Effect of zinc supplementation on chronic hepatorenal toxicity following oral exposure to glyphosate-based herbicide (Bushfire®) in rats

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Abstract

Objectives: To assess the effects of zinc pretreatment on hepatorenal toxicity following chronic exposure to glyphosate-based herbicides in male rats.

Methods: Following zinc pretreatment (50 mg/kg and 100 mg/kg), 14.4 to 750 mg/kg of oral glyphosate (Bushfire® herbicide) was administered daily for 36 weeks. Thereafter, serum samples were obtained following jugular venipuncture. Liver and kidney samples were processed for histopathological examination.

Results: Serum aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase activity as well as levels of bicarbonate, calcium, creatinine were significantly increased following chronic exposure to Bushfire®. Serum levels of sodium, potassium, chloride, total protein,
albumin, globulin and urea were unchanged. Moderate to severe coagulative necrosis of hepatocytes as well as glomerular and renal tubular necrosis were observed in herbicide-treated rats. Zinc pretreatment reduced the elevation of serum enzymes associated with hepatobiliary lesions, abrogated hypercalcemia and metabolic alkalosis, and mitigated serum accumulation of creatinine following Bushfire® exposure, but was ineffective in completely preventing histological lesions.

**Conclusion:** Chronic Bushfire® exposure in rats caused hepatorenal toxicity. The effects of exposure on serum parameters were ameliorated by zinc pretreatment, but the histopathological changes associated with toxicity persisted in milder forms in zinc-pretreated animals.

**Keywords**
Zinc, liver, kidney, dysfunction, glyphosate-based herbicide, chronic exposure, toxicity, Wistar rat

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**Introduction**
Glyphosate is a non-selective, post-emergent, systemic herbicide that inhibits 5-enolpyruvylshikimate 3-phosphate synthase in the shikimic acid pathway, preventing aromatic acid synthesis in plants. Low toxicity of glyphosate in humans and animals was anticipated because the shikimic acid pathway is restricted to plants and some microorganisms. In animals, glyphosate interferes with mitochondrial oxidative phosphorylation leading to increased generation of reactive oxygen species and induction of oxidative stress. These effects occur even at permissible daily intake levels. Human and animal exposure to the herbicide occurs through consumption of residues in foodstuffs and drinking water, which emanate from surface runoff and leach into the soil following vegetation sprays. Over the past decade, an accumulating body of evidence has illustrated the toxic effects of glyphosate. Hepatorenal toxicity of glyphosate in animals has been reported in previous studies at low and ultralow doses. Zinc is an essential component of the structure of the enzyme, superoxide dismutase, catalyzing the conversion of two superoxide radicals into hydrogen peroxide and molecular oxygen, thereby reducing the toxicity of reactive oxygen species. Zinc also influences the activity of glutamate-cysteine ligase, the rate limiting enzyme for de novo synthesis of glutathione, and acts as a cofactor for glutathione peroxidase in the antioxidant defence system. Therefore, zinc supplementation has been used in some studies to mitigate oxidative stress induced by environmental toxins. Frequent dietary intake of or supplementation with zinc is required to meet physiological needs, especially with respect to its function in antioxidant defence system, because this trace element is not stored in substantial amounts in tissues.

Our previous study demonstrated that oral zinc supplementation ameliorated the serum biochemical and histological aberrations associated with subchronic glyphosate toxicity (10% LD50) to the liver and kidney following oral exposure of rats for 8 weeks. In this subsequent study, the effect of zinc supplementation on serum biochemical changes and the histological lesions was investigated in rats chronically
exposed to glyphosate (up to 20% LD<sub>50</sub>) for a period of 36 weeks. Our goal was to ascertain whether prolonged zinc supplementation could mitigate the chronic toxicity induced by increasing doses of glyphosate in a herbicide.

**Materials and Methods**

**Experimental animals**

Approval was obtained from the Ethics Committee on Animal Use and Care of Ahmadu Bello University, Zaria, Kaduna State, Nigeria prior to commencement of the study. Twenty-eight adult male Wistar rats, with initial weights of 140 to 150 g, were obtained from the National Institute for Trypanosomiasis and Onchocerciasis Research (NITOR), Vom, Nigeria. The housing, feeding, and handling of the animals were as previously described.

**Herbicide composition and zinc supplementation**

The glyphosate-based herbicide (Bushfire®, Ningbo Agro-star Industrial Co., Ltd., Zhejiang, China) contained 360 g/L of glyphosate in the form of 441 g/L of the potassium salt. A 2% glyphosate solution was prepared by diluting 1 mL of the glyphosate-based herbicide solution with 49 mL of distilled water. The herbicide was used for toxicity studies at the standard concentration applied in agricultural spraying for a herbicidal effect (2% solution of the herbicide in distilled water).

The oral zinc supplement was prepared as a 2% aqueous solution of zinc chloride (1 g of zinc chloride crystal dissolved in 50 mL of distilled water).

**Chronic toxicity study**

Eighty rats were randomly divided into eight groups of 10 animals as follows:

- **Group I (DW) rats** served as controls and were administered 2 mL/kg of distilled water daily.
- **Group II (Z) rats** were administered zinc chloride at a dose of 50 mg/kg body weight.
- **Group III (G1) rats** were administered glyphosate orally at 14.4 mg/kg body weight (<1% LD<sub>50</sub>).
- **Group IV (G2) rats** were administered glyphosate orally at 375 mg/kg body weight (10% LD<sub>50</sub>).
- **Group V (G3) rats** were administered glyphosate at 750 mg/kg body weight (20% LD<sub>50</sub>).
- **Group VI (ZG1) rats** were pretreated with zinc chloride (50 mg/kg) and then administered glyphosate at 14.4 mg/kg body weight 1 hour later.
- **Group VII (ZG2) rats** were pretreated with zinc chloride (50 mg/kg) and then administered glyphosate at 375 mg/kg body weight (10% LD<sub>50</sub>) 1 hour later.
- **Group VIII (ZG3) rats** were pretreated with zinc (100 mg/kg) and then administered glyphosate at 750 mg/kg (20% LD<sub>50</sub>) 1 hour later.

Treatments were administered per os by gavage once daily for 36 weeks as recommended for standard toxicological studies. Body weight changes were monitored weekly using a digital electronic balance (Hangzhou Gongheng, Hangzhou, China) to enable appropriate dosing. Blood and tissue (liver and kidney) samples were collected from each rat at the end of the 36-week study period to evaluate hepatorenal toxicity via serum biochemical and histopathological evaluations.

**Serum biochemical analysis**

Blood samples (about 5 mL) were collected without anticoagulant from the jugular vein of each rat. The blood samples were allowed to clot at room temperature for 30 minutes, then centrifuged at 800 × g for
10 minutes to obtain sera. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) activities and levels of total protein, albumin, electrolytes (sodium, chloride, potassium, bicarbonate, calcium), urea and creatinine were determined using the Bayer Express plus Clinical Chemistry Autoanalyser (Bayer®, Leverkusen, Germany). Globulin concentration was calculated by subtracting the albumin concentration from the total protein concentration.

**Histopathological examination**

Tissue samples of the liver and kidney were collected and fixed in 10% neutral buffered formalin. The samples were dehydrated in graded concentrations of alcohol (70%, 80%, 95% and 100%), cleared using xylene, impregnated with paraffin wax, incubated in a vacuum air oven at 60°C, embedded in plastic embedding rings, cut into 5-μm sections using a microtome, deparaffinized with xylene, rehydrated in graded concentrations of alcohol (100%, 95%, 80% and 70%) and stained with haematoxylin and eosin (H&E). The tissue sections were examined by light microscopy to assess the presence of lesions and photomicrographs were taken.

**Statistical analysis**

Data were presented as means ± standard errors of the means. Differences among multiple groups were evaluated by one-way analysis of variance followed by Tukey’s post-hoc test using GraphPad Prism Version 4.0 for Windows (GraphPad Inc., La Jolla, CA, USA). Values of \( p < 0.05 \) were considered statistically significant. Any relative difference of >10% was noted even in the absence of statistical significance.

**Results**

**Effects of zinc and glyphosate treatments on serum electrolyte concentrations**

There was no significant difference in sodium (\( \text{Na}^+ \)), chloride (\( \text{Cl}^- \)) or potassium (\( \text{K}^+ \)) concentrations between any of the groups (Figure 1a). However, there were decreasing trends in \( \text{K}^+ \) concentrations observed in groups ZG1 (13.1%), G1 (13.8%), G3 (15.4%) and G2 (18.0%) compared with the DW group (Figure 1a). There was a significant (\( p = 0.02 \)) increase in the bicarbonate (\( \text{HCO}_3^- \)) concentration in the G3 group compared with the DW group. There were increased trends in \( \text{HCO}_3^- \) concentrations in groups Z (25%), ZG3 (50%), ZG1 (85%), ZG2 (102.8%), G1 (131%) and G2 (150%) compared with the DW group (Figure 1a).

There was a significant (\( p = 0.002 \)) increase in calcium (\( \text{Ca}^{2+} \)) concentrations in the G2 group compared with the DW group (Figure 1b).

**Effects of zinc and glyphosate treatments on serum enzyme activities**

There were significant (\( p < 0.001 \)) increases in AST activity in the G3, G2 and G1 groups compared with the DW group. There were increasing trends in AST activity in the Z (26%), ZG1 (104%), ZG2 (109%) and ZG3 (109%) groups compared with the DW group (Figure 2).

ALT activity was significantly (\( p < 0.001 \)) higher in the G2 and G3 groups compared with the DW group. There was an increasing trend in ALT activity in the G1 group (80.9%) compared with the DW group and a decreasing trend in ALT activity in the ZG1 (12%), ZG3 (29%) and Z (54%) groups compared with the DW group (Figure 2).
Figure 1. (a) Serum electrolyte concentrations in Wistar rats treated with 2 mL/kg distilled water (DW), 50 mg/kg zinc (Z), 14.4 mg/kg glyphosate (G1), 375 mg/kg glyphosate (G2), 750 mg/kg glyphosate (G3), 50 mg/kg zinc + 14.4 mg/kg glyphosate (ZG1), 50 mg/kg zinc + 375 mg/kg glyphosate (ZG2), or 100 mg/kg zinc + 750 mg/kg glyphosate (ZG3) for 36 weeks. a, p = 0.02. (b) Serum calcium concentrations in Wistar rats treated with 2 mL/kg distilled water (DW), 50 mg/kg zinc (Z), 14.4 mg/kg glyphosate (G1), 375 mg/kg glyphosate (G2), 750 mg/kg glyphosate (G3), 50 mg/kg zinc + 14.4 mg/kg glyphosate (ZG1), 50 mg/kg zinc + 375 mg/kg glyphosate (ZG2), or 100 mg/kg zinc + 750 mg/kg glyphosate (ZG3) for 36 weeks. a, p = 0.002.
There was a significant ($p < 0.001$) increase in ALP activity in the G3 group compared with the DW group. There was an increasing trend in ALP activity in the ZG1 (16%), G1 (46%), ZG3 (55%), ZG2 (58%) and G2 (65%) groups compared with the DW group. There was a decreasing trend in ALP activity in the Z group (23%) compared with the DW group (Figure 2).

**Effects of zinc and glyphosate treatments on serum total protein concentration**

There were no significant differences in total serum protein between any of the groups. There were decreasing trends in total serum protein in the ZG2 (10%), ZG1 (13%), G1 (14%), G2 (16%) and G3 (21%) groups compared with the DW group (Figure 3).

**Effects of zinc and glyphosate treatments on serum albumin concentration**

Levels of serum albumin showed no significant changes between any of the groups. There were decreasing trends in albumin concentrations in the ZG2 (16%), ZG1 (18%), ZG3 (19%), G1 (19%), G2 (20%) and G3 (29%) groups compared with the DW group (Figure 3).

**Effects of zinc and glyphosate treatments on serum globulin concentration**

There were no significant differences in serum globulin concentration among the groups. There was an increasing trend in globulin concentrations in the ZG2 (13%) group compared with the DW group and relative decreasing trends in the serum globulin concentrations of the ZG3 (18%), G1
(18%), G3 (18%) and G2 (37%) groups compared with the DW group (Figure 3).

**Effects of zinc and glyphosate treatments on serum urea concentration**

There was no significant difference in the serum urea concentrations between any of the groups. There were increasing trends in the serum urea concentrations of groups Z (13%), ZG1 (42%), G1 (79%), ZG2 (102%), ZG3 (122%), G2 (172%) and G3 (265%) compared with the DW group (Figure 4).

**Effects of zinc and glyphosate treatments on serum creatinine concentration**

There was a significant (p = 0.01) increase in the serum creatinine levels of the G2 group compared with the DW group. There were increasing trends in the serum creatinine concentrations of the ZG1 (55%), ZG2 (109%), ZG3 (128%), G1 (126%) and G3 (181%) groups compared with the DW group (Figure 5).

**Histopathological findings**

The livers of rats in group I (DW) showed no observable lesions (Figure 6a). The livers of rats in group II (Z) showed moderate fatty degeneration, coagulative necrosis of hepatocytes and mononuclear cell infiltration (Figure 6b). Severe fatty degeneration was observed in the livers of rats in group III (G1) (Figure 6c). The livers of rats in group IV (G2) showed severe fatty degeneration and coagulative necrosis of hepatocytes (Figure 6d). Moderate fatty degeneration, congestion and coagulative necrosis of hepatocytes were observed in the livers of rats in group V (G3).
Figure 6e. Fatty degeneration, congestion, coagulative necrosis of hepatocytes and mononuclear cell infiltration were observed in the livers of rats in group VI (ZG1) (Figure 6f). Histopathological changes observed in the livers of rats in group VII (ZG2) included fatty degeneration, congestion, coagulative necrosis of hepatocytes and mononuclear cell infiltration, especially at the perivascular areas (Figure 6g). Fatty degeneration and coagulative necrosis were observed in the livers of rats in group VIII (ZG3) (Figure 6h).

The kidneys of rats in group I (DW) and group II (Z) showed no observable lesions (Figures 7a and 7b, respectively). Mild coagulative necrosis of the glomeruli and renal tubules and moderate mononuclear cell infiltration into the periglomerular spaces and interstices of the tubules were observed in the kidneys of rats in group III (G1) (Figure 7c). Moderate coagulative necrosis of the glomeruli, mild renal tubular coagulation necrosis, mild mononuclear cell infiltration into the periglomerular spaces and interstices of the tubules, and shrunken glomeruli were observed in the kidneys of rats in group IV (G2) (Figure 7d). Severely shrunken glomeruli, severe coagulation necrosis of the glomeruli and renal tubules, and mild mononuclear cell infiltration into the interstices of the tubules were observed in the kidneys of rats in group V (G3) (Figure 7e). The kidneys of rats in group VI (ZG1) showed coagulative necrosis of the glomeruli and renal tubules and mononuclear cell infiltration into the periglomerular spaces and interstices of the tubules (Figure 7f). The kidneys of rats in group VII (ZG2) showed pyknosis of the
glomerular epithelial cells, coagulative necrosis of the glomeruli and renal tubules, and mononuclear cell infiltration into the periglomerular spaces and interstices of the tubules (Figure 7g). Pyknosis of the glomerular epithelial cells, coagulative necrosis of the glomeruli and renal tubules, and mononuclear cell infiltration into the periglomerular spaces and interstices of the tubules were observed in the kidneys of rats in group VIII (ZG3) (Figure 7h).

**Discussion**

We observed significant increases in serum parameters associated with damage to the liver (AST, ALT, ALP) and kidney (creatinine) following chronic oral exposure of rats to glyphosate present in the herbicide, Bushfire®. These serum changes occurred in tandem with degenerative and necrotic changes in the tissues as shown by histopathological examination. Our earlier report showed that subchronic glyphosate exposure caused relative changes (>10%) in serum parameters that were biomarkers of hepatorenal damage and mild histomorphological lesions in the organs. The current study suggests that chronic exposure caused more severe hepatorenal lesions than subchronic exposure at 10% LD$_{50}$. Increasing the exposure dose to 20% LD$_{50}$ appeared to further increase hepatorenal damage in a dose-dependent manner.
Figure 6. Photomicrographs of liver of rats administered distilled water (DW) (a), zinc (Z) (b), glyphosate 14.4 mg/kg (G1) (c), glyphosate at 375 mg/kg (G2) (d), glyphosate at 750 mg/kg (G3) (e), zinc 50 mg/kg + glyphosate 14.4 mg/kg (ZG1) (f), zinc 50 mg/kg + glyphosate 375 mg/kg (ZG2) (g), or zinc 100 mg/kg + glyphosate 750 mg/kg (ZG3) (h) for 36 weeks (H&E). No observable microscopic lesions were observed in panel (a). Moderate fatty degeneration (fd) can be observed in panels (b), (e), (g) and (h) and severe fd can be observed in panels (c) and (d). Hepatocyte degeneration (hd) can be observed in panel (c) and congestion (c) can be observed in panel (d). Perivascular mononuclear cell infiltration (mnc) can be observed in panels (e) and in milder form in (f). Coagulative necrosis of hepatocytes (nhc) can be observed in panel (e) and severe hepatocellular coagulation (hcn) can be observed in panel (f).
Figure 7. Photomicrographs of kidneys of rats administered distilled water (DW) (a), zinc (Z) (b), glyphosate 14.4 mg/kg (G1) (c), glyphosate at 375 mg/kg (G2) (d), glyphosate at 750 mg/kg (G3) (e), zinc 50 mg/kg + glyphosate 14.4 mg/kg (ZG1) (f), zinc 50 mg/kg + glyphosate 375 mg/kg (ZG2) (g), or zinc 100 mg/kg + glyphosate 750 mg/kg (ZG3) (h) for 36 weeks (H&E). No observable microscopic lesions were observed in panels (a) or (b). Coagulative necrosis of the glomerulus (n) can be observed in mild form in panel (c), in moderate form in panel (d), and in panel (f). Renal tubular necrosis (tn) can be observed in mild form in panels (c) and (d), in severe form in panel (e), and in panels (f), (g) and (h). Moderate and mild periglomerular mononuclear cell infiltration (pgmnc) can be observed in panels (c) and (d), respectively, as well as in panel (f) where it is indicated as (mnc). Mononuclear cell infiltration into the interstices of the tubules (mnc) can be observed in panel (g). A severely shrunken glomerulus (sg) and edematous fluid (e) are present in panel (e). Pyknosis of the glomerular epithelial cells (pyk) can be observed in panels (g) and (h).
Tissue damage induced by glyphosate has been reported after glyphosate exposures that were acute and subacute, sub-chronic, and chronic. The hepatotoxicity of glyphosate is associated with cell membrane damage, resulting in leakage of intracellular enzymes (ALT, AST) into the circulation. Hepatic inflammation characterized by increasing numbers of mononuclear cells and proliferation of collagen fibres in the liver may be responsible for the increased serum ALP activity; in line with this, hepatic enzyme secretion is elevated following cholestasis in the presence of glyphosate-induced steatohepatitis. Damage to the liver was expected to affect the functional capacity of the liver to synthesize proteins, thereby influencing serum total protein and albumin levels. However, chronic glyphosate exposure did not significantly affect these values, indicating that the compensatory synthetic capacity of the liver was sustained despite any toxicity. It has been reported that the global proteome of rapidly-dividing cells was not impacted by exposure to glyphosate, implying that glyphosate might not impair protein synthesis and protein metabolism.

Renal damage resulting from chronic glyphosate toxicity caused reduction of glomerular filtration as shown by elevated serum creatinine concentrations, without any effect on serum urea concentrations. Elevations of both serum creatinine and urea concentrations in subchronic glyphosate toxicity was previously reported following 12 weeks of exposure in rats. Serum creatinine level was reported as the better biomarker of nephrotoxicity following glyphosate exposure and its association with renal damage was sustained until the chronic stage as shown by the present study. Factors that prevented the elevation of serum urea concentrations during chronic renal impairment may include excretion of urea from the blood circulation into the intestinal lumen and failure of intestinal microbiota to metabolize urea to absorbable ammonia. This potential loss of blood urea through the intestine might not have resulted in negative nitrogen balance affecting the plasma protein concentration. In addition, renal loss of nitrogen affecting the plasma protein concentration might not also have occurred because proteinuria is not a prominent feature of glyphosate-induced glomerular damage, even during acute glyphosate poisoning.

Renal impairment was accompanied by significant elevation of serum calcium and bicarbonate levels, implying that chronic glyphosate exposure caused hypercalcemia and metabolic alkalosis without affecting other serum electrolytes. Hypercalcemia could result from parathormone action stimulated by the hypocalcemic tendency associated with preceding subchronic glyphosate toxicity. Metabolic alkalosis could arise from altered renal tubular function caused by hypercalcemia. This is at odds with reports of metabolic acidosis and hyperkalaemia in acute and subacute glyphosate poisoning. Following subchronic glyphosate exposure in rats, there was slight and insignificant depression of serum calcium concentrations. The ability of glyphosate to decrease serum calcium levels may arise from reduced intestinal calcium absorption when calcium is chelated by glyphosate in tandem with decreased production of active vitamin D following kidney damage. Mild and chronic hypocalcemic stimulation of the parathyroid gland may induce secondary upregulation of parathormone action resulting in calcium mobilization from the bone into the blood stream. This hypercalcemia induces renal tubular excretion of sodium, with fluid loss and bicarbonate retention, leading to dehydration and metabolic alkalosis. Pretreatment with zinc ameliorated glyphosate-induced serum biochemical changes following chronic exposure. A
similar ameliorative effect of zinc was reported previously following subchronic glyphosate exposure.\textsuperscript{14,15} Hepatorenal protection by zinc was attributed to its antioxidant properties,\textsuperscript{16,17} which might have promoted the integrity of cell membranes and reduced the impact of oxidative stress; this is considered to be the principal factor promoting toxic injury following glyphosate exposure.\textsuperscript{3,4,6,7,30,33,47} In addition, the inflammatory effect induced by glyphosate\textsuperscript{5,34,36} might be counteracted by the anti-inflammatory properties of zinc.\textsuperscript{17} During a chronic toxicity study, zinc pretreatment may not be capable of preventing the metabolic effects of glyoxylate, a metabolite of glyphosate.\textsuperscript{48} Therefore, lesions caused by glyoxylate may persist in rats exposed to glyphosate even with zinc pretreatment. Glyoxylate inhibits metabolic enzymes containing cysteine, downregulates fatty acid oxidation, and promotes cellular lipid accumulation and lipidotic cell damage.\textsuperscript{48} The pro-oxidant action of zinc may occur in hepatorenal cells overloaded with zinc during pretreatment, such that intracellular mobilization of zinc may promote some level of lesion development via aberrant zinc homeostasis.\textsuperscript{49} Slight oxidative stress arising from mobilized zinc may be responsible for the mild microscopic lesions observed in zinc-pretreated rats that were unexposed to glyphosate.

In conclusion, zinc pretreatment of rats chronically exposed to a glyphosate-based herbicide ameliorated hepatorenal toxicity by minimizing serum biochemical changes without preventing morphological lesions. These effects might have been derived from the antioxidant and anti-inflammatory properties of zinc. The persistence of some lesions may be related to the metabolic effects of a glyphosate metabolite (glyoxylate) against which zinc has no activity.

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**Declaration of conflicting interest**

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