



## **Exploring the Impacts of Glyphosate on Liver Health: An Advanced Approach with Digital Image Processing**

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#### Abstract

**Introduction:** Glyphosate (N-phosphonomethyl-glycine) is a non-selective, systemic herbicide widely used in crops. Its extensive use aims to increase productivity, making the process more profitable. Exposure to the herbicide causes damage to flora and fauna and may be a potential harmful agent to the hepatic epithelium, causing pathological changes. **Study Model:** Experimental study. **Objective:** To analyze the effects of chronic oral and inhalatory exposure to different concentrations of the herbicide glyphosate on the hepatic tissue of rats, using digital image processing. A total of 112 Wistar rats were used for the research, distributed into 2 exposure groups (inhalational and oral) with 14 rats in each cage. The inhalational exposure group received daily nebulization, while the oral exposure group received food nebulized with glyphosate. In both groups, we used three concentrations of the herbicide, characterized as high, medium, and low concentrations. After 180 days of the experiment, the animals were euthanized. Microscope images were taken, hepatic nuclei were selected and delineated, and a database was created that enabled the digital processing analysis of 200,000 hepatocytes. **Results:** It was observed that both the routes of exposure and the dosages exert predominant changes, suggestive of hepatic damage with a value of ( $p < 0.05$ ). **Conclusion:** Chronic inhalatory exposure to glyphosate presents a high potential for harm to hepatic cells.

**Keywords:** Agrochemicals. Glyphosate. Toxicity. Liver. Hepatocyte. Animal models.

## 1 INTRODUCTION

Glyphosate is currently the most widely used herbicide in Brazil and worldwide, and its increased consumption is well established, especially in countries in North America, South America, Europe, and Asia. The herbicide market is a significant financial aggregator, generating approximately US\$ 17 billion annually, with a tendency for exponential growth (GIANESSI, 2013; BRASIL, 2018). Glyphosate is a potent herbicide that works by inhibiting the synthesis of amino acids, effectively eradicating weeds (SILVA; SOARES; MELO, 2023). According to more recent estimates, herbicides represent nearly US\$ 24 billion in trade value (BRASIL, 2023; MARTINS-GOMES et al., 2022).

The application process of agrochemicals occurs in various ways, such as manual application, tractors, and airplanes over crops. However, in this process, besides reaching the desired target, it spreads into the environment and can contaminate the soil, lakes, water sources, air, and food, as well as directly affect workers, residents, and animals living around these sprayed areas (LOPES; ALBUQUERQUE, 2018; SOARES et al., 2021).

Previous studies corroborate findings demonstrating the high toxic potential of glyphosate for both humans and animals. In humans, it has been observed to be harmful, presenting carcinogenic, mutagenic, teratogenic, neuroendocrine effects, respiratory difficulties, memory and skin problems, depression, among others (KWIATKOWSKA; JAROSIEWICZ; BUKOWSKA, 2013; BAEK et al., 2021). In animals, studies have shown that fish experience spermatogonia alterations, reducing fecundity, and in bee populations, it causes behavioral changes and alterations in the gut microbiota, making them more susceptible to diseases and death (ANTUNES et al., 2023; SANTOS et al., 2011).

Despite the fact that Brazilian research on the impact of pesticide use on human health has grown in recent years, it is still not sufficient to understand the extent of the chemical burden of occupational exposure and the magnitude of health damage. One of the problems pointed out is the lack of information about pesticide consumption and the insufficiency of data on poisonings caused by these products (TOSETTO; ANDRIOLI; CHRISTOFFOLI, 2021; FIGUEIREDO; TRAPE; ALONZO, 2011).

In this study, the morphological alterations in the liver of rats chronically exposed to glyphosate, both orally and inhalationally, at different concentrations of the chemical agent were analyzed using digital image processing as a quantitative measurement tool. Additionally, the respective alterations according to concentration and exposure route were evaluated, establishing comparison pathways for human exposure to glyphosate. As far as we know, this study is innovative, as it is the only one that evaluates such a large number of hepatic cells after exposure to glyphosate.

## 2 METHODS

### 2.1 Ethical Considerations

O projeto foi submetido ao Comitê de Ética no uso de Animais da Universidade do Oeste Paulista para ser realizado de acordo com o Guia para o Cuidado e Uso de Animais de Laboratório do Instituto Nacional de Saúde (USA). Todo o material biológico usado na elaboração do presente estudo encontra-se armazenado no laboratório da universidade, proveniente de um outro estudo já aprovado e realizado, intitulado ‘COMPARAÇÃO MORFOLÓGICA PULMONAR DE RATOS EXPOSTOS CRONICAMENTE POR VIA INALATÓRIA E ORAL AOS HERBICIDAS GLIFOSATO E ÁCIDO 2,4 DICLOROFENOXIACÉTICO’, aprovado em 13/11/2019 sob protocolo 5684, de responsabilidade da Dra. Renata Calciolari Rossi.

### 2.2 Experimental Design

The experimental protocol included two boxes (32 x 24 x 32 cm), each connected to an ultrasonic nebulizer of the brand Pulmosonic Star®, which administered the following concentrations diluted in 10 ml of sodium chloride.

- Control group: Solution composed of only 10 ml of sodium chloride;
- Low concentration group: Solution composed of  $3.71 \times 10^{-3}$  grams of active ingredient per hectare, diluted in sodium chloride;
- Medium concentration group: Solution composed of  $6.19 \times 10^{-3}$  grams of active ingredient per hectare, diluted in sodium chloride;
- High concentration group: Solution composed of  $9.28 \times 10^{-3}$  grams of active ingredient per hectare, diluted in sodium chloride.

The exposure time was approximately 15 minutes for the entire solution to be nebulized. The animals exposed via inhalation were nebulized daily. The feed of the animals exposed orally was changed every two days, with the feed being nebulized 24 hours before being offered to the animals. All animals underwent 180 days of experimentation.

### 2.3 Sample Collections

After the experimental protocol was completed, the animals were euthanized using sodium thiopental (30 mg/kg of body weight) via intraperitoneal injection. Following this step, the livers of these animals were removed and subsequently processed and embedded in paraffin blocks.

## 2.4 Data Analysis

Liver samples were sectioned at 5 $\mu$ m and stained with Hematoxylin and Eosin for subsequent selection of the most appropriate hepatic areas for morphological evaluation. Liver slides with preserved histological architecture were included in the study. The Hematoxylin and Eosin staining protocol consists of first deparaffinizing, alcoholizing (absolute alcohol), and hydrating. Following this procedure, the slides are immersed in Harris Hematoxylin for 5 minutes and washed in running water. After that, the slides are dipped in a differentiator for 4 seconds (hydrochloric acid alcohol) and quickly washed in running water. Subsequently, the tissue fragment is immersed in Eosin for 5 minutes, followed by absolute alcohol, and then mounted in synthetic medium with Entellan. The Image-Pro Plus<sup>®</sup> software (Version 7.0, Media Cybernetics, Silver Spring, MD, USA) was used to quantify hepatic cells throughout the entire extent of the liver tissue sections from the rats.

For each image, the count of intact and damaged hepatocytes was performed automatically using a methodology based on digital image processing techniques. Initially, a pre-processing was carried out on the images for the segmentation of each hepatocyte; subsequently, the segmented hepatocytes were classified as intact or damaged, following some metrics obtained from the hepatocytes in the images and some empirically defined parameters:

- Hepatocyte area (count of the interior pixels of the segmented hepatocyte region) calculated using the region filling algorithm (MARQUES FILHO; NETO, 1999).
- Hepatocyte contour points (perimeter) using the Border Following algorithm (SUZUKI et al., 1985).
- Bounding rectangle of the hepatocyte from the Cartesian planes.
- Minimum bounding rectangle of the segmented hepatocyte (possibly rotated) using the contour points obtained by the Border Following algorithm (SUZUKI et al., 1985).

With the use of image processing, it was possible to provide greater robustness to the number of cells studied; 25,000 hepatic cells from each group were evaluated, making a total of 200,000 cells assessed.

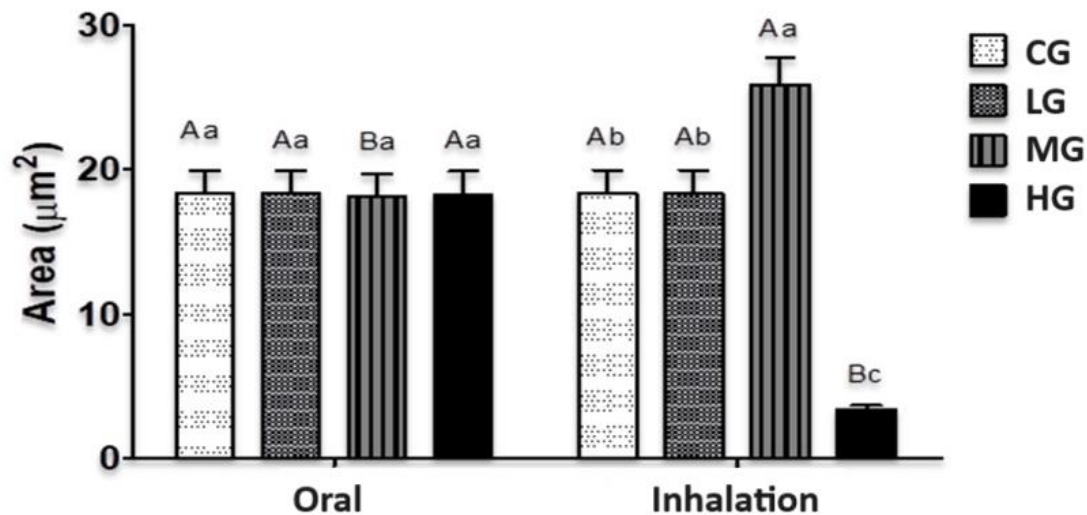
The classifications of hepatocytes as intact or damaged were performed following certain rules and some minimum and maximum parameters empirically assigned to the metrics defined and obtained from the segmented hepatocytes in the images. It was determined that all hepatocytes found at the edges of the images, or smaller than a minimum size of 43x43, or with an area smaller than a minimum area of 725, or a perimeter smaller than a minimum perimeter of 175, or a width smaller than a minimum width of 37 would be excluded from the classification.

After the preparation of the slides, images were captured using a microscope with an attached camera (Leica ICC50 HD) for morphometric analysis. The Image-Pro Plus<sup>®</sup> software (Version 7.0, Media Cybernetics) was used. An electronic database (Excel<sup>®</sup>) was created for the analysis of the results.

## 3 RESULTS

Exposure to glyphosate resulted in alterations in the hepatic epithelium of animals exposed via inhalation, while the oral exposure group did not show such alterations. The results of the two-factor ANOVA indicate a significant difference ( $p < 0.05$ ) in the route of administration (oral and inhalation), as shown in Figure 1.

Figure 1 – Comparison between exposure groups and herbicide concentrations.



Caption: Uppercase letters represent comparisons between groups (Inhalation and Oral). Lowercase letters represent comparisons between doses (CG=Control Group; LG=Low Concentration Group; MG=Medium Concentration Group; HG=High Concentration Group). Different letters indicate statistical difference ( $p < 0.05$ ). Source: Authors, 2024.

Another significant finding was in the inhalation exposure group, which showed alterations in the medium concentration inhalation group. This group stood out among all, with the highest number of altered cells and reduced nuclear size. The route of administration and dose were effective in causing harmful effects. The results obtained through the two-factor ANOVA support this finding with a  $p$ -value  $< 0.05$ , confirming that the interaction between the route of administration and dose also shows a significant effect.

In the multiple comparisons analysis (Bonferroni), the mean of the MG group in the inhalation route of administration was higher compared to the other groups. This group also showed a higher mean compared to the oral route of administration. Conversely, the mean of the HG group in the inhalation route of administration was lower compared to the other groups in the same route. This could have led to the interaction result.

The significant interaction between Dose and Route of administration indicates that the effect of Dose on the results varies depending on the Route of administration. This means that the influence of Dose is not consistent across all levels of Route of administration. Both Dose and Route of administration have significant and independent effects on the results. Due to the significant interaction, the effects of Dose and Route of administration on the results cannot be interpreted independently; the effect of one depends on the level of the other.

#### 4 DISCUSSION

Although the use and interaction of glyphosate is a widely discussed topic, in terms of alterations to hepatic cells, more studies are needed to define the interaction mechanism and its long-term implications in the development of pathologies (CHRISTENSEN, 2023). This research addresses an extremely important topic for society, with robust results; 200,000 hepatic cells were analyzed to achieve the results obtained.

Thus, the present study provides new and relevant insights on the subject. The research collected important data on both oral and inhalational exposure to glyphosate. The findings indicate that there is a greater interaction when glyphosate contamination occurs via inhalation. Additionally, the study revealed that a medium concentration dosage administered via inhalation shows a significant interaction, capable of causing hepatic damage.

The inhalation route with a medium concentration dose presented greater alterations than the high concentration route. This finding may be attributed to a probable higher sensitivity to medium concentrations of the active ingredient (PARIZI et al., 2020). These findings underscore the relevance of the topic, considering the large areas of spraying that expose workers to glyphosate. The drift of the product also indirectly exposes residents of nearby areas to the harmful active ingredient.

Our results indicate that the concentrations of the active ingredient, as well as the route of administration, are predominant factors for the observed alterations. However, chronic exposure, even at high doses, did not result in lethality. It is possible that a longer observation period would reveal the development of conditions and complications leading to lethality. Other studies with similar exposure periods have yielded comparable results without the death of the studied subjects, underscoring the need for long-term observational studies to monitor the development of complications suggested by chronic exposure (MAIA et al., 2021).

As important as the chronicity of exposure is the route of contamination. The route of exposure to the active ingredient can provoke more exacerbated immune responses, resulting in different types of responses. In the present study, it was observed that the inhalation route presented greater alterations compared to the oral administration route. The group exposed to a medium concentration of the active ingredient via inhalation showed greater injury to hepatic cells. The different concentrations of the active ingredient administered orally did not result in significant alterations, suggesting that oral exposure is less harmful. This finding contrasts with a study conducted on carp that were contaminated orally through feed, which showed significant hepatic damage, with alterations in the levels of the enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT), indicating hepatic injury (ABDELMAGID et al., 2023).

The process of chronic exposure to glyphosate occurs mainly via inhalation, either directly through handling and application of the product or indirectly through drift that carries the droplets beyond the spraying area. The present study showed that medium concentration inhalational dosage is responsible for scientifically significant alterations, being capable of inducing substantial changes in hepatic cells, which suggests liver damage. The chronicity of exposure results in significant changes in various physiological processes. As found in this research, another study that collected urine samples from rural workers exposed to glyphosate in recent years during the spraying process detected glyphosate excretion in the urine (CHANG et al., 2023).



Different exposure concentrations have been observed to establish safety levels regarding contact with glyphosate. In the present research, it was not possible to observe significance in low exposure levels, both in oral and inhalational exposure. Low concentrations did not show significant disparities in alterations. In contrast, a prospective cohort study conducted with individuals exposed to inhalational contamination due to drift followed pregnant participants exposed to glyphosate for a period of 18 years, during which the children were monitored. All were about 1.6 km away from the glyphosate application site. This study inferred that chronic exposure exacerbated the risk of hepatic injury, among other diseases (ESKENAZI et al., 2023).

In the group exposed to a high inhalational concentration of the active ingredient, a lower mean was observed compared to the other groups of the same route. This reinforces the idea that the route of administration influences the effect of doses differently, highlighting how an interaction process can manifest. This contrasts with a study conducted with a similar methodology and exposure time, which observed greater alterations at high concentrations and changes in the esophagus, small intestine, and large intestine, regardless of sex (MARIA SERRA; PARIZI; ODORIZZI et al., 2021).

## 5 CONCLUSIONS

The results of the present study show that the herbicide glyphosate causes alterations in the hepatic epithelium when administered via inhalation at medium concentration over a chronic period.

Oral exposure did not show suggestive results of alterations in hepatic tissue, regardless of the administered doses, whereas the group exposed via inhalation at medium concentration showed alterations suggestive of hepatic damage. A longer follow-up period would be necessary to observe the progression of the damage and thus understand to what extent the injuries to hepatic tissue could lead to a state of homeostatic inefficacy.

Chronic exposure to glyphosate at different concentrations and through different routes, whether oral or inhalational, causes alterations in the dimensions of the hepatic tissue nucleus, and these alterations can lead to the development of pathological conditions. It is undoubtedly necessary to conduct more extensive studies over longer periods and with larger populations to monitor alterations over time.

There is a long way to go to demonstrate the deleterious effect of glyphosate, not only on human health but also on the entire ecosystem. It is crucial to seek safer alternative methods for the exposed population and the environment. Therefore, more comparative studies between groups and body systems are needed to mitigate the risks that glyphosate exposure can cause. It is suggested to expand research on the effects of long-term exposure on animal, human, and environmental health.

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