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Original article

# A glyphosate-based herbicide disrupted hematopoiesis and induced organ toxicities, ameliorated by vitamin $B_{12}$ in a mouse model

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ABSTRACT

Glyphosate-based herbicides (GBH) are widely used worldwide. Their negative impact on human health is a matter of debate by regulatory bodies and the public. The present study sought to determine the impact of a GBH on the vital organs; and the potential protective effects of vitamin B12 (cyanocobalamin) supplementation. Sixty white Swiss mice were randomly assigned to five treatment groups, each containing twelve mice. Group one represented the normal control; Group two mice were treated with 375 mg/kg of GBH for 56 days; Group three mice received 10 mg/kg of cyanocobalamin for 56 days; Group four mice were administered with 375 mg/kg of GBH and 10 mg/kg cyanocobalamin for 56 days and Group five received 10 mg/kg cyanocobalamin first for 7 days, then continued thereafter co-administered together with 375 mg/kg of GBH for 56 days). Oral administration of GBH induced severe anemia in mice, which was attenuated by cyanocobalamin. Moreover, GBH resulted in a very significant alteration of platelets, WBCs, and its sub-types. Once again, cyanocobalamin stabilized the levels of platelets and WBCs in the presence of GBH. GBH-induced elevation of triglycerides and HDL was nullified by the administration of cyanocobalamin. Further studies showed evidence for GBH-induced inflammation represented by an imbalance in serum levels of the TNF-α: IL-10 and IFN-γ ratios. The GBH severely depleted GSH levels in the liver. A GBH-induced rise in GSH in the kidney, lungs and brain was noted; and is an indicator of antioxidant capacity enhancement in response to a GBH-induced oxidant challenge. Moreover, cyanocobalamin supplementation abrogated GBH-induced oxidative stress as depicted by stabilized GSH levels in the liver, kidney, lungs, and brain. In the presence of cyanocobalamin, the GBH-induced liver injury depicted by elevation of AST, ALT, and bilirubin, was attenuated. From the results, we conclude that the capacity of cyanocobalamin to assuage GBH-induced inflammatory responses, hepatotoxicity, and hematological alteration as well as oxidative stress may be attributable to its antioxidant and anti-inflammatory properties. The current findings provide a solid foundation for further scrutiny of this phenomenon, with vital implications in GBH exposure and the role of potent antioxidant supplementation in the management of GBH-induced toxicity.
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## 1. Introduction

The need to meet the food demand for the world's growing population has resulted in a rise in use of agrochemicals. Glyphosate-based herbicides (GBHs) are among the economically important agrochemicals widely used. Glyphosate, chemically referred to as N-phosphonomethyl glycine is the active ingredient in GBHs, which include formulations such as Roundup (Djaber et al., 2020; Panzacchi et al., 2018).

With increasing use in agriculture, the contamination of air, water, and food is inevitable. Therefore, non-target organisms such as humans are most susceptible to GBH exposure, resulting in various health issues (Turkmen et al., 2019; Samsel & Seneff, 2013). In 2015, the World Health Organization's International Agency for Research on Cancer (IARC), identified glyphosate as group 2A chemical – whose constituents have probable carcinogenic effects

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on humans (Kogevinas, 2019; Tarazona et al., 2017). Across the globe and in particular Africa, use of herbicides to control weeds is rampant in the absence of vital data on their composition and safety. This study sought to profile the toxicity of one such product (Roundup) – a GBH-based herbicide commonly used in the control of weeds.

Related studies have shown that oxidative stress probably contributes to the toxic mechanism of GBH in tissues (Webster & Santos, 2015; Menezes et al., 2011). Notably, glyphosate as probable antioxidant disruptor, stimulates the production of reactive oxygen species (ROS) which occasions the imbalance in antioxidant system culminating in oxidative stress and consequent damage to vital functional compounds such as DNA, proteins, carbohydrates, and lipids (Mesnage et al., 2015).

The oxidative modification of these functional compounds has been implicated in many age-related diseases such as schizophrenia, type-2 diabetes, Alzheimer's disease, Parkinson's disease, and cardiovascular disease. Primarily, such age-related diseases share a commonality; oxidative stress is probably a major important factor in their pathophysiology (van de Lagemaat et al., 2019; Birch et al., 2009). These potential targets for the ROS are particularly important biomolecules, demonstrating their destructive potential. However, organisms have evolved effective antioxidant defense systems (enzymatic and non-enzymatic) to reduce the effects of oxidative metabolism (Rainio et al., 2019; Isaac et al., 2007). Related studies have demonstrated that Roundup and other pesticides induce over-production of ROS in the intracellular and extracellular spaces resulting in the disruption of antioxidant/pro-oxidant balance, leading to oxidative stress (Martínez et al., 2009; El-Shenawy, 2009).

Changes or interference with hematological parameters such as packed cell volume, red blood cells, white blood cells, hemoglobin, and platelets can be used to gauge toxicity; and have potential application in forensic, occupational and environmental GBH exposure monitoring (Jasper et al., 2012). Chemical toxicants often have the potential for interfering with inflammation and the immune response via cytokine derangement. Cytokines act on signaling molecules and cells, attracting them towards sites of inflammation and trauma. Proinflammatory cytokines such as TNF-$\alpha$ and INF-$\gamma$ are primarily involved in up-regulation of inflammatory reactions (Tisoncik et al., 2012). Chronic overproduction of proinflammatory cytokines has been associated with disorders such as atherosclerosis and cancers. Anti-inflammatory cytokines such as IL-4 and IL-10 on the other hand, act in a feedback loop to inhibit proinflammatory cytokines responses thus limiting the potentially damaging effects of inflammatory reactions. A balance between proinflammatory and anti-inflammatory cytokines is critical in maintaining a healthy life (Ferreira et al., 2019).

It therefore made sense to test the ability of vitamin $B_{12}$, an antioxidant, known to decrease with age, to assuage GBH toxicity. From the recent studies, the power of cyanocobalamin as an antioxidant is evident. The antioxidant properties of cyanocobalamin probably result from a combination of direct and indirect effects; stimulation of the methionine synthase activity, direct scavenging for the reactive oxygen species, glutathione sparing effect, and modulation of signaling molecules. These properties suggest that cyanocobalamin might have potential in management of pathological conditions in which oxidative stress is a crucial component (van de Lagemaat et al., 2019; Birch et al., 2009). Consequently, the present study was conducted to assess various physiological and biochemical parameters in mice administered with GBH. In addition, the ameliorative effects due to GBH exposure was determined.

## 2. Materials and methods

### 2.1. Animals and ethical statement

All the experimental procedures and protocols involving mice in this study were reviewed and approved by the Institutional Review Committee (IRC) of the Institute of Primate Research, Karen, Kenya (IRC/03/16). Accordingly, all experiments were conducted in compliance with the recommendations of the Helsinki declaration on guiding principles on care and use of animals. Sixty, male swiss white mice, 3–4 weeks old were obtained from Biotechnology Research Institute (BRI), Muguga, Nairobi. The animals were housed in standard cages at a temperature (21-25$^0$c) and were provided *ad libitum* access to water and standard mice cubes (Unga Feeds Ltd, Nairobi, Kenya) Wood chippings were used as bedding material. Mice were dewormed with 0.02 ml of Ivermectin (Ivermectin®, Anupco, Suffolk, England) injected subcutaneously to each mouse to eradicate endoparasites and ectoparasites infestation.

### 2.2. Experimental groups

Mice were randomly assigned to five treatment groups of 12 mice each (n = 12). Group 1 served as the normal control; Group 2 mice were treated with 375 mg/kg b.w. of GBH for 56 days; Group 3 mice received 10 mg/kg b.w. of cyanocobalamin for 56 days; Group 4 mice were administered with 375 mg/kg b.w. of GBH and 10 mg/kg b.w. cyanocobalamin for 56 days and Group 5 received 10 mg/kg b.w. cyanocobalamin first for 7 days, then continued thereafter together with 375 mg/kg b.w. of GBH for 56 days). All treatments were administered orally. In this study treatment and evaluation of the health status of mice were performed sequentially. At the end of the experimental period (56 days), for ex vivo analysis mice were euthanized using Rompun (2%) and ketamine (50 mg/ml) through intramuscular injection followed by perfusion with sterile PBS.

### 2.3. Preparation of cyanocobalamin and GBH

Cyanocobalamin powder (98%) was purchased from Sigma Aldrich (St. Louis, MO) and was prepared by dissolving it in distilled water. A dose of 6 mg/Kg was selected for the this study based on previous findings that 6 mg/Kg enhance protection against xenobiotic induced toxicity (Hajihashemi et al., 2017). Glyphosate based herbicide (GBH) 375 mg/kg was prepared by dissolving it in distilled water. A dose of 375 mg/kg GBH was chosen for this study based on the findings that 375 mg/kg is able to induce sub-chronic toxicity (Turkmen et al., 2019).

### 2.4. Sample collection and preparation

At the end of the experiment (56 dpt), mice were euthanized using Rompun (2%) and ketamine (50 mg/ml) through intramuscular injection, whole blood was obtained intra-cardially and placed either in heparinized tubes (for full hemogram analysis) or in tubes without the anti-coagulant (for biochemical and immunological analysis). The blood samples blood was left to settle at room temperature for 30 min then centrifuged for 5 min at 10,000 rpm and 4 °C (Centurion Scientific Ltd K240R, UK) to obtain serum. To obtain vital organs for reduced glutathione assay, the mice were perfused with sterile PBS after obtaining blood. The samples for reduced glutathione (GSH) were homogenized in ice-cold conditions in a homogenizing buffer (0.5 ml of 0.25 M sucrose, 5 mM Hepes-Tris pH 7.4 with protease inhibitor) and aliquoted into 0.5 cryovial tubes. The homogenates were kept at −80 °C until further analyses.

### 2.5. Determination of the body and organ weights

The body weights were measured before the start of the experiment and the last day of the experiment. The weights of the organs (brain, liver, kidney, and spleen) were measured at the end of the experiment. This was followed by the determination of the relative organ weights. Individual organ weight divided by the respective body weight at 56 days post treatment (56 dpt) multiplied by 100 provided the relative organ weight. All the weights were measured using an analytical electronic balance (Mettler PM34, DoltaRange®).

### 2.6. Determination of reduced glutathione

The reduced glutathione levels (GSH) in the liver, kidney, lungs, and the brain were determined quantitatively according to the method of Griffith (1980) with slight modification. The homogenates were mixed with 4.31% w/v prepared solution of sulphosalicylic acid together with 0.25 mM EDTA. The GSH from the liver, kidney, lungs, and the brain homogenates was then determined by reacting the GSH therein with Elman's reagent and measuring the absorbance of the reaction product at 412 nm using a multi-detection microplate reader (Bio-Tek Synergy HT, Winooski, VT, USA).

### 2.7. Determination of hematological and biochemical parameters

For hematological and biochemical assays, the samples of blood were collected into heparinized tubes using automated Benchman Coulter counter (Benchman, Indianapolis, USA). Serum, Aspartate Amino Transferase (AST), Alanine Amino Transferase (ALT), Gamma-glutamyl transferase (GGT), Direct Bilirubin (DBIL) and Total Bilirubin (TBIL) was measured following the manufacturer's instructions of enzymatic colorimetric assay kits (Roche's Reflotron test system). To obtain full hemogram blood from different experimental groups were measured using automated analyzer (COBAS Integra-400 plus analyzer, Roche, Basel, Switzerland).

### 2.8. Cytokine assays

Serum cytokine levels of interleukin-10 (IL-10), interferon-gamma (IFN-$\gamma$) and tumor necrosis factor-alpha (TNF-$\alpha$) were measured by sandwich enzyme-linked immune sorbent assay (sandwich-ELISA) using cytokine specific kit from Invitrogen (Thermofischer Scientific, California, USA) according to the manufacturer's instructions. Measurement was done using ELISA optical reader (Multiskan ex-355, Thermo electron corporation, Waltham, Massachusetts, USA) with absorbance set at 450 nm.

### 2.9. Statistical analysis

The data obtained from the experimental animals were expressed as means of standard error and analyzed benefiting from the technique of one-way ANOVA, followed by Tukey post-test on Graph pad prism 5.0 statistical software package. Results were given as mean + SEM with the significance level set at $P < 0.05$.

## 3. Results

### 3.1. Exposure to GBH induced general body weight loss without significant change in relative organ weight

Exposure to GBH has the potential to alter various metabolic process with concomitant impairment of the body physiological functions. Additionally, change in the general body weight as well as relative organ weight in mice following exposure to toxic compounds serves as an important indicator for evaluating the developmental processes and general health status. Thus, this study sought to determine the effects of oral administration of cyanocobalamin on general change in body weight following GBH induced toxicity. Significant changes in general body weight in mice administered with GBH were noted, when compared to the control (Fig. 1A). Cyanocobalamin treatment did not rescue or reverse the GBH-induced decrease in the bodyweight. The relative organ weight (ROW) of the Heart, kidney, spleen, brain, liver and lungs were unaffected upon exposure to GBH (Fig. 1B–G).

### 3.2. GBH exposure markedly induced suppression of hematocrit

Packed cell volume (PCV) is the percentage of red blood cells (RBCs) in the circulating blood. RBCs transports oxygen throughout the body. A low PCV is an indication for RBCs loss due to cell destruction, blood loss, and/or the failure in bone marrow function. An increased PCV generally means dehydration or abnormal increase in RBC production. Lower or higher than normal PCV is a primary indicator of a disease process or toxicity resulting from chemical exposure. The GBH-exposed mice showed marked suppression in PCV level compared to the control ($P \leq 0.001$). However, treatment with cyanocobalamin before and after GBH exposure, significantly stabilized the PCV levels in the presence of GBH (Fig. 2).

### 3.3. Cyanocobalamin supplementation restored GBH-induced RBC and hemoglobin suppression

Results for the mean hematological profile for red blood cells (RBCs) and hemoglobin are presented in Fig. 3A and B. Statistical analysis revealed significant suppression of RBCs count in the GBH-treated mice compared to the group administered with cyanocobalamin and the control group (Fig. 3A). Similarly, comparison of the means revealed significant suppression of the hemoglobin level in GBH-treated mice relative to post-cyanocobalamin treated mice and the control ($p < 0.05$) (Fig. 3B). Notably, cyanocobalamin administration before or after GBH, stabilized RBCs and HGB levels.

### 3.4. Cyanocobalamin assuaged GBH driven microcytic-hypochromic anaemia

The RBC indices provide information about the hemoglobin content and the size of RBCs. This is essential in diagnosing the various types of anemia. From the current study, it was observed that the mice exposed to GBH showed significantly low levels of hemoglobin, red blood cells, and the packed cell volume. An attempt was made to further analyze and determine the type of anemia induced by the GBH. The results showed insignificant alterations in mean corpuscular volume (MCV) levels (Fig. 4A). Nevertheless, the mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) levels and red blood cells distribution width (RDW) were significantly suppressed ($p < 0.05$) in GBH administered mice, relative to the control group (Fig. 4B–D); while the levels of an indication that microcytic-hypochromic type of anemia was predominantly induced by GBH. In the presence of cyanocobalamin red blood cell indices were stabilized.

### 3.5. The effect of GBH and cyanocobalamin on WBCs and its subtypes

Leukocytes are an important part of the immune system. They fight infections by attacking bacteria, viruses, and other pathogens that invade the body or exposure to toxic compounds. Statistical analysis of WBCs using one-way ANOVA showed

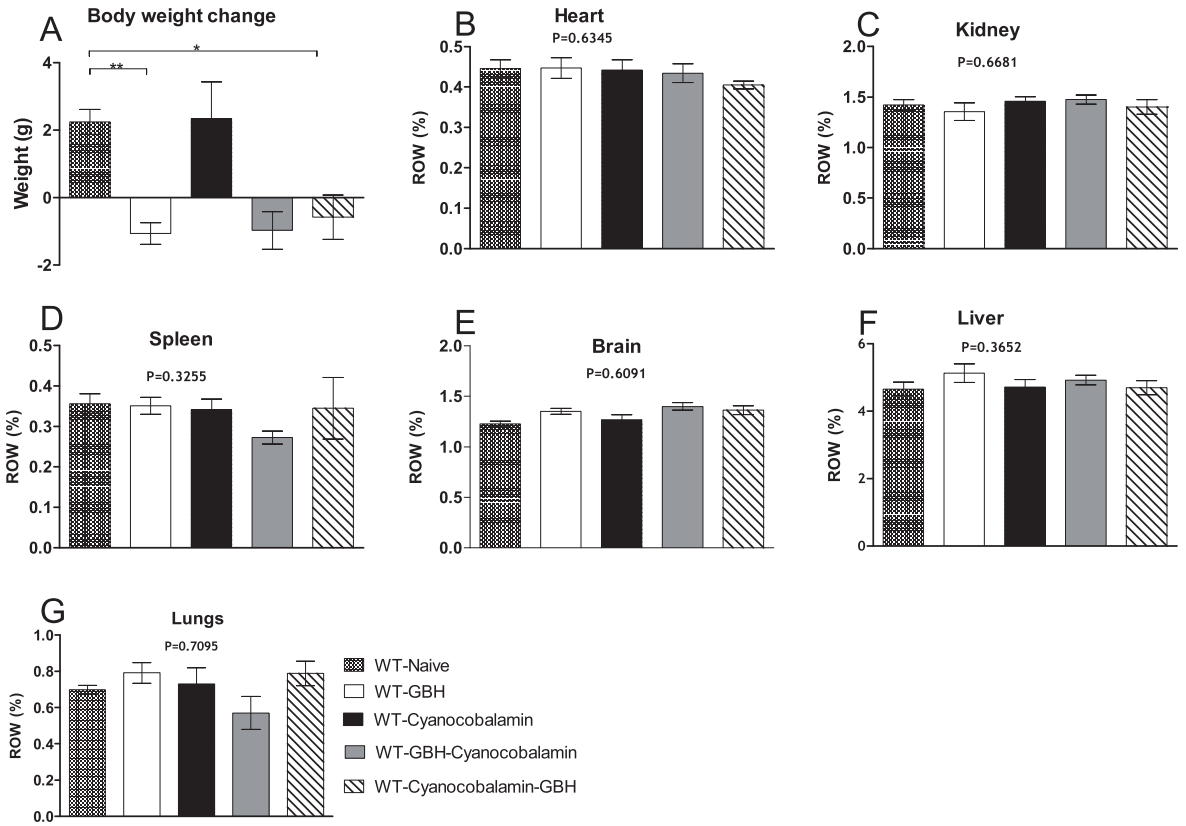


**Fig. 1.** Changes in general body weight relative organ weight of mice administered with GBH and /or cyanocobalamin. Data sets are presented as the mean of each group ± SEM. The figures show the change in body weight (A), Heart (B), Kidney (C), Spleen (D), Brain (E), Liver (F) and Lungs (G). The bodyweights were analyzed by One-way ANOVA, followed by Tukey's post-test. n = 12, with the level of significance: $^{*}P \leq 0.05$.

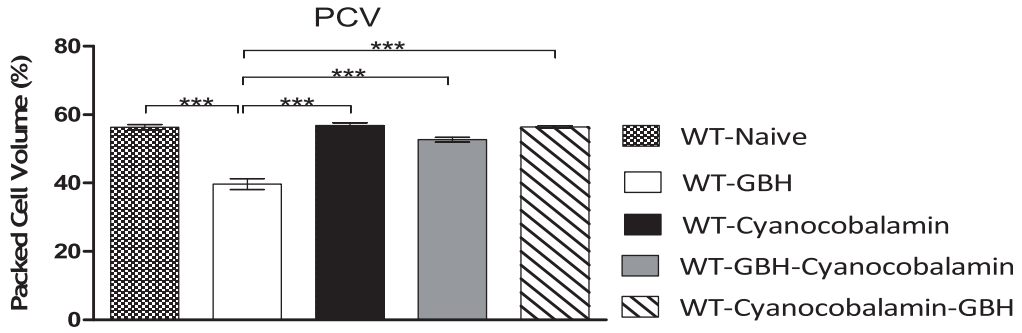


**Fig. 2.** The effects of GBH and / or cyanocobalamin treatment on packed cell volume levels. Data sets are presented as a mean of each group ± SEM. The PCV values were analyzed by one-way ANOVA, followed by Tukey's multiple comparison post hoc test. n = 12. Indicated level of significance: $^{***}P \leq 0.001$.

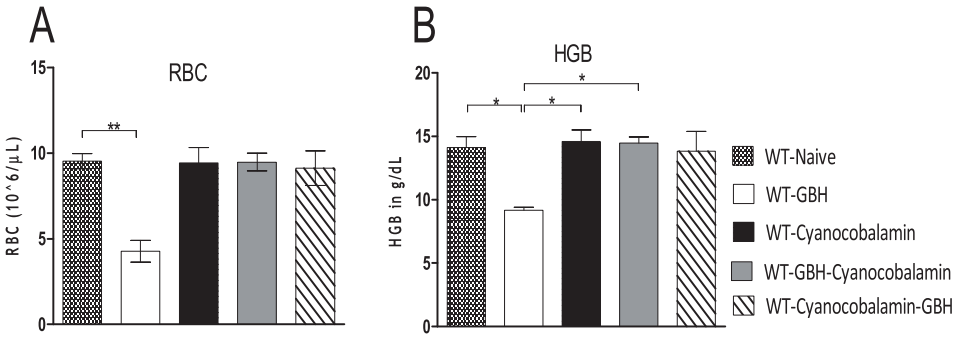


**Fig. 3.** The effects of GBH and cyanocobalamin on RBCs and hemoglobin levels. Data sets are presented as a mean of each group ± SEM. The RBCs (A), and hemoglobin (B) values were analyzed by One-way ANOVA, followed by Tukey's multiple comparison post hoc test. n = 12. (Indicated level of significance $^{*}P \leq 0.05$; $^{**}P \leq 0.01$. u/L: microlitres per Litre, g/dL: grams per decilitre.

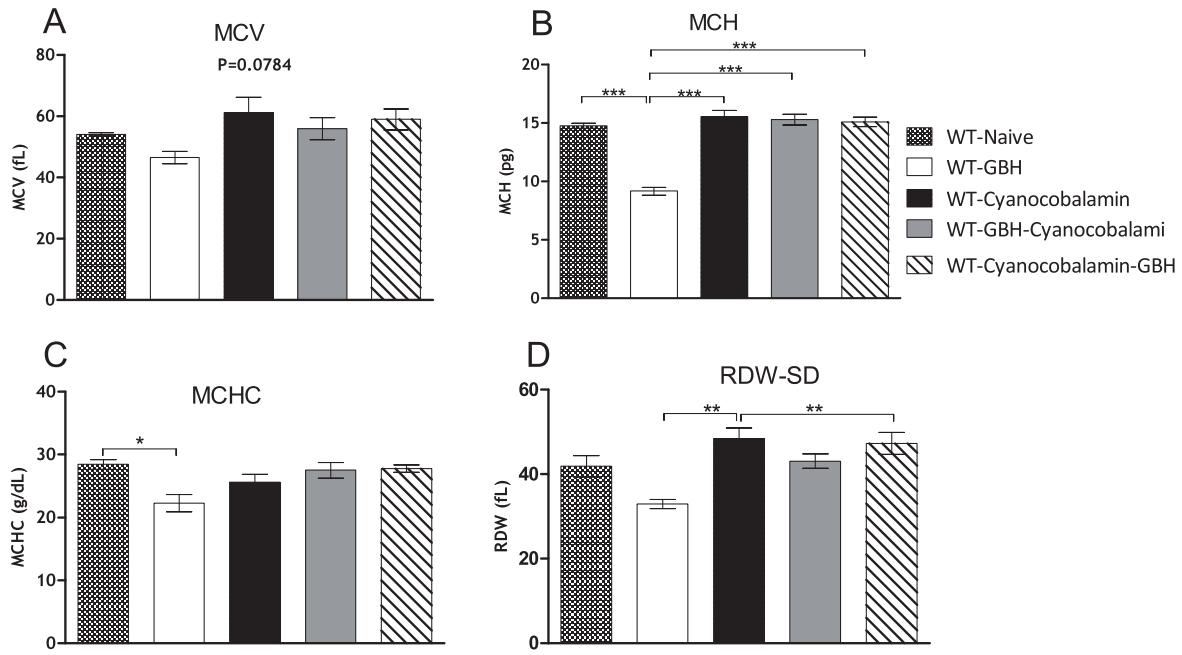


**Fig. 4.** The effects of GBH and/or cyanocobalamin on RBC indices: MCV (mean corpuscular volume) (A), MCH (mean corpuscular concentration) (B), MCHC (mean corpuscular hemoglobin concentration) (C), and RDW (red cell distribution width) (D) Data sets are presented as a mean of each group ± SEM. statistical analysis was done by One-way ANOVA, followed by Tukey's multiple comparisons post hoc test. (n = 12. Indicated level of significance: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. fL: femtoliters, pg: picograms, g/dL: grams per decilitre).

marked suppression in total WBC count among the GBH-treated mice ($p < 0.01$) relative to the cyanocobalamin and control groups. In the presence of GBH, cyanocobalamin was effective in blocking GBH-induced leukopenia (Fig. 5A). Further experiments were carried out to elucidate any negative effects of GBH on WBC phenotypes. Specifically, the impact of GBH on the expression levels of WBC phenotypes was determined. The levels of neutrophils, lymphocytes. Monocytes and basophils were determined as shown in Fig. 5B–E. Oral administration of GBH was found to significantly suppress neutrophils count ($p < 0.05$) (Fig. 5B). In contrast, the levels of lymphocytes were not significantly changed (Fig. 5C). Interestingly, after administration of cyanocobalamin significantly blocked GBH-induced leukopenia and neutropenia ($p < 0.05$). Furthermore, GBH exposure markedly suppressed the monocytes count in the blood (Fig. 5D). Notably, before, and after cyanocobalamin treatment significantly alleviated the GBH-induced monocytopenia. Additionally, GBH administration significantly elevated the basophils count in the blood. In the presence of cyanocobalamin, the effects of GBH on basophils were nullified (Fig. 5E).

### 3.6. The effect of GBH and cyanocobalamin on platelet count

Platelets are primarily involved in stopping the bleeding during injury, through intricate blood clotting cascades. Platelets abnormalities may be reflected by variation in the number and function. The results showed marked suppression of platelets count in GBH-exposed mice ($p < 0.001$) compared to the control (Fig. 6A). The implication of this finding is a clear possibility for suppression of the ability for coagulation, and a risk factor for patients on blood thinning medications. Notably, pre-, and post cyanocobalamin treatment stabilized the GBH-induced suppression in platelets count. Mice exposed to GBH however did not show significant change in mean platelet volume (MPV) and platelet distribution width (PDW) relative to the control (Fig. 6B–C).

### 3.7. The effect of GBH and cyanocobalamin on the lipid profile

Findings further revealed that GBH did not alter total cholesterol levels (Fig. 7A). However, changes were noted for tri-gricerides. It was observed that the levels of triglycerides were significantly higher ($p < 0.05$) in GBH treated mice in comparison to the control group (Fig. 7B). In the serum, the mice administered with GBH showed marked elevation in HDL levels ($p < 0.05$) when compared to the control group. In the presence of GBH, cyanocobalamin stabilized the HDL levels (Fig. 7C).

### 3.8. Cyanocobalamin supplementation markedly modulates GBH-induced inflammatory response

The secretion of proinflammatory cytokines which is observed in inflammatory conditions has been observed to cause deleterious effects that modulate hemodynamic and metabolic processes when over-produced. In the present study, inflammation-driven by GBH toxicity and the effects of administering cyanocobalamin pre-, or post-treatment with GBH were determined by measuring the levels of proinflammatory cytokines, interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α). Using enzyme-linked immunosorbent assay (ELISA), a significant elevation in TNF-α ($p < 0.001$) was observed in the serum of the GBH-treated mice in comparison to the cyanocobalamin and control group (Fig. 8A). However, cyanocobalamin treatment before, or after GBH exposure resulted in stabilization of TNF-α serum levels ($p < 0.001$) as clearly shown in the Fig. 8A. GBH or cyanocobalamin did not have significant effect on the levels of the pro-inflamatory cytokine, interferon gamma (IFN-γ) (Fig. 8B). The current study further determined how the levels of serum anti-inflammatory cytokine; interleukin-10 (IL-10) vary upon GBH exposure. Indeed, IL-10 is overly critical in regulating the levels of pro-inflammatory cytokines. Exposure to GBH

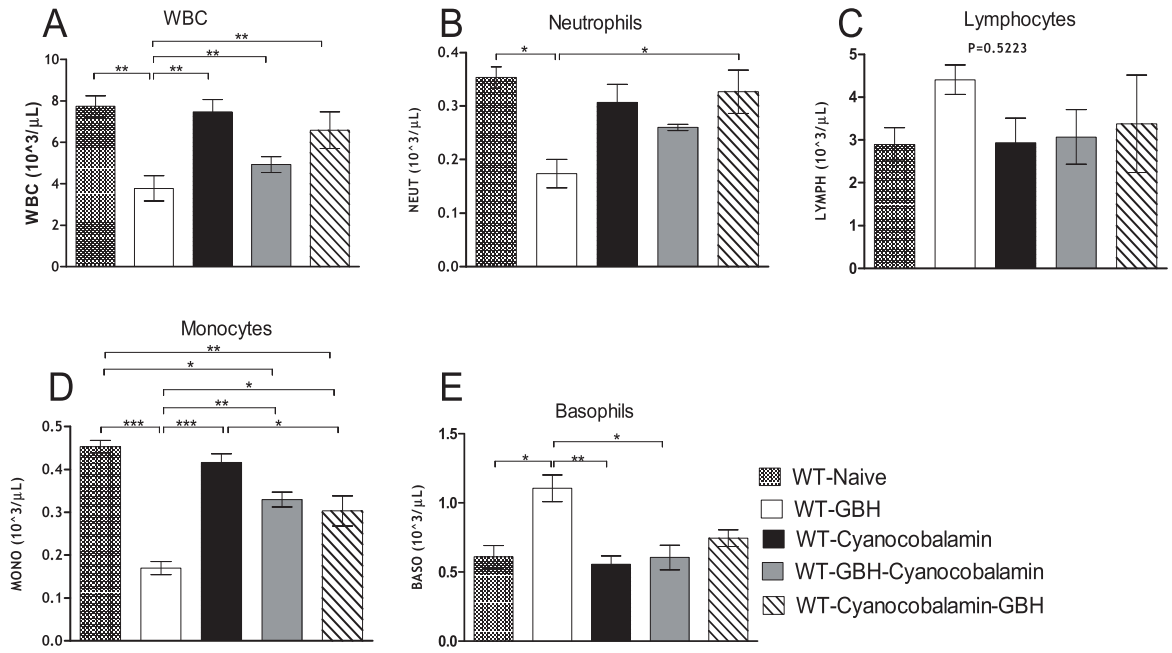


**Fig. 5.** The figure shows the effects of GBH and cyanocobalamin on WBC and its sub-types count. The WBC (A), Neutrophils (B), Lymphocytes (C), Monocytes (D), and Basophils (E) were measured using an automated Bechman Coulter Counter (Coulter A -T diff TM) and their values analysed by one-way ANOVA followed by Tukey's multiple comparisons post hoc test. (Data sets are presented as a mean of each group ± SEM. Indicated level of significance: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$). n = 12. u/L: microlitres per Litre).

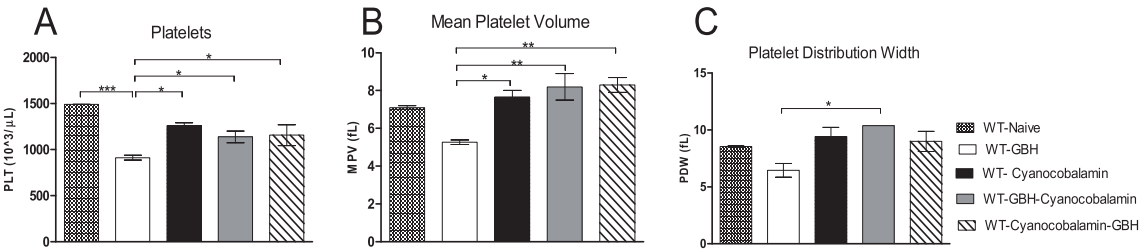


**Fig. 6.** The effects of GBH and cyanocobalamin on platelets and platelet indices. This figure above show platelets levels (A) mean platelet volume (MPV) (B) and platelet distribution width (PDW) (C) were measured using an automated Bechman Coulter Counter (Coulter A -T diff TM) and their values analysed by One-way ANOVA followed by Tukey's multiple comparisons post hoc test. (Data sets are presented as a mean of each group ± SEM. Indicated level of significance: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$); n = 12. fL: femtoliters, u/L: microlitres per Litre).

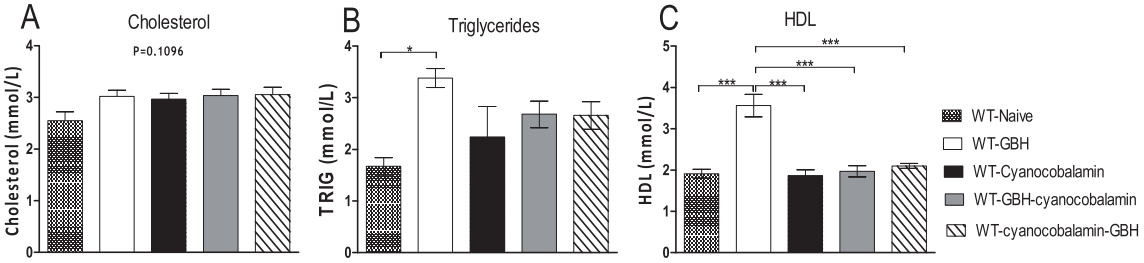


**Fig. 7.** The figure shows the effects of GBH and cyanocobalamin treatment on the lipid profile. The lipid parameters, Cholesterol (A), Trigricerides (B), and HDL (C), were measured by automatic hemoanalyzer and the values analysed by One-way ANOVA followed by Tukey's multiple comparison post hoc test. Data sets are presented as a mean of each group ± SEM. Indicated level of significance: $^{*}P \leq 0.05$; $^{***}P \leq 0.001$). n = 12. mmol/L: millimole per litre.

resulted in a marked suppression ($p < 0.05$) of serum IL-10 levels, while pre-, and post-cyanocobalamin administration resulted in stabilization of IL-10 levels ($p < 0.001$) in the serum (Fig. 8C). Fig. 8D and E below, demonstrate the imbalanced pro-inflammatory and anti-inflammatory cytokines in the serum of the GBH-exposed mice in favor of pro-inflammatory cytokines, (TNF-α and INF-γ).

### *3.9. Cyanocobalamin supplementation abrogated GBH-induced oxidative stress in the liver, kidney, lungs, and brain.*

Reduced Glutathione (GSH) is an important cellular endogenous antioxidant that plays a fundamental role in maintaining the cell's redox status. Augmentation or a significant depletion of GSH is a clear indicator of induction of oxidative stress. The levels of cellular

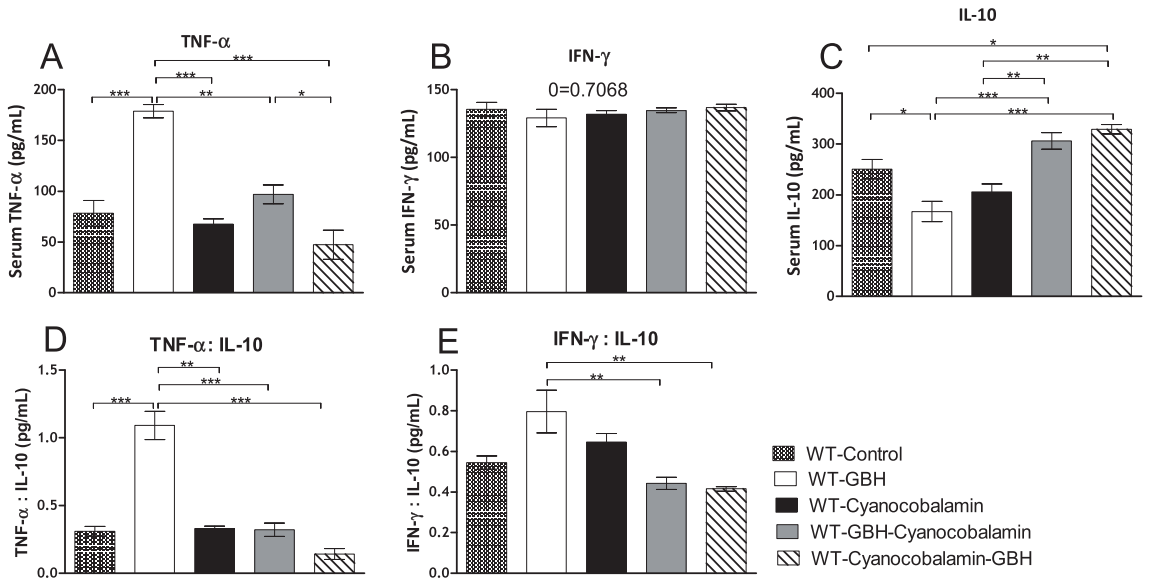


**Fig. 8.** The effect of GBH and cyanocobalamin on the levels of pro-inflammatory cytokines and anti-inflammatory cytokines in the serum. The levels of the cytokines Tumor Necrosis Factor-alpha (TNF-α) (A), Interferon gamma (IFN-γ) (B), Interleukin-10 (IL-10) (C), TNF-α to IL-10 (D) and INF-γ to IL-10 (E). The serum levels of cytokine was measured using sandwich-ELISA and the values analyzed by One-way ANOVA followed by Tukey's multiple comparison post hoc test. (Data sets are presented as a mean of each group ± SEM. Indicated level of significance: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$, n = 12. pg/mL: picograms per millilitre).

GSH were measured on the liver, kidney, lungs, and brain samples from all treatment groups, respectively. In the GBH-treated group, there was significant decrease in the level of GSH in the liver (Fig. 9A). On the contrary, a significant increase was observed in the kidney, lungs, and brain (Fig. 8B–D). Supplementation with cyanocobalamin resulted in a marginal increase in the level of GSH in the liver. While co-administration of cyanocobalamin and GBH resulted in restoration of the cellular levels of GSH in the kidney, lungs, and brain.

### *3.10. Cyanocobalamin protected the liver from GBH-induced hepatotoxicity*

Administration of GBH resulted in statistically significant increase of the levels of the liver aminotransferases; alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Fig. 10A–B). The heightened levels of liver enzymes is an indication of GBH-induced hepatotoxicity. Indeed, it was also observed that the ratio of AST: ALT in the present study was augmented

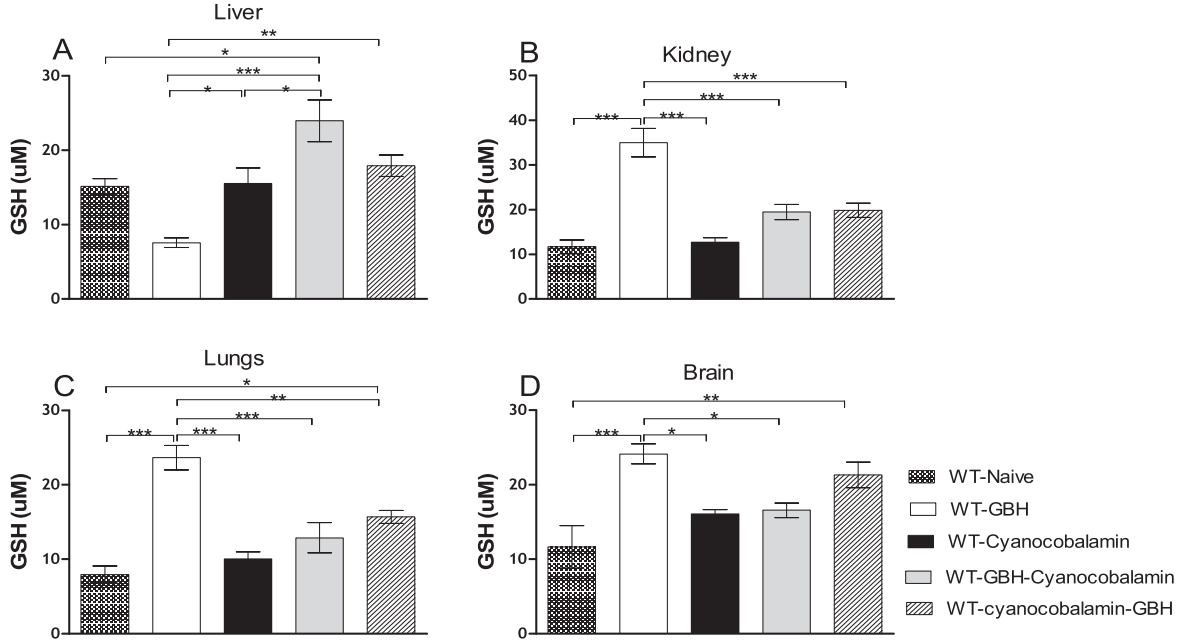


**Fig. 9.** Comparison of the effect of GBH and cyanocobalamin on the levels of GSH in the Swiss Albino mice. GBH administration suppressed GSH levels in the liver (A), and increase in the kidney (B), Lungs (C) and Brain (D. The concentration of reduced glutathione in the liver was determined using enzymatic colorimetric assay and values analyzed by One-way ANOVA followed by Tukey's post-test. Data sets are presented as the mean of each group ± SEM. Level of significance: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. n = 12.

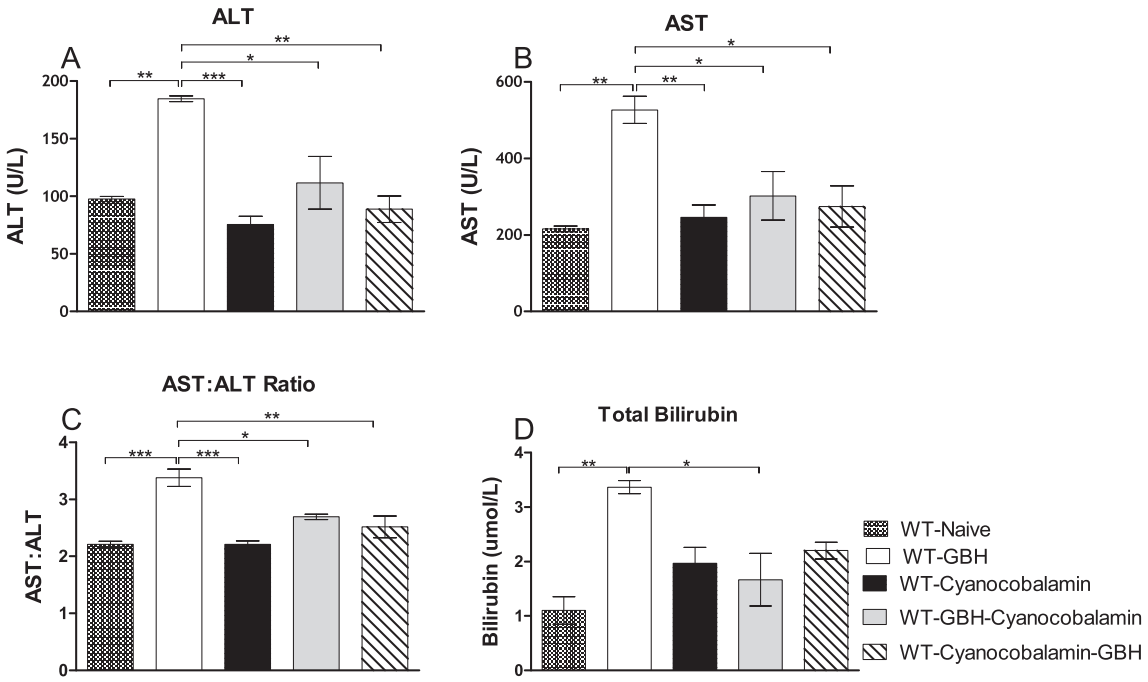


**Fig. 10.** Effects of GBH and cyanocobalamin administration on the liver transaminases enzymes and total bilirubin in Swiss albino mice. (A–D) shows changes in the activity of ALT (Alanine aminotransferase) (A), AST (aspartate aminotransferase) (B), the ratio AST: ALT (C) and Total Bilirubin (D). The values were analysed by One-way ANOVA followed by Tukey's post-test. Data sets are presented as the mean of each group ± SEM. level of significance: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. n = 12.

among GBH administered mice, which further confirmed the presence of liver injury (Fig. 10C). Remarkably, Cyanocobalamin supplementation restored the levels of AST and ALT; a strong indication of ameliorative effect by cyanocobalamin against GBH-induced liver hepatotoxicity. A significant elevation of serum bilirubin levels was recorded in the GBH-treated group while co-supplementation with cyanocobalamin resulted in normal levels of bilirubin (Fig. 10D).

### *3.11. The effects of cyanocobalamin on levels of creatinine following GBH administration*

GBH is known to interfere with creatinine excretion, resulting in renal injury. This phenomenon prompted this study to determine the effect of cyanocobalamin on GBH-altered serum creatinine. The results showed no significant change in levels of the serum creatinine among mice treated with GBH or with cyanocobalamin in comparison to the control group (Fig. 11).

## 4. Discussion

Indeed, herbicides are essential inputs in agricultural production. However, they persist in agricultural products and the environment posing serious health risks to humans and other living organisms (Dallegrave et al., 2007). The toxic effects of these herbicides are associated with the potential to stimulate the production of reactive oxygen species, which results in the oxidation of cellular functional compounds (Turkmen et al., 2019). The use of antioxidants has shown potential in managing the oxidative events which arise due to chemical toxicity (Mesnage et al., 2015). However, no study has previously investigated the possibility of cyanocobalamin to protect from herbicide-induced toxicity. Cyanocobalamin is a potent antioxidant that scavenges for oxygen

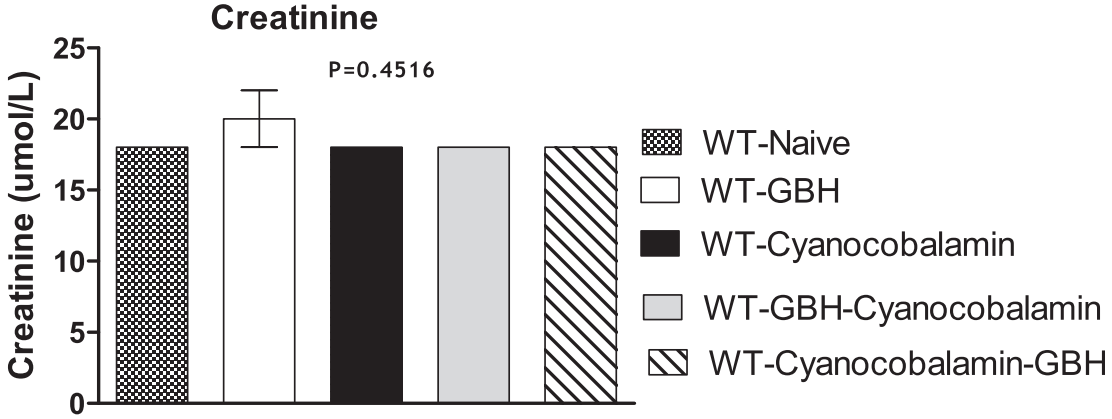


**Fig. 11.** The figure shows the effects of GBH and/or cyanocobalamin on creatinine. The values were analyzed by One-way ANOVA followed by Tukey's post-test. Data sets are presented as the mean of each group ± SEM. level of significance: n = 12.

radicals and has been shown to preserve glutathione and modulate cytokine production (van de Lagemaat et al., 2019). The current study clearly demonstrates that, GBH are highly toxic and that cyanocobalamin, is quite potent in nullifying GBH-induced haematological changes, hepatotoxicity, inflammatory responses, and oxidative stress.

The findings from the current study demonstrated that GBH exposure in mice induced a significant alteration in body weight. This finding has been noted before in adult mice in a previous study by (Jasper et al., 2012). Other studies have implicated reduction in body weight upon GBH exposure as the main important indicator of toxicity which may be associated with the capacity of GBH to provoke oxygen radical production and consequent oxidant damage (Namratha et al., 2019). Similar findings have been noted in animals exposed to pesticides such as diazinon (Kalender et al., 2006).

The current study also investigated the negative physiological and biochemical effects of a GBH and potential rescue by cyanocobalamin. One of the key aspects elucidated, was the effects of GBH on the haematopoiesis, which is an overly critical process in the formation of blood cellular components. The current study shows marked suppression of packed cell volume, red blood cells, and hemoglobin levels upon GBH exposure. However, treatment with cyanocobalamin before, and after GBH exposure stabilized the GBH-induced suppression of hematological parameters.

The study further showed depressed RBC indices; mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) levels in the group of mice administered with GBH. There was no significant changes on the mean corpuscular volume (MCV) among the GBH group. These features are suggestive of severe anemia, characterised as normocytic hypochromic anemia. A previous similar study demonstrated significant decrease in RBC and haemoglobin in a group of rats exposed to Roundup (Owagboriaye et al., 2019). The GBHs have been shown to stimulate the production of reactive oxygen species (ROS), with a significant potential for hematological alterations and induction of anemic syndrome. Furthermore, depletion of hemoglobin in the current study, possibly confirmed a decrease in RBCs which is consistent with the elevation of the total bilirubin level in the plasma; perhaps due to GBH-induced hemolysis. Reduction of GSH and increased lipid peroxidation of RBCs has been reported to result in cellular lysis (Kennedy et al., 2020; Jasper et al., 2012).

Previous studies have shown that changes in leukocyte count after exposure to toxicants may be associated with a decrease in non-specific immunity of the mice (Kavitha et al., 2010). Leukocytes are involved in the regulation of immunity and depletion of WBCs is characteristic of a decline in the level of immune function. In the current study, mice exposed to GBH showed marked suppression of WBC levels. Remarkably, cyanocobalamin administration before or after GBH exposure, stabilized the WBC values. Further experiments showed that exposure to GBH had varied effects on leukocyte phenotypes; a significant reduction in neutrophils and monocytes, and elevation of basophils and lymphocytes was reported. A prior study on common carp (*Cyprinus carpio* L) exposed to Roundup for 7 days demonstrated suppression in white blood cell levels (Kondera et al., 2018). Similar findings were observed on Piava (*Leporinus obtusidens*) subjected to varying concentrations of Roundup (Glusczak et al., 2006). Conversely, a study on albino rats orally exposed to Roundup demonstrated marked elevation in white blood cells (Owagboriaye et al., 2019). A study by Martinez and others on *Prochilodus lineatus* subjected to Roundup for 96 h showed significant elevation in white blood cells and lymphocytes count (Martínez et al., 2009). Another study suggested that the reduction in white blood cells in glyphosate-exposed Common carp (*Cyprinus carpio* L) could be related to the GBH toxicity (Kondera et al., 2018). The current study, for the first time showed that vitamin B12 (cyanocobalamin) was beneficial in maintaining normal white blood cell balance during GBH exposure. This finding has tremendous implication in the ability of those exposed to maintain a robust and functional immune response.

Further investigations revealed suppression of platelet levels in the GBH-exposed mice, perhaps due to platelet breakdown and the diminished bone marrow function owing to GBH toxicity. Related studies on human subjects show that GBH treatment inhibited platelets aggregation (Neiva et al., 2010); posing a significant risk factor for patients on blood thinning medications.

While it is clear that the antioxidant system is compromised by GBHs, no study has investigated the mitigative potential of antioxidants. We chose to study cyanocobalamin in this regard, partly because it decreases with age, and the fact that it has potent antioxidant capabilities. Moreover, most users of GBH are adults with declining cyanocobalamin levels, potentially deficient and exposed.

On the other hand, GBH-treated mice exhibited marked elevation in triglycerides and high-density lipoprotein (HDL) compared to the cyanocobalamin and control groups. The hypertriglyceridemia and high HDL may be attributed to increase in the lipid rate of peroxidation and free radical release. These findings corroborate similar studies by Djaber et al., (2020) and El-Shenawy (2009). Again, the findings from the current study showed that cyanocobalamin treatment before or after GBH exposure was effective in stabilizing the cholesterol levels in the blood.

Further analysis of GBH toxicity demonstrated significant alteration of pro-inflammatory and anti-inflammatory markers. GBH induced significant elevation of pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF-$\alpha$). On the contrary, a substantial reduction in the anti-inflammatory cytokine, interleukin-10 (IL-10) was noted in the serum. There was no significant change in proinflammatory cytokine, interferon-gamma (INF-$\gamma$) among the treated groups. TNF-$\alpha$ is a pleiotropic pro-inflammatory cytokine produced by various cells such as macrophages, monocytes, T and B lymphocytes, and natural killer cells. TNF-$\alpha$ is involved in cell proliferation, differentiation, inflammation, and apoptosis as well as stimulation of various environmental challenges in the immune system (Ma & Li, 2015).

Previous studies in rats and carp fish reinforce current findings. In such a study, Roundup, an herbicide, resulted in elevation of pro-inflammatory cytokines such as TNF-$\alpha$; which stimulated the deposition of collagen in the hepatic tissue thus causing modifications in the diffusion of solutes between hepatocytes and plasma (Ma & Li, 2015; El-Shenawy, 2009; Benedetti et al., 2004).

Interferon gamma (IFN-$\gamma$) plays an important role in modulating immune responses and inhibiting cellular proliferation as well as in repressing tumor growth (Wunnapuk et al., 2014). The results from the current study show that GBH did not significantly alter pro-inflammatory cytokine, IFN-$\gamma$. However, previous studies have reported marked elevation in INF-$\gamma$ level in carp fish exposed to glyphosate. Further, the current study showed IL-10 was depleted in mice exposed to GBH. However, cyanocobalamin treatment before and after GBH exposure, stabilized the IL-10 levels. It is important to conduct further studies to determine the cause for varied findings among various studies, which in part, maybe due to the difference in the formulations of GBH used in different countries. From the current study and other previous studies, it is fairly reasonable to conclude that GBH interferes with the normal functioning of the immune system by altering key molecules such as TNF-$\alpha$ and IL-10. Alterations of the immune system has grave implications in the ability of the body to respond to infections and fight proliferation of abnormal cells that can cause cancer. Perhaps, this phenomenon may explain the reported cases of cancer linked to Roundup use. Hence, the GBH-induced modulation of

the immune and inflammatory systems has profound implications in the well-being of those exposed.

Maintaining a robust antioxidant capacity is particularly important in any biological system. A normal and functional antioxidant defence is dedicated to protection of cellular structures by removal of reactive molecules. The consequence of a disruption of this balance in the body is oxidative stress which results in the damage to nucleic acids and functional groups of vital biomolecules including enzymes. The oxidant/antioxidant balance changes in favor of oxidants in the presence of environmental toxicants or disease process. The oxygen radicals are considered an important indicator of oxidative damage (Owagboriaye et al., 2019; Kisaoglu et al., 2013).

Reduced glutathione is a powerful antioxidant vital for metabolism and protection from oxidative stress. GSH is also critical component of the biochemical pathways, centrally involved in detoxification or removal of xenobiotics including toxic chemicals and drugs from the body. This is mostly accomplished in the liver. In the current study, investigations were conducted to determine how GBH exposure might affect its levels. The results demonstrated significant depletion in GSH in the liver of mice exposed to GBH. However, cyanocobalamin treatment before and after GSH exposure reversed the GBH-induced depletion of GSH in liver tissue. This is a vital finding, showing that antioxidants can protect from GBH-induced organ damage. The liver is the main organ of detoxification and biotransformation, thus the excessive release of ROS during GBH metabolism may have overwhelmed the GSH antioxidant activity as reflected by its depletion. These findings on GSH and GBH are consistent with a previous study, in which albino male rats were intraperitoneally administered with Roundup and glyphosate, and the levels of GSH in the liver showed a significant decrease after one week of exposure. Furthermore, this study demonstrated that there was a direct relationship between increasing the duration of exposure and the extent of GSH depletion in the liver (El-Shenawy, 2009). In addition, we observed a substantial elevation of GSH in the brain, lungs, and kidney of mice administered with GBH. The significant elevation of GSH concentration in the brain, lungs, and kidney is an indication of the activation of the antioxidant system, in response to rising oxidative stress. Similar findings were noted in rats exposed to 375 mg/kg GBH for a period of 56 days (Turkmen et al., 2019).

As a consequence of oxidative damage that is observed in regard to extreme oxidative stress and insufficiency of antioxidant potential, the level of GSH decreases due to depletion, and pathological situations arise depending on the free radical damage (Tang et al., 2020; Yildiz et al., 2002). All these findings on GSH depletion by GBHs raise an important question; is GSH depletion a critical event in GBH-induced liver damage? The consequence is that restoration or stabilization of GSH among herbicide users via supplements such as cyanocobalamin, provides great opportunity for intervention.

The liver is a very crucial organ that protects the body from xenobiotics. The leakage of the enzymes such as ALT and AST into the bloodstream is a sign of hepatocellular damage and hepatic dysfunction (Djaber et al., 2020). AST and ALT play a crucial role in protein and carbohydrate metabolism and act as an indicator of tissue damage and cell lysis in the liver (Jasper et al., 2012). The current findings show a significant elevation in the activity of ALT and AST relative to the control. This elevation is attributed to a certain level of damage to hepatocytes, and perhaps other cell types (Djaber et al., 2020). These observations are consistent with other published studies which demonstrated that glyphosate and its commercial formulation Roundup, at a concentration of 50 mg/kg and 100 mg/kg, induced significant liver damage as indicated by increased leakage of liver enzymes, ALT and AST in both male and female mice (Jasper et al., 2012). Other studies on rats have shown a dose dependent rise in serum AST and ALT on exposure to Roundup. The leakage is suggestive of damage to hepatocytes and serves as an index of liver toxicity or injury (El-Shenawy, 2009; Benedetti et al., 2004). Such an effect on the liver has been known to lead to changes in the cellularity of the tissues with an increase in connective tissue and fibrous expansion of some portal areas. Liver injury may occasion production of mitochondria enzymes whose toxic effects could be lipolytic in nature and which leads to the dissolution of cell and organelle membranes, releasing enzymes into the blood stream (Prasanna et al., 2020; Jyothi & Narayan, 1999). Thus, the elevation of the liver enzymes in the current study may be a result of hepatocyte cell death.

Similarly, a significant increase in blood bilirubin levels was observed in the mice administered with GBH relative to the control group, confirming liver damage. The current study findings are also consistent with previous studies by (Djaber et al., 2020; Owagboriaye et al., 2019). Inhibition of heme oxygenase was a possibility since catabolism of heme takes place in the microsomal fraction of cells through heme oxygenase, which catalyzes the conversion of the heme to bilirubin (Seddik et al., 2010). Under toxic conditions due to GBH exposure, bilirubin conjugation may be halted thus leading to bilirubin elevation in the serum (Owagboriaye et al., 2019). However, the antioxidant power of cyanocobalamin decreased the damage and normalized the bilirubin serum levels which is also a hepatic function biomarker.

The serum level of creatinine was used as an indicator of renal function. The findings of the current study did not demonstrate significant alterations in the creatinine level in the group of mice exposed to GBH. Other studies have shown contrasting results (Djaber et al., 2020; Turkmen et al., 2019; Wunnapuk et al., 2014; Jasper et al., 2012).

## 5. Conclusion

The results of the present study reveal that cyanocobalamin has the capacity to assuage GBH-induced inflammatory responses hepatotoxicity, hematological alteration as well as oxidative stress. These findings demonstrate the potential for cyanocobalamin to reverse deleterious effects of GBH and therefore should be considered as part of therapeutic intervention for countering GBH toxicity. Supplementation with cyanocobalamin for farm or factory workers chronically exposed to GBH might protect them from harmful toxic effects of GBH. The current findings provide solid foundation for further scrutiny of this phenomenon, which may result in the pharmacological and/or mitigative interventions to reverse or prevent toxicity from GBHs.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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