*https://doi.org/10.31925/farmacia.2023.6.5* **ORIGINAL ARTICLE**

# EXPOSURE TO GLYPHOSATE AND ITS MIXTURE WITH DICAMBA AND 2,4-D FROM GESTATIONAL DAY 6 UNTIL WEANING IN RAT DAMS REVEALS SIGNS OF NON-ALCOHOLIC FATTY LIVER DISEASE

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**Abstract**

The growing usage of glyphosate, dicamba, and 2,4-D herbicide mixtures to combat glyphosate-resistant weeds raises concerns about human health and environmental consequences. The purpose of this study was to see how developmental exposure to glyphosate and a herbicide mixture combining glyphosate, dicamba, and 2,4-D affected rat dams' liver structure and function. A mixture of glyphosate, dicamba, and 2,4-D each at their European Union acceptable daily intake (ADI) (0.5, 0.002, and 0.3 mg/kg bw/day, respectively) and glyphosate at the ADI and NOAEL levels (0.5 and 50 mg/kg bw/day), were administered to pregnant Wistar rats from day-6 of gestation until weaning. The dams were sacrificed after weaning, and blood and liver tissue collected. In dams, glyphosate exposure resulted in the induction of non-alcoholic liver disease in a dose-dependent manner. In addition, exposure to the herbicide mixture resulted in effects similar to those observed with glyphosate at the NOAEL dose, suggesting at least an additive effect of the herbicide mixture at regulatory doses considered individually safe for humans.

**Rezumat**

Utilizarea din ce în ce mai frecventă a amestecurilor de erbicide pentru combaterea plantelor dăunătoare ridică probleme în ceea ce privește sănătatea umană și a mediului. Scopul studiului a fost evaluarea afectării hepatice a femelelor de șobolan, după expunere la un amestec de erbicide care combină glifosatul, dicamba și 2,4-D, în perioada gestațională. Amestecul de erbicide a avut un efect toxic aditiv asupra țesutului hepatic.

**Keywords**: pesticides, mixtures, glyphosate, NAFLD, dicamba, 2,4-D, hepatotoxicity

## Introduction

Non-alcoholic fatty liver disease (NAFLD) is a syndrome defined by fat buildup (steatosis) in more than 5% of hepatocytes in the absence of other factors such as alcohol abuse [1]. Nowadays, NAFLD is the main cause of chronic liver disease, with a high prevalence in patients with metabolic syndrome, affecting around 25% of adults worldwide [2] and more than 25% of European adults, with an increasing trend in children who suffer from obesity or who are overweight [3]. Liver disease during pregnancy should be evaluated

by a multidisciplinary team as it can contribute to complications that puts the lives of mothers and foetuses at risk [4]. NAFLD has been linked to a two-fold increase in the chance of developing hypertensive disorders during pregnancy [4], preterm birth and postpartum haemorrhage [5]. In recent years, the prevalence of NAFLD during pregnancy has increased three-fold and statistics have shown that pregnancies affected by NAFLD are associated with all metabolic co-morbidities and caesarean deliveries [5]. These findings highlight the need for enhanced awareness of the hazard of NAFLD in pregnant women, as well as its connection to high-risk obstetric treatment.

Pregnancy has a direct effect on the physiology of the liver, and hepatic diseases can have a negative impact on pregnancy outcomes. Thus, when hepatic diseases appear in pregnancy, the risks for the mother and foetus are very high and pregnancy counselling and post-partum follow-up are recommended, especially as it is a symptom of metabolic syndrome.

The liver is an important organ in the metabolism, distribution, and excretion of exogenous substances, as well as in the maintenance of metabolic homeostasis. Chemical-induced hepatotoxicity is a worldwide health concern because hepatocytes are exposed to a considerable influx of diverse exogenous substances. Exposure to environmental toxicants has been linked to dysregulation of amino acid and lipid metabolism, which is one of the causes of liver damage that influences the development of NAFLD [6]. Pesticides, including herbicides, are two potential environmental factors that can contribute to the pathophysiology of NAFLD [7]. Glyphosate, the main weed-killing chemical in Roundup® and other word-wide used glyphosate-based herbicide (GBH) formulations is sprayed on genetically modified (GM) crops as well as many non-GM grain crops, and is detected in these crops at harvest [7]. With the massive increase in glyphosate-tolerant weeds, glyphosate is increasingly used in combination with other herbicides such as dicamba and 2,4-dichlorophenoxyacetic acid (2,4-D). This has been exacerbated by the launch of GM food crops tolerant to glyphosate plus dicamba or glyphosate and 2,4-D [8]. Studies in rodents have shown that chronic exposure to low doses of Roundup GBH results in hepatotoxicity, fatty liver, congestion of liver cells, necrosis, and even DNA damage [9-12] The health effects of other herbicides used in conjunction with glyphosate are unknown, and this has become a greater concern since the advent of GM crops resistant to multiple herbicides as this is expected to increase exposure to herbicide combinations. In addition, studies have emphasized the fact that exposure of individuals is not only to a single potentially toxic chemical but to a wide range of compounds that, when combined, can cause harmful effects even at very low doses, including those considered safe by regulators. As a result, greater efforts are being undertaken to assess the possible risk of exposure to combinations of chemicals using a real-life simulation approach [13, 14]. Furthermore, data from animal studies have shown detrimental effects after exposure to very-low doses of environmentally relevant chemical mixtures [15-20].

Exposure to liver toxicants during pregnancy can influence not only maternal health and pregnancy outcomes [5], but can also contribute to the further development of NADFL in offspring [21], which is very crucial to avoid. In this study, we aimed to evaluate the potential implication of NADFL appearance during pregnancy on dams exposed during gestation and lactation to low doses of glyphosate alone and in combination with 2,4-D and dicamba.

## Materials and Methods

*Animal experiment*

The Animal House of the University of Medicine and Pharmacy of Craiova, Romania, provided 20 female Wistar rats aged three months. The animals were acclimatized to conventional laboratory settings for two weeks prior to the start of the experiment. Temperatures 21 ± 2°C and humidity 50 ± 10%, free access to filtered tap water and standard animal feel (Cantacuzino Institute, Bucharest, Romania) were among the requirements. Female rats were mated after acclimatization, and on gestational day 1 (GD1) were taken from the males and housed two animals *per* cage. Dams were randomly assigned to one of four experimental groups (n = 5 *per* group) on GD6. From GD6 through weaning (postnatal day (PND) 28), these animal groups received daily treatment as summarized in Figure 1. The detailed design of the animal experiment has already been described elsewhere [22]. The animal experiment was carried out in accordance with the standards for the use of animals in toxicology, and it was approved by the Ethical Committee of Craiova University of Medicine and Pharmacy (Romania).

On PND28, pups were separated from their mothers. The dams were sacrificed after being anesthetized with a mixture of xylazine and ketamine (Alfasan Int., Woerden, Netherlands) and sacrificed by exsanguination. The blood collected from the heart was used for the determination of biochemical and haematology parameters. The liver was collected, weighed and used for histo-pathological evaluation.

*Treatment*

All the chemicals used for the treatment were of analytical grade Sigma-Aldrich, Merck KGaA, Darmstadt, Germany. We used for treatment Glyphosate Prestanal®, ≥ 98.0% purity, Dicamba Prestanal®, ≥ 98.0% purity and 2,4-dichlorophenoxyacetic acid, 97% purity.

*Blood biomarkers*

Biochemistry parameters, namely alanine amino-transferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), cholesterol (CHOL), triglycerides

(TRIG); alkaline phosphatase (ALP), were quantified using an Spotchem EZ SP-4430 veterinary automated analyser (Arkay, Japan).

Haematological parameters, namely leukocytes count (WBC), red blood cell number (RBC), haemoglobin (HGB), haematocrit (HCT), platelet count (PLT); platelet crit (PCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), mean platelet volume (MPV), platelet distribution width (PDW), were determined using BC-500 automated haematology analyser (Mindray, North America).

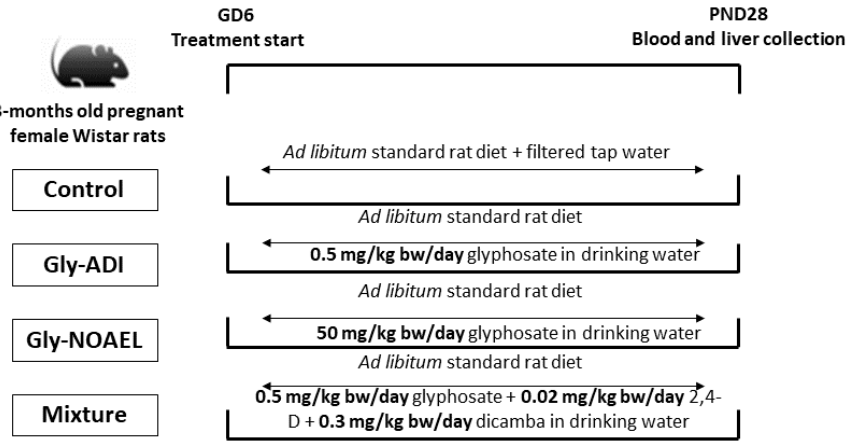


**Figure 1.**

Flowchart of the study design and treatments in each exposure group

GD6 – gestational day 6; PND28 – postnatal day 28; Gly-ADI – glyphosate acceptable daily intake; Gly-NOAEL – glyphosate no observed adverse effect level; Mixture – glyphosate, 2,4-D and dicamba each at their acceptable daily intake

*Histopathological evaluation of the liver*

Histology was performed on the liver. Organs were preserved in formalin solution (10%) and stained with haematoxylin and eosin as previously described [20]. Samples were examined using a Panthera L light microscope (Motic Europe, S.L.U) by two experienced pathologists, who were blinded to the experimental groups. In the case of opposing interpretation of results, the two pathologists discussed the results and arrived at a consensus.

Histopathological alterations were scored according to their severity and the number of animals in which the changes appeared. The severity of the damage was graded as follows: (-) none, (+) mild, (+++) moderate, (+++) severe).

*Statistical analysis*

SPSS 20 (SPSS Inc., Chicago, USA) was used for all analyses. The mean ± standard deviation of the mean was used to express continuous data. Data analysis was performed using one-way ANOVA test, followed by a Dunnett's test for multiple comparisons. Values of $p < 0.05$ were considered for statistical significance.

## Results and Discussion

*Exposure to low doses of glyphosate and its mixture with dicamba and 2,4-D determined changes in blood liver health biomarkers in dams*

Exposure to the glyphosate ADI dose resulted in a significant decrease in blood ALT and AST levels and a significant increase in CHOL, TRIG and ALP levels. At the higher glyphosate NOAEL dose also gave rise to a significant increase in ALT, AST, TBIL, CHOL, TRIG and ALP levels compared with the control group. In animals exposed to the mixture of glyphosate, 2,4-D and dicamba an additive effect on hepatotoxicity was observed as serum ALT and AST levels were significantly increased compared to control and Gly-ADI groups. Moreover, the TRIG and ALP levels are also significantly increased compared with the control group (Table I).

**Table I**

Liver health biomarkers determined in the serum of dams after treatment

| Parameter | Control | Gly-ADI | Gly-NOAEL | Mixture |
|---|---|---|---|---|
| **ALT (U/L)** | 66.50 ± 01.14 | 39.66 ± 1.86** | 91.66 ± 3.25** | 87.94 ± 3.27** |
| **AST (U/L)** | 112.76 ± 03.09 | 99.76 ± 3.45* | 157.06 ± 6.93** | 156.20 ± 12.77** |
| **TBIL (mg/dL)** | 00.08 ± 00.01 | 0.09 ± 0.01 | 00.06 ± 0.02* | 0.07 ± 0.02 |
| **CHOL (mg/dL)** | 55.70 ± 04.49 | 66.20 ± 4.15** | 63.20 ± 3.42* | 59.40 ± 3.21 |
| **TRIG (mg/dL)** | 28.70 ± 03.81 | 95.14 ± 1.68** | 46.60 ± 3.01** | 105.64 ± 3.20** |
| **ALP (U/L)** | 69.44 ± 09.34 | 100.14 ± 7.29** | 108.96 ± 4.91** | 105.52 ± 10.80** |

* $p < 0.05$ compared with control; ** $p < 0.001$ compared with control; ALT – alanine aminotransferase; AST – aspartate aminotransferase; TBIL – total bilirubin; CHOL – cholesterol; TRIG – triglycerides; ALP – alkaline phosphatase

*Exposure to a low dose of glyphosate and its mixture with dicamba and 2,4-D correlates with changes in haematological parameters in dams*

An increasing trend of leukocyte count (WBC), red blood cell number (RBC), haemoglobin (HGB), haematocrit (HCT) and platelet crit (PCT) in all the treatment groups compared with the control was observed, but the only group that reached statistical significance was the herbicide mixture group.

Mean corpuscular volume (MCV), mean corpuscular haemoglobin concentrations (MCHC) and platelet distribution width (PDW) showed a significant decrease in the mixture group compared with the control group, while mean platelet volume (MPV) levels significantly increased in all three treatment groups compared to control (Table II).

**Table II**

Haematological parameters determined in the blood of dams after treatment

| Parameter | Control group | Gly-ADI group | Gly-NOAEL group | Mixture group |
|---|---|---|---|---|
| **WBC ($10^3$/mm$^3$)** | 2.80 ± 0.22 | 4.92 ± 1.83 | 4.66 ± 1.97 | 5.12 ± 0.76* |
| **RBC ($10^6$/mm$^3$)** | 6.54 ± 0.47 | 6.89 ± 0.33 | 6.77 ± 0.86 | 7.92 ± 0.29** |
| **HGB (g/dL)** | 14.66 ± 0.53 | 15.10 ± 0.16 | 15.68 ± 0.89* | 16.60 ± 0.49*** |
| **HCT (%)** | 41.90 ± 3.62 | 43.16 ± 1.11 | 46.60 ± 5.01 | 52.20 ± 5.81** |
| **PLT ($10^3$/mm$^3$)** | 593.20 ± 19.93 | 503.60 ± 99.01 | 596.40 ± 189.34 | 637.40 ± 56.57 |
| **PCT (%)** | 0.38 ± 0.02 | 0.38 ± 0.07 | 0.44 ± 0.13 | 0.52 ± 0.04* |
| **MCV (µm$^3$)** | 64.60 ± 1.14 | 63.20 ± 1.48 | 64.00 ± 2.12 | 60.40 ± 1.14*** |
| **MCH (pg)** | 22.48 ± 1.22 | 22.12 ± 1.15 | 22.12 ± 1.33 | 21.26 ± 0.55 |
| **MCHC (g/dL)** | 35.10 ± 1.84 | 35.36 ± 1.00 | 34.08 ± 2.01 | 32.50 ± 0.97* |
| **RDW (%)** | 12.76 ± 0.89 | 12.90 ± 0.55 | 13.60 ± 0.37 | 12.98 ± 0.28 |
| **MPV (µm$^3$)** | 6.74 ± 0.34 | 8.32 ± 0.08*** | 7.70 ± 0.38*** | 7.94 ± 0.23*** |
| **PDW (%)** | 8.96 ± 0.36 | 8.48 ± 0.78 | 8.50 ± 0.10 | 7.06 ± 0.15*** |

* $p < 0.05$ compared with control; ** $p < 0.01$ compared with control; *** $p < 0.001$ compared with control; WBC – leukocytes count; RBC – red blood cell number; HGB – haemoglobin; HCT – haematocrit; PLT – platelet count; PCT – platelet crit; MCV – mean corpuscular volume; MCH – mean corpuscular haemoglobin; MCHC – mean corpuscular haemoglobin concentration; RDW – red cell distribution width; MPV – mean platelet volume; PDW – platelet distribution width

*Liver histopathological evaluation*

Regarding the relative weight of the liver, we observed a significant increase in the mixture group (4.49 ± 1.01 g/100 g bw) compared with the control group (4.35 ± 0.45 g/100g bw) ($p < 0.001$). There was no difference in the relative weight of the liver in the Gly-ADI group (3.61 ± 0.26 g/100g bw) and Gly-NOAEL group (4.28 ± 1.03 g/100g bw) compared with the control group.

The control group, as expected, was found to have normal liver architecture. The polygonal shape of the lobule is preserved with portal areas in the corners and in the centre the central vein. Normal hepatocytes are arranged in linear hepatic cords separated from each other by sinusoids (Table III; Figure 2A).

In contrast, the Gly-NOAEL treatment group started to show modifications to normal architecture. There was a noticeable slight increase in sinusoidal space dilatation, but not uniformly distributed throughout the liver. The central vein appeared also dilatated with marked vascular congestion. We identified rare swollen hepatocytes and small areas of necrosis (Table III; Figure 2B).

The Gly-ADI exposure group also presented with histopathological changes but retained periphery small areas with normal appearance. Similarly, to Gly-NOAEL group, there was central vein dilatation, but without vascular congestion. Regarding the sinusoids, there were elements of dilatations but only focally and only slight, thus presenting a distortion in terms of normal architecture. For the hepatocytes, we observed a slight variation in cell size, granular degeneration and even the presence of microvacuolar cytoplasm, especially next to the vein (Table III; Figure 2C).

In the herbicide mixture treated group, important modified aspects in liver architecture were evident. There was a diffuse marked vascular congestion in the central veins but with no associated dilatated lumen. The sinusoidal spaces were also dilatated, and in general irregularly distributed presenting congestion. Regarding the hepatic cords, we observed an alternation regarding the linear pattern, The hepatocytes showed histological alterations with granular degeneration or with a microvacuolar cytoplasm, and focally we noticed a tendency towards clear contouring and merging of microvacuoles. In this group, binuclear hepatocytes and a chromatin margination phenomenon were observed (Table III; Figure 2D).

We used a scoring system to evaluate histopathological changes after reviewing their intensity and the number of specimens involved. The intensity of the alterations was classified from absent to intense as showed in Table III.

**Table III**

The severity and frequency of histological abnormalities in liver from the four groups as observed in histological evaluation of liver tissue

| Parameters | Experimental groups | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | | Gly-ADI | | Gly-NOAEL | | Mixture | |
| | Intensity | Animals affected | Intensity | Animals affected | Intensity | Animals affected | Intensity | Animals affected |
| **Liver architecture changes** | - | 5 | + | 3/5 | + | 2/5 | ++ | 3/5 |
| **Central vein dilatation and congestion** | - | 5 | ++ | 2/5 | +++ | 4/5 | + | 3/5 |
| **Sinusoidal spaces dilatation** | - | 5 | + | 1/5 | + | 2/5 | ++ | 3/5 |
| **Modified hepatocytes** | - | 5 | ++ | 3/5 | + | 2/5 | +++ | 3/5 |

(-) absent; (+) mild; (++) moderate; (+++) intense

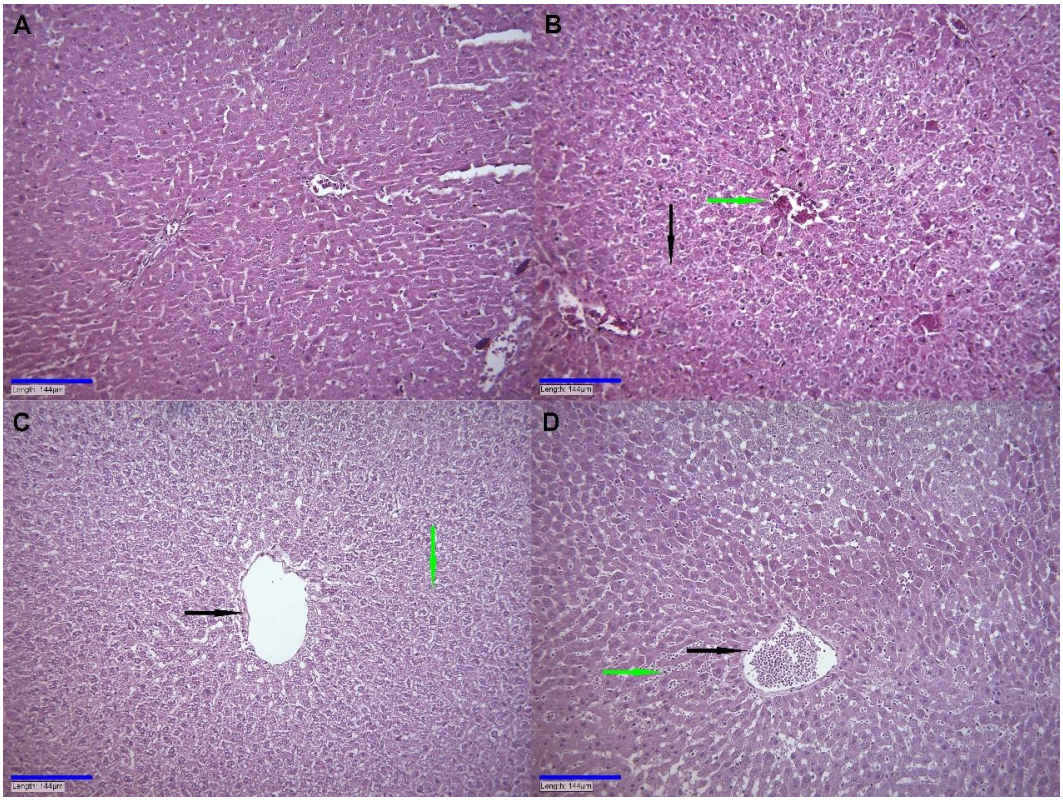


**Figure 2.**

Liver, HE staining, X200. A – Control group: normal architecture, linear arranged hepatocytes; B – Gly-NOAEL group: areas of necrosis with lack of nuclei-black arrow, dilated central vein with marked vascular congestion-green arrow; C – Gly-ADI group: central vein with important dilatation-black arrow, hepatocytes with modified histological aspects-green arrow; D – Mixture group: central vein showing marked vascular congestion-black arrow, dilatated sinusoidal spaces-green arrow

The physiological changes that appear during pregnancy in order to support the growth and development of the foetus, also affect the liver due to an increase in oestrogen and progesterone levels and hemodynamic changes [23]. Serum albumin levels can decrease, while ALP can increase in pregnancy, while other liver function biomarkers are kept in a normal range [24].

Liver diseases that can appear during pregnancy can increase the risks both for mothers and foetuses [5]. Previous studies have shown that exposure to low and ultra-low doses of glyphosate and a Roundup GBH formulation at levels below the European NOAEL and ADI, can induce oxidative stress in liver tissue, leading to steatosis and necrosis, and influencing the development of NAFLD [9, 25]. The increase in the

cultivation of GM crops tolerant to multiple herbicides is a genuine public health concern, especially if exposure occurs during pregnancy, affecting the health of the mother and consequently the development of the foetus [26-28]. To our knowledge, this is the first study that has evaluated the effects of exposure to low levels of a mixture of glyphosate, dicamba and 2,4-D during gestation and lactation on rat mother liver function. The changes in liver biomarkers reveal the liver injury. The necrosis of liver cells is highlighted by an increase in the levels of serum AST and ALT, whilst the increase in TBIL levels correlates with overall liver function. Another important marker of liver injury is ALP, which increases in serum when biliary epithelial cells or canalicular membranes are affected [29]. In our study, we observed that exposure to the European Union glyphosate NOAEL [30] produces clear signs of NAFLD as revealed by a significant increase in liver injury biomarkers such as ALT, AST and ALP, a significant increase in cholesterol and triglycerides levels compared with the control group (Table I) and histopathological changes of the liver with small areas of necrosis (Table III; Figure 2B). Moreover, the decrease in total bilirubin observed in the Gly-NOAEL group (Table I) can be associated with a higher risk for NAFLD and cardiometabolic symptoms [31, 32]. The mechanism through which bilirubin can be inversely associated with NAFLD is related to its potential antioxidant and cytoprotective properties identified in both *in vitro* and *in vivo* studies [33] and partially validated by population-based investigations, which showed bilirubin's antioxidant capacity by reducing oxidative stress markers in patients with Gilbert's syndrome [34]. Another significant change observed in the Gly-NOAEL treatment group is a significant increase in serum haemoglobin levels compared with the control group of animals. This is in accordance with previous findings showing increased levels of haemoglobin in patients with NAFLD, which correlates with the severity of steatosis [35]. Exposure to the European Gly-ADI, which is 100 times lower than the NOAEL, [30] produced less severe changes in the liver, with histopathological changes being minor, but nevertheless following the same pattern suggesting a dose dependent effect (Table III; Figure 2C). At a biochemical level, the Gly-ADI dose was observed to produce a significant increase in cholesterol, triglycerides and ALP levels compared with the control group (Table I).

Interestingly, exposure to a mixture of glyphosate, dicamba and 2,4-D with each at their European ADI, produced additive toxic effects on the liver, with observed changes providing evidence for severe NAFLD. In this group, there is a significant increase in serum ALT, AST, TRIG and ALP levels (Table I) associated with a significant increase in haematological parameters such as WBC, RBC, HGB, PCT, MCHC, MPV and a significant decrease in PDW compared with the control untreated group of animals (Table II). It has been shown that hepatic steatosis is connected with high WBC and platelet counts and that higher WBC counts are a prognostic marker for the severity of hepatic steatosis, which can also be associated with chronic inflammation that can appear during disease progression [36]. In the same manner, an increase in haemoglobin in NAFLD patients correlated with the severity of steatosis [35]. With respect to the liver histological changes observed in this herbicide mixture group, the detrimental effects of the treatment were more pronounced compared with the other groups (Figure 2D; Table III).

Mesnage *et al*. have demonstrated that a Roundup formulation disrupted liver mitochondrial oxidative phosphorylation, resulting in proteome disturbances illustrating peroxisomal proliferation, steatosis, and necrosis, a pattern that fits NAFLD and its development to non-alcoholic steatohepatitis [9]. Additional research suggests that glyphosate reduces fatty acid oxidation and boosts fat and cholesteryl ester levels in mouse livers, resulting in a higher lipid composition per gram of liver [37].

To our knowledge, this is the first study that evaluated the effects of the combined exposure of glyphosate, dicamba and 2,4-D from gestational day-6 until postnatal day 28 on the liver status of dams. We previously showed that this mixture of herbicides at the same nontoxic levels (European ADI) considered safe by the regulators, produced thyroid and kidney disturbances, showing an additive effect of the 2,4-D and dicamba with glyphosate [38]. In this study, we showed hepatotoxicity induced by the combination of glyphosate, dicamba and 2,4-D at regulatory and environmentally relevant levels of exposure. The peroxisome proliferator-activated receptor (PPAR), a receptor connected to the transcriptional control of a range of peroxisome-specific enzymes, has been reported to be transcriptionally upregulated by dicamba, along with a significant increase in the activity of peroxisomal beta-oxidation, fatty acylCoA-oxidase, lauric acid hydroxylase in liver homogenates [39]. The modulation of lipid metabolism, inflammation and oxidative stress was demonstrated to be the mechanism by which hepatotoxic effects can be induced by pesticides [40]. Hepatic liver toxicity induced by 2,4-D is associated with the induction of oxidative stress and a decrease in antioxidant enzyme activity [41].

Pregnant women are particularly vulnerable to liver dysfunction following environmental toxicant exposure due to the effects this can have on pregnancy outcomes and foetal development [5]. Because NAFLD during pregnancy can interfere with the normal progression of gestation, efforts need to be made to decrease or eliminate pesticide exposure during gestation to avoid negative health outcomes for both mother and foetus. Our research has the capability of replicating real-world human exposure; *i.e.*, long-term low-dose

exposure situations to combinations of chemical pollutants. Despite the fact that equivalent results have been observed in all treatment groups in this study, the experiment's small sample size of only 5 pregnant rats *per* group is a limiting factor. Given the low statistical power provided by the sample size in this study for the impacts identified in dams, this work should be considered a pilot study. Nonetheless, the fact that we were able to obtain statistically significant results with such a small number of animals implies that a larger-scale investigation is warranted considering its consequences for public health.

## Conclusions

Exposure of dams from GD6 till PND28 to subtoxic doses of glyphosate produced increases in biological markers of liver function and dose-dependent blood lipid levels reflective of NAFLD. Histopathological changes such as central vein dilatation, vascular congestion, granular degeneration and the presence of microvascular cytoplasm were also seen to be dependent on glyphosate dose. Exposure to the mixture of glyphosate, dicamba and 2,4-D produced NAFLD-inducing effects greater than those observed in the group treated with glyphosate NOAEL and ADI doses, suggesting an additive effect of the three herbicides at doses considered safe by government regulatory authorities. In this herbicide mixture group, changes in haematological markers such as increases in WBC, RBC, HGB, HCT and PCT were also observed, and which are reflective of a more advanced stage of NAFLD. At a histological level, liver pathological changes in the herbicide mixture group were found to be greater than those observed in the Gly-NOAEL or Gly-ADI groups. In summary, our results highlight the importance of revising the standard process for safety testing of chemical contaminants, which is currently based on toxicological tests using high doses of individual compounds. The current approach does not take into account the risks that may arise from exposure to a mixture of chemicals even at low doses.

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## Conflict of interest

The authors declare no conflict of interest.

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