Lasers in Medical Science (2020) 35:1703–1709
https://doi.org/10.1007/s10103-020-02961-z

ORIGINAL ARTICLE

Effects of consumption of contaminated feed with 2,4-dichlorophenoxyacetic acid (2,4-D) on the rat tibia: analysis by Raman spectroscopy and mechanical properties

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Received: 15 October 2019 / Accepted: 10 January 2020 / Published online: 17 January 2020
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Abstract

Studies reported the harmful effects of 2,4-D on body tissues, provoking changes in the anatomy and physiology of the kidneys, liver, and testicles. Thus, the objective was to evaluate if there were alterations in the bone quality of the tibia of rats submitted to feed consumption that were exposed to three different 2,4-D doses. Male *Wistar* rats were divided into four groups: oral control group (C: feed consumption without 2,4-D contamination); low oral concentration group (G3: contaminated feed with low concentration of 2,4-D); medium concentration group (G6: contaminated feed with medium concentration of 2,4-D); and high concentration group (G9: contaminated feed with high concentration of 2,4-D). The results demonstrated alterations of the mechanical properties and Raman ratios of the tibias of the contaminated groups. The maximum load, maximum stress, elastic modulus, and the cortical area were lower in the G6 and G9 compared to C group. The mineral-to-matrix ratio (relative mineral to organic content) was lower in the G6 and G9 groups compared to C group, but carbonate-to-matrix ratio (indicator of bone turnover) was higher in both groups. Thus, it is possible to suggest that the 2,4-D herbicide performed deleterious effects on the bone quality of male *Wistar* rats.

Keywords Herbicide · 2,4-D · Contamination · Tibias · Bone remodeling

Introduction

Dichlorophenoxyacetic acid (2,4-D) is a type of herbicide widely used for weed control and also in cereal crops, forestry control, and especially sugarcane plantations [1]. As this compound is toxic, careful handling and application are required to avoid environmental and worker contamination, since it is rapidly distributed to body tissues and easily absorbed into the gastrointestinal tract [1].

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The neurotoxic [2], hepatotoxic [3, 4], and cytotoxic effects [3, 5, 6] of the 2,4-D have been well-documented. This organic compound has also shown harmful effects on reproductive organs by interfering with their reproductive capacities. Joshi et al. [5] studied male rats exposed to 2,4-D and observed degenerative changes on the seminiferous tubules of the testicles, decreased fertility rates and decreased testicular, epididymis, seminal vesicle, and ventral prostate weights. In addition, they observed increased and decreased levels of serum cholesterol and testosterone, respectively. According to the authors, testosterone reduction clearly demonstrates a sign of disturbance in its biosynthesis and also an inhibitory effect on gonadotropic hormones. Thus, it can be suggested that the 2,4-D compound promoted the onset of acquired hypogonadism, which is a situation in which the gonads do not produce adequate amounts of sex hormones [7].

According to Carsote et al. [8], hypogonadism can cause sexual dysfunction, decrease muscle strength, and compromise bone mineralization. There are many articles in the literature that have related this disease to bone health impairment [7, 9, 10]. The association of the results of these studies with those of Joshi et al. [5] suggest that prolonged 2,4-D contamination may indirectly influence bone metabolism.

Other studies have demonstrated that 2,4-D contamination exerts a teratogenic effect, such as fetal skeletal malformation [11, 12]. In the study by Alpöz et al. [11], pregnant rats were fed a diet containing 2,4-D compound during the gestational period and during the breastfeeding period. After 30 days, the pups were sacrificed and it was found that exposure to 2,4-D compromised tooth development (odontogenesis), affected the function of odontoblasts and ameloblasts, and showed a lack of dentin matrix mineralization. In addition, Mazhar et al. [12] also studied offspring of contaminated female by 2,4-D mixed with corn oil and administered by gavage. A significant increase in the number of brood with morphological and skeletal defects was observed in the herbicide-treated group when compared to the control group. Among the defects, the authors observed reduced ossification of vertebrae, absence of long bone in limbs, and absence of ossification of metatarsals and phalanges bones.

There is evidence, therefore, that 2,4-D may compromise bone metabolism. However, there are no studies in the literature that have analyzed the effects of this compound on the bones of animals or even humans that have been exposed to direct or indirect contact with the herbicide. Thus, the aim of this study was to evaluate the bone quality of the tibias of Wistar rats submitted to the consumption of feed contaminated with the herbicide 2,4-D in three different concentrations.

Materials and methods

Animals

Male Wistar rats (n = 40) were used and provided by the Central Animal Facility of the University of Western São Paulo (UNOESTE), Presidente Prudente, SP, Brazil. The average weight was 150–200 g. The animals were kept in plastic cages (30 x 16 x 19 cm) in an experimental room with controlled temperature (22 ± 2 °C) and luminosity (12-12 h light-dark cycle). This study was approved by the Animal Experimentation Ethics Committee under protocol number 5224.

Division of groups and experimental protocol

The feed was nebulized for 15 min with 2,4-D herbicide by Nortox S.A. (Arapongas, Paraná, Brazil), registered with the Ministry of Agriculture, Cattle and Supplying (MAPA) under number 03009, with the following composition: 806 g/l (80.6% m/v) of 2,4-dichlorophenoxyacetic acid dimethylamine salt; 670 g/l (67.0% m/v) of acid equivalent of 2,4-D; 424 g/l (42.4% m/v) of inert ingredients.

The animals were randomly divided into four experimental groups (10 animals per group):

1) Control Group (C): animals daily consumed feed that was previously nebulized with sodium chloride solution (NaCl)
2) Low Concentration Group (G3): animals daily consumed feed that was previously nebulized with the herbicide 2,4-D at a concentration of 3.71×10^{-3} g of active ingredient per hectare
3) Medium Concentration Group (G6): animals daily consumed feed that was previously nebulized with the herbicide 2,4-D at a concentration of 6.19×10^{-3} g of active ingredient per hectare
4) High Concentration Group (G9): animals daily consumed feed that was previously nebulized with the herbicide 2,4-D at a concentration of 9.28×10^{-3} g of active ingredient per hectare

Exposure protocol to 2,4-D herbicide

The handling of the 2,4-D herbicide was carried out using the following personal protective equipment (PPE): rubber gloves, goggles, and gas filter masks. The experimental protocol used two plastic boxes (32x24x32 cm) each one connected to a Pulmosonic Star ultrasonic nebulizer [13]. The feed was exposed for 15 min to 2,4-D nebulization since this is the time required for all solutions to be nebulized. The feed was changed every 2 days and nebulization was performed 1 day before being placed for the animals. The experimental procedure was performed for 6 months and, after this period, the animals were euthanized and their tibias removed. Euthanasia was performed with sodium thiopental at a dose of 100 mg/kg of weight and administered to the peritoneal cavity. Clinical signs of death were the absence of respiratory movements, heartbeat, and loss of reflexes.

Mechanical test and morphometry of the diaphysis

For the mechanical test, tibias (n = 10 per group) were submitted to three-point bending testing until complete fracture at a velocity of 3 mm/min. An Instron (EMIC 23-2S) apparatus with a load cell of 100 kgf was used. The distance between the two lower supports was 17 mm. After testing of the specimens in three-point bending, the failure sites of all bone specimens were photographed, together with a measurement standard, by a high-resolution digital camera at a standardized distance according to Huang et al. [14]. The parameters of cortical thickness and cross-sectional cortical bone area of the diaphysis were measured on the images using the NIS-Elements 3.0 software (Advanced Research, USA).

The load and displacement data were obtained directly from the MTS system and recorded with a computer coupled to the testing machine. These data were used for the acquisition and calculation of the structural properties: maximum load, yield load, and extrinsic stiffness. The extrinsic stiffness was calculated as the slope of the most linear portion of the elastic region of the load–displacement curve [14–17]. The material properties were obtained from the structural properties [14, 17]. The following material properties were evaluated: maximum stress, maximum strain, and elastic modulus).

Raman spectroscopy

After euthanasia, the tibia was immersed in saline solution and stored at − 20 °C for subsequent Raman analysis. At the time of analysis, intact bones were stripped of the periosteum from the mid-shaft by lightly scraping with a scalpel and were wetted with phosphate-buffered saline. Thus, Raman spectra were then either acquired from the diaphysis of the tibia and were not processed/treated (baseline or offset correction), but the "spikes or cosmic rays" were removed from each one, if necessary. These samples were analyzed in a dry state.

Raman spectroscopy measurements were obtained with a micro-Raman spectrograph (inVia model from Renishaw), equipped with Leica optical microscope. A laser wavelength of 785 nm [18] with a 1200 lines per mm diffraction grating was used. The exposure time adopted was 10 s, with 5 accumulations and spectral range from 200 to 1800 cm^{-1}. Spectra were collected using a 50X objective lens. The analysis was obtained from the cortical bone of the midpoint of the diaphysis of non-decalcified tibias.

In this analysis, we used relative peak intensities for certain pairs of bands (430, 960, 1070, 1272, 1660) from the Raman spectrum (Table 1). Then the following ratios were used: $\nu_2PO_4^{3-}$/amide III (430/1272) [19], $\nu_1PO_4^{3-}$/amide I (960/1660) [20], and $\nu_1CO_3^{2-}$/amide I (1070/1660) [21]. The 430/1272 ratio is associated with calcium content [19], 960/1660 ratio indicates the amount of mineralization, and 1070/1660 ratio may indicate bone turnover and remodeling activity [20, 21].

Statistical analysis

As normality was found in the sample distribution, parametric tests were used to analyze the mechanical parameters of the four independent groups. Thus, one-way analysis of variance (ANOVA) was used, followed by Tukey's a posteriori test. Results were expressed as mean ± standard deviation. All tests were performed with 5% significance level.

Results

Statistical analysis of structural properties demonstrated that the maximum load was higher in the control group compared to the medium (C vs G6, $p < 0.05$) and high (C vs G9, $p < 0.01$) concentration groups but was similar to the low concentration group (G3). For this property, there was no difference between G6 and G9 groups. The maximum displacement showed no difference when comparing the four groups ($p = 0.9988$). Extrinsic stiffness only differed between the C and G9 groups ($p < 0.01$) (Fig. 1a, b, c).

In the study of material properties, the maximum stress of the C group was significantly different of the G6 and G9 groups (C vs G6, $p < 0.05$; C vs G9, $p < 0.01$), but the maximum strain was similar among all groups ($p = 0.3108$). The elastic modulus was higher compared to the G6 and G9 groups (C vs G6, $p < 0.01$; C vs G9, $p < 0.01$) (Fig. 1d, e, f).

The cross-sectional area (CSA) of the diaphysis of tibia presented higher value compared to the G6 (C vs G6, $p < 0.05$) and high concentration (GCO vs GACO = $p < 0.01$) compared to control group. The CSA of the G3 group was higher than the G9 group ($p < 0.01$) (Fig. 2). However, measurements for thickness showed no difference among all groups ($p = 0.8051$) (Fig. 3).

The 430/1272 ratio was similar for all groups analyzed ($p = 0.1743$), but the 960/1660 ratio (mineral-to-matrix) was lower in groups G6 ($p < 0.05$) and G9 ($p < 0.05$) compared to the control group. However, the 1070/1660 ratio (carbonate-to-matrix) was higher in groups G6 ($p < 0.05$) and G9 ($p < 0.01$) compared to the control group (Table 2). Figure 4 shows a representative Raman spectrum from the diaphysis of tibia of C, G3, G6, and G9 groups.

Table 1 Assigned Raman spectra bands to bone tissue

Raman shift, (cm^{-1})	Assignment of the band
430	Symmetrical stretching of $\nu_2PO_4^{3-}$ (phosphate HA)
960	Symmetrical stretching of ν_1PO4^{3-} (phosphate HA)
1070	$\nu_1CO_2^{3-}$ (overlaps with components of ν_3PO4^{3-})
1272	Amide III (protein α-helix)
1660	Amide I (strongest amide I ν(C=O) component, polarization sensitive)

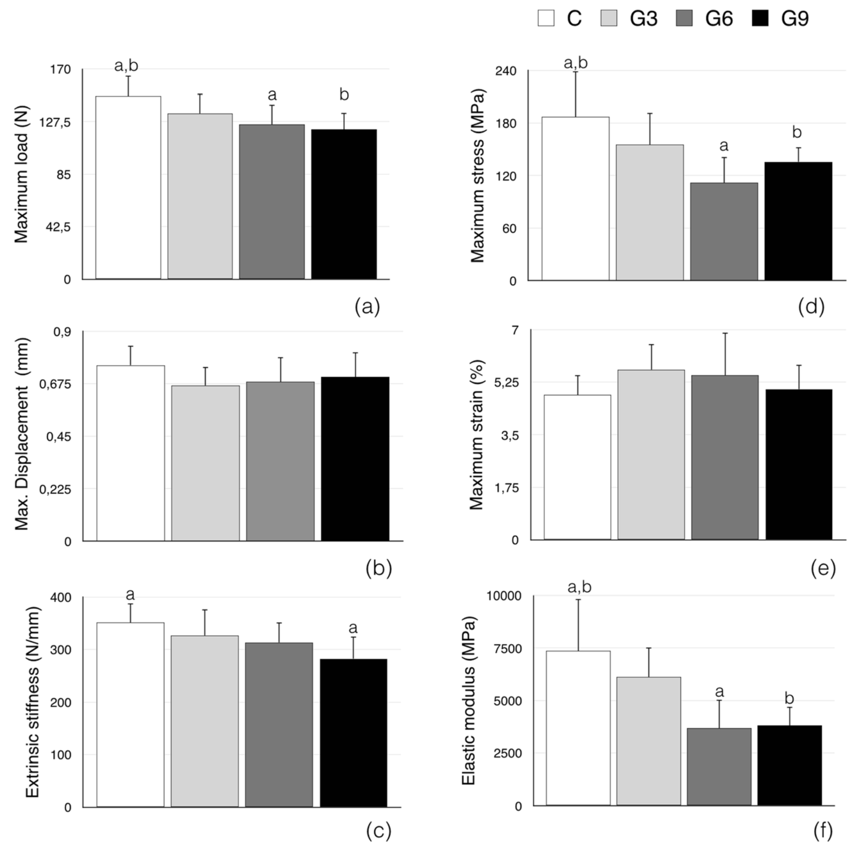

Fig. 1 Structural properties (maximum load, maximum displacement and extrinsic stiffness) and material properties (maximum stress, maximum strain and elastic modulus) of the tibias of C, G3, G6, and G9 groups. Equal letters indicate a statistically significant difference between the two groups

Discussion

There is a wide use of pesticides in Brazil and particularly Western of state of São Paulo presents a high rate of use of 2,4-D and atrazine [22], allowing contamination of people, animals, and the environment. This contamination can occur through direct (product handling) or indirect contact (respiration and food consumption) [23], and, therefore, many studies have been interested in the effects of 2,4-D under the physiological systems. Studies have reported anatomical and physiological impairment of the kidneys [24], liver [3, 25], and testicles [26, 27], demonstrating that 2,4-D can promote harmful effects on testicular function and, consequently, on testosterone production. Thus, it is interesting to investigate how foods exposed to different doses of 2,4-D could compromise tibias of Wistar rats, since there is no similar study in the literature.

The mechanical properties of bone can be classified into structural and material properties. Structural properties are related to bone geometry (architecture, shape, size, and

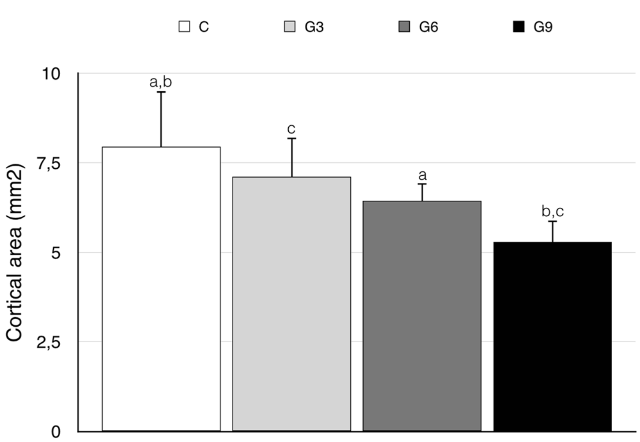

Fig. 2 Morphometric measurements of the cross-sectional area (CSA) of C, G3, G6, and G9 groups. Equal letters indicate a statistically significant difference between the two groups

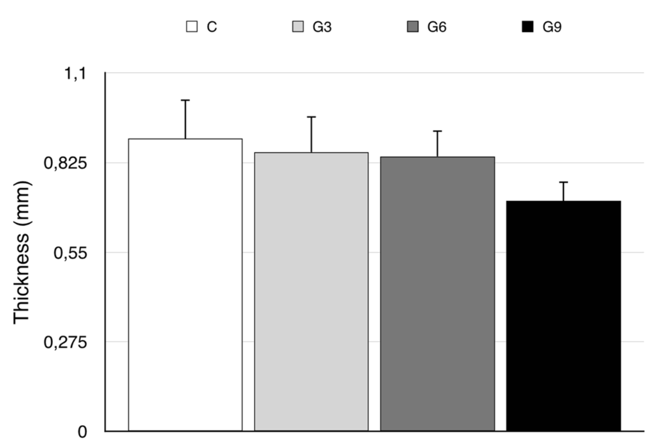

Fig. 3 Morphometric measurements of the thickness of C, G3, G6, and G9 groups. Equal letters indicate a statistically significant difference between the two groups

Table 2 Peak ratio in each Raman property (mean ± SD) among C, G3, G6, and G9 groups for the cortical bone of the tibial shaft. The letters A and B indicate statistical difference to the control group. The *p* values for A and B are 0.05 and 0.01, respectively

Ratio	C Group	G3 group	G6 group	G9 group
$v_2PO_4^{3-}$/amide III	9.29 ± 2.34	7.34 ± 1.44	7.47 ± 2.01	6.13 ± 2.55
$v_1PO_4^{3-}$/amide I	40.12 ± 7.61	31.48 ± 4.25	29.96 ± 2.36^A	29.28 ± 6.15^A
$v_1CO_3^{2-}$/amide I	2.66 ± 0.43	3.32 ± 0.46	3.82 ± 0.63^A	4.03 ± 0.73^B

dimension), while material properties refer to properties related to bone tissue composition, regardless of size and shape, and can be measured in homogeneous bone samples of unknown geometry [28]. Thus, material properties reflect the state of organization and constitution of macromolecules and cells in bone tissue and also its mineral content [17, 28].

In the present study, it was found that the tibias of the C group were more resistant to the load (force) applied during the mechanical test when compared to those of the groups that consumed contaminated feed with medium and high concentration of 2,4-D. In addition, a decrease in cortical area was also observed in all groups exposed to 2,4-D. Thus, there is sufficient evidence to suggest that the 2,4-D herbicide promoted a decrease in bone volume in the tibial shaft and interfered in the development and maturation of the skeleton of growing rats. This fact corroborates the study by Mazhar et al. [12]. These researchers studied the effect of 2,4-D contamination on pregnant rats and found impaired fetal growth and a high incidence of skeletal malformation, which was attributed to 2,4-D-induced oxidative stress. According to the authors, oxidative stress appears to have damaged embryos, causing peroxidation of cell membrane phospholipids and altering cell molecules such as lipids, proteins, and nucleic acids.

The analysis of the material properties demonstrated lower values of maximum tension and elastic modulus for the contaminated groups, indicating impairment to the bone quality of rats that consumed contaminated feed with medium and high doses of 2,4-D, making the bone more susceptible to the fracture. This possibly suggests that the tibias of the contaminated groups presented alterations of cell types, in the composition and organization of bone matrix molecules and in mineral content. Thus, 2,4-D contamination may have promoted osteoblast suppression and increased osteoclast activity per bone tissue area, since there is greater bone volume in the diaphysis of the control group. It is also possible to suggest that the contaminated groups (medium and high concentration) had some impairment in the process of differentiation of mesenchymal osteoprogenitor cells [29]. This hypothesis may be supported by the study by Miyakoshi et al. [30], in which it was verified that material properties are regulated by bone remodeling rate, cellular activity, and oxidative stress.

According to Marouani et al. [27], 2,4-D can cause damage to various body systems. They observed that oral exposure to 2,4-D for 30 days caused a decrease in serum testosterone level, histological changes in the testicles, and a decrease in sperm count. Thus, there is another factor that may have contributed to the impairment of the quality and mechanical resistance of the tibias of the contaminated groups. This other factor would be the level of testosterone found in animals since it is an essential hormone for maintaining bone integrity for both trabecular and cortical bone [31] by preventing osteoblast apoptosis and stimulating osteoclast apoptosis [29]. Another research group from our university found changes related to spermatic quality, such as reduced sperm cell production, reduced progressive motility, and also changes in sperm morphology (Ana Paula Alves Favareto - personal

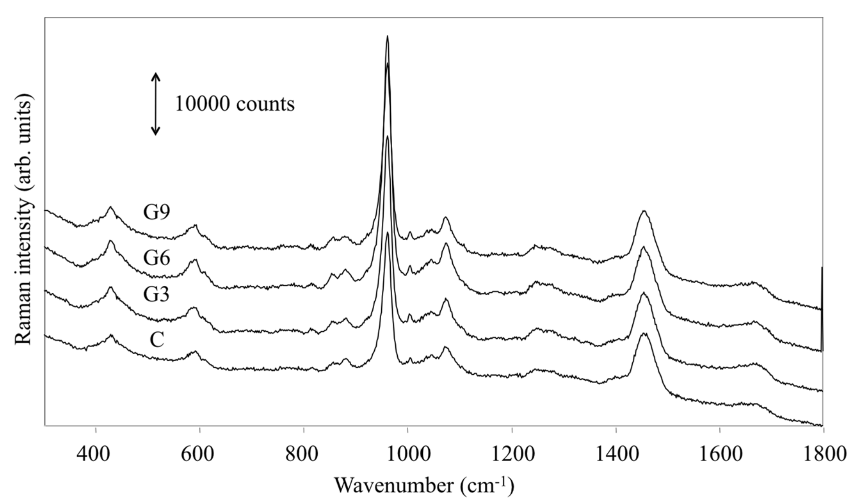

Fig. 4 Raman spectra of rat tibia performed in the C, G3, G6, and G9 groups

communication). These parameters may have been affected by the change in testosterone levels, but it cannot be stated that its reduction directly caused the changes mentioned.

According to Morris and Mandair [21], mechanical properties are related with mineral-to-matrix and carbonate-to-matrix ratios. The mineral-to-matrix (960/1660) ratio "represents the amount of mineral per amount of collagen per volume analyzed" [20]. The animals that consumed contaminated feed with medium and high concentration of 2,4-D demonstrated lower mineral-to-matrix ratios and higher carbonate-to-matrix ratios. This indicates that the herbicide promoted the decrease of the amount of mineralization of bone. However, these groups also demonstrated higher carbonate-to-matrix ratios. This can be attributed to high levels of bone remodeling since this ratio is an indicator of bone turnover, and it also has been associated with increased risk of fracture [21]. Thus, this might explain the lower values found for maximum stress and elastic modulus.

Considering the facts presented and the limitations of this study, such as number of analyses (cell count and hormonal dosage), it can be concluded that the herbicide 2,4-D had deleterious effects on the mechanical properties of tibias from male Wistar rats in the growing process and biological maturation. It should be considered that the amount of 2,4-D to which the organism is exposed may be a crucial factor for bone tissue strength.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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