

ORIGINAL ARTICLE

Effect of Short-Term Inhalation of The Herbicide 2,4D on Cardiac Remodeling: Morphological Aspects

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Abstract

Background: Brazil is the worldwide leader in the long-term use of pesticides and herbicides. This compromises the health of handlers by causing harmful neurological, respiratory, and cardiovascular changes. The herbicide 2.4D has been shown to cause cardiac overload with subsequent pathological remodeling.

Objective: To analyze the cardiac morphological repercussions on the left ventricle (LV) of mice submitted to nebulization by the herbicide 2.4D.

Methods: Fifteen mice were divided into three groups: control group (CG; n = 5) exposed to nebulization with sodium chloride solution; low concentration group (LCG; n = 5) exposed to nebulization of the herbicide 2.4D with 3.71×10^{-3} grams; and high concentration group (HCG; n = 5) exposed to nebulization of the herbicide 2.4D with 9.28×10^{-3} grams for 15 minutes. The fractal dimension analysis was performed through the box-counting method. Later, the ImageJ program was used to calculate the fractal dimension of each group. To evaluate cardiac remodeling, histological slides were prepared and stained with Hematoxylin-Eosin (HE). Fifty areas of cardiomyocytes were analyzed per animal. The comparisons between groups were performed by ANOVA One-Way with Tukey's posttest ($p < 0.05$).

Results: There was no change in fractal dimension values between the CG = 1.37 ± 0.02 , LCG = 1.33 ± 0.04 and the HCG = 1.33 ± 0.07 groups. However, cardiac hypertrophy occurred in the HCG = $303.9 \pm 38.80 \mu\text{m}^2$ when compared to the CG group = $236.9 \pm 61.71 \mu\text{m}^2$ ($p = 0.034$).

Conclusion: The herbicide 2.4D used for 72 hours did not promote cardiotoxicity when evaluated by fractal dimension. However, cardiomyocyte hypertrophy was observed in the LV. (Int J Cardiovasc Sci. 2019; [online]. ahead print, PP.0-0)

Keywords: Mice; Pesticides / adverse effects; Herbicides / adverse effects; Agrochemicals; Neurologic Manifestations; Respiratory Tract Diseases; Hypertrophy, Left Ventricular.

Introduction

Herbicides are pesticides used in agriculture, plants and crops (green vegetables, fruits and vegetables) used to protect, repel and fight the proliferation of living organisms (pests, fungi, insects, weeds, birds, mollusks, microbes and arachnids) that are harmful to the agricultural production.¹⁻⁵ They are classified

according to their toxicity, which may range from low to extreme, constituting a great risk to the health and the environment.^{1,3-5}

According to the Brazilian Department of the Environment, Brazil is the worldwide leader in the consumption of agrochemicals, due to the vast culture and source of labor in agriculture.⁶ Since 2003, the agrochemical market has increased its use

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by 93%, with Brazil being the leader in consumption worldwide, a position previously occupied by the United States.⁷ The Ministry of Health considers that there are more than 100,000 chemical substances capable of causing chemical, physical, morphological and pathophysiological damage to the health of populations.⁸⁻¹² Among those who suffer the most from the exaggerated use of agrochemicals are rural workers, when these substances are utilized incorrectly.^{6,9}

In 1990, the World Health Organization (WHO) estimated that approximately 3,000,000 cases of pesticide poisoning occurred per year in the world, with 220,000 deaths per year.¹³ Only in 2009, more than 1 billion liters of agrochemicals were utilized in Brazil; it is the same as if each one of the Brazilian individuals consumed an average of five liters of poison per year.¹⁴ In Brazil, pesticide poisoning is a public health problem and a matter of compulsory notification.¹⁵

The toxicity can be fast or slow and it can take from months to years for symptoms to appear in the worker exposed to the pesticide. Among the harmful effects are allergic reactions, skin and eye irritation, neurological, respiratory, hematopoietic, hepatic, nephrological, gastrointestinal, pancreatic, and cardiovascular alterations, as well as several toxic reactions.⁹⁻¹² The effects of several types of pesticides have already been well established, resulting in cardiotoxicity with mitochondrial alterations, oxidative stress, hypertrophy, and molecular alterations of gene expression related to cardiac calcium.¹⁶⁻¹⁹

One of the herbicides frequently used to fight weeds in sugarcane plantations is 2,4D (2,4-dichlorophenoxyacetic acid),⁶ which has harmful effects when ingested, absorbed by the skin and/or inhaled. This herbicide can cause peripheral neuritis and transient diabetes during exposure to it, as well as loss of appetite, skin irritation, nausea, gastrointestinal tract irritation, vomiting, chest and abdominal pain, exhaustion, weakness, muscle twitching, mental confusion, seizures and even coma. If 2,4 D is used at high concentrations, it may cause degenerative liver and kidney lesions.²⁰ However, to the best of our knowledge, there have been no studies evaluating the cardiotoxic effects of 2,4 D using a nebulizing spray containing a sodium chloride (NaCl) solution during a 72h-period. The aim of this study was to analyze the cardiac morphological effects on the left ventricle (LV) of mice submitted to daily nebulizing sprays containing the 2,4D herbicide for 15 minutes. It is

hypothesized that exposure to high concentrations of 2,4D herbicide will lead to cardiac remodeling.

Methods

Ethical aspects

This was an experimental cross-sectional study submitted to the Animal Ethics Committee of *Universidade do Oeste Paulista* and evaluated by the Animal Use Ethics Commission (CEUA, from the Portuguese *Comissão De Ética do Uso De Animais*), and approved under protocol n. 3331 to be performed according to the Guide for the Care and Use of Laboratory Animals of the Institutes of Health (USA).

Sample characterization

For the experiment, 15 male Swiss mice (30-40 g) were provided by the Animal Center of *Universidade do Oeste Paulista*, where they were housed in collective plastic cages (5 animals per cage) measuring 30x16x19 centimeters, under a mean temperature of $22 \pm 2^\circ\text{C}$, with 12-hour cycles of light and dark, i.e., light from 7AM to 7PM and dark from 7PM to 7AM. To perform the experiment, the 15 animals were randomly divided into three groups, using the simple randomization method (random sequence performed through Excel):

- CG: control group (n = 5) exposed to nebulization with sodium chloride solution (NaCl) for 15 minutes/day, for 3 consecutive days.

- LCG: low concentration group (n = 5) exposed to nebulization of 2,4D herbicide with 3.71×10^{-3} grams of active ingredient per hectare (g.i.a./ha) for 15 minutes/day, for 3 consecutive days.

- HCG: high concentration group (n = 5) exposed to nebulization of 2,4D herbicide with 9.28×10^{-3} grams of active ingredient per hectare (g.i.a./ha) for 15 minutes/day, for 3 consecutive days.

2,4D Herbicide Exposure Protocol

The experimental protocol used two boxes of 2,4D (32x24x32 cm), each attached to a Pulmosonic Star^{®9} ultrasonic nebulizer, and the following concentrations were administered after being diluted in 10 mL of 0.9% sodium chloride:

- CG: 10 mL of 0.9% sodium chloride;

- LCG: Solution consisting of 3.71×10^{-3} grams of active

ingredient per hectare (g.i.a/ha) of 2,4D, diluted in 10 mL of 0.9% sodium chloride. The time of exposure consisted of 15 minutes/day on 3 consecutive days.

- HCG: Solution consisting of 9.28×10^{-3} grams of active ingredient per hectare (g.i.a/ha) of 2,4-D diluted in 10 mL of 0.9% sodium chloride. The time of exposure was 15 minutes/day for 3 consecutive days.

On the first day of exposure, all 15 animals were submitted to the nebulization with the recommended concentration for each group. After the 3 consecutive days of nebulization, they were anesthetized with Thiopental Sodium, at a dose of 100 mg/kg of weight, administered in the peritoneal cavity. Soon after being euthanized, the animals had their hearts fixed in 10% buffered formalin for 72 hours.

Histological and histomorphometric analyses

After being fixed in formalin, the tissue was embedded in paraffin blocks to obtain 4-micrometer thick coronal histological sections. The histological sections were stained on a slide with the Hematoxylin-Eosin (HE) solution to measure cardiomyocyte cross-sectional areas using a LEICA microscope (model DM750, Germany), which sends digital images to the computer using the LeicaApplication Suite LAS 4.2.0 image analysis system (Media Cybernetics, Silver Spring, Maryland, USA).^{21,22}

The images were obtained using a binocular optical microscope. All images were captured by video camera at 400x magnification (40x objective). Four LV sections were obtained from each animal and different field captures were analyzed, which were chosen according to the place where more cells could be visualized in a cross-sectional view.^{22,23} Fifty cells were measured per analyzed ventricle. The selected cardiomyocytes were transversely sectioned and exhibited a round shape, with a nucleus visible in the center of the cell and were located in the subendocardial layer of the LV muscle wall.^{22,23} The mean sectional areas obtained for each group were used as indicators of cell size.²³ We also performed the fractal dimension analysis through the box-counting method, in which 3 images were captured per each analyzed slide; after that, the ImageJ program was used to calculate the fractal dimension of each group (Figure 1). This careful technique aims to standardize the set of cardiomyocytes from the different groups to the maximum. The mean sectional areas obtained for each group were used as indicators of cell size.²³

Statistical analysis

The software GraphPadPrism was used for the statistical analysis. The Shapiro-Wilk test was performed to analyze data normality. The data showed normal distribution and were presented as mean \pm standard deviation. One-Way ANOVA followed by Tukey test ($p < 0.05$) were performed for comparison between the groups.

Results

There was no alteration in the fractal dimension parameter between the CG = 1.37 ± 0.02 , LCG = 1.33 ± 0.04 and HCG = 1.33 ± 0.07 groups ($p > 0.05$). The animals submitted to a high dose of the herbicide had cardiomyocyte hypertrophy (HCG = $303.9 \pm 38.80 \mu\text{m}^2$) when compared to the control group (CG = $236.9 \pm 61.71 \mu\text{m}^2$; $p = 0.034$). (Figure 2).

Discussion

To the best of our knowledge, this is the first study showing that the inhalation of 2,4D herbicide at high concentrations for a short time in mice causes cardiac remodeling.

The 2,4D herbicide, when inhaled, is absorbed and distributed by the lungs, kidney and circulatory system. Enzymes promote the metabolism of the herbicide in these organs and tissues, which results in the formation of free radicals, which can be often more toxic than the herbicide itself.²⁴ Oxidative stress is caused by the release of free radicals, and it is an important mechanism that causes cell mutations.²⁴⁻²⁶ There is strong evidence that oxidative stress plays a prominent pathophysiological role in cardiac remodeling.²⁷⁻²⁸ The remodeling process is associated with changes in different mechanisms related to cardiac dysfunction, such as alterations in LV geometry, wall thickness, cavity diameter and normal configuration.²⁴⁻²⁶

The animals of this study that inhaled 2,4D herbicide at a high concentration showed left ventricular hypertrophy, which suggests the development of cardiotoxicity and confirms the action of the 2,4D in the development of cardiac remodeling. Other studies using the same experimental model in rats indicate that the ingestion of a dose equivalent to 10% of the lethal dose (LD50) of oral pyrethroid insecticides causes changes in ventricular depolarization and repolarization, suggesting cardiotoxicity.^{27,28}

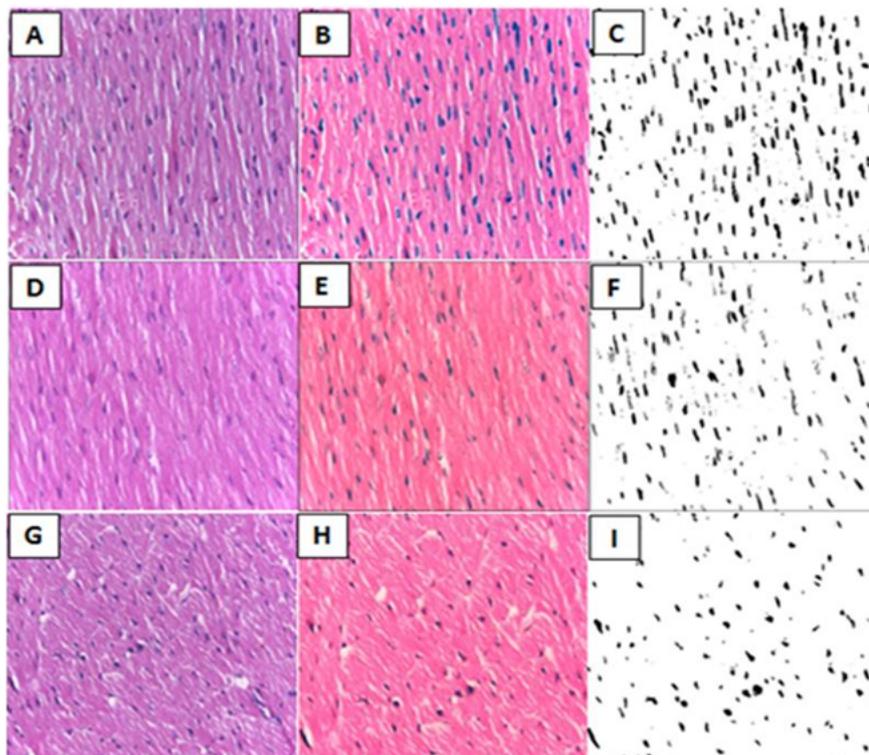


Figure 1 - Letter: A, D, G) Histological section of the left ventricle stained with Hematoxylin and Eosin (HE), 40x increase in the Control Group (CG, n = 5), Low Concentration Group (LCG, n = 5) and High Concentration Group (HCG n = 5). B, E, H) Binarization process in the Control Group (CG, n = 5), Low Concentration Group (LCG, n = 5) and High Concentration Group (HCG n = 5). C, F, I) HE image after the binarization process in the Control Group (CG, n = 5), Low Concentration Group (LCG, n = 5) and High Concentration Group (HCG n = 5). The cell nuclei can be observed in black and the rest of the cell (cytoplasm, plasma membrane and other elements), in white.

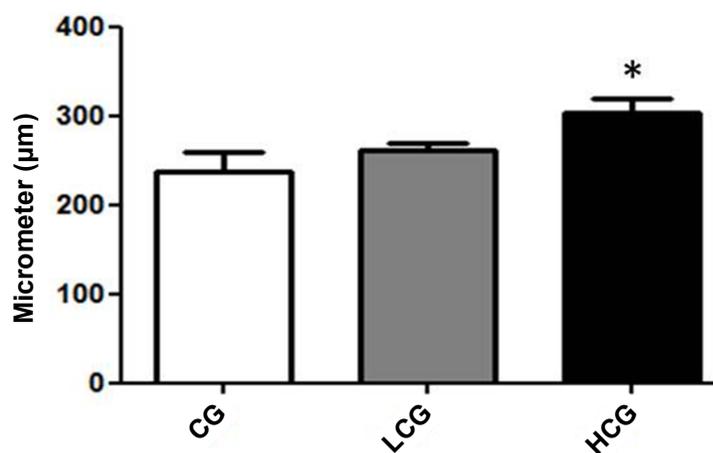


Figure 2 - Analysis of cardiomyocyte hypertrophy.

CG: control group, LCG: low concentration group, HCG: high concentration group. (* $p < 0.05$ when comparing HCG versus CG).

In another study, Razavi²⁶ shows that exposure to Diazinon (DZN) through a catheter once a day, during a 4-week period, also resulted in cardiac lesions in rats and cardiac hypertrophy in the DZN group, showing that this exposure chronically induces infiltration of inflammatory cells and necrosis in cardiac tissue, where they play an important role in cardiotoxicity.

The analysis of the fractal dimension did not demonstrate a significant increase in the animals exposed to the 2,4D herbicide in the HCG (n = 5) when compared to the CG animals (n = 5), suggesting that there was no cardiotoxicity when it evaluated by the fractal dimension method. This method reveals the irregularities in the histological slides, and the increase in the inflammatory process and the collagen can cause these alterations, as it occurs in cardiac remodeling.^{29,30} Possibly, these changes did not occur in the short term. Further studies involving the inhalation route and chronic exposure might disclose these alterations.

This study becomes extremely relevant from the clinical point-of-view, as it demonstrates the acute potential for short-term cardiac damage when herbicides are inhaled. Considering that the workload of rural workers exceeds this period, it is important to use personal protective equipment (PPE), and this information must be disclosed to farmers, alerting them to the risks of heart disease.^{30,32}

As a study limitation, we can cite sample size, with only 5 animals per group.

The results of this study, which is a translational research, show the possible heart damage that can be caused by pesticide exposure in the body of rural workers during their work routine. The pesticides can result in damage to the cardiac function and thus cause signs and symptoms of intolerance to exertion, such as fatigue and dyspnea. Control strategies to prevent the inhalation of these substances by rural workers are necessary to prevent progression to pathological cardiac remodeling.⁸ Moreover, further analyses may also be used to characterize other pathological cardiac

alterations, such as molecular analyses of the heart and evaluations of systolic and diastolic functions by echocardiography. The mechanisms involved in cardiac hypertrophy should also be elucidated.³³

Conclusion

The 2,4D herbicide at acutely inhaled concentrations promoted cardiomyocyte hypertrophy in mice, showing its potential in causing pathological cardiac remodeling.

Author contributions

Conception and design of the research: Pacagnelli FL, Mariano TB, Sabela AKDA, Silva RCR, Nai GA. Acquisition of data: Negrão ALR, Oliveira B, Gonçalves MG, Oliveira TFS. Analysis and interpretation of the data: Negrão ALR, Oliveira B, Gonçalves MG. Statistical analysis: Mantovani RO. Obtaining financing: Pacagnelli FL. Writing of the manuscript: Pacagnelli FL, Negrão ALR, Oliveira B, Gonçalves MG. Critical revision of the manuscript for intellectual content: Silva RCR, Nai GA. Supervision / as the major investigator: Pacagnelli FL.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Sources of Funding

There were no external funding sources for this study.

Study Association

This study is not associated with any thesis or dissertation work.

Ethics approval and consent to participate

This study was approved by the Ethics Committee on Animal Experiments of the University of Western São Paulo (UNOESTE) under the protocol number 3331.

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