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Effect of zinc supplementation on immunotoxicity induced by subchronic oral exposure to glyphosate-based herbicide (GOBARA®) in Wistar rats

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Abstract

Objectives: To evaluate the effect of zinc supplementation on immunotoxicity induced by subchronic oral exposure to glyphosate-based herbicide (GBH).

Methods: Sixty adult male Wistar rats randomly divided equally into six groups were exposed to GBH by gavage daily for 16 weeks with or without zinc pretreatment. Group DW rats received distilled water (2 mL/kg), group Z rats received zinc (50 mg/kg), and group G1 and G2 rats received 187.5 and 375 mg/kg GBH, respectively. Group ZG1 and ZG2 rats were pretreated with 50 mg/kg zinc before exposure to 187.5 and 375 mg/kg GBH, respectively. Tumor necrosis factor alpha (TNF-α) and immunoglobulin (IgG, IgM, IgE) levels were measured by enzyme-linked immunosorbent assay. Spleen, submandibular lymph node, and thymus samples were processed for histopathology.

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Results: Exposure to GBH (G1 and G2) significantly increased serum TNF-α concentrations and significantly decreased serum IgG and IgM concentrations compared with the control levels. Moderate-to-severe lymphocyte depletion occurred in the spleen, lymph nodes, and thymus in the GBH-exposed groups. Zinc supplementation mitigated the immunotoxic effects of GBH exposure.

Conclusions: GBH exposure increased pro-inflammatory cytokine responses, decreased immunoglobulin production, and depleted lymphocytes in lymphoid organs in rats, but zinc supplementation mitigated this immunotoxicity.

Keywords

Glyphosate, herbicide, subchronic toxicity, immunoglobulin, cytokine, zinc supplementation, immunotoxicity

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Introduction

Glyphosate is a broad-spectrum herbicide that inhibits the synthesis of aromatic amino acids necessary for protein anabolism in susceptible plants.[1] The use of glyphosate-based herbicide (GBH) formulations has tremendously increased in recent times, indicating a possible need to update safety standards because of rising human exposure and toxicological risks.[1,2] Amidst the debate on whether glyphosate has carcinogenic effects, recent assessments of toxicological data raised no cause for concern,[3,4] but the genotoxicity and cytotoxicity of glyphosate have been reported, pointing to aberrations in cell biology that could arise from glyphosate exposure in respect to lymphocyte viability, growth, and proliferation.[5–11] There is strong evidence that glyphosate induces the progression of malignant lymphoma in male and female CD-1 mice[12] and multiple myeloma in Vk*MYC mice.[5,9] The immune systems of fish and rodents exposed to glyphosate had alterations in the activities of complement proteins, functions of lymphocytes and phagocytes, and production of pro-inflammatory cytokines.[6,9,10,13–15] Wild-type mice treated with glyphosate were reported to have increased serum immunoglobulin G (IgG) levels because of benign monoclonal gammopathy.[5] In fish, glyphosate exposure was reported to cause lymphocyte apoptosis along with altered expression of cytokines related to pro-inflammatory responses.[6] Increased susceptibility to viral infection likely attributable to decreased antibody responses was observed,[13] and cell-mediated immunity and humoral immunity were suppressed.[16,17] Glyphosate formulations in fish ecosystems promoted inflammation and decreased the immunity of fish, as indicated by the downregulation of genes for IgM and mRNA expression of nuclear factor-kappa B2, tumor necrosis factor alpha (TNF-α), and interleukin-1 beta (IL-1β).[18] Evidence regarding the immunotoxicity of glyphosate and GBH in animal models is limited, and further investigation is required.[19]

Zinc supplementation was used in our previous studies to ameliorate the toxic effects of GBH[20–26] based on the premise that zinc can enhance anti-oxidant and anti-inflammatory responses.[27] In the present study, we focused on the immunotoxicity of GBH exposure with respect to pro-inflammatory cytokine and immunoglobulin responses

and the stability of lymphocyte populations in lymphoid tissues of the spleen, lymph nodes, and thymus. Lymphocytes from the lymphoid organs produce immunoglobulins required for adaptive immunity.[28] Pro-inflammatory cytokines such as TNF-α and IL-1β modulate inflammatory and immune responses[29] and potentially trigger lymphocyte apoptosis.[30] The effect of GBH-induced toxicity on these immune variables in rats needs to be further investigated[19] along with the mitigating impact of zinc supplementation. The objective of the study was to evaluate the effect of zinc supplementation on immunotoxicity induced by sub-chronic oral exposure to glyphosate-based herbicide in Wistar rats.

Materials and methods

Animals

Ethical approval was obtained from the ethics committee of the Institutional Animal Care and Use, University of Jos (reference number: F17-00379; approval date: 3 September 2019). Sixty adult male Wistar rats weighing 140 to 150 g were purchased from the National Veterinary Research Institute Vom and allowed to acclimatize for 2 weeks prior to commencement of the research. The rats were fed standard rat chow (Livestock Feed, Jos, Nigeria), and water was provided *ad libitum*.

Chemicals

GBH (GOBARA®, Saro Agrosciences, Ibadan, Nigeria) containing 360 g/L glyphosate isopropylamine salt in 100% surfactant and anhydrous zinc chloride (LOBA Chemie Pvt Ltd, Mumbai, India) were used for the research.

Experimental design

The rats were randomly selected and assigned to six groups (n = 10/group) as follows: group DW (control), each rat was orally administered 2 mL/kg of distilled water daily; group II (Z), each rat was orally administered zinc chloride (2%) at 50 mg/kg;[26] group G1, each rat was orally administered 187.5 mg/kg glyphosate in GBH) (5% of the LD_{50});[20] group G2, each rat was orally administered 375 mg/kg glyphosate in GBH (10% of the LD_{50});[20] group ZG1, each rat was orally pretreated with 50 mg/kg zinc chloride 1 hour before the oral administration of 187.5 mg/kg GBH; and group ZG2, each rat was orally pretreated 50 mg/kg zinc chloride 1 hour before the oral administration of 375 mg/kg GBH.

The treatments were administered by gavage once daily for 16 weeks. The rats were weighed weekly using an electronic weighing balance (OHAUS, Shanghai, China) to monitor the weight changes and ensure appropriate dosing.

Estimation of serum immunoglobulins and cytokines

Blood samples (3 mL) were collected by jugular venipuncture under ketamine anesthesia from each rat into clean test tubes without anti-coagulant. The blood samples were kept at room temperature for 30 minutes and then centrifuged at $80 \times g$ for 10 minutes to obtain the sera.

Serum immunoglobulin (IgG, IgM and IgE) and cytokine (TNF-α and IL-1β) levels were estimated using enzyme-linked immunosorbent assay kits following specified procedures in the kits. Bioassay Technology Laboratory kits (Shanghai, China) were used for IgG, IgM, and TNF-α estimation, whereas IgE was assayed using a kit from Calbiotech (El Cajon, CA, USA). The IL-1β assay was performed using a kit from Elabscience (Houston, TX, USA).

Pathological examination

Necropsy was conducted after sacrifice by cervical exsanguination under deep ketamine anesthesia to grossly examine the carcass with special attention paid to the lymphoid organs.[31] Tissue samples from the spleen, submandibular lymph nodes, and thymus were collected and fixed in 10% neutral buffered formalin. The fixed tissues were processed, embedded in paraffin wax, and cut into sections that were stained with hematoxylin and eosin and examined using a light microscope.[32,33] Photomicrographs were taken at $\times 400$ magnification. During histological examination of the lymphoid organs, lymphocyte depletion in the cortical areas was semi-quantitatively described as none (normal), mild, moderate, or severe and assigned an ordinal category of 0, 1, 2, or 3, respectively, based on the position of the papers on immunotoxicity screening from the National Toxicology Program[34] and Society of Toxicologic Pathology.[35] The mean lymphocyte depletion score of an organ was obtained by dividing the total score of all rats by the number of rats in the group.

Data analysis

Data were summarized as the mean ± standard error of the mean, and variations in means were analyzed by one-way analysis of variance followed by Tukey's post-hoc test using Graph Pad Prism version 5.0 for Windows (GraphPad, San Diego, CA, USA). Significant variations were accepted at $P < 0.05$, but in the absence of significant differences ($P > 0.05$), the mean difference between groups (expressed as percentage) was reported when the value was $\geq 10\%$. The ordinal scores of lymphocyte depletion were expressed as means for groups, and differences in means between groups were indicated as percentages.

Results

Effects of treatments on serum cytokine and immunoglobulin concentrations

The effects of the treatments on cytokine concentrations are presented in Figure 1. Serum TNF-α concentrations were significantly increased in the G1 ($P = 0.0043$) and G2 ($P = 0.0426$) groups compared with that in the DW group, and these increases were mitigated in the ZG1 and ZG2 groups, indicating that zinc supplementation suppressed the effects of GBH exposure. There was no significant or reportable relative difference in the serum IL-1β concentration between the treatment groups and the DW group.

Figure 2 presents the effects of treatment on immunoglobulin (IgG, IgM, IgE) concentrations. Serum IgG concentrations were significantly decreased in the Z ($P = 0.0130$), G1 ($P = 0.0142$) and G2 ($P = 0.0229$) groups compared with that in the DW group, but these values reverted toward the control value in the zinc-supplemented groups (ZG1 and ZG2), with a relative decrease in the ZG2 group (12.6%) and a relative increase in the ZG1 group (10.3%) compared with the value in the DW group.

Serum IgM concentrations were significantly higher in the G1 ($P = 0.0139$) and G2 ($P = 0.0031$) groups than in the DW group. The value was also significantly lower in the G1 ($P = 0.0243$) group than in the Z group, whereas a relative decrease in the serum IgM concentration was observed in the Z (15.2%) group compared with that in the DW group. However, the IgM concentration was significantly higher in the ZG2 ($P = 0.0455$) group than in the G2 group and relatively higher in the ZG1 (26.4%) group than in the DW group.

Serum IgE concentrations were significantly decreased in the G1 ($P = 0.001$) and G2 ($P = 0.0003$) groups compared with that in the ZG2 group, but the values in the G1

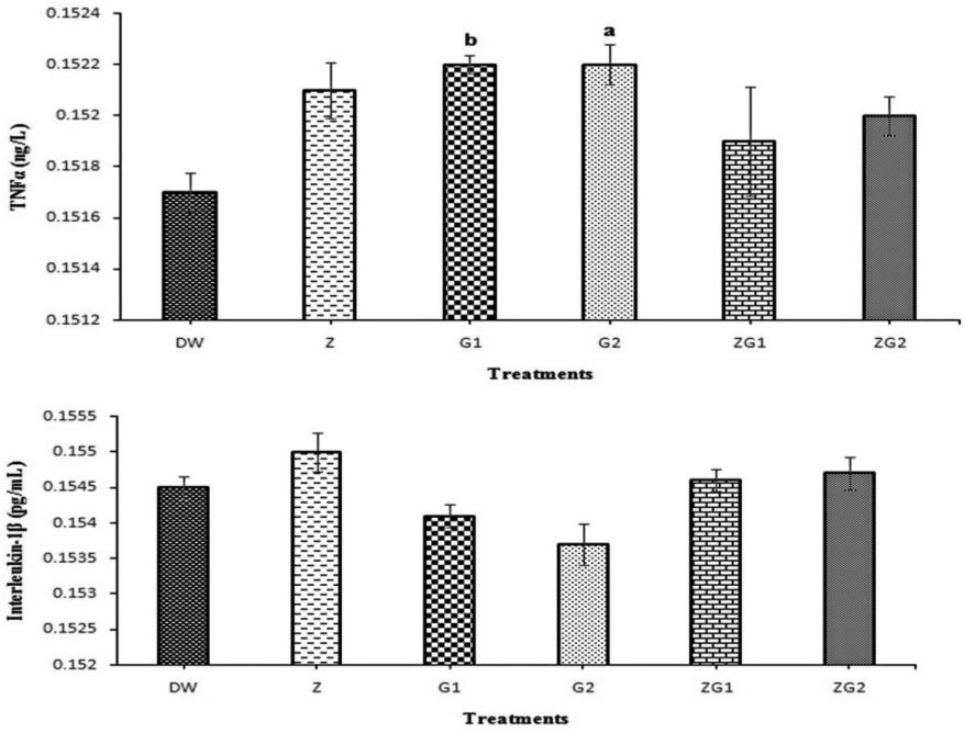

Figure 1. Effect of oral zinc pretreatment on serum TNF-α concentrations (a) and IL-1β concentrations (b) in rats orally exposed to glyphosate-based herbicide on daily basis for 16 weeks. Treatment groups: DW, control (2 mL/kg distilled water); Z, zinc chloride (50 mg/kg); G1, glyphosate (187.5 mg/kg); G2, glyphosate (375 mg/kg); ZG1, zinc chloride (50 mg/kg) with glyphosate (187.5 mg/kg); and ZG2, zinc chloride (50 mg/kg) with glyphosate (375 mg/kg). Letters indicate increases in the G1 (b, $P = 0.0043$) and G2 (a, $P = 0.0426$) groups compared with the DW group.
TNF-α, tumor necrosis factor alpha; IL-1β, interleukin-1 beta.

and G2 groups did not differ significantly from that in the DW group. Similarly, the serum IgE concentration was significantly decreased in the G2 ($P = 0.0238$) group compared with that in the ZG1 group but significantly increased in the ZG2 ($P = 0.0287$) group compared with that in the ZG1 group.

Effects of treatment on the lymphoid tissues of the spleen, submandibular lymph nodes, and thymus

There was no observable gross lesion in the spleen, lymph nodes, and thymus. In the spleen, there were no observable histopathological changes in rats in the DW, Z, ZG1, and ZG2 groups, but moderate and severe lymphocyte depletion in the red and white pulp was observed in the spleens of rats in the G1 and G2 groups, respectively (Figure 3a). The lymph nodes of rats in the DW, Z, ZG1, and ZG2 groups exhibited no observable lymphocyte depletion, whereas the lymph nodes of rats in the G1 and G2 groups displayed moderate-to-severe lymphocytic depletion (Figure 3b). The thymi of rats in the DW, Z, ZG1, and ZG2 groups also exhibited no observable

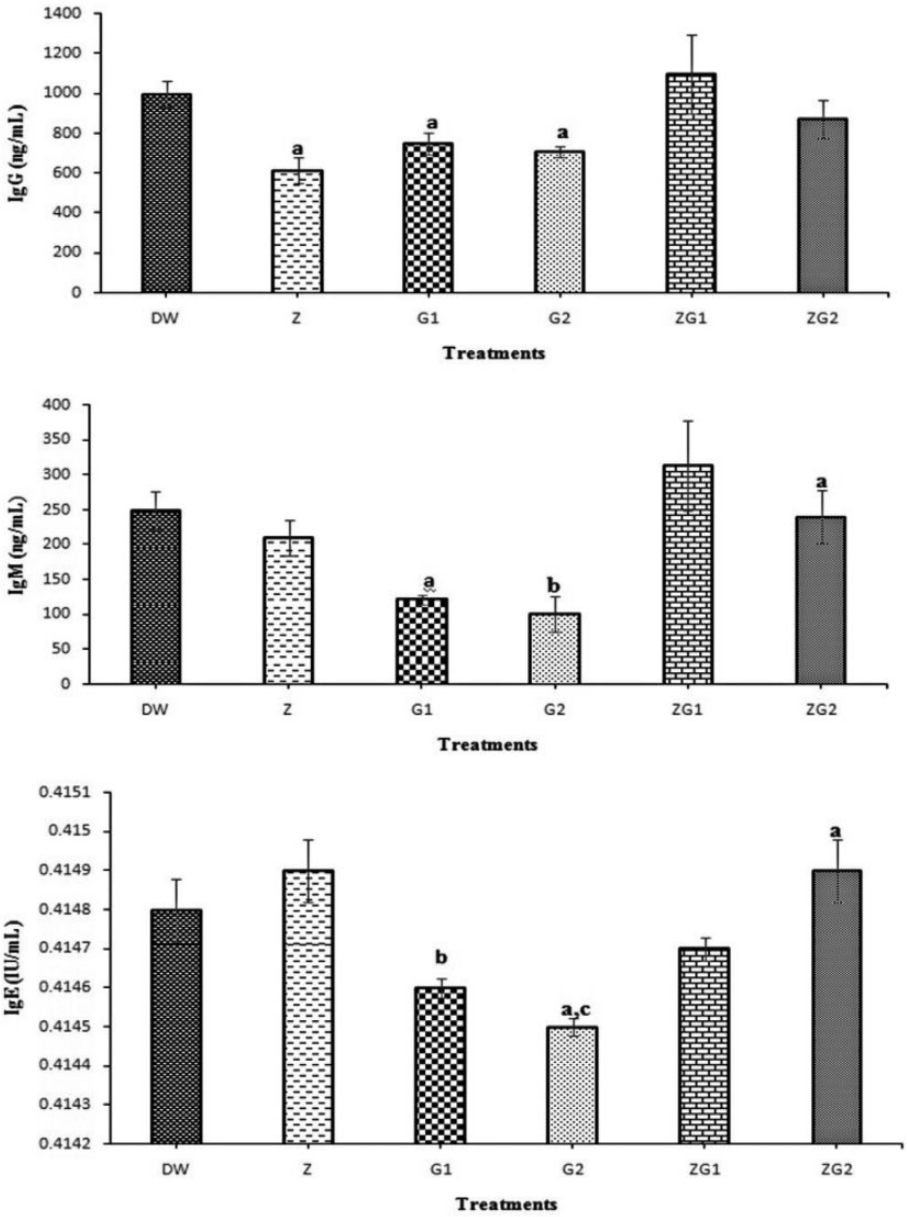

Figure 2. Effect of oral zinc pretreatment on serum immunoglobulin concentrations (IgG, IgM, IgE) concentrations in rats orally exposed to glyphosate-based herbicide daily for 16 weeks. Treatment groups: DW, control (2 mL/kg distilled water); Z, zinc chloride (50 mg/kg); G1, glyphosate (187.5 mg/kg); G2, glyphosate (375 mg/kg); ZG1, zinc chloride (50 mg/kg) with glyphosate (187.5 mg/kg); and ZG2, zinc chloride (50 mg/kg) with glyphosate (375 mg/kg). IgG: the letter (a) indicates decreases in the Z ($P = 0.0130$), G1 ($P = 0.0142$), and G2 ($P = 0.0229$) groups versus the DW group. IgM: letters indicate decreases in the G1 (a, $P = 0.0139$) and G2 (b, $P = 0.0031$) groups versus the DW group, a decrease in the G1 (a, $P = 0.0243$) group versus the Z group, and an increase in the ZG2 (b, $P = 0.0455$) group versus the G2 group. IgE: letters indicate decreases in the G1 (b, $P = 0.001$) and G2 (c, $P = 0.0003$) groups versus the ZG2 group, a decrease in the G1 (a, $P = 0.0238$) group versus the ZG1 group, and an increase in the ZG2 (a, $P = 0.0287$) group versus the ZG1 group.
IgG, immunoglobulin G; IgM, immunoglobulin M; IgE, immunoglobulin E.

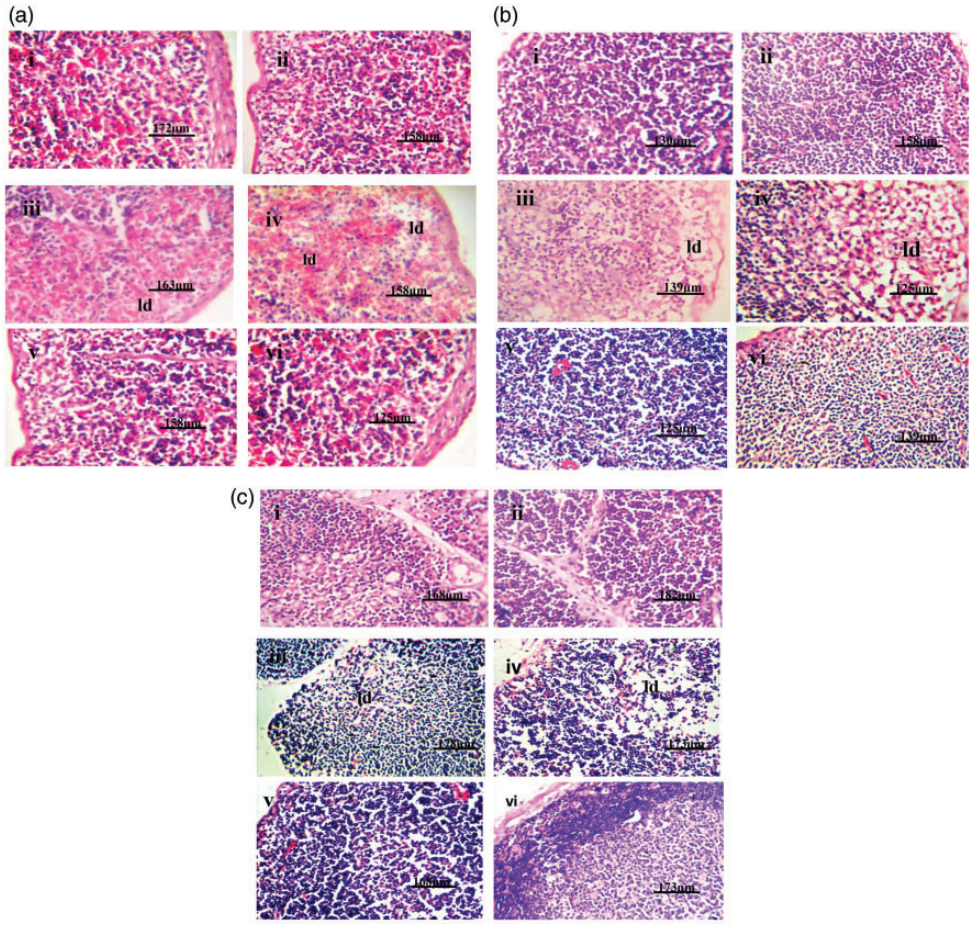

Figure 3. Hematoxylin and eosin staining of tissues in rats treated with glyphosate-based herbicide daily for 16 weeks with zinc pretreatment. (a) Photomicrographs of the spleens of rats. No lymphocyte depletion was observed in the DW (i) and Z groups (ii), moderate lymphocyte depletion (ld) was detected in the G1 group (iii), severe lymphocyte depletion (ld) was observed in the G2 group (iv), and no lymphocyte depletion was detected in the ZG1 (v) and ZG2 groups (vi). (b) Photomicrographs of the submandibular lymph nodes of rats. Severe lymphocyte depletion (ld) was present in the G1 (iii) and G2 groups (iv), but no lymphocyte depletion was observed in the DW (i), Z (ii), ZG1 (v), and ZG2 groups (vi). (c) Photomicrographs of the thymi of rats. No lymphocyte depletion was detected in the DW (i), Z (ii), ZG1 (v), and ZG2 groups (vi), but moderate and severe lymphocyte depletion (ld) was detected in the G1 (v) and G2 groups (vi), respectively. Treatment groups: DW, control (2 mL/kg distilled water); Z, zinc chloride (50 mg/kg); G1, glyphosate (187.5 mg/kg); G2, glyphosate (375 mg/kg); ZG1, zinc chloride (50 mg/kg) with glyphosate (187.5 mg/kg); and ZG2, zinc chloride (50 mg/kg) with glyphosate (375 mg/kg).

lymphocyte depletion, whereas moderate-to-severe lymphocyte depletion was observed in the G1 and G2 groups (Figure 3c). The lymphocyte depletion scores are summarized in Table 1. The mean scores of lymphocyte depletion in the lymphoid organs ranged 2.0 to 2.4 in the G1 group and 2.7 to 3.0 in the G2 group, indicating that lesion score in the submandibular lymph nodes, thymus and spleen was increased by 25%, 35%, and

Table 1. Effect of oral zinc pretreatment on semiquantitative histological ordinal scores* (mean of 10) of lymphocyte depletion in the lymphoid organs of rats orally exposed to glyphosate-based herbicide daily for 16 weeks.

Treatment group[a]	Spleen	Submandibular lymph nodes	Thymus
I (DW)	0*	0	0
II (Z)	0	0	0
III (G1)	2.0*	2.4	2.0
IV (G2)	3.0	3.0	2.7
V (ZG1)	0	0	0
VI (ZG2)	0	0	0

[a]DW, control (2 mL/kg distilled water); Z, zinc chloride (50 mg/kg); G1, glyphosate (187.5 mg/kg); G2, glyphosate (375 mg/kg); ZG1, zinc chloride (50 mg/kg) with glyphosate (187.5 mg/kg); and ZG2, zinc chloride (50 mg/kg) with glyphosate (375 mg/kg).
*Semiquantitative histological ordinal score of lymphocyte depletion in lymphoid organs: normal (0), mild depletion (1), moderate depletion (2), and severe depletion (3).

50%, respectively, in the G2 group compared with those in the G1 group.

Discussion

The results of this study indicated that GBH (GOBARA) as a vehicle for glyphosate can potentially induce immunotoxicity in rats by altering cytokine and immunoglobulin levels in circulating blood and causing lymphocyte depletion in the spleen, submandibular lymph nodes, and thymus. This implies that B cell and T cell subsets of the lymphocyte population might experience toxic injury under GBH exposure, and both cells are expected to cooperate in generating adaptive immune responses.[36] The implication of immunosuppression in rodents exposed to GBH might be relevant to the renewed quest for assessing the public health impact of xenobiotic immunotoxicity,[37] contrary to the earlier contention that GBH did not affect the immune system.[38]

GBH exposure increased serum TNF-α concentrations, but it did not affect serum IL-1β levels in this study. There are reports that GBH induced TNF-α and IL-1β production in fish[18] and in the liver[39] and jejunum[40] of rats. The production of other cytokines (IL 6, IL-4, and IL-17A) was also induced by glyphosate exposure in intestinal mucosal cells and peripheral blood mononuclear cells.[41,42] Interferon gamma production was reduced in blood mononuclear cells exposed to glyphosate.[42] IL-1β expression was reported to be unchanged in the intestines and decreased in the gills of sea bass fish,[43] suggesting that the effects of glyphosate on cytokine production could be bidirectional. The reason why GBH had no effect on serum IL-1β levels could not be identified, but it might be related to pathways of T cell differentiation and cytokine production.[42] The increasing levels of pro-inflammatory cytokines, especially cytotoxic TNF-α, might induce apoptosis in lymphocytes in the lymphoid organs.[44]

Exposure to GBH decreased serum IgG and IgM concentrations in the rats, indicating decreased production of these immunoglobulins. However, serum IgE concentrations were not affected compared with the control level, suggesting that there was no allergic reaction to actively drive IgE switching during GBH exposure. The GBH-induced reduction in serum IgM concentrations might be attributable to the reduction in the number of lymphocytes mobilized for its production, their impaired secretion from rough endoplasmic reticula, and undetected abnormal molecular forms attributable to genomic damage and transcriptome aberration.[6,45,46] Glyphosate or GBH was reported to suppress IgM expression and reduce antibody responses in fish as components of immunosuppression.[13,14,16,18] The histopathological observation of lymphocyte depletion in lymphoid tissues of the spleen, submandibular lymph nodes, and thymus might be largely responsible for the reduced serum immunoglobulin concentrations, and these changes

could have arisen from lymphocyte apoptosis induced by the cytotoxic effects of GBH exposure and GBH-induced cytokine release.[8–11,44]

Zinc supplementation in normal rats significantly decreased serum IgG concentrations, and this effect was presumed to be attributable to the reduction of antigen translocation to immune sites arising from the enhancement of intestinal tight junction and improvement of mucosal barriers that prevent trans-mucosal seepage or the migration of antigenic stimulants from the intestinal lumen to lymphoid locations.[47] During GBH exposure, zinc supplementation prevented the increase in serum TNF-α concentrations, the decreases in serum IgG and IgM concentrations, and the depletion of lymphocytes in lymphoid tissues. This feat was possible because of the anti-oxidant and anti-inflammatory effects of zinc in rats,[27] which counteract the toxigenic actions of glyphosate induced by oxidative stress[48] and oxidant-induced inflammatory processes.[39] Supra-physiologic levels of zinc reverse the increase of cytokine secretion induced by lower zinc bioavailability.[49,50] The suppression of immunoglobulin production and secretion during GBH exposure was abated by zinc supplementation because of the capacity of zinc to enhance lymphocyte proliferation and boost immunoglobulin production.[51,52] Although IgE levels were not negatively affected by GBH exposure, we observed that zinc supplementation exaggerated its secretion, indicating that zinc could worsen allergic responses in glyphosate-exposed individuals.[53]

In this study, the dose rate of GBH as the toxicant was based on its glyphosate content without consideration of any other components of the herbicide, which was not disclosed. GBH is an isopropylamine salt in 100% surfactant. It was presumed to contain glyphosate as the only active toxicant, and all other chemical components were considered inert.[54] We could not determine whether other unidentified components of GBH played any role in the observed toxic outcomes. The surfactant component of GBH was considered relevant in the human toxicity of GBH,[55,56] and surfactants with other co-formulants in GBH might contribute to its toxicity.[54,57] However, the recently approved surfactants used to formulate GBH are reported to have extremely low toxicity.[54] The limitations of the study include the lack of measurements of cytokine and immunoglobulin levels in the tissues of GBH-exposed rats and a lack of immunohistochemistry staining of inflammatory biomarkers and the lymphocyte distribution in GBH-exposed rats, which would have added additional evidence to the research data. This study was a preliminary immunotoxicity screening providing directions for further detailed investigations of the components of the immune system as proposed previously[35].

In conclusion, our data supported the previously observed immunotoxic effects of GBH exposure, indicating the induction of pro-inflammatory cytokine release (TNF-α), suppression of immunoglobulin production and release, and depletion of lymphocytes in the lymphoid tissues of the spleen, submandibular lymph nodes, and thymus. Meanwhile, the immunostimulating effect of zinc supplementation might have mitigated these aspects of GBH-induced immunotoxicity. Therefore, physiologic zinc supplementation in GBH-exposed individuals might prevent probable GBH-induced immune dysfunction in agricultural communities in which the herbicide is used, and this observation could represent the clinical relevance of this study.

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of all parameters involving the use of ELISA test kits.

Authors' contributions

- **TIZHE, Emmanuel Vandi:** Conceptualization, literature review, experimental data collection, data analysis, writing draft manuscript, and funding.
- **IGBOKWE, Ikechukwu Onyebuchi:** Conceptualization, supervision, literature review, data analysis, critical review and rewriting of manuscript.
- **NJOKU, Celestine Onwu-Ibe:** Conceptualization, supervision, critical review.
- **FATIHU, Mohammed Yakasai:** Conceptualization, supervision, critical review.
- **TIZHE, Ussa Delia:** Literature review, data analysis.
- **IBRAHIM, Najume Dogon-Giginya:** Conceptualization, supervision, critical review.
- **UNANAM, Essienifiok Saturday:** Literature review, data analysis and interpretation.
- **KORZERZER, Rachel Mngu-suur:** Literature review, data analysis and interpretation.

Data availability statement

The data for this manuscript are available upon request by the Journal.

Declaration of conflicting interests

The authors declared that there was no conflict of interest.

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