



Haematological changes induced by subchronic glyphosate exposure: ameliorative effect of zinc in Wistar rats

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Abstract

The aim of this study was to determine the haematological changes induced by subchronic glyphosate exposure in Wistar rats and the ameliorative effect of zinc. Sixty adult male and female Wistar rats were used for the study. Twelve of them were used for the LD₅₀ which was evaluated to be 3750 mg kg⁻¹ with clinical signs of respiratory distress, diarrhoea, rough hair coat and subsequently death observed. The remaining 48 rats were divided into six groups of four males and four females each. The agent(s) administered are as follows: Group I (DW), control, distilled water (2 ml kg⁻¹), group II (Z), zinc (50 mg kg⁻¹), group III (G), glyphosate (375 mg kg⁻¹, 10 % of the LD₅₀), group IV (Z + G), zinc (50 mg kg⁻¹) and glyphosate (375 mg kg⁻¹) while group V (GC), glyphosate (14.4 mg kg⁻¹) and group VI (Z + GC), zinc (50 mg kg⁻¹) and glyphosate (14.4 mg kg⁻¹). The treatment regimens were administered orally by gavage once daily for eight weeks. At the end of the study, blood samples were collected and analysed but there were no statistical different ($p > 0.05$) among the treatment groups. However, the haematological parameters were relatively higher in the groups treated with glyphosate alone except the lymphocyte which was relatively low in the glyphosate treated groups compared to the control group. These changes were suggestive of haematological toxicity induced by oxidative stress caused by glyphosate exposure. In conclusion, the alterations in the haematological parameters such as packed cell volume, hemoglobin concentration, total white blood cell count, neutrophils and lymphocyte counts induced by subchronic glyphosate exposure were found to be differentially ameliorated by pre-treatment with zinc.

Keywords: Ameliorate, Glyphosate, Haematology, Subchronic, Wistar rat, Zinc.

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Introduction

Glyphosate (phosphonomethylglycine) was first reported as herbicide in 1971, and is said to be the world's biggest selling herbicide (Anon, 2013). It is a broad-spectrum herbicide widely used to kill unwanted plants both on agricultural and non-agricultural landscapes (Temple & Smith, 1992). As herbicide, glyphosate works by being absorbed into the plant mainly through its leaves, but also through soft stalk tissues. It is then transported throughout the plant where it acts on various enzyme systems, inhibiting amino acid metabolism in what is known as shikimic acid pathway. This pathway exists in higher plants and microorganisms but not in animals.

Plants treated with glyphosate slowly die over a period of days or weeks, and because the chemical is transported throughout the plant, no part survives (Malik *et al.*, 1989; Cox, 1995).

In animals, mechanisms of toxic action have not been fully elucidated. A reduced respiratory control ratio, enhanced ATPase activity and stimulated oxygen uptake rate were observed in liver mitochondria obtained from rats given glyphosate. Based on these results, the authors suggested that these toxicological effects may be primarily due to the uncoupling of oxidative phosphorylation (Olorunsogo *et al.*, 1979). Oxidative stress have been

implicated in the molecular mechanisms of glyphosate toxicity (Beuret *et al.*, 2004). The body responds to oxidative stress by evoking the enzymatic defence system within the body (Vivian & Claudia, 2007).

In pure chemical terms, glyphosate is an organophosphate (OP) in that it contains carbon and phosphorus. However, it does not affect the nervous system in the same way as organophosphate insecticides, and is not a cholinesterase inhibitor (Rebecca *et al.*, 1991). Most glyphosate containing products are either made or used with a surfactant. The surfactant used in a common glyphosate product (Roundup®) is more acutely toxic than glyphosate itself, but the combination of the two is yet more toxic (Santillo *et al.*, 1989). The most widely used surfactant in glyphosate formulation is an ethylated amine. Polyoxy-ethyleneamine has been frequently mentioned as a surfactant, but in fact it refers to a group of ethylated amine products used in glyphosate formulations (WHO, 1994). Organophosphates are the most widely used pesticides owing to their high efficacy, less persistence and more biodegradable nature than the organochlorines (Jirachayabhas *et al.*, 2004). Although there are benefits to the use of pesticides, there are also setbacks such as potential toxicity to humans and other animals (FAO, 2002).

Zinc is an essential trace mineral, which means that it must be obtained from the diet since the body cannot produce enough. It is the second most abundant mineral in the body, stored primarily in the muscle; it is also found in high concentrations in red and white blood cells, the retina of the eye, bones, skin, kidneys, liver and pancreas (Belongia *et al.*, 2001). Zinc plays important role in the immune system, regulation of appetite, stress level, taste and smell (McClain *et al.*, 1992). Two antioxidant mechanisms of Zinc have been identified: Zinc ions may replace redox active molecules such as iron and copper at critical sites in cell membranes and proteins; alternatively, Zinc ions may induce the biosynthesis of metallothionein, sulfhydryl-rich proteins that protect against free radicals (Rostan *et al.*, 2002). Owing to its mechanisms of action, Zinc has been used in the amelioration of organophosphate (Chlorpyrifos) induced alterations in haematological and serum – biochemical changes in Wistar rats (Ambali *et al.*, 2010a). It has also been used to attenuate oxidative stress in arsenic and cadmium exposed rats (Amara *et al.*, 2008; Kumar *et al.*, 2010).

Materials and methods

Experimental animals

Sixty Wistar rats, 160 – 200 g body weight, were purchased from the Animal House of the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria. They were housed in the animal room of the Department of Veterinary Pathology and Microbiology for two weeks before the commencement of the research which lasted for eight weeks. The rats were fed appropriately using standard rat chow and water was provided *ad libitum*.

Chemical source

Glyphosate (Bushfire®, Monsanto Europe S. A) which contains 360 g glyphosate/litre in the form of 441 g/litre potassium salt and zinc chloride crystals were used for the experiment.

Determination of median lethal dose (LD₅₀)

The median lethal dose (LD₅₀) was determined through a two phase approach as described by Lorke (1983). The first phase involved 9 Wistar rats divided in to 3 groups of 3 Wistar rats each. Each group was administered glyphosate at doses of 4000 mg/kg, 5000 mg/kg and 6000 mg/kg body weight *per os* respectively. Signs of toxicity and death were observed over a period of 48 hours. The doses of the second phase which depended on the result obtained from phase 1, consisted of three Wistar rats divided into three groups of one Wistar rat each, administered with glyphosate at 3500 mg/kg, 4000 mg/kg and 4500 mg/kg respectively. The LD₅₀ was then calculated to be 3750 mg/kg by evaluating the average of the highest dose that killed the rats and the lowest dose that did not kill the rats (table 1 and 2).

Subchronic toxicity study

Forty eight adult male and female Wistar rats were randomly divided into six groups of eight rats each so as to mimick what is obtainable in their natural environment as described below:

Group I (DW): Served as the control and were administered 2ml / kg of distilled water daily.

Group II (Z): Were administered Zinc at the dose rate of 50mg/kg body weight (Ambali *et al.* 2010a).

Group III (G): Were administered glyphosate (10% of the LD₅₀)

Group IV (Z + G): Were administered Zinc at 50mg/kg + glyphosate (10% of the LD₅₀).

Group V (GC): Were administered glyphosate at the concentration of 1:50 glyphosate and distilled water respectively.

Group VI (Z + G): Were administered Zinc at 50 mg/kg + glyphosate at the concentration of 1:50 glyphosate and distilled water respectively.

The dose regimens were administered *per os* once daily for a period of 8 weeks. The rats were monitored for clinical signs and death.

Haematological analysis

At the end of the study period, the rats were euthanized by severing the jugular vein after light chloroform anesthesia. Blood samples (3ml each) were collected into heparinized sample bottles and assayed for packed cell volume, haemoglobin concentration, total white blood cell count, absolute and relative leucocytes count using an auto analyzer (Abbot Haematological analyzer, cell-Dyn 1700; Abbot park, Illinois, U.S.A) at Ahmadu Bello University Teaching Hospital, Haematology Laboratory, Zaria - Nigeria.

Statistical analysis

Data obtained were expressed as Mean \pm SEM. They were subjected to one-way Analysis of Variance followed by Tukeys' post test using Graph Pad Version 4. Values of $p < 0.05$ were considered significant (Graphpad, 2000).

Results

Clinical signs

The rats given distilled water and Zinc ($ZnCl_2$) and Zinc + glyphosate at the two different concentrations (10% of the LD_{50} and 1:50 glyphosate and water respectively) did not show any sign of toxicity. The toxic signs that were observed in the rats administered with glyphosate included respiratory distress, diarrhoea, rough hair coat and death of one rat in the group on the first day of the LD_{50} determination.

Effect of the treatments on packed cell volume (PCV)

There were no significant changes ($P > 0.05$) in the Packed Cell Volume (PCV) between the groups. However, there was a relatively high (5.9%) PCV in the group administered glyphosate at 1:50 glyphosate and water respectively (GC) compared to the group administered distilled water (DW) group. On the other hand, there was a relatively low PCV in

the (Z + G) group pretreated with zinc at 50 mg/kg body weight and glyphosate at 10 % of the LD_{50} (7.6%), (Z + GC) the group pretreated with zinc and glyphosate at 1:50 glyphosate and water respectively (9.0%), (Z) the group administered with zinc at the rate of 50 mg/kg body weight (10.0%) or (G) the group administered with glyphosate at 10 % of the LD_{50} (11.5%) group when compared to DW group (Figure 1).

Effect of the treatments on haemoglobin (Hb) concentration

The effect of the treatments on haemoglobin concentration is shown in Figure 2. There were no significant changes ($p > 0.05$) in the Hb concentration in all the groups. However, there was a relatively high (5.8%) Hb concentration in GC group compared to DW group, on the other hand, there was a relatively low Hb concentration in the Z + G (7.6%), Z + GC (8.9%), Z (10.0%) and G (11.4%) groups, respectively when compared to DW group.

Effect of the treatments on total white blood cell (WBC) counts ($\times 10^9/L$)

The effect of the treatments on WBC counts in all the groups was not significantly different from each other. However, there was a relatively low WBC count in the Z group when compared to DW group. On the other hand, the WBC count was relatively high in the Z + GC (5.4), Z + G (5.1), GC (5.1) and G (5.2) groups, respectively, when compared to those recorded in the DW group (4.9)(Figure 3).

Effect of the treatments on differential leucocyte counts ($\times 10^9/L$)

The effect of the treatments on the differential leucocyte counts is shown in Figure 4. There were no significant changes in the neutrophils count of the groups. However, there were relatively higher neutrophils count in the Z + GC (3.9), GC (3.6), G (3.0), Z (3.1) and Z + G (3.6) groups, respectively compared to those recorded in the DW group (2.8).

The differences in the lymphocyte count between the groups were not significant ($p > 0.05$). However, a relatively low lymphocyte count was recorded in the Z + GC (1.8), GC (1.5), Z + G (1.6), Z (1.5) or G (1.7) groups (Figure 4) compared to those recorded in the DW group (2.1). Therefore, the differential leucocyte count showed higher neutrophils with lower lymphocytes counts.

Table 1: Median lethal dose (LD₅₀) for glyphosate – Phase I

Group	Dose mg/kg	No_ dead/ No_ dosed
1	4000	1/3
2	5000	0/3
3	6000	0/3

Table 2: Median lethal dose (LD₅₀) for glyphosate – Phase II

Group	Dose (mg/ kg)	No_ dead/ No_ dosed
1	3500	0/1
2	4000	0/1
3	5000	0/1

Thus, based on the method of Lorke (1983), the result of the LD₅₀ = 4000 + 3500/2 = 3750 mg /kg.

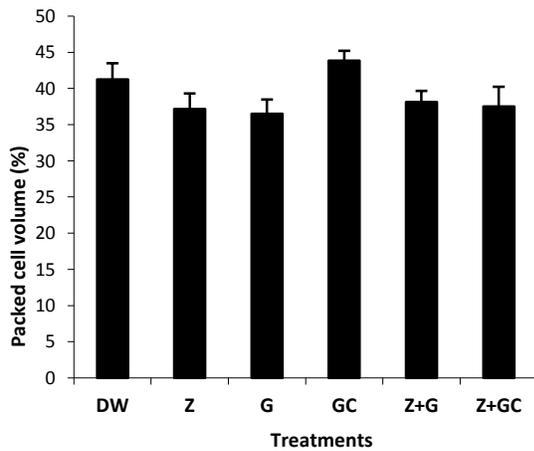


Figure 1: Effect of sub – chronic exposure to distilled water (DW), zinc (Z) and glyphosate at 10% of the LD₅₀ (G) and at 1:50 glyphosate and distilled water (GC) on packed cell volume in Wistar rats.

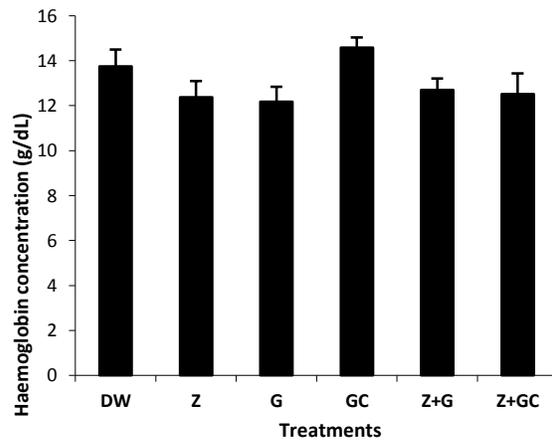


Figure 2: Effect of sub – chronic exposure to distilled water (DW), zinc (Z) and glyphosate at 10% of the LD₅₀ (G) and at 1:50 glyphosate and distilled water (GC) on haemoglobin concentration in Wistar rats.

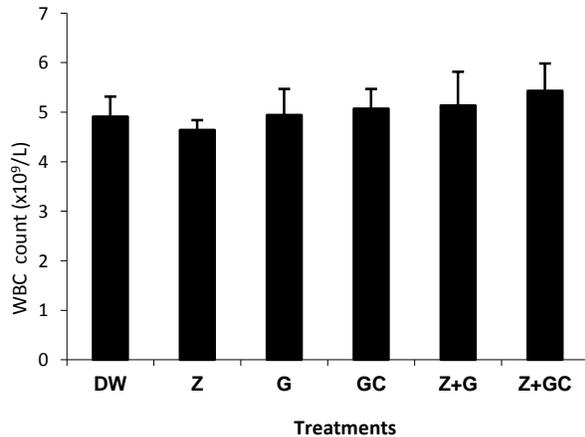


Figure 3: Effect of sub – chronic exposure to distilled water (DW), zinc (Z) and glyphosate at 10% of the LD₅₀ (G) and at 1:50 glyphosate and distilled water (GC) on white blood cell concentration in Wistar rats.

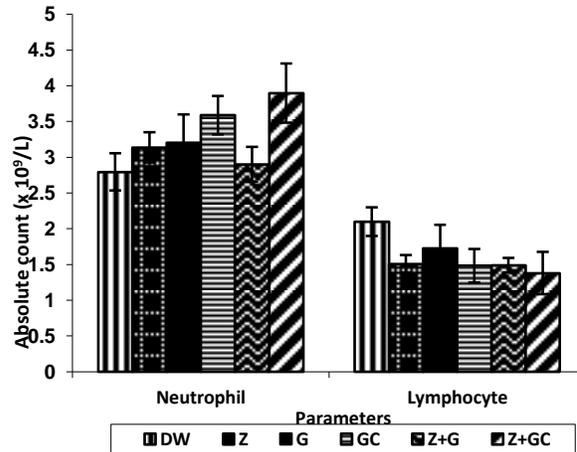


Figure 4: Effect of sub – chronic exposure to distilled water (DW), zinc (Z) and glyphosate at 10% of the LD₅₀ (G) and at 1:50 glyphosate and distilled water (GC) on neutrophils and lymphocytes in Wistar rats.

Discussion

The LD₅₀ of 3750 mg/kg obtained in this study showed that glyphosate is in the category of chemicals with low toxicity, since chemicals with LD₅₀ of 500 – 5000 mg/kg have been categorized as such (NPIC, 2010). This result is slightly different from the earlier report by Rodwell (1980) which placed glyphosate under very low toxicity category, > 5000 mg/kg. The reason for the difference is not clear but might be attributed to some factors relating to the chemical composition of the glyphosate preparation (Busfire®) used in this study, the species of the rats used and the environment. The clinical signs observed during the LD₅₀ evaluation included respiratory distress, diarrhoea, rough hair coat and death in one animal. Respiratory signs and diarrhoea have been previously reported following glyphosate poisoning (Olorunsogo *et al.*, 1979; Adam *et al.*, 1997).

The observed toxic signs in the subchronic toxicity study in the group exposed to glyphosate alone were respiratory distress, diarrhoea and death. Similarly, respiratory distress and diarrhoea were documented by Olorunsogo *et al.* (1979) and were said to be due to uncoupling of oxidative phosphorylation. The diarrhoea observed in this study upholds the observations of Adam *et al.* (1997) who demonstrated that glyphosate – induced toxic signs included diarrhoea. The fact that zinc pretreated groups did not manifest any toxic signs signifies the amelioration of the toxic signs induced by subchronic glyphosate exposure (McClain *et al.*, 1992; Powell, 2000).

In the present study, the PCV and Hb concentration were lowest in the group exposed to glyphosate alone at the dose rate of 375 mg/kg (G) which indicates that sub – chronic exposure to glyphosate may induce anemia (decreased PCV and Hb). The anemia observed might have been due to the ability of the herbicide to cause extravascular hemolysis or it might be due to its ability to cause oxidative stress (Modesto & Martinez, 2010). The erythrocyte membrane was reported to be highly vulnerable in an oxidative stress condition because it contains high amounts of lipid, iron and is bathed in serum that has low antioxidant properties (Ambali *et al.*, 2010d). The result is similar to that of Gluszczak *et al.* (2006) who observed a decrease in PCV and Hb concentration of fish, *Leporinus obtusidens*, exposed to different concentrations of Roundup® (3, 6, 10 and 20 mg l⁻¹) for 96 hours. On the other hand, the rats that were exposed to glyphosate at the dose rate of 14.4 mg/kg (GC), had a relatively high PCV

and Hb concentration compared to the group that was administered distilled water (DW). The relatively high PCV and Hb concentration in this group might be as a result of the release of immature RBC in to the circulation or due to dehydration as a result of diarrhoea observed in the group. The increase in PCV and Hb concentrations previously observed were said to be caused by the release of erythrocytes from blood depots and/or from haemopoetic tissues into the blood stream (Svodova *et al.*, 1994). The group supplemented with zinc + glyphosate at the dose rate of 50 mg/kg and 375 mg/kg respectively (Z + G) and the group supplemented with zinc + glyphosate at the dose rate of 50 mg/kg and 14.4 mg/kg respectively (Z + GC) had relatively high PCV and Hb concentrations which might be as a result of its role in metabolism serving as a cofactor of many enzymes, coupled with its antioxidant effects (Powell, 2000; Kraus *et al.*, 1997). The relatively low PCV and Hb concentrations in the group administered zinc alone at the dose rate of 50 mg/kg (Z) was probably due to its redox status, since at higher concentration, without a corresponding oxidative challenge, zinc is capable of acting as pro – oxidant, promoting oxidative stress by eliciting a decline in erythrocyte Cu – Zn superoxide dismutase activity as reported by Abdallah & Samman (1993). The study revealed a slight increase in WBC count in G and GC group compared to the DW group. This result is similar to the finding of Modesto & Martinez (2010) who reported increase in the total WBC count in oxidative stress condition in fish due to roundup® toxicity. This alteration could be as a result of the activation of the immune system in the presence of contaminants, which in turn may be an adaptive response of the rat resulting in a more effective immune defence (Barreto – Medeiros *et al.*, 2005). The WBC counts in the Z + G and Z + GC groups were relatively higher than what were observed in the other groups, which indicated that supplementation with zinc before glyphosate administration in both cases caused a relatively high WBC count. The protective effect of zinc on the WBC obtained in this study is similar to the earlier study by Powell (2000) who reported that the protective effect of zinc on WBC might be attributed to its antioxidant properties either as a vital component of enzymatic antioxidant Cu – Zn superoxide dismutase (SOD) or due to its ability to antagonize the catalytic properties of redox – reaction of active transition metals namely iron and copper, with respect to production of hydroxyl from hydrogen peroxide

and superoxide. Zinc also causes inhibition of both endogenous lipid peroxidation as well as induced lipid peroxidation, thereby resulting in stabilization of biomembranes (Dhawan *et al.*, 1992; Srivastava *et al.*, 1993). This study revealed a relatively low WBC count in the Z group when compared to the DW group which might be due to its redox status, since at higher concentration without a corresponding oxidative challenge; zinc is capable of acting as pro – oxidant, promoting oxidative stress (Abdallah & Samman, 1993).

In this study, the neutrophil count in the G and the GC groups were relatively higher than that of the DW group. This finding is similar to the normal physiological responses, higher percentage of neutrophils and lower percentage of lymphocytes differential count that were found when fishes were subjected to an array of toxicants (Witneska, 2005). However, this result contradicts the result of Modesto & Martinez (2010) who reported a decrease in the absolute neutrophils count which might be due to differences in species of the rats and the formulations of the glyphosate – based herbicide, Roundup® Transorb and Bush fire®. In the Z + G group, there was relatively low neutrophil count due to the ameliorative effect of zinc, since high neutrophil : lymphocyte ratio was reported to be indicative of stress in rats (Goel *et al.*, 2006; Ambali *et al.*, 2010b). The reason for the relatively high neutrophils count in the Z + GC group is not known, but it might be due to higher neutrophils

count in the GC group and might therefore require higher dosage of zinc. The relatively high neutrophils count in the Z group might be due to the pro – oxidant action of zinc on the haemopoietic organs in the rats (Abdallah & Samman, 1993).

The rats in the G and the GC groups in this study showed a relatively higher lymphocyte counts when compared to the rats in Z + G and Z + GC groups respectively. Similar to previous reports in studies on oxidative stress (Goel *et al.*, 2006; Ambali *et al.*, 2010a) in which lymphocytic leucopaenia which might be due to either decreased production and / or increased rate of removal due to rapid destruction of lymphocytes. The ameliorative effect of zinc following supplementation with zinc in the Z + G and Z + GC groups are similar to the protective effect of zinc documented by Powell (2000) who reported the protective effect of zinc on WBC count which was attributed to the antioxidant properties either as a vital component of enzymatic antioxidant Cu – Zn (SOD) or due to their ability to antagonize the catalytic properties of redox – reaction of active transition metals namely iron and copper, with respect to the promotion of hydroxyl radicals formation from hydrogen peroxide and superoxide. In conclusion, the alterations in the haematological parameters, packed cell volume, hemoglobin concentration, total white blood cell count, neutrophils and lymphocytes counts recorded in this study were differentially ameliorated by pretreatment with zinc.

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