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Original Article

Hematological, biochemical, antioxidant and histopathological alterations in kidneys of Wistar rat pups exposed to glyphosate herbicide during lactation period

Amel A. Refaie[1], Amal Ramadan[2,*] and Hanan F. Aly[3]

[1]Environmental Toxicology Research Unit (ETRU), Pesticide Chemistry Department;
[2]Department of Biochemistry, Genetic Engineering and Biotechnology Research Division;
[3]Department of Therapeutic Chemistry, Pharmaceutical and Drug Industries Research Division, National Research Centre (NRC), El Behouth St., P. O. 12622, Giza, Egypt.

ABSTRACT

Information about the adverse effects caused by glyphosate herbicide transferred during lactation to pups is important for human health. So, in the present study, we demonstrated the effects of glyphosate administered during lactation period of pup rats at a daily dose of 0.3 mg/kg body weight [the acceptable daily intake (ADI)], 31 mg/kg body weight [no-observed-adverse-effect level (NOAEL)] and 56 mg/kg body wt. [1/100 LD50] for 21 days. At the end of each treatment, the suckling pups were separated into male and female, and the relative kidney weights were measured. The levels of creatinine and urea were determined in serum. Oxidative stress was measured using lipid peroxidation and the activities of glutathione peroxidase (GPx) and catalase (CAT) in kidney tissue were also measured. Red blood corpuscle (RBC) and white blood cell (WBC) counts and hemoglobin content (Hb) were measured. Histopathological examination was also performed in kidney tissues. The pups showed increased relative kidney weight with the highest increment in 1/100LD50 dose followed by NOAEL and ADI doses. Also, marked increment in malondialdehyde (MDA) level along with significant inhibition in catalase (CAT) and glutathione peroxidase (GPx) activities were detected in pup's kidney tissues. Counts of WBCs and RBCs and Hb content exhibited significant reduction in the blood of pups that received 1/100 LD 50 dose which is more than the NOAEL and ADI doses. Histopathological examination of kidney tissue demonstrated focal fibrosis with inflammatory cells between glomeruli and tubules of male and female pup kidney tissue (lower scoring severity (+) in ADI and NOAEL doses than 1/100LD50 (+++)). Additionally, at 1/100LD50 dose focal hemorrhages, degradation in tubular lining epithelial cells (+) and renal arteries congestion (++) were observed. These results revealed that even exposure to minimal glyphosate levels can have dangerous adverse effects on renal tissues.

KEYWORDS: kidney, oxidative status, glyphosate, rats, lactation period.

1. INTRODUCTION

Glyphosate (N-(phosphonomethyl)glicine), also known by the trade names Roundup and Rodeo is considered as an organophosphate herbicide used in controlling undergrowth prior to seeding. This herbicide is widely applied for controlling weeds in areas of non-crop land, and control of aquatic weed in fish ponds [1, 2].

In rodents, the toxic action mechanisms of glyphosate have not been completely understood. A lower ratio of respiratory control, enhancement

*Corresponding author: amalramadan2006@yahoo.com

of activity of ATPase and rate of oxygen consumption were noticed in hepatic rats' mitochondria exposed to glyphosate [3]. Depending on these findings, it was suggested that these effects of glyphosate may be attributed to the uncoupling oxidative phosphorylation process. Additionally, oxidative stress has been involved in the toxicological molecular mechanisms of glyphosate [4].

The exposure to and the metabolism of glyphosate in renal tissue can stimulate the production of malonaldehyde (MDA), and oxidative stress through reactive oxygen species (ROS) generation. ROS in turn can induce damage of lipids, proteins, DNA and modified functions of gene essentially for growth [2].

The production of ROS is regulated by the antioxidant enzymes' defense because these enzymes' level may reflect the alteration in physiological states during pregnancy [5]. Also in developing countries, breastfeeding may be the only source of nutrition for infants in the first critical weeks of life. So, it is important for human health to study the adverse effects caused by glyphosate herbicide transferred during lactation to pups.

Hence, the present study aimed to evaluate the antioxidant enzymes GPx and catalase as antioxidant biomarkers after glyphosate exposure. Besides, lipid peroxidation was measured in male and female pups. Additionally, the effects on haematological profile of pup rats following 21 days of exposure to the glyphosate along with the toxic effect of glyphosate on kidney function, blood urea and creatinine were determined. Histopathological examination of kidney tissue was also investigated.

2. MATERIALS AND METHODS

2.1. Chemicals

Analytical grade glyphosate [*N*-(phosphonomethyl) glycine], (96%), was purchased from Sigma-Aldrich (St. Louis, MO, USA). The kits used for biochemical studies of creatinine, urea, catalase, glutathion peroxidase, 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.

2.2. Animals

The pregnant albino rats of the Wistar strain weighing 220-245 g were obtained from the animal house, National Research Centre of Egypt. The local committee approved the design of the experiments, and the protocol corresponds to the guidelines of the National Research Centre (NRC, 2011). The rats were acclimatized to the laboratory conditions for one week prior to the experiment. After acclimation period, the pregnant female rats (n = 8) were housed in stainless steel cages (one pregnant female in each cage) in breeding animals' room at 25 ± 2 °C with 45% relative humidity, dark/light cycle (12-h), and were fed standard pellet diet and tap water *ad libitum*. The number of male and female pups in each litter was recorded and weighed. To maximize the lactation performance, male and female pups in each litter were randomly divided into five male and female pups [6].

2.3. Experimental design

Eight adult pregnant female Wistar rats were randomly divided into four groups of two rats each. Glyphosate was dissolved in corn oil and administrated *via* the oral route to dams at a fixed volume of 0.5 ml/dam from the first day after parturition for 21 days (lactation period). Dams were weighed weekly to adjust the dose of glyphosate. The suckling pups were grouped as follows: **G1**: male and female pups (five each) of the two mother female rats who 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 who were administered glyphosate at 0.3 mg/kg body weight for acceptable daily intake (ADI) [7], **G3**: five male and female pups of the two mother female rats who were administered glyphosate at no-observed-adverse-effect level (NOAEL) for 21 days (31 mg/kg body weight) [8], **G4**: Five male and five female pups suckling from the two mother rats who were administered glyphosate at 1/100 of acute oral toxicity (1/100LD50) (56 mg/kg b. wt) [8].

2.4. Blood sample collection and tissue preparation

Post lactation cycle of pups (21 days), rats were fasted overnight and blood samples were obtained by puncturing the retero-orbital venous plexus of animals with a fine sterilized glass capillary. The blood sample was divided into two portions; the first portion was collected in an anticoagulant (EDTA) tube and used for blood cell count and Hb content measuring. The second portion of blood was collected in centrifugal tubes without anticoagulant, and left to clot in these dry tubes and centrifuged at 3000 rpm (600 x g) for 10 min at 4 °C using Heraeus Labofuge 400R (Kendro Laboratory Products GmbH, Germany) to get the sera. Sera were stored at -20 °C for further biochemical analysis including blood urea, and creatinine determination. Then, the rats were sacrificed by decapitation. Kidney was removed immediately after sacrification, weighed, washed in saline and relative kidney weight was calculated. A small part of the kidney was homogenized in 10% (w/v) ice cold 100 mM phosphate buffer (pH 7.4) and centrifuged at 10,000 rpm for 15 min at 4 °C, then the supernatant was obtained and used for oxidative stress (lipid peroxidation) and antioxidant enzyme measurements (CAT and GPx). Then, small pieces of kidney samples were cut and kept in 10% natural formalin and used for histopathological investigations.

2.5. Biochemical parameter determination

2.5.1. Oxidative stress biomarkers in kidney tissues

Kidney glutathione peroxidase and catalase enzyme activities were determined according to the method of Paglia and Valentin [9] and Abei [10]. Lipid peroxidation (LPO) was estimated by determining thio-barbituric acid reactive substances (TBARS) and was expressed in relation to malondialdehyde (MDA) content using a colorimetric technique according to Satoh [11]. The MDA values were expressed as nanomoles of MDA per gram tissue. Total protein content in homogenate was measured according to the method described by Gornal *et al*. [12].

2.5.2. Kidney function in serum

Urea and creatinine were measured according to the method of Tietz [13].

2.6. Measurements of blood constituents

Red blood corpuscles (RBC) and white blood cells (WBC) were counted according to Britton [14] and Seivered [15]; Heamoglobin (Hb) measurement was carried out as per Wintrobe [16].

2.7. Histopathological examination

Kidney specimens of male and female pups were cut and dehydrated in graded series of alcohol, cleaned in xylene and fixed in paraffin wax. Five micrometer thick pieces were cut and stained by hematoxylin and eosin (H&E). Two slides were prepared for this organ; each slide contained ten field areas and two sections were examined for histopathological changes. The examination was done using a light microscope (Olympus BX50) with a digital camera (Olympus E-410). According to Michael (2008), the histopathological alterations in kidney tissues were scored as follows: normal appearance (−), mild (+), moderate (++) and severe (+++) [17].

2.8. Statistical analysis

Data were reported as mean ± SE. Data were analyzed using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test as a post-hoc test. The level of significance between mean values was set at $P \leq 0.05$. All statistical analyses were performed using SPSS software (Version 18.0).

3. RESULTS AND DISCUSSION

The present results indicated significant increase in relative kidney weight (%) post exposure of male and female pups to glyphosate during lactation period with G4 >G3 >G2, compared to control group (G1) (Table 1). In this context, Mossa and Abbassy [18], explained that the variation in the body weights post exposure to the insecticide is an important factor determining the toxicity of insecticide. The decrement in body weight and the elevation of relative organ weight may be related to the degradation in the lipids and proteins as a direct effect of glyphosate as well as herbicide accumulation in renal tissues [19-21].

The effects of glyphosate on renal function revealed significant increase in creatinine and urea levels in both male and female pups in all treated groups compared with control group, where G4

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

Treatments	Creatinine (mg/dl)		Urea (mg/dl)		Relative kidney weight (%)	
	Female	Male	Female	Male	Female	Male
G1	1.25 ± 0.04^{a}	1.54 ± 0.011^{a}	34.34 ± 0.32^{a}	36.14 ± 0.42^{a}	0.52 ± 0.005^{a}	0.63 ± 0.006^{a}
G2	$1.35 \pm 0.01^{a\,b}$	1.63 ± 0.005^{b}	41.71 ± 0.39^{b}	44.91 ± 0.33^{b}	0.63 ± 0.006^{b}	0.73 ± 0.007^{b}
G3	1.43 ± 0.03^{b}	1.73 ± 0.012^{c}	45.95 ± 0.38^{c}	53.95 ± 0.58^{c}	0.74 ± 0.005^{c}	0.77 ± 0.008^{c}
G4	1.78 ± 0.02^{c}	1.77 ± 0.008^{d}	55.90 ± 0.67^{d}	66.10 ± 0.43^{d}	0.78 ± 0.002^{d}	0.83 ± 0.005^{d}

G1: control group, G2: glyphosate (ADI), G3: glyphosate (NOAEL), G4: glyphosate (1/100LD50). Values expressed as a mean of 5 animals ± S.E.; $^{a, b, c, d}$ values having different letters significantly different at $p \leq 0.05$.

exhibited more noticeable significant increase in both blood urea and creatinine levels than G3 and G2 (G4 >G3 >G2) at $p \leq 0.05$ (Table 1). The significant increase observed in our work suggested the nephrotoxic effect of the herbicide. A previous work has reported elevation in blood creatinine and urea levels, as the exposure time to glyphosate increased [22]. Also, an elevation in serum creatinine was found in rats treated with sumithion [23]. Abu-El-Zahab *et al*. [24] and Sakr *et al*. [25], showed similar results in pyrethroid-treated rats. Blood urea and creatinine levels have been used as indicative markers for the diagnosis of kidney injury [3].

Our results indicated that, the sub-chronic exposure to glyphosate (21 days) caused high level of serum urea in all selected doses of treated pups compared to control. This finding is in agreement with Tizhe *et al*. [2], Mossa and Abbassy [18], and Caglar and Kolankaya [26] who examined the doses 56 and 560 mg/kg/day of Roundup® (glyphosate) and declared that the level of blood urea reached 2.1-fold and 1.9-fold, respectively compared to control. Further, the high creatinine level in the glyphosate-treated pups is in agreement with the result of Tizhe *et al*. [2], Caglar and Kolankaya [26] who found high level of creatinine post Roundup® exposure in rats, which may be attributed to renal dysfunction.

Mossa and Abassay [18] explained that the elevated level of blood urea appears to be consistent with an increase in protein degradation. It may also be associated with the stimulation process of ammonia transformation to urea as a result of increase in the enzyme synthesis involved in the urea production. Other researchers reported increase in total blood urea and creatinine in chicks and rats exposed to acute and chronic toxicity of 2,4-D cypermethrin [27, 28]. Additionally, creatinine is a metabolite of creatine and is completely discharged in urine through filtration process of glomeruli. So, the increase of its level in blood is regarded as an indication of renal dysfunction [29]. Following toxicant exposure, it is transferred to different organs through blood circulation, including hepatic and renal tissues where they may ultimately produce damaging effects [30].

In our study, the significant reduction in the RBC count and haemoglobin level post 21 days of lactation is indicative of anemic condition (Figure 1). This is consistent with previous results of Holy *et al*. [31], who found reduction in the RBC count post ip injection of dichlorvos. On the contrary, Celik *et al*. [32] did not notice any change in RBC count and haemoglobin values in rats that ingested 5 and 10 ppm of dichlorvos. The indices of red blood cells reflect the size of MCV and content of haemoglobin (MCH and MCHC) in the blood cells and help in the diagnosis of anemia. This could be attributed to the high damage of red blood cells by the glyphosate beyond the capacity of bone marrow production [32], and the drop in the iron level in the body. The reduction in WBC count ($p \leq 0.05$) post 21 days in our study is in contradiction with Holy *et al*. [31] and Celik *et al*. [32] who found that the elevation in the WBC and platelet counts are indicative of leukocytosis and

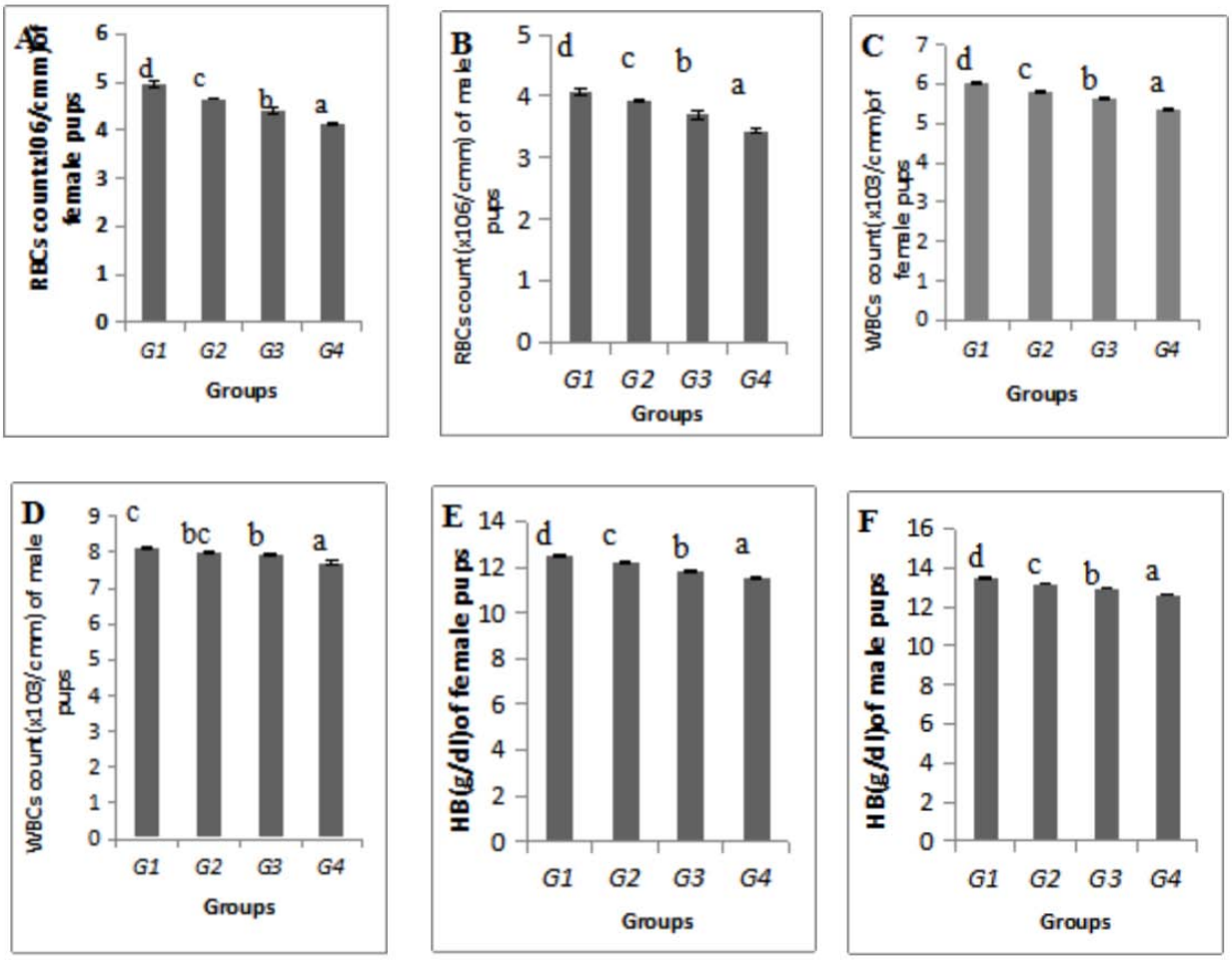

Figure 1. Effect of glyphosate herbicide-induced alteration in red blood cells (RBC count) (A, B), white blood cells (WBC count) (C, D) and heamoglobin) (Hb) (g/dl) (E, F) in female and male pups. G1: control group, G2: glyphosate group (ADI), G3: glyphosate group (NOAEL), G4: glyphosate group (1/100LD50). Each value is a mean of 5 rats ± SE; $^{a, b, c, d}$ values not sharing superscript letters (A, B, C, D) differ significantly at $p \leq 0.05$.

thrombocytosis, respectively. WBCs are synthesized using bone marrow and their low count may be due to viral infections, inflammatory disease, and hemolytic anemia, and also may be due to cancer and autoimmune disease [3]. In this regard, Akomas *et al*. [33] reported that the hypochronic anemia is related to the drop in the body iron content due to oxidative stress.

In concomitant with the present results Mossa and Abbassy [18] declared chlorpyrifos methyl as a formulated organophosphorous insecticide that produced a noticeable reduction in the Hb level and packed cell value (PCV%), which are markers of red blood cell volume, RBC and WBC counts. A previous work of Mossa [34] significantly correlated the pesticides' effects with the decrease in number of erythrocytes, PCV and Hb content. It was suggested that, these alterations were related to the increase in RBC breakdown and/or to the direct toxicological effect of pesticides on bone marrow. Further, the low content of Hb may be attributed to the high level of RBC breakdown and/or low RBC synthesis [34]. Shakoori *et al*. [35] previously documented that the low RBC count is either a predictive factor for severe erythrocyte damage or inhibition in the rate of RBC synthesis.

Pups exposed to glyphosate herbicide showed significant inhibition in antioxidant enzyme activities CAT and GPx with G4 >G3 >G2,

as compared to control levels ($p \leq 0.05$), while, significant increase in MDA level in all treated groups was recorded (G4 >G3 >G2) (Table 2). Bhardwaj *et al*. [36] found that exposure to glyphosate induced oxidative stress by ROS production. The generation of oxidative stress starts when the free radicals rise beyond the body's antioxidant defence capabilities. The inhibition in CAT and GPx activities recorded in the current study is in disagreement with earlier results of Edem *et al*. [37], Dwivedi and Flora [38]. However, the inhibition in these enzymes post dichlorvos ingestion or intravenous injection has been documented [22]. Beuret *et al*. [39] explained that the high level of peroxidation is associated with the low antioxidant defense system, while, the higher adverse effects in fetal than pregnant animals confirmed the present results.

The histopathological examination clearly indicated focal fibrosis with inflammatory cells between glomeruli and tubules of renal tissue with severity score (+) in G2 and G3 and (+++) in G4. Also, focal hemorrhages and degradation in tubular lining epithelial cells were noticed in G4 (+). Besides these, renal artery congestion (++) was noticed (Table 3, Figure 2). The finding in our study is parallel with that of Al-Sahhaf [23], who explained that treating animals with the organophosphorous insecticide sumithion for 12 days showed diverse histopathological alterations in the renal tissue, such as degeneration of kidney tubules, atrophied glomerulus, high damage in renal tubules, loss in their identified shape and corrosion in the walls of Bowman's capsule. Similarly, Abdeen *et al*. [40] observed that mice treated with fenvalerate showed renal tubule degeneration and enlargement of the glomerulei. Additionally, subchronic feeding of the pesticide decarboxy fenvalerate was found to induce glomerulonephrosis in rats' renal tissue [41].

Table 2. Effect of glyphosate on kidney antioxidant enzyme activities and lipid peroxidation level in the kidney tissue of female and male pups.

Treatments	Catalase (u/mg protein)		Glutathion–peroxidase (u/mg protein)		Lipid peroxidation (nmol MDA/g tissue)	
	Female	Male	Female	Male	Female	Male
G1	38.46 ± 0.36^{d}	39.86 ± 0.35^{b}	4.54 ± 0.01^{c}	6.24 ± 0.05^{d}	0.48 ± 0.004^{a}	0.68 ± 0.004^{a}
G2	36.41 ± 0.07^{c}	37.86 ± 0.54^{d}	4.51 ± 0.02^{c}	5.41 ± 0.09^{c}	0.53 ± 0.006^{b}	0.69 ± 0.004^{a}
G3	34.66 ± 0.70^{b}	34.06 ± 0.43^{c}	4.25 ± 0.03^{b}	5.10 ± 0.02^{b}	0.57 ± 0.006^{c}	0.71 ± 0.006^{b}
G4	31.98 ± 0.28^{a}	25.19 ± 0.71^{a}	3.78 ± 0.01^{a}	4.19 ± 0.03^{a}	0.72 ± 0.003^{d}	0.91 ± 0.004^{c}

Control (G1), Glyphosate (ADI) (G2), Glyphosate (NOAEL) (G3), and 1/100LD50 (G4). Values are means ± SE, n = 5; values having different letters are significantly different from each other.

Table 3. Histopathological changes in the kidney of female and male pups exposed to glyphosate based on scoring severity of injury.

Organ	Observation	G1	G2	G3	G4
Kidney	Focal fibrosis with inflammatory cells between glomeruli and tubules	-	+	+	+++
	Focal hemorrhages & degeneration in tubular lining epithelial cells	-	-	+	+
	-Congestion	-	-	-	++

Normal (−), mild (+), moderate (++), severe (+++). G1: control group, G2: glyphosate (ADI), G3: glyphosate (NOAEL), G4: glyphosate (1/100LD50).

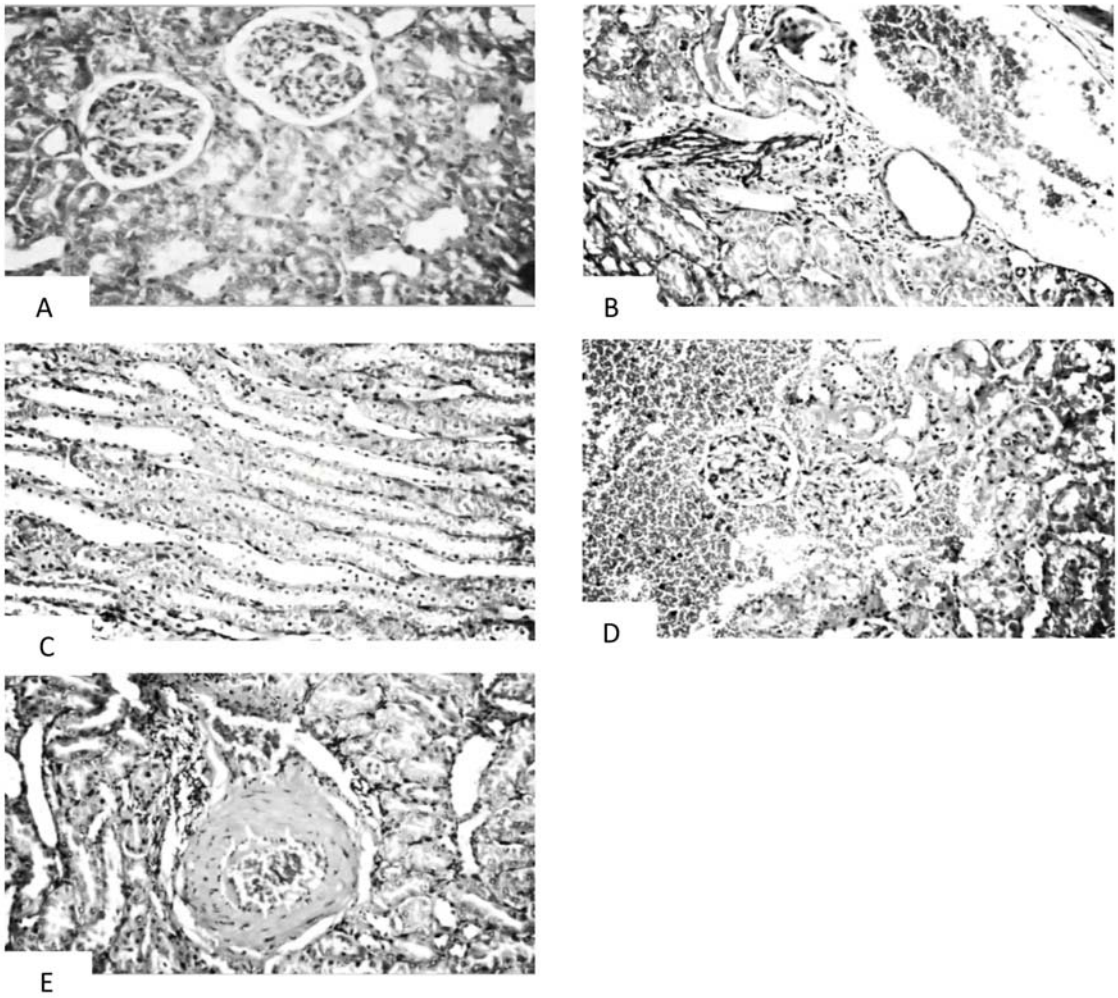

Figure 2. (A-E): Photomicrography of kidney sections of control pups (A) showing normal renal architecture of the glomeruli and tubules at the cortex. Glyphosate- treated pups at dose 0.3 mg/kg body wt. showing focal fibrosis with inflammatory cell infiltration in between the tubules at the cortex (B). Glyphosate-treated pups at dose 31 mg/kg body wt. showing architecture degeneration in the tubular lining epithelium (C) and at dose 56 mg/kg. body wt. showing (D&E) focal haemorrhage at the cortex and disrupted congestion in the sclerotic blood vessels with perivascular inflammatory cell infiltration (H&E 200x).

Thus, the elevation in creatinine and total urea together with the histopathological results proved that glyphosate induced renal injury in pups.

4. CONCLUSION

The exposure of pups to glyphosate herbicide *via* breastfeeding showed increase in relative kidney weight and kidney function, while, it lowered hematological values and induced perturbations in antioxidant status in male and female pups. Severe histopathological alterations were also examined in renal tissue of pups. Glyphosate showed a dose-dependent relationship, where 1/100LD50 showed more drastic effects than the other selected two doses.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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