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Research Article

Atherogenesis associated with chronic exposure to glyphosate-based and dichlorophenoxyacetic acid herbicides in rats

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Abstract

Glyphosate-based herbicides (GBH) and dichlorophenoxyacetic acid (2,4-D) are the most commonly used pesticides worldwide. Both have the ability to increase oxidative stress, which can lead to atherogenesis. The aim of this study was to establish and compare the atherogenic potential and histological damage to the arterial wall in chronic exposure to GBH and 2,4-D. A total of 140 adult male Wistar rats were allocated into 14 groups: 2 control groups (exposed to distilled water), 6 groups exposed to GBH and 6 exposed to 2,4-D (n=10/group). The animals were exposed to three concentrations of each herbicide orally (contaminated feed) and by inhalation for 6 months. The aortas were collected for histopathological examination. Fatty streaks were observed only in animals exposed to herbicides ($p < 0.0001$), with no difference in the exposure route and concentration. Animals exposed to GBH had twice as many cases with fatty streaks as those exposed to 2,4-D ($p < 0.05$). Animals exposed to 2,4-D showed the largest fractal dimension of the wall nuclei, and those exposed to GBH showed the smallest fractal dimensions. GBH exhibited a greater atherogenic potential than 2,4-D. Structural disorganization of the arterial wall was observed in both herbicides using fractal dimension analysis.

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1. Introduction

There is much evidence of the relationship between pesticide exposure and increased rates of chronic diseases such as cancer, diabetes, neurodegenerative disorders, congenital and reproductive disorders, chronic obstructive pulmonary disease, cardiovascular diseases, chronic kidney disease, autoimmune diseases, chronic fatigue syndrome and aging [1].

Chronic diseases usually progress slowly and are long-lasting. They are considered the leading cause of mortality worldwide accounting for more than 60% of all deaths. Most premature deaths (in patients under 60 years of age) associated with chronic diseases occur in low- and middle-income countries. [1].

Studies have shown an association between exposure to pesticides and cardiovascular diseases, such as acute myocardial infarction, increased blood pressure, and arrhythmia. [2, 3] Heavy metals, arsenic, trimethylarsine and dimethylarsinic acid are most associated with the development of atherosclerosis [2]. Atherosclerosis is a chronic systemic inflammatory disease that develops gradually in blood vessels and can culminate in important cardiovascular manifestations, such as myocardial infarction, stroke and lower limb ischemia [4].

The association between pesticide exposure and atherosclerosis remains unclear, although pesticide exposure has been associated with cardiovascular disease and various forms of atherosclerosis [5]. This occurs for several reasons, such as the fact that atherosclerosis is multifactorial and can be caused by underlying diseases (diabetes and arterial hypertension) and addictive habits (smoking); occupational and nonoccupational exposure to more than one pesticide, which does not allow the assessment of which agent is the cause of atherosclerosis; and low specificity of metabolites and technological limitations for the evaluation of pesticide biomarkers [5]. Some studies have evaluated the association between atherosclerosis and pesticides through ultrasound measurements of carotid intima-media thickness and the assessment of pesticide exposure based on questionnaires in humans, [5], through the atherosclerotic index in animals [6], or *in vitro* functional assessments of the arterial wall [7].

Atherosclerosis is associated with the accumulation and oxidation of low density lipoproteins (LDLs) in the arterial walls. Lipoprotein oxidation stimulates the local inflammatory process [8]. Elevated levels of oxidative stress and changes in lipid homeostasis may explain the atherosclerosis process associated with exposure to pesticides. There is experimental evidence that organochlorines and organophosphates are associated with atherosclerosis because they cause increased levels of reactive oxygen species, depletion of the antioxidant defense system and increased lipid peroxidation [9]. Studies have shown that both glyphosate-based herbicides (GBHs) [10] and 2,4-dichlorophenoxyacetic acid (2,4-D) [6] cause oxidative stress, which can contribute to aging diseases, atherosclerosis, cancer, and premature aging [10]. Increased induction of oxidative stress can trigger several pathologies, including chronic inflammatory diseases [11] and can contribute to the formation of atheromatous plaques [12].

Glyphosate [N-(phosphonomethyl)glycine] was discovered in 1950, and from 1974, it began to be marketed as an herbicide. Since then, it has been widely used to combat weeds in crops, mainly grains, and in urban gardens [13]. Cytotoxic and genotoxic effects, increased oxidative stress, changes in brain function and correlations with some types of cancer are associated with GBH and glyphosate [14]. Conduction blocks and cardiac arrhythmias have been associated with glyphosate exposure in *in-vitro* [15], in animal [16] and in human [17] studies. Likewise, arterial changes associated with glyphosate exposure were observed in *in vitro* studies [7].

2,4-D is a synthetic auxin and was the first chemical for selective control of broadleaf plants, sparing grasses and narrowleaf crops such as wheat, corn, rice and other cereal crops [18]. 2,4-D was first commercialized in 1944 and is one of the most widely used herbicides in the world due to its general applicability and low cost [19]. "Agent Orange", a 50:50 mixture of 2,4-D and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), was widely used during the Vietnam War in the 1960s as a defoliant [20]. Several diseases have been associated with "Agent Orange", such as dyslipidemia, hypertension, ischemic heart disease, diabetes, multiple sclerosis, Parkinson's disease, peripheral neuropathy, chloracne and porphyria cutanea tarda [20, 21]. Cardiotoxicity was reported in rats subjected to chronic exposure to 2,4-D [22].

Agriculture with a lower environmental impact is important for maintaining the ecosystem and, therefore, human health. As stated, previous studies have evaluated arterial function and radiological changes upon exposure to pesticides, but few have analyzed the arterial wall histologically to assess microscopic damage. Assessment of the histology of the arterial wall may contribute to a better understanding of the arterial damage associated with exposure to pesticides. Although GBH and 2,4-D are the most commonly used herbicides in crops

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worldwide, to our knowledge, our group is the first to perform a histological analysis of the arterial wall to determine the possible histological changes that may occur upon exposure to these herbicides. In addition, we used concentrations and exposure routes similar to those involved in human exposure. The aim of this study was to evaluate and compare histologically the atherogenic potential and the arterial wall damage caused by chronic oral and inhalation exposure at different concentrations of GBH and 2,4-D in rats.

2. Materials and methods

The study was designed and monitored according to the ARRIVE guidelines [23].

2.1. Herbicides used

Exposure to GBH was performed with glyphosate [N-(phosphonomethyl) glycine] (Roundup Original DI, Monsanto, São Paulo, Brazil) with the following composition: diammonium salt of N-(phosphonomethyl)glycine (GLYPHOSATE): 445 g/L (370 g/L acid equivalent), N-(phosphonomethyl) glycine (GLYPHOSATE) acid equivalent: 370 g/L (37.0% m/v) and other ingredients: 751 g/l (75.1% m/v).

The 2,4-D herbicide (Nortox® S.A., Arapongas, Paraná, Brazil) had the following composition: dimethylamine salt of 2,4-D: 806 g/liter (80.6% m/v), acid equivalent of 2,4-D: 670 g/liter (67.0% m/v) and inert ingredients: 424 g/liter (42.4% m/v).

2.2. Animal protocol

To determine the minimum sample size for comparing scores for 14 groups, the "pwr" package was used, available in the R program to calculate sample sizes for conducting the analysis of variance. After obtaining the minimum number of elements per sample, 15% more elements per sample were added to adapt them to a possible non-parametric distribution. The following parameters were used: test power = 80%; significance level = 5%; number of groups to be compared = 14 and effect size (Cohen's d) = 0.50. From these data it was concluded that at least 8 elements per group are necessary. After adding 15% more elements, we chose to use 10 animals per group.

For the experiments, 140 adult male Albino Wistar rats, weighing between 200-250 g were supplied by the Central Animal Facility of the Universidade do Oeste Paulista (UNOESTE). The animals were housed in collective large plastic cages in an experimental vivarium at an average temperature of 22 ± 2 °C with a dark/light cycle (12 h).

The animals were randomly allocated into 14 groups (n=10/group), two control groups, six groups exposed to GBH and six groups exposed to 2,4-D (Table 1).

2.3. Exposure protocol

The inhalation control (CI) and oral control (CO) groups were exposed to nebulization with distilled water and nebulized feed with distilled water, respectively. The experimental groups were exposed to three concentrations of each herbicide as described in the manufacturer's package insert, by oral administration (nebulized feed) and inhalation: 3.71 x 10^{-3} grams of active ingredient per hectare (g.a.i./ha) [corresponding to 20.69 parts per million (ppm) of 2,4-D and 27.05 ppm of GBH], considered low concentration; 6.19 x 10^{-3} g.a.i./ha (corresponding to 34.63 ppm of 2,4-D and 45.27 ppm of GBH), considered medium concentration; and 9.28 x 10^{-3} g.a.i./ha (corresponding to 51.66 ppm of 2,4-D and 67.54 ppm of GBH), considered high concentration. Each type of crop requires a different concentration of herbicides, therefore, we chose the lowest, medium and highest concentrations of each herbicide described in the package inserts. Furthermore, we adapted the amount of product used according to the area of the box used for nebulization.

For nebulization, three boxes (32x 24x 32 cm) were used, one for the control group, one for group exposed to GBH and one for group exposed to 2,4-D. Each box was connected to an ultrasonic nebulizer (Pulmosonic Star®, Soniclear Ind. Com. Imp. and Exp. Ltda., São Paulo, Brazil). The animals and feed were exposed until the entire solution was nebulized (approximately 15 min) [24].

The animals exposed by inhalation were nebulized daily for five consecutive days during the week (simulation of occupational exposure). The feed of animals exposed orally was nebulized one day before being offered and was changed every two days (simulation of alimentary route exposure) [25]. The amount of feed consumed per cage was calculated by subtracting the amount of residual feed found at each change from the amount of feed offered to the animals. This value was divided by the number of animals in

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Table 1. Study groups exposed to GBH and 2,4-D herbicide.

Exposure Intensity	GBH		2,4-D	
	Inhalation exposure	Oral exposure	Inhalation exposure	Oral exposure
Low concentration [3.71 x 10^{-3} grams of active ingredient per hectare (g.a.i./ha)]	GLI	GLO	DLI	DLO
Medium concentration (6.19 x 10^{-3} g.a.i./ha)	GMI	GMO	DMI	DMO
High concentration (9.28 x 10^{-3} g.a.i./ha)	GHI	GHO	DHI	DHO

the cage to obtain the amount of feed intake per animal.The animals were weighed monthly until the end of the experiment. Animals from all groups were exposed for 6 months. Euthanasia was performed at the end of the experiment with sodium thiopental (Syntec, USA) at a dose of 100 mg/kg of body weight intraperitoneally [26].

2.4. Histopathological analysis

After euthanasia, the aorta was removed from each animal and fixed in 10% buffered formalin (Cinetica Indústria Química, São Paulo, Brazil) for 24 h. Three aortic fragments after cross-sectioning (proximal, middle and distal thirds) were fixed in formalin and underwent usual histological processing with paraffin embedding (Dinâmica Reagentes Analíticos, São Paulo, Brazil). Serial sections (5 μm thick) were obtained using a LEICA RM2265 microtome (Leica Biosystems Nussoch GmbH, Germany) and stained by the hematoxylin-eosin (HE) method (Dolles, São Paulo, Brazil).

The histopathological analysis of the slides was blinded for the treatments using a common optical microscope (NIKON Labophot, Japan). The presence of atherosclerosis was evaluated according to the following scoring scheme: 0= absent, 1= fatty streaks, 2= mild atherosclerosis, 3= moderate atherosclerosis, and 4= severe atherosclerosis [27]. The following parameters were also evaluated: presence and intensity of the inflammatory process (mild, moderate or severe) and type of inflammatory cell present; and presence of apoptotic cells counted in 10 high power fields (HPF), which corresponded to 1 mm^2 of area.

2.4.1. Aortic thickness measurement

One HE-stained histological image of the aortic wall per animal was captured at 200x magnification using a LEICA DM750 optical microscope (Leica Microsystems, Germany), and measurements were taken in two areas of the wall using ImageJ software [National Institutes of Health (NIH) of the United States, available at http://rsbweb.nih.gov/ij/].

2.4.2. Fractal analysis of the aortic wall nuclei

Nuclei were analyzed by fractal dimension using ImageJ software (NIH, USA). One HE-stained histological image of the aortic wall per animal was captured at 400x magnification using a LEICA DM750 optical microscope (Leica Microsystems, Germany). The original HE-stained images were treated with contrast adjustment to highlight the nuclei and were later binarized, becoming black and white, a necessary procedure since fractal analysis measures the black area of the image. Fractal dimension analysis was performed using the box-counting method, as illustrated in Fig. 1. The program considers two dimensions, allowing the quantification of the pixel distribution in this space, without considering the image texture. The calculated fractal dimension will always be between 0 and 2, which does not distinguish different textures [28].

2.5. Statistical analysis

For comparisons between groups, analysis of variance was used when the assumptions of normality (verified by the Komogorov-Smirnov test) and homogeneity of variances (verified by the Levene test) were observed, followed by the minimum significant difference test. When the variable did not agree with the assumptions of the analysis of variance, the Kruskal–Wallis test was used, followed by multiple comparisons with the Tukey–Kramer-Nemenyi test. Pearson's correlation between the thickness and fractal dimension of the nuclei of the aortic wall was also used. For qualitative variables, the likelihood ratio test was used. Data were processed with SPSS v. 23. All statistical tests were performed at a significance level of $p < 0.05$.

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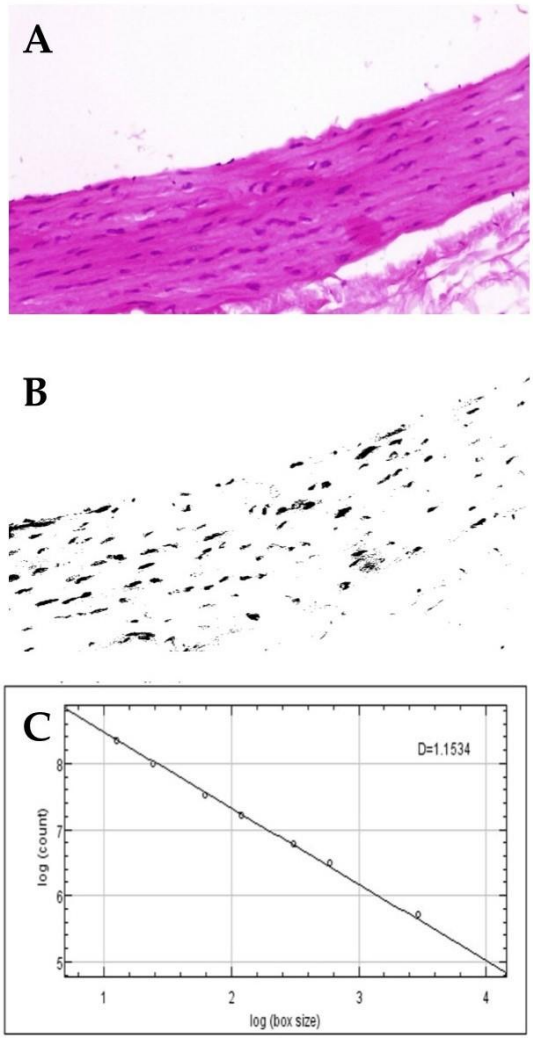

Figure 1. A – Photomicroscopy of the aorta – animal from the GBH high inhalation concentration group (hematoxylin-eosin, 400x magnification). B – Binarized image. C – Box-counting of the fractal dimension analysis.

The effect size was also evaluated by Cohen's d, where values <0.19 are considered insignificant, values between 0.20-0.49 are considered small, values between 0.50-0.79 are considered medium, values between 0.80-1.29 are considered large and values > 1.30 are considered very large [29].

3. Results

3.1. Mortality

One animal in the group exposed to a high oral concentration of 2,4-D died during the study due to ear canal infection.

3.2. Animal weight and feed consumption

There were no differences in feed intake or weight gain between the groups ($p > 0.05$).

3.3. Histopathological analysis of the aorta

Fatty streaks were observed in the aortas only in animals exposed to herbicides ($p < 0.001$) (Fig. 2).

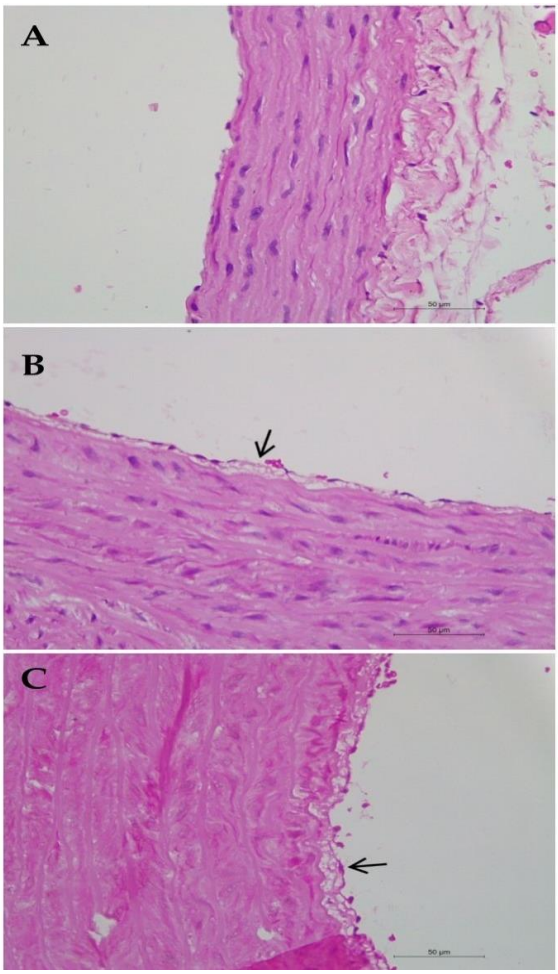

Figure 2. Photomicroscopy of the aorta. A – Normal aorta (animal from the inhalation control group). Note the endothelium attached to the internal elastic lamina. B – Aorta with fatty streaks (arrows) (animal from the GBH low inhalation concentration group). C - Aorta with fatty streaks (arrows) (animal from the 2,4-D low inhalation concentration group). In B and C, note foam cells elevating the endothelium (arrows). Hematoxylin-eosin, 400x magnification. Scale bar: 50 μm.

There was no difference between the different concentrations of GBH, in both the oral and inhaled routes ($p = 0.062$), or for 2,4-D ($p = 0.688$) (Table 2). Animals exposed to GBH had twice as many cases with fatty streaks as those exposed to 2,4-D ($p < 0.05$). No inflammatory processes or apoptotic cells were observed in any of the evaluated animals.

3.4. Aortic thickness measurement

There was no significant difference in the

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Table 2. Incidence of fatty streaks by study group (n=139).

Inhalation groups	Fatty streaks	Oral groups	Fatty streaks
CI	0/10 (0%)^{Aa}	CO	0/10 (0%)^{Aa}
GLI	3/10 (30%)^{Ab}	GLO	2/10 (20%)^{Ab,c}
GMI	1/10 (10%)^{Ab,c}	GMO	3/10 (30%)^{Ab}
GHI	3/10 (30%)^{Ab}	GHO	4/10 (40%)^{Ab}
DLI	1/10 (10%)^{Ac}	DLO	2/10 (20%)^{Ac}
DMI	1/10 (10%)^{Ac}	DMO	1/10 (10%)^{Ac}
DHI	1/10 (10%)^{Ac}	DHO	2/9 (22.2%)^{Ac}

Capital letters compare groups on the same line. Lowercase letters compare groups at the same time and in the same column. Different letters indicate $p < 0.05$ (Anova), where A ≠ B; a ≠ b, c; b ≠ c. Groups: GLI - glyphosate-based herbicide (GBH) low inhalation concentration; GLO - GBH low oral concentration; GMI - GBH medium inhalation concentration; GMO - GBH medium oral concentration; GHI - GBH high inhalation concentration; GHO - GBH high oral concentration. DLI - 2,4-D low inhalation concentration; DLO - 2,4-D low oral concentration; DMI - 2,4-D medium inhalation concentration; DMO - 2,4-D medium oral concentration; DHI - 2,4-D high inhalation concentration; DHO - 2,4-D high oral concentration.

measurement of aortic thickness in relation to the control groups and exposure to GBH (p=0.560) or between the control groups and exposure to 2,4-D ($p > 0.05$) (Fig. 3).

The mean thickness of the aortic wall in the unexposed group was 304,182 (± 54,064.8) pixels, and in the group exposed to herbicides, it was 319,629 (± 96,552.3) pixels. With a Cohen's d of 0.197412, exposed animals showed an insignificant difference from those not exposed to herbicides for aortic thickness.

When we evaluated only those exposed to herbicides, the mean aortic thickness in the groups exposed to GBH was 303,635 (± 78,681) pixels, and in the groups exposed to 2,4-D, it was 335,895 (± 110,158.8) pixels ($p > 0.05$). With a Cohen's d of 0.337015, animals exposed to GBH showed a small difference from those exposed to 2,4-D.

3.5. Fractal analysis of the aortic wall nuclei

Animals exposed to 2,4-D showed a higher fractal dimension than animals exposed to GBH and animals in the control group ($p < 0.001$). The animals exposed to GBH did not differ from those in the control group ($p = 0.058$).

In the exposure to GBH, significant differences were observed between the low inhalation and low oral concentration groups, between low oral concentration and high oral concentration groups, between high inhalation concentration and high oral concentration

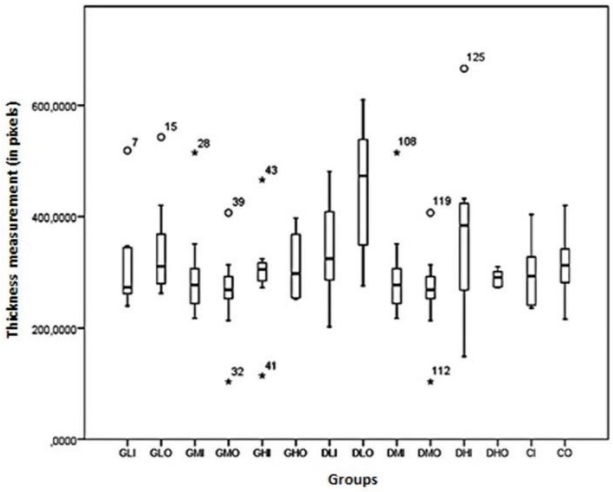

Figure 3. Medians and interquartile range of aortic wall thickness measurement (in pixel) by study group (n=139). ($p = 0.368$ (Kruskal–Wallis test). *,°: outlier and animal number. Groups: GLI - glyphosate-based herbicide (GBH) low inhalation concentration; GLO - GBH low oral concentration; GMI - GBH medium inhalation concentration; GMO - GBH medium oral concentration; GHI - GBH high inhalation concentration; GHO - GBH high oral concentration. DLI - 2,4-D low inhalation concentration; DLO - 2,4-D low oral concentration; DMI - 2,4-D medium inhalation concentration; DMO - 2,4-D medium oral concentration; DHI - 2,4-D high inhalation concentration; DHO - 2,4-D high oral concentration).

groups and between high oral concentration and oral control groups ($p < 0.001$). The group exposed to a high oral concentration of GBH had the smallest nuclear fractal dimension. In the exposure to 2,4-D, a significant difference was observed between the low

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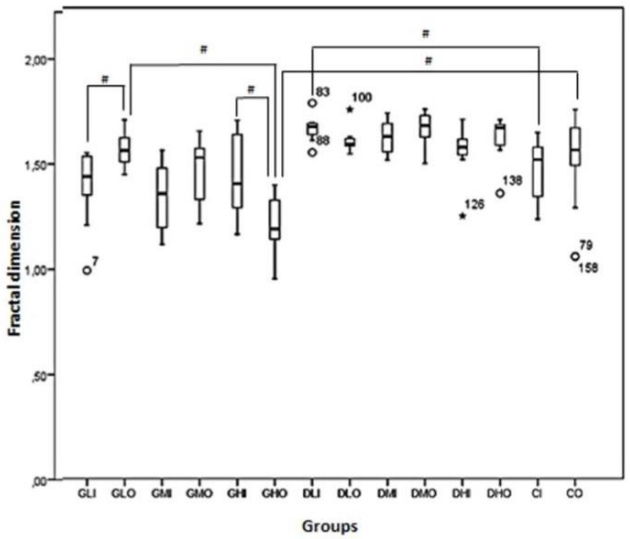

Figure 4. Medians and interquartile ranges of fractal dimensions of aortic wall nuclei by study group (n=139).

(#: $p < 0.05$ (Kruskal–Wallis test). *,°: outlier and animal number. Groups: GLI - glyphosate-based herbicide (GBH) low inhalation concentration; GLO - GBH low oral concentration; GMI - GBH medium inhalation concentration; GMO - GBH medium oral concentration; GHI - GBH high inhalation concentration; GHO - GBH high oral concentration. DLI - 2,4-D low inhalation concentration; DLO - 2,4-D low oral concentration; DMI - 2,4-D medium inhalation concentration; DMO - 2,4-D medium oral concentration; DHI - 2,4-D high inhalation concentration; DHO - 2,4-D high oral concentration).

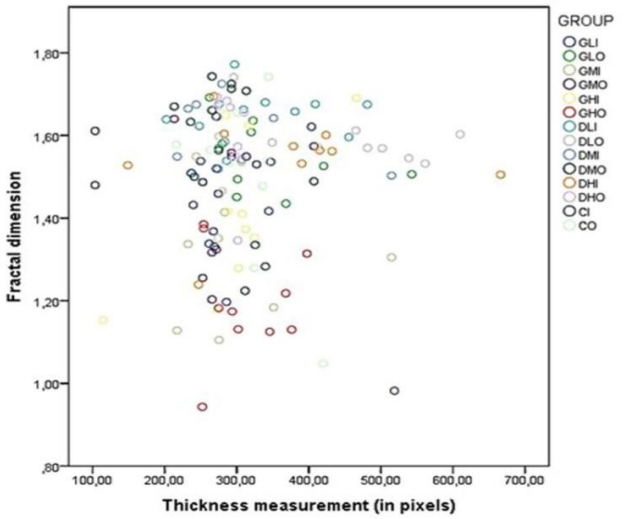

Figure 5. Correlation between thickness (in pixels) and fractal dimension of aortic wall nuclei by study group ($r = 0.021$; $p = 0.809$).

(Groups: GLI - glyphosate-based herbicide (GBH) low inhalation concentration; GLO - GBH low oral concentration; GMI - GBH medium inhalation concentration; GMO - GBH medium oral concentration; GHI - GBH high inhalation concentration; GHO - GBH high oral concentration. DLI - 2,4-D low inhalation concentration; DLO - 2,4-D low oral concentration; DMI - 2,4-D medium inhalation concentration; DMO - 2,4-D medium oral concentration; DHI - 2,4-D high inhalation concentration; DHO - 2,4-D high oral concentration).

inhalation concentration and inhalation control groups ($p < 0.05$) (Fig. 4).

When we evaluated only those exposed to herbicides, the mean fractal dimension of nuclei in the groups exposed to GBH was 1.387 (± 0.182), while in the groups exposed to 2,4-D, it was 1.608 (± 0.093) ($p < 0.001$). With a Cohen's d of 1.529183, animals exposed to GBH showed a very large difference from those exposed to 2,4-D.

3.6. Correlation between aortic wall thickness and fractal dimensions of the nuclei

There was no correlation between the measurement of aortic wall thickness and the fractal dimensions of the nuclei ($r = 0.021$; $p = 0.809$) (Fig. 5).

4. Discussion

There is evidence that persistent organic pollutants (i.e., those that accumulate in the environment, animals and humans) of most different chemical classes are associated with lipid alterations, carotid atherosclerosis and cardiovascular diseases, such as myocardial infarction and stroke [30].

At the concentrations used in our study over six months, both herbicides caused the formation of fatty streaks, and animals exposed to GBH had a 50% higher incidence than those exposed to 2,4-D. We did not observe any difference in the measurement of the aortic thickness of animals exposed to herbicides in relation to those not exposed. While the animals exposed to 2,4-D had a higher fractal dimension of the arterial wall nuclei, those exposed to GBH had a lower fractal dimension.

Our study had four differences from previous studies: 1. We used different concentrations that are normally used in crops according to the manufacturer's package insert; 2. The exposure routes used were those to which humans are usually exposed; 3. We used commercial formulations based on glyphosate and 2,4-D and not only the active product; and 4. We performed histological analysis of the aortas of the animals.

2,4-D is a selective herbicide used to control weeds in

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crops such as wheat, corn, soybeans, rice, oats, sorghum, coffee and *Brachiaria* pastures [18]. GBH can be used to eradicate narrow- and broad-leaf weeds and is used in cotton, irrigated rice, coffee, sugarcane, citrus, corn and soybean crops, as well as in domestic and urban gardens [13]. For each type of weed in a given crop, there is an ideal spray concentration indicated in the manufacturer's package. In this study, we chose the lowest, medium and highest concentrations recommended in the product package insert for spraying crops. To simulate a scenario of environmental exposure of humans as close to reality as possible, we nebulized the animals for a time sufficient for the entire solution to be nebulized for five consecutive days a week to simulate the workday recommended by labor law in our country.

There is continuing concern about pesticide residues in food and water, even in small concentrations; with long-term cumulative exposure, they can cause damage to health. More frequent consumption of organic (pesticide-free) products has been shown to be associated with lower levels of pesticide metabolites in human urine [31]. Thus, in this study, we evaluated the possible arterial damage, not only from inhalation exposure (which is more associated with occupational exposure) but also from oral exposure through feed nebulized with the evaluated herbicides (more associated with the ingestion of food and water with pesticide residues). Oral exposure was not performed by gavage, because this is not what happens in human oral exposure. Also, the feed was offered one day later the nebulization, because food is not ingested immediately after being sprayed.

The active ingredients of herbicides are never used alone but are always formulated with other compounds and adjuvants, such as solvents and surfactants. Adjuvants increase the solubility, stability and adhesion of active ingredients. The expansion and penetration of active ingredients in the target species are indispensable [32]. Adjuvants are normally an industrial secret and are not described in the package insert. Additionally, each manufacturer has a specific formulation for herbicides [33]. Thus, the toxicity of an herbicide cannot be attributed only to the active ingredient but also to the adjuvants [7]. Therefore, we evaluated formulations based on glyphosate and 2,4-D and not just the active ingredient.

The study by Yi et al. [21] on Korean Vietnam veterans showed that dyslipidemia and circulatory diseases are associated with high exposure to Agent Orange. Several studies have associated 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which is formed in the 2,4,5-T production (another component of the Agent Orange), with the accumulation of lipids and changes in lipid transfer and metabolism [20, 21] and cardiovascular damage [20]. In a study by Nakbi et al., [6] an increase in the atherosclerotic index (based on dosage of serum lipids) was observed in rats treated with 2,4-D by gavage (5 mg/kg body weight). Our findings of fatty streaks in animals exposed to 2,4-D show that not only the other components of Agent Orange, but also this herbicide, is associated with atherosclerotic risk, even when used at doses recommended by the manufacturer.

In a previous study by our group, we exposed Wistar rats for 75 days (subchronic exposure) to concentrations of GBH similar to those in this study, and observed fatty streaks without association with the route of exposure or GBH concentration [34]. We corroborated our previous hypothesis that GBH probably has a dose/concentration where it triggers its maximum atherogenic effect and higher doses do not potentiate the appearance of more lesions or their worsening. The same hypothesis can be raised for the 2,4-D herbicide, as we also did not observe a change in the incidence of fatty streaks depending on the concentration and exposure route.

Fatty streaks formed by the accumulation of foam cells (macrophages that phagocytized LDL cholesterol) in the intimal layer, are the first histological changes that occur in atherogenesis. Foam cells recruit lymphocytes into the intimal layer only after a long period of aggression to the endothelium [4]. This data justifies the fact that we did not observe inflammation in the animals evaluated. Also, the process of complete atherogenesis with the formation of atheroma plaques can take decades to occur in humans [4]. Therefore, a longer time may be necessary for atheromatous plaque formation to occur because even after six months, we did not observe well-formed plaques upon exposure to the two herbicides.

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As a way to evaluate structural changes, the use of fractal analysis has been proposed. The term fractal was proposed by Mandelbrot, a French mathematician, in 1975. The word comes from the adjective "fractus", from the Latin verb "frangere" (to break) [35]. A fractal is a shape made up of parts that are similar to the whole, and "fractal dimension" is a term that describes the space-filling properties of irregular objects, that is, the degree of occupation of the object in the space that contains it [35, 36].

There are several methods exist for calculating the fractal dimension of a given digital image. We chose the box-counting method for analysis because it is one of the most commonly used methods and quantifies the visual complexity of an object. This method has been widely studied and applied in science and mathematics [36]. In medicine, it has been widely used for the analysis of several pathologies, such as the evaluation of trabecular bone, [37] atypical nuclei in tumors, [35] neurosciences, [38] microbiology [39] and myocardial analysis, [40].

Although, we did not observe changes in arterial wall thickness in both herbicides exposures, there were differences in the nuclear fractal dimension in the groups exposed to 2,4-D and GBH compared to the control. Changes in the fractal dimension of the nucleus have been described during physiological development, growth and aging, and may be associated with epigenomic changes and a topographic redistribution of the nuclear architecture [41]. An increase in the fractal dimension of the nucleus has been observed in the carcinogenesis and tumor progression of several epithelial and lymphoid neoplasms. This is believed to be due to an increase in the complexity of the chromatin structure, loss of heterochromatin and less perfect self-organization of the nucleus in aggressive neoplasms. [41, 42]. The change in the fractal dimension observed in our study represents an architectural change in the nucleus that can result in structural/organizational alterations of the arterial wall which can lead to functional alterations.

Also, while animals exposed to 2,4-D had larger fractal dimensions of the arterial wall nuclei, the animals exposed to GBH had smaller fractal dimensions, and those exposed to high oral concentrations of GBH had the smallest ones among all groups. The opposition in the fractal dimensions between the two herbicides shows that each acts differently in the nucleus. In a study with ultraviolet irradiation of cell cultures, there was a significant decrease in the fractal dimension during early apoptosis, even before DNA fragmentation and increased cell membrane permeability could be visualized to define the diagnosis of apoptosis [43]. This decrease in the nuclear fractal dimension of animals exposed to GBH may represent an early phase of cellular apoptosis, which cannot yet be visualized microscopically. In contrast, 2,4-D appeared to affect the chromatin structure, altering the concentrations of heterochromatin and euchromatin, thus leading to an increase in the nuclear fractal dimension. This may also explain why we did not observe apoptotic cells.

Reactive oxygen species, derived from oxidative stress, function as signaling molecules for several signaling pathways involved in cardiovascular diseases associated with pesticide exposure [4]. Prolonged exposure to high concentrations of reactive oxygen species can damage various molecules, including nucleic acids, and exposure to low or intermediate concentrations effects cell signaling [44]. Exposure to high concentrations of reactive oxygen species can also lead to cell death via apoptosis and necrosis [45]. Therefore, these alterations in the fractal dimension of the nuclei may be associated with damage caused by increased production of reactive oxygen species in the arterial wall due to exposure to these herbicides.

A study performed with the aorta of Sprague-Dawley rats showed lower relaxation of the aorta after treatment with glyphosate, illustrating the direct effect of this herbicide on the arterial wall [7]. Another study that evaluated the offspring of pregnant Wistar rats exposed to 0.2% of a commercial formulation of glyphosate in drinking water at 3, 6 and 12 months of age showed that the animals did not present changes in blood pressure or in the histological analysis of the aorta, however, there was an alteration in arterial relaxation in exposed animals [46]. The decrease in the nuclear fractal dimension the animals exposed to GBH in our study may explain this change in

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relaxation observed in the aforementioned studies.

The toxicity of glyphosate is associated with the salts used in its formulation. Studies have shown that glyphosate isopropylamine (IPA) salt is more harmful to the cardiovascular system than the herbicide based on glyphosate ammonium salt [47]. In our study, we used an herbicide based on ammonium glyphosate salt and observed arterial damage. Identifying toxic symptoms associated with specific pesticide formulations is important for improving treatment and increasing the chances of a better prognosis for poisoned patients [47].

More studies that jointly evaluate the biochemical, histological and physiological changes and oxidative stress in the aorta of animals of different age groups are necessary to better establish all the possible damage caused by these herbicides to the arterial wall and its implications.

5. Conclusions

In conclusion, both herbicides have atherogenic potential, regardless of the exposure route (oral or inhalation) and the concentration used. However, the higher incidence of atherosclerosis and smaller nuclear fractal dimensions in animals exposed to GBH show that this herbicide has a greater potential for arterial wall damage than 2,4-D. The high agricultural production rates achieved require the intensive use of pesticides with varied chemical compositions, the most commonly used of which are 2,4-D and GBHs. The major concern is the risk of contamination of humans with residues of these herbicides in water or food or even due to post-spray drift of crops and the possible damage that this contamination causes. Our data show that 2,4-D and GBHs should be used with caution and handled with individual protective equipment to minimize the risk of contamination. Also, the risk of food and drinking water contamination by these herbicides must be constantly evaluated by regulatory agencies to avoid oral contamination of humans and animals and further cardiovascular damage.

Ethical statement

All experimental procedures were carried out in accordance with the national and international animal management guidelines. This study was evaluated and approved by the Institutional Research Advisory Committee and Ethics Committee for Animal Use of Universidade do Oeste Paulista (UNOESTE) (Protocol n° 6724) on March, 11, 2021.

Authors' contributions

Conceptualization, G.A.N., R.C.R.; Methodology, G.A.N., R.C.R.; Formal analysis, F.M.B.V.T., G.H.R., M.E.S.S.; Investigation, F.M.B.V.T., G.H.R., M.E.S.S.; Resources, G.A.N., R.C.R.; Data curation, F.M.B.V.T., G.A.N.; Writing – original draft preparation, F.M.B.V.T.; Writing – review & editing, G.A.N.; Visualization, F.M.B.V.T., G.A.N.; Supervision, G.A.N., R.C.R.; Project administration, G.A.N.; Funding acquisition, G.A.N.

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Availability of data and materials

All data will be made available on request according to the journal policy.

Conflict of interest

The authors declare no conflict of interest.

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