ABSTRACT
Glyphosate is an organophosphorus, broad-spectrum herbicide used to kill weeds. In the present study, we studied the effects of glyphosate on suckling rat pups. We assessed the antioxidant biomarkers and lipid peroxidation in the liver tissue along with the activity of liver enzymes following the administration of glyphosate. In addition, we determined the histopathological alterations in the liver in suckling pups and the percentage of DNA damage in blood cells. Glyphosate treatment resulted in reduced liver weight, with the maximum decrease observed with 1/100 LD50 dose. All doses of glyphosate significantly increased the activity of hepatic enzymes, i.e., aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) (1/100 LD50 dose>NOAEL>ADI dose). Moreover, a marked increment in malondialdehyde level (MDA) along with significant inhibition in catalase (CAT) and glutathione peroxidase (GSHPx) activity was detected in the liver tissue. Severe DNA damage was detected in pups treated with 1/100 LD50 dose. Histopathological examination of the liver demonstrated that all doses induced dilatation and congestion in the portal vein, whereas 1/100 LD50 dose resulted in fibrotic portal tracts. Based on these results, we conclude that exposure to even minimal levels of glyphosate exerts detrimental effects on the liver.

KEYWORDS: glyphosate, oxidative stress, DNA damage, liver enzymes, histopathological examination, rat.

INTRODUCTION
Glyphosate (N-(phosphonomethyl)glycine) is a post-emergence herbicide used for controlling weeds in different crops, such as soybean, maize, and rice. Several commercial formulations containing glyphosate as the active ingredient have become common worldwide due to their high efficiency and comparatively minimal toxic effect on mammals [1]. However, studies have speculated that long-term exposure to chemical compounds can affect pregnant rats due to their toxic action and could result in bone deficiency in the fetus [1], alterations in cell metabolism [1], cutaneous lesions [2], and elevated risk of non-Hodgkin’s lymphoma [1]. Further, researchers have reported that even minimal glyphosate-Biocarb® doses can produce marked hepatic alteration, as well as bleeding from the nose without aggregation of platelets [3]. Biochemical
markers of liver and renal function and that of oxidative stress are used to monitor the effects of exposure to environmental pollutants [1]. Numerous studies have demonstrated no effects of exposure to minimal levels of environmental pollutants on the metabolism of mammalian organs. An in vitro study suggested that exposure to low glyphosate levels affected mitochondrial functions by altering hepatic mitochondrial oxidative phosphorylation [4], enhancing mitochondrial membrane permeability for protons and calcium ions, and inhibiting succinate dehydrogenase [4]. Moreover, glyphosate can elicit oxidative stress causing damage to lipids, proteins, and DNA [4], particularly to the membranes of erythrocytes and lymphocytes [5]. Furthermore, it affects glutathione content, levels of aromatase, and antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSHPx), and glutathione reductase (GR) [6].

Short-term studies in rodents did not show toxic effects of glyphosate [4]. However, life-long exposure to glyphosate demonstrated dysfunction of hepatic and renal organs, toxic manifestations, ossification deficiencies in the fetus of pregnant rats [7], elevated cancer risk, and short lifespan. The alterations in the urine and liver function markers and proteomic and metabolomic profiles were detected after two years in rats [8]. Moreover, glyphosate toxicity can be both species- and dose-dependent [9].

However, several pollutants can promote liver damage in mammals, which is the principle detoxification organ and the site for major biotransformations, including the formation of reactive oxygen species (ROS); superoxide anions (O2−), hydrogen peroxide (H2O2), hydroxyl radical (OH) [10]. Environmental pollutants induce damage via different pathways involving lipids, proteins, carbohydrates, and nucleic acids [11], leading to harmful effects. The antioxidant defense mechanism in animals, which consists of several antioxidant enzymes such as SOD, CAT, GSHPx, and GR, besides antioxidants of non-enzymatic nature, particularly glutathione (GSH), is activated to neutralize the ROS. A deficiency in this defense mechanism results in oxidative destruction and membrane lipid peroxidation [1]. This has been detected in several fish species [12]. Moreover, antioxidant enzyme activities and oxidative damage serve as oxidative stress indicators [13]. Because of the increasing use of glyphosate, along with lack of information on its toxic effects in pregnant rodents, we aimed to demonstrate its effects on specific biomarkers, including liver function enzymes, study oxidative and DNA damage, and histopathologically examine the hepatic tissue of male and female rat pups exposed to glyphosate through mother’s milk during lactation.

MATERIALS AND METHODS

Chemicals

Analytical grade glyphosate [N-(phosphonomethyl) glycine], (96%), was purchased from Sigma-Aldrich (St. Louis, MO, USA). The kits used for biochemical studies of AST, ALT, ALP, catalase (CAT), glutathion peroxidase (GSHPx), malondialdehyde (MDA) and total protein were obtained from Biodiagnostic company, 29 Tahrir Street, Dokki, Giza, Egypt. All other chemicals were of reagent grades and obtained from local scientific distributors in Egypt.

Animals

The pregnant female albino rats of the Wistar strain, weighing 220 to 245 g, were obtained from the animal house, National Research Centre of Egypt. The animal experiments were approved by the local committee, and the protocol complied with the National Research Centre (NRC, 2011) guidelines. The rats were acclimatized to the laboratory conditions for one week before the experiments. After the acclimation period, pregnant female rats (n = 8) were transferred and housed in eight stainless steel cages (one pregnant female in each cage) in the animal breeding room at 25 ± 2 °C with 45% relative humidity and dark/light cycle (12 h). The animals were fed a standard pellet diet and tap water ad libitum. The day of birth and the number of male and female pups in each litter were recorded and weighed. To maximize the lactation performance, male and female pups in each litter were randomly divided into five male and five female pups [14].
Experimental protocol
Eight adult pregnant female Wistar rats were randomly divided into four groups of two rats each.

Glyphosate was dissolved in corn oil and administered via the oral route to dams at a fixed volume of 0.5 mL/dame from the first day after parturition for 21 days (lactation period). Dams were weighed weekly to adjust the dose of glyphosate; suckling pups were grouped into G1: male and female pups (five each) of the two mother female rats that were administered 0.5 mL corn oil/dam daily (served as control); G2: male and female pups (five each) of the two mother female rats that were administered glyphosate at 0.3 mg/kg body weight for acceptable daily intake (ADI) [15]; G3: five male and female pups of the two mother female rats that were administered glyphosate 31 mg/kg body weight at no-observed-adverse-effect level (NOAEL) [15, 16] for 21 days; and G4: five male and five female pups suckling from the two mother rats that were administered glyphosate at 1/100 of acute oral toxicity (1/100 LD50) (56 mg/kg body weight) [15, 16].

Blood samples collection and tissue preparation
Post lactation period of pups (21 days), rats were made to fast overnight, and blood samples were obtained by puncturing the retro-orbital venous plexus of animals with a fine sterilized glass capillary and divided into two tubes. The first tube contained anticoagulant, and the blood was used for the comet assay. In the second tube not containing the anticoagulant, blood was allowed to clot and centrifuged at 3,000 rpm (600 g) for 10 min at 4 °C using Heraeus Labofuge 400R (Kendro Laboratory Products GmbH, Germany) to get the serum. The serum was stored at −20 °C for further biochemical analyses, such as ALT, AST, and ALP. The liver was removed immediately after the rats were sacrificed. It was weighed, washed in saline, and the relative liver weight was calculated. A small part of the liver was homogenized in 10% (w/v) ice-cold 100 mM phosphate buffer (pH 7.4) and centrifuged at 10,000 rpm. The supernatant obtained was used to measure oxidative stress (lipid peroxidation) and antioxidant enzymes (CAT and GSHPx). The other part of the liver was placed in 10% formalin and used for histopathological studies.

Biochemical parameters
Oxidative stress biomarkers in liver tissue
The activity of liver GSHPx and CAT was determined according to the method described by Paglia and Valentin [17] and Abei [18], respectively. Lipid peroxidation was estimated by determining thiobarbituric acid reactive substances (TBARS) and was expressed relative to MDA content using a colorimetric technique, according to Satoh [19]. Protein concentration in the homogenate was measured according to the method described by Gornal et al. [20].

Liver biomarkers in serum
Serum AST and ALT were measured according to the methods of Reitman and Frankel [21] and ALP according to Young et al. [22].

Determining DNA damage percentage using comet assay
The modified single-cell gel electrophoresis or comet assay was used to determine the percentage of DNA damage [23]. Blood samples collected from control and groups exposed to different doses of glyphosate were centrifuged at 200 rpm for 5 min. The pellet obtained was washed with an excess of ice-cold Hank’s balanced salt solution (HBSS), then immersed in it and easily minced into approximately 1 mm³ pieces. The minced blood cells were washed several times with cold phosphate-buffered saline (PBS) and dispersed into single cells using a pipette. The isolated cells were inserted into the agarose gel on microscopic slides. The cells were lysed with detergent at high salt concentrations overnight (in cold). To denature the DNA, the cells were treated with alkali for 20 min and electrophoresed under alkaline conditions (30 min) at 300 mA, 25 V. Ethidium bromide was used to stain the slides that were examined under a fluorescence microscope using a green filter at ×40 magnification. Approximately 100 cells were examined for each sample to determine the percentage of cells with DNA damage that appeared like comets. We selected the non-overlapping cells and visually ascribed a score on
Values were considered significant at $p \leq 0.05$. All statistical analyses were performed using SPSS software (version 18.0).

RESULTS AND DISCUSSION

Researchers have previously demonstrated the toxicity of glyphosate formulations on liver cells, including damage to the hepatic tissue and oxidative destruction [26].

The present results demonstrated a significant reduction in the relative liver weight in both male and female suckling pups following administration of 1/100 LD₅₀ dose, which was more than the reduction observed following administration of ADI and NOAEL doses (Figure 1A, B). The results suggested that the treatment with glyphosate affected the growth in rats. Tang et al. [26] observed low gain in the body weight in adult male rats following treatment with 5 to 500 mg kg⁻¹ body weight glyphosate for 35 days. The alteration in hepatic tissue weight following exposure to glyphosate has also been reported by other studies [27]. This could be hypothesized based on the concentration of glyphosate and the species exposed to it [4]. However, studies have reported no changes in liver weight [28] or even indicated an increase in it [4]. This increase could be

an arbitrary scale of 0 to 3 (class 0 = no detectable DNA damage and no tail; class 1 = tail with a length less than the diameter of the nucleus; class 2 = tail with a length between 1× and 2× the nuclear diameter; and class 3 = tail greater than 2× the diameter of the nucleus) based on the observed comet tail length migration and the relative ratio of DNA in the nucleus [24].

Histopathological studies

The liver was cut and dehydrated in a graded series of alcohol and fixed in paraffin wax. Five micrometer thick pieces were cut and stained by hematoxylin and eosin (H&E). Slides were prepared; each slide contained 10 field areas and two sections that were examined for histopathological changes under a light microscope (Olympus BX50) fitted with a digital camera (Olympus E-410). The histopathological alterations in liver tissues were scored as follows: normal appearance (―), mild (+), moderate (++), and severe (+++) [25].

Statistical analysis of results

Data are expressed as mean ± standard error (SE). One-way analysis of variance (ANOVA) was used to analyze the data, followed by the least significant difference (LSD) as a post-hoc test.
associated with non-alcoholic fatty liver disease and its evolution to non-alcoholic steatohepatitis [8, 29].

The current findings showed that the activity of ALT, AST, and ALP significantly increased in male and female pups following exposure to all doses \( (p \leq 0.05) \) with severe side-effects in G4 (Table 1). Parallel to these results, Benedetti et al. [7] stated that male Wistar rats exposed to glyphosate-Biocarb\(^b\) for 75 days displayed elevated activity of hepatic enzymes ALT and AST; enhanced cellular modifications manifested as excessive connective tissue and collagen fiber deposition in the liver. Identical results were achieved in our study, following treatment of both genders of pups with glyphosate for 21 days. Similarly, Cavusoglu et al. [30] intraperitoneally injected mice with a single dose (50 mg/kg body weight) of glyphosate-Roundup\(^b\) and demonstrated significant hepatic destruction post 15 days, with alterations in the activity of ALT and AST, and urea and creatinine levels, representative of hepatic and renal damage. These changes could be attributed to low glutathione levels and elevated lipoperoxidation.

For example, Kumar et al. [31] and Tang et al. [26] reported that exposure to glyphosate elevated the mRNA expression of inflammatory biomarkers and suggested that glyphosate-induced toxic effects on hepatic tissue were caused by inflammation and oxidative damage, particularly involving several lipid pathways. However, these findings warrant further work.

The liver is considered as the primary vital organ implicated in the biotransformation of xenobiotics such that it is the principal site for multiple oxidative reactions. Therefore, it has a high antioxidant activity, but this activity was insufficient to prevent the damage caused by the minimal dose (50 mg/kg body weight) of glyphosate-Roundup\(^b\). Elevated lipid peroxidation and inclusion of low levels of non-protein thiols in liver tissue strengthen the hypothesis that herbicides exert their toxic effects by generating ROS. According to Hazarika et al. [32] and Kavitha and Rao [33], lipoperoxidation occurs through the reaction between organophosphorus compounds and the cell membrane. These biochemical findings are well documented by the histopathological examination of hepatic tissue of glyphosate-treated pups that reveals dilatation and congestion in portal vein for all doses. Proliferated bile ducts and focal necrosis in the hepatic parenchyma was established in G3 and G4 in addition to fibrosis surrounding the bile ducts in G4 (glyphosate 1/100 LD\(_{50}\)) (Table 2, Figure 2A-D).

The results of the current study demonstrated significant inhibition of CAT and GSHPx activities along with a significant increase in lipid peroxidation, suggesting oxidative stress following exposure to all doses of glyphosate, with a more drastic effect for G4 (1/100 LD\(_{50}\) dose) (Table 3). Literature links high oxidative stress levels to low doses of glyphosate [34], indicating a high level of bioavailability, high concentrations of glyphosate in the blood over low-term, low-rate distribution/

Table 1. Liver dysfunction biomarkers in serum of male and female pups exposed to glyphosate during lactation period.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>ALP (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>G1</td>
<td>40.08±0.36(^a)</td>
<td>38.48±0.46(^a)</td>
<td>45.18±0.36(^a)</td>
</tr>
<tr>
<td>G2</td>
<td>46.10±0.73(^b)</td>
<td>43.95±0.50(^b)</td>
<td>52.30±0.62(^b)</td>
</tr>
<tr>
<td>G3</td>
<td>54.24±0.66(^c)</td>
<td>51.98±0.52(^c)</td>
<td>61.13±0.40(^c)</td>
</tr>
<tr>
<td>G4</td>
<td>64.04±0.46(^d)</td>
<td>63.86±0.42(^d)</td>
<td>73.13±0.65(^d)</td>
</tr>
</tbody>
</table>

Control (G1), glyphosate (ADI) (G2), glyphosate (NOAEL) (G3), and 1/100 of LD\(_{50}\) (G4). Values are means ± SE, \(n = 5\); values having the different letters are significantly different from each other at \(p \leq 0.05\).
transporting agents or open ion gates and that the exposure route (oral administration) and examined doses were extremely low to ascertain adequate availability for lipoperoxidation to occur. However, Astiz et al. [35] intraperitoneally administered glyphosate and documented high availability as elimination from the blood to different organs, and ROS generation that exhausts the antioxidants. Contrary to this, Milić et al. [4] did not detect elevated lipid peroxidation and attributed this finding to the fact that glyphosate could not be transported across the lipid membrane without

Table 2. Histopathological changes in the liver of male and female pups exposed to glyphosate and scoring severity of injury.

<table>
<thead>
<tr>
<th>Observation</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammatory cells in the portal area</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Focal necrosis in the hepatic parenchyma</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Diffuse kupffer proliferation</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
</tbody>
</table>

Normal (−), mild (+), moderate (++) severe (+++), Control (G1), glyphosate (ADI) (G2), glyphosate (NOAEL) (G3), and 1/100 LD50 dose (G4).

Figure 2 (A-D). Photomicrography of liver sections showing (A) normal liver tissue in control group with normal hepatocytes and central vein, (B) glyphosate-treated pup at 0.3 mg/kg body wt. showing dilatation and congestion in the portal vein as well as multiple newly formed bile ducts, (C) glyphosate-treated pup at 31 mg/kg body wt. with the portal area showing multiple newly formed bile ducts with congestion in the portal vein and focal necrosis in the hepatic parenchyma. (D) gluphosate-treated pup at 56 mg/kg body wt. with the portal area showing fibrosis specifically surrounding the bile ducts (H&E200x).
Glyphosate induces liver injury in suckling rat pups

The antioxidant defense system by elevating the activity of GSHPx in the hepatic tissue. Our results indicated severe DNA damage and increased comets in blood of lactational pups treated with the 1/100 LD50 dose of glyphosate (G4) than that observed in pups treated with ADI and NOAEL doses (G2 and G3, respectively) (Table 4, Figure 3A-C). This could be attributed to the fact that this compound damaged DNA, resulting from the higher activity of caspases 3 and 7, responsible for initiating cellular apoptosis following high levels of ROS [35]. The hypothesis of oxidative stress has been strengthened by the findings of Jasper et al. [1], who recorded adducts of DNA in the mice renal and hepatic tissues and activated CAT in the hepatic tissue of rats that were administered glyphosate. The same authors reported inhibitory activity of cytochrome P-450 and monooxygenase in the hepatic tissue of rats treated with glyphosate-Roundup®. In good agreement with our findings, Milić et al. [4] reported the presence of DNA damage using comet assay. Our study suggested that exposure to glyphosate highly affected the DNA in the blood cells.

**CONCLUSION**

The exposure of pups to even low levels of glyphosate during lactation for a short-term duration enhanced histopathological alterations in hepatic tissue and DNA deterioration due to elevated oxidative stress. The comprehensive

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**Table 3.** Effect of glyphosate on liver antioxidant enzymes CAT, GSH-Px and lipid peroxide activities in the liver tissue of female and male pups.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>CAT (u/mg protein)</th>
<th>GSH-Px (u/mg protein)</th>
<th>Lipid peroxidation (nmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>G1</td>
<td>43.41±0.61b</td>
<td>45.41±0.37b</td>
<td>6.28±0.06d</td>
</tr>
<tr>
<td>G2</td>
<td>42.16±0.40b</td>
<td>44.01±0.49a</td>
<td>4.54±0.05c</td>
</tr>
<tr>
<td>G3</td>
<td>39.43±0.36a</td>
<td>43.81±0.52a</td>
<td>4.10±0.02b</td>
</tr>
<tr>
<td>G4</td>
<td>39.03±0.38a</td>
<td>43.41±0.43a</td>
<td>3.58±0.01a</td>
</tr>
</tbody>
</table>

Control (G1), glyphosate (ADI) (G2), glyphosate (NOAEL) (G3), and 1/100 of LD50 (G4). Values are means ± SE, n = 5; values having the different letters are significantly different from each other at p ≤ 0.05.
and water must be performed as this could result in serious health problems, including liver damage, inflammation, cancer, and neurodegenerative diseases — all linked to ROS production.

universal use of several glyphosate formulations emphasizes the principle of our detection. Further studies on the effects of long-term exposure to low concentrations of glyphosate in polluted soil and water must be performed as this could result in serious health problems, including liver damage, inflammation, cancer, and neurodegenerative diseases — all linked to ROS production.

Table 4. Visual score of DNA damage in blood cells collected from pups exposed to glyphosate through mother’s milk.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No of samples</th>
<th>No. of cells Analyzed*</th>
<th>Class**</th>
<th>DNA damaged cells % (Mean±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Comets</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>4 400</td>
<td>33</td>
<td>367</td>
<td>31</td>
</tr>
<tr>
<td>G2</td>
<td>4 400</td>
<td>37</td>
<td>363</td>
<td>25</td>
</tr>
<tr>
<td>G3</td>
<td>4 400</td>
<td>51</td>
<td>349</td>
<td>27</td>
</tr>
<tr>
<td>G4</td>
<td>4 400</td>
<td>83</td>
<td>317</td>
<td>24</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>4 400</td>
<td>35</td>
<td>365</td>
<td>32</td>
</tr>
<tr>
<td>G2</td>
<td>4 400</td>
<td>40</td>
<td>360</td>
<td>24</td>
</tr>
<tr>
<td>G3</td>
<td>4 400</td>
<td>54</td>
<td>346</td>
<td>28</td>
</tr>
<tr>
<td>G4</td>
<td>4 400</td>
<td>80</td>
<td>320</td>
<td>23</td>
</tr>
</tbody>
</table>

*: Number of cells examined per a group. **: Class 0 = no tail; 1 = tail length < diameter of nucleus; 2 = tail length between 1X and 2X the diameter of nucleus; and 3 = tail length > 2X the diameter of nucleus.

Figure 3 (A-C). Visual score of DNA damage in blood cells collected from pups exposed to glyphosate through mother’s milk.

A: Visual score of normal DNA (class 0) using comet assay in blood samples.
B: Visual score of DNA damage (classes 1 and 2) using comet assay in blood cells.
C: Visual score of DNA damage (class 3) using comet assay in blood cells.
ACKNOWLEDGEMENT
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CONFLICT OF INTEREST STATEMENT
The authors declare no conflict of interest.

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