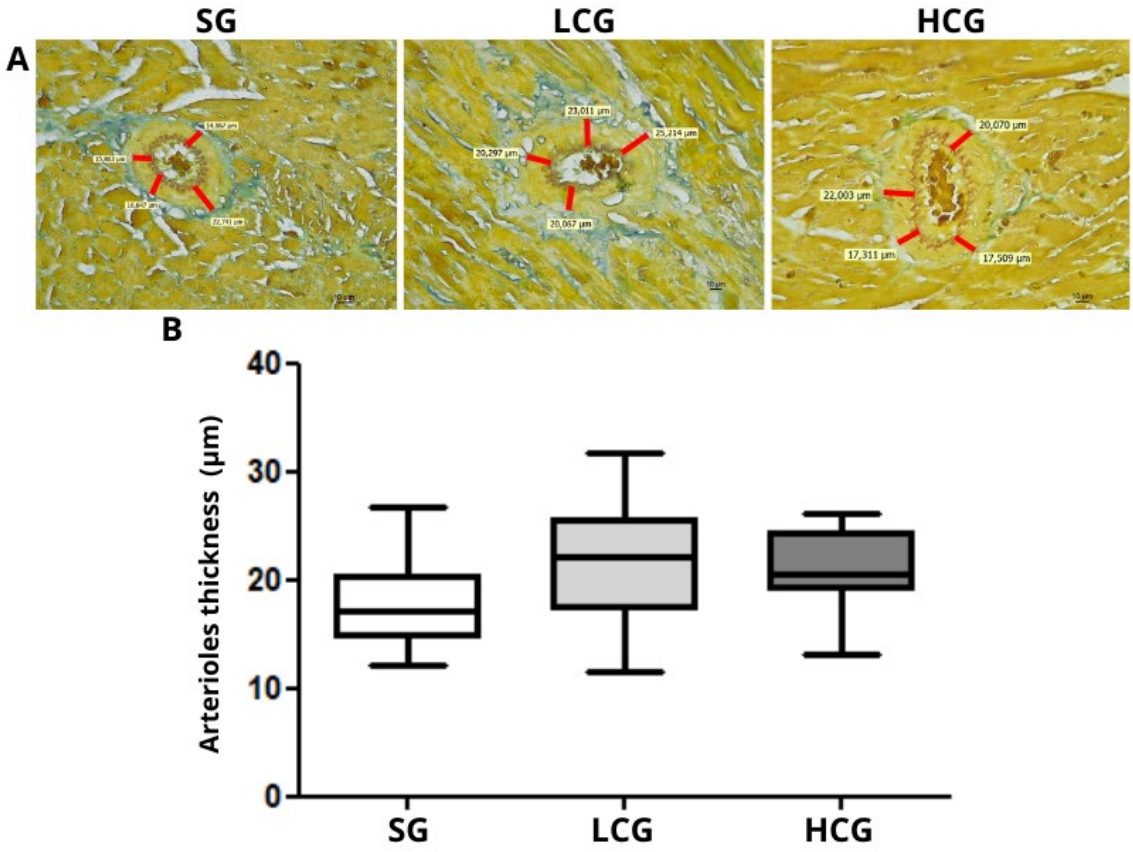

Fig. 3 A Transverse sections of cardiac arterioles from rats exposed to chronic inhalation of 2,4-D were stained using the VVG technique (Verhoeff) and visualized with a 40x objective and 400x magnification. The red lines illustrate the four measurements of the thickness of the cardiac arterioles for subsequent calculation of the average. B Quantitative analysis of arteriolar thickness. SG: Saline group; LCG: Low concentration group; HCG: High concentration group; µm: micrometers. Data expressed as median and interquartile range. Kruskal-Wallis followed by Dunn.

Analysis of cardiac fibrosis induced by 2,4-dichlorophenoxyacetic acid

Figure 4 (A) shows interstitial collagen stained with Picrosirius Red, to assess the presence of cardiac fibrosis. There was an increase in collagen in animals exposed to a low dose of 2,4-D ($p<0.0001$). When evaluating the VE using the fractal dimension in figure 4 (B), a reduction in the fractal dimension was found in the HCG when compared to the SG ($p=0.010$). Figure 4 (C) shows the image with polarized light, the results show type I collagen in red and type III

collagen in green, there was no difference in the amount of these two types of collagen.

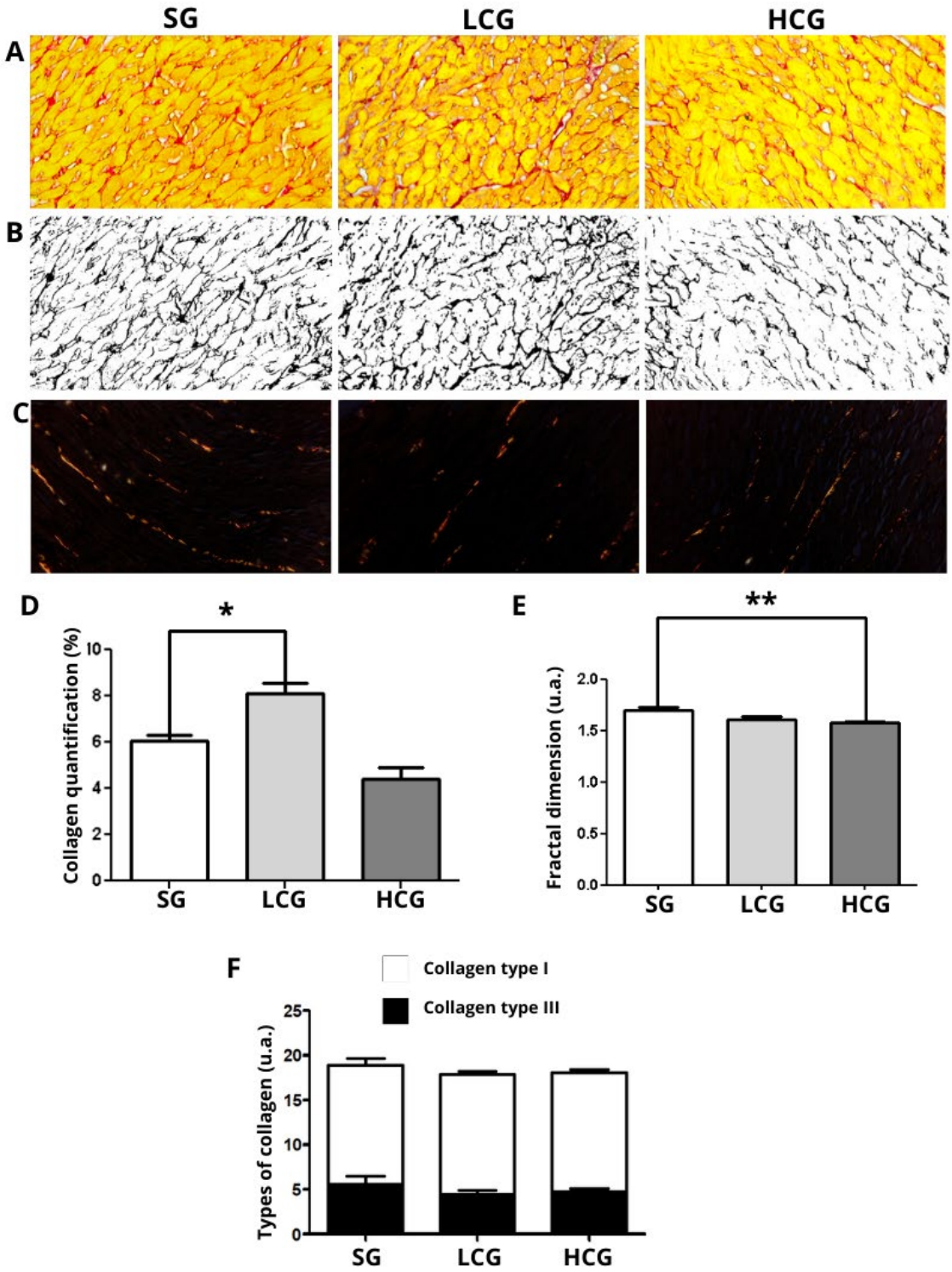

Fig. 4 A Transverse sections of the left ventricle of rats exposed by inhalation to the herbicide 2,4-D were stained using the Picrosirius Red (PSR) technique and

visualized with a 40x objective and 400x magnification for the quantification of type I collagen (red) and type III (green) in ImageJ® Software. B Cross sections of the LV after binarization to measure the fractal dimension in ImageJ® software. C PSR observed under polarized light and quantified by ImageJ® software, type I collagen red and type III collagen green. D Collagen quantification analysis. E Analysis of the fractal dimension of collagen. F Analysis of the quantification of type I and type III collagen). SG: Saline group; LCG: Low concentration group; HCG: High concentration group; μm: micrometers. Data expressed as mean and standard deviation. One-Way ANOVA followed by Tukey and Kruskal-Wallis followed by Dunn.

Discussion

This is an unprecedented study that showed that chronic inhalation exposure to 2,4-D caused cardiotoxicity in rats by promoting reorganization of the extracellular matrix, demonstrated by the increase in collagen in the group with low concentration exposure and a decrease in fractal dimension in the high concentration group. concentration. There were no changes in anatomical parameters, cardiomyocyte area, thickness of cardiac arterioles and quantification of collagen types I and III.

In the present study, no change in left ventricular weight or cardiomyocyte area was identified. In study withrats that fed food contaminated with 2,4-D pelthe same period of 180 days showed a reduction in cardiomyocyte area and this change was related to the pro-apoptotic protein BAX and anti-apoptotic Bcl-2 degradation that leads to apoptosis [15]. However, in another study with Swiss rats, there was cardiac hypertrophy upon acute exposure to 2,4-D by nebulization for 3 days as a probable compensatory mechanism [31]. These data from Mantovani et al. (2020) [15] and Negrão et al. (2019) [31] suggest that different types of environmental exposure and duration may influence the different results, as the dose applied to the animals was the same as in the present study.

Based on our results, we did not observe changes in arteriole thickness. Although there is a paucity of studies on these changes in mice, two studies highlight changes in zebrafish. In Lee's et al. (2020) [32] study on the toxicity of

chlorpropham, it is highlighted that vascular morphogenesis, crucial for the cardiovascular circulatory system, was interrupted by chlorpropam, resulting in a decrease in the expression of specific regulators. Another study, using Bifenox in zebrafish embryos, suggests that Bifenox induces hepatotoxicity and vascular toxicity by generating reactive oxygen species and modifying signaling pathways, which in turn inhibits blood vessel growth [33].

In this study we observed that the percentage of interstitial collagen increased, which indicates that environmental exposure to low doses can already cause cardiac toxicity. Studies report that increased collagen is related to fibrosis, which causes changes in ventricular relaxation and significant contractile impairments [34, 35]. In our study, the increase in collagen infers reorganization of the extracellular matrix (ECM). The ECM is a three-dimensional network responsible for organizing, structuring, and providing function to cellular processes [36], and in the heart, not only to structure, but also transmits contractile force, being essential to maintain adequate ventricular ejection fraction. This is made up of glycoproteins, glycosaminoglycans and collagen, with types I and III being the most common [34]. The impairment of these components causes changes in cardiac remodeling [37], thus inferring a specific cardiotoxic action of this herbicide.

Shortly after an acute myocardial infarction, fibroblasts are activated and transform into myofibroblasts, playing a crucial role as the main source of extracellular matrix during the healing process. This transformation is essential for the formation of scars, which help maintain the structural integrity of the heart, although it can also contribute to cardiac stiffness and dysfunction if collagen production is excessive [38, 39]. Adequate modulation of this fibrous response is essential to promote efficient healing and minimize adverse remodeling of cardiac tissue.

A study with cardiac fibroblasts in vitro, administered Nitrofen, a herbicide from the diphenyl ether class, observed changes in the remodeling of the extracellular matrix and proliferation of cardiac fibroblasts. Nitrofen stimulated collagen mRNA in fibroblast cells, also influencing growth factor and tropoelastin, which altered the extracellular matrix [38].

In two weeks, Rotenone, a pesticide derived from plants, was applied intraperitoneally and induced cardiac fibrosis through the loss of energetic

mechanisms, by increasing reactive oxygen species through mitochondrial damage, which accelerates the fibrosis process [40]. However, Dinis-Oliveira et al. 2009 used Paraquat, a herbicide that belongs to the chemical groupbipyridylium, considered highly toxic, which was administered by gavage, at a dose of 125 mg/kg, and no cardiac changes were observed in relation to fibrosis, only in other organs. Therefore, the type of herbicide, its chemical composition, mechanism of action, may be selective in causing cardiac fibrosis. One of the mechanisms that may be involved in the increase in cardiac fibrosis is the increase in reactive oxygen species, which accelerated the fibrosis process [22, 24].

We analyzed the fractal dimension of collagen due to the importance of the technique in highlighting tissue organization. There was a reduction in fractability in the high dose group, which indicates a reorganization of the extracellular matrix in this tissue. In the research by Mantovani et al. 2020 [15], 2,4-D chronically through oral exposure did not cause interstitial fibrosis and changes in fractal dimension, which contradicts our findings. This may indicate that the inhaled form of administration of 2,4-D influences this aspect of the extracellular matrix more than the oral form. The reduction in fractal dimension has already been identified in other studies and has been related to functional impairments. Aminuddin et al., 2022 [41] showed that reduced cerebral vascular fractal dimension was a potential biomarker of cerebral small vessel disease in asymptomatic individuals. Smallest retinal vascular fractal dimension assessed from 97,895 individuals it was also associated with a high risk of other complications such as hypertension, heart failure and other changes [42].

In the study by Zouein et al. 2014 [43] wild-type hypertensive mice showed cardiac fibrosis, with an increase in collagen and a significant decrease in DF. The authors suggest collagen packaging that could lead to myocardial stiffening and diastolic dysfunction. However, in a study with C57BL/6NJ mice induced to pulmonary fibrosis with bleomycin or recombinant adenovirus Ad-TGFβ1, histological images of fibrotic lung tissue were analyzed and they found that fibrotic regions have greater fractal dimensions compared to non-fibrotic regions, indicating that the Tissue remodeling in fibrotic regions is associated with increased spatial complexity [44]. Therefore, quantifying changes in tissue structure through a fractal dimension may provide an alternative metric of

phenotypic changes in fibrosis to complement the conventional metric of total collagen, as well as demonstrating that both increases and decreases in DF indicate changes in tissue complexity.

The study carried out had limitations such as not evaluating cardiac function in vivo and the molecular mechanisms involved in these changes.

Conclusions

Based on data from this study, we conclude that chronic inhalational exposure to 2,4-D in rats resulted in cardiotoxicity, evidenced by the reorganization of the extracellular matrix in cardiac tissue. This was indicated by the increase in the amount of collagen with the administration of a low concentration of the herbicide and the decrease in the fractal dimension with exposure to a high concentration of the herbicide. The observed changes were correlated with the different concentrations of the 2,4-D herbicide.

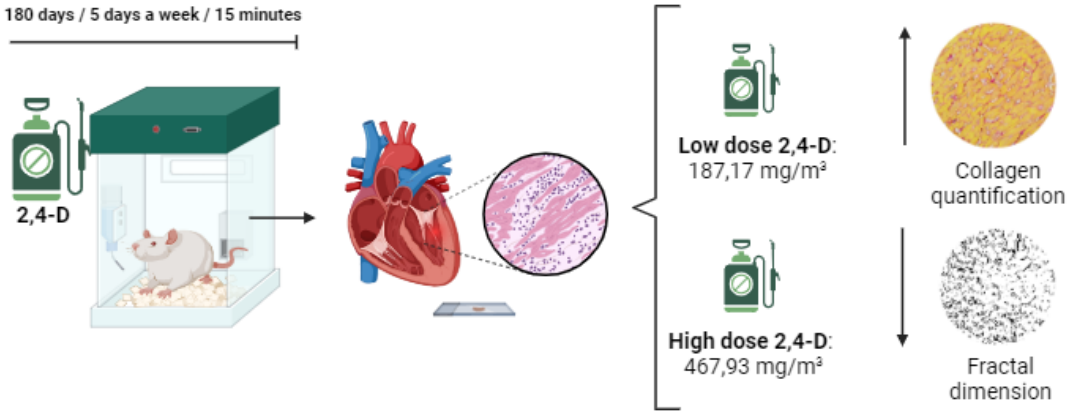

Fig. 5 Schematic figure summarizing the main results. 2,4-D: 2,4-Dichlorophenoxyacetic; mg/m3: milligram per square meter. Created with BioRender.com.

Abbreviations

2,4-D: dichlorophenoxyacetic acid; SG Saline group; LCG: Low concentration group; HCG: High concentration group; HE: hematoxylin-eosin method; DF: fractal dimension; PSR: Picro Sirius Red; VVG: Verhoeff

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Authors' contributions

L.R.M. conceived of the study. L.R.M., performed data collection, data analysis, and produ-ced the figured and scripts, with overall guidance from F.L.P. LRM, TBM, LEE, RSF, MJG, GAN e FLP histopathological analysis, investigation, and contribution to writing the original project. L.R.M, M.J.G., RSF. and F.L.P research and resources. All authors wrote the ma-nuscript. L.R.M., G.A.N. and F.L.P. deposited the data.

Conflict of interests

The authors have no conflict of interest to disclose.

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ANEXO A- APROVAÇÃO ÉTICA

UNOESTE - Universidade do Oeste Paulista

PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO

PPG - Programa de Pesquisa de Pós-Graduação
PEIC - Programa Especial de Iniciação Científica

Parecer Final

Declaramos para os devidos fins que o Projeto de Pesquisa intitulado **"ANÁLISE DA REMODELAÇÃO CARDÍACA DE RATOS SUBMETIDOS A EXPOSIÇÃO CRÔNICA AO HERBICIDA 2,4 D"**, cadastrado na Coordenadoria de Pesquisa, Desenvolvimento e Inovação (CPDI) sob o número nº **7782** e tendo como participante(s) **LARISSA FERREIRA ROS MARIANO (discente), CAMILY COLNAGO RIBEIRO (discente), VINICIUS LUZ SALES (discente), GLAURA SCANTAMBURLO ALVES FERNANDES (participante externo/voluntário), GISELE ALBORGHETTI NAI (docente), FRANCIS LOPES PACAGNELLI (orientador responsável)**, foi avaliado e **APROVADO** pelo **COMITÊ ASSESSOR DE PESQUISA INSTITUCIONAL (CAPI) e COMISSÃO DE ÉTICA USO DE ANIMAIS (CEUA)** da Universidade do Oeste Paulista - UNOESTE de Presidente Prudente/SP.

Este Projeto de Pesquisa, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de Outubro de 2008, do Decreto nº 6.899, de 15 de Julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), tendo sido APROVADO em reunião realizada em 07/12/2022.

MATERIAL ARMAZENADO/DOADO

Protocolo(s)	Data Aprovação	Armazenado (local)	É doação	Detalhes armazenamento
3761	10/05/2017	UNOESTE	SIM	Laboratório de Histologia

Presidente Prudente, 5 de Fevereiro de 2024.

Prof. Dr. Jair Rodrigues Garcia Jr.
Docente Responsável pela CPDI

Prof. Dr. Felipe Rydygier de Ruediger
Coordenador da CEUA - UNOESTE

ANEXO B- NORMAS DE PUBLICAÇÃO

General Instructions

Toxicology Research has partnered with Enago to offer a free trial of their Trinka AI Editing tool for pre-submission language editing. Please see the Language editing pre-submission section for full details.

Scope

Toxicology Research aims to publish cutting edge research that is excellent and innovative, that drives toxicology and has international impact. Articles should cover chemical or biological aspects of the toxic response and the mechanisms involved.

The journal's scope includes the following.

Carcinogenicity, including studies on mode of action of carcinogens, genotoxicity and mutation.
Biomarkers of toxicity, including studies on their identification, validation, and utilization.
Computational and predictive toxicology, including *in vitro*, *in vivo* and *in silico* studies of toxicity and the development of predictive tools and models. Work considering alternative methods to in vivo studies is encouraged.
Systems toxicology, studies that describe the toxicological effects of chemicals on specific organs or systems (for example, immune, nervous, reproductive, respiratory), including studies that incorporate genomic, metabonomic and proteomic data.
Risk assessment, studies which provide toxicological data or information—such as hazard identification, and dose-response assessment.
Exposure assessment for the development of risk assessments or regulation.
Environmental toxicology, studies reporting toxicology data for organisms within an ecosystem are encouraged if there is a wider benefit to human health. This includes studies on lower organisms as models for the human toxic response, studies which provide cross-species perspective, or studies on organisms which lie within the human food chain. Studies where the toxicological conclusions are only relevant for a specific lower organism should be submitted to a more specialized journal.
Clinical toxicology, studies relating to clinical trials or medicinal applications of toxicological research, including translational toxicology, and studies translating a molecular understanding of the toxic response to clinical application.
Nanotoxicology, an adequate characterization of relevant physico-chemical properties of the nanoparticles is required for studies on the biochemical or molecular mechanisms of toxic responses to nano materials. Studies on common nanomaterials which are of direct

relevance to human health are strongly encouraged. For studies reporting the synthesis and characterization of novel nanomaterials, the rationale for reporting the toxicological effect of these materials must be justified.
Food toxicology, toxicological studies of chemical extracts related to food and nutrition. Studies that involve uncharacterized extracts or in which the food substance is only of limited or local interest should be submitted elsewhere.
Analytical metrology, including studies of new analytical methods and applications.
The following areas are not within the scope of *Toxicology Research*:

- Studies that focus only on toxic contaminant levels in the environment or in populations, and the sources, transport or fate of these contaminants.
- Biomarkers for the detection of contaminants in the environment.
- Environmental contaminant studies that do not discuss toxic effects on a molecular level.

Ethical policies

Authors should observe high standards with respect to publication best practice. Falsification or fabrication of data, plagiarism, including duplicate publication of the authors' own work without proper citation, and misappropriation of work are all unacceptable practices. Any cases of ethical or publication malpractice are treated very seriously and will be managed in accordance with the Commission on Publication Ethics (COPE) guidelines. Further information about OUP's ethical policies is available.

Data policies

Availability of Data and Materials

Where ethically feasible, *Toxicology Research* strongly encourages authors to make all data and software code on which the conclusions of the paper rely available to readers. We suggest that data be presented in the main manuscript or additional supporting files, or deposited in a public repository whenever possible. Information on general repositories for all data types, and a list of recommended repositories by subject area, is available here.

Data and Software Citation

Toxicology Research supports the Force 11 Data Citation Principles and requires that all publicly available datasets be fully referenced in the reference list with an accession number or unique identifier such as a digital object identifier (DOI). Data citations should include the minimum information recommended by DataCite:

[dataset]* Authors, Year, Title, Publisher (repository or archive name), Identifier

*The inclusion of the [dataset] tag at the beginning of the citation helps us to correctly identify and tag the citation. This tag will be removed from the citation published in the reference list.

Software citations should include the minimum information recommended by the FORCE11 Software Citation Implementation Group:

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OUP's policy on data and privacy.

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Toxicology Research requires submitting authors to provide an ORCID iD at submission to the journal. More information on ORCID and the benefits of using an ORCID iD is available. If you do not already have an ORCID iD, you can register for free via the ORCID website.

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Article types and submission

Toxicology Research publishes:

Original Article

Communications

Reviews

Viewpoints

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OUP author FAQs

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Typical articles would be between three and seven pages in length, and articles would not normally exceed 10 pages. Authors are encouraged to supply any additional procedures, data and other material in an electronic supplementary information file.

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Toxicology Research Communications contain novel scientific work of such importance that rapid publication is desirable (typically 50% faster than a full paper). Authors should briefly indicate in a covering letter the reasons why they feel that publication of their work as a Communication is justified.

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Toxicology Research Reviews are concise and critical appraisals of specialist areas throughout toxicology. Reviews will be easy-to-read articles (typically 6–10 journal pages

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Authors are encouraged to include their own perspective on developments, trends and future directions; however Reviews should not solely cover the author's own work, but should include key developments from across the field. Reviews are normally published by invitation of the *Toxicology Research* editorial office. However, direct submissions from authors are welcome. Reviews are subject to the journal's standard peer review process and an invitation to submit is not a guarantee of acceptance. Enquiries regarding the submission of Reviews should be directed to the editorial office.

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Comments and Replies are a medium for the discussion and exchange of scientific opinions between authors and readers concerning material published in *Toxicology Research*.

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A brief description of the procedures used for the handling and care of animals

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Guidelines about conflicts of interest.

Experimental details and data guidelines

Sufficient details of experimental procedures should be included such that a scientist skilled in the art would be able to reproduce the results presented. The synthesis of all new compounds must be described in detail. Extensive experimental procedures and data (more than two journal pages) should be placed in an electronic supplementary information file.

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Synthetic procedures must include the specific reagents, products and solvents and must give the amounts (g, mmol, for products: %) for all of them, as well as clearly stating how the percentage yields are calculated. The final physical state (solid; amorphous; liquid; solution) should be disclosed. Where compounds are synthesized as part of an array or library a representative synthesis will be sufficient. Characterization levels should be consistent with the importance of the structure to the conclusion of the work.

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Data is not required for known compounds synthesized by a known method, but an original reference should be cited.

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Chemical structures should be reported in the manuscript if that structure is necessary to understand the paper or repeat an experimental or computational procedure. Chemical structures should not be blanked out. In certain cases the non-disclosure of chemical structures may be acceptable, and these are considered on a case-by-case basis by the associate editor.

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Doses and concentrations should be expressed as molar quantities (for example, mol kg^{-1}, mmol kg^{-1}, M, mM). Forms of administration as well as physical states and formulations should be noted. Biological test methods should be described in sufficient detail such that a scientist skilled in the art would be able to reproduce the results presented.

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Characterization data should include the following:

size and size distribution (in both dry and wet states)

elemental composition including impurities

morphology.

Dosing

Authors should give nanomaterial dosing as nanomaterial surface area per volume and number of particles per volume. Justification of the doses of nanomaterials used should be given, particularly when doses are beyond realistic exposure and application scenarios.

In vitro dosing parameters such as exposure length, cell seeding density, an estimate of the actual cellular dose and the relevance of the cell types used should be given.

Additional information

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Author names

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Introduction

This should give clearly and briefly, with relevant references, both the nature of the problem under investigation and its background.

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Descriptions of methods and/or experiments should be given in detail sufficient to enable experienced experimental workers to repeat them.

Results

Only strictly relevant results should be presented and figures, tables, and equations should be used for purposes of clarity and brevity.

Discussion

This section should discuss the significance of the results reported.

Conclusion

This section is for interpretation of the key results and to highlight the novelty and significance of the work. The conclusions should not summarize information already present in the article or abstract. Plans for relevant future work can also be included.

Acknowledgements

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These should be listed at the end of the manuscript in numerical order.

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