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ORIGINAL ARTICLE

Humoral and Cellular Immune Regulation in Albino Rat Exposed to Glyphosate and Aluminum Phosphide

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Abstract

Background: Glyphosate (GLP) and Aluminum phosphide (ALP) pesticides remain vital for managing pests and disease vectors in both agriculture and public health. However, humans may experience chronic exposure to complex mixtures of pesticides from diverse environmental or dietary sources. The potential effects of this exposure on humoral and cellular immune processes are yet to be fully understood.
Method: The expression and potential regulation of T cells, specifically $CD4^+$ helper T cells and $CD8^+$ cytotoxic T lymphocytes, were evaluated in groups of male and female albino rats treated with oral distilled water; a low dose of glyphosate (1000 mg/kg/day); a high dose of glyphosate (3500 mg/kg/day); a low dose of ALP (1.1 mg/kg/day); and a high dose of ALP (5.0 mg/kg/day). This assessment was conducted using flow cytometry (Cyflow).
Results: The results indicated that the pre-exposure stage exhibited a $CD4^+$ to $CD8^+$ ratio of 2:1. Subsequently, there was a significant increase in $CD8^+$ values at the first, second and third months after exposure to GLP and ALP ($p<0.001$ for all). Additionally, there was a notable reduction in CD4+ levels at the second and third months post-exposure ($p=0.001$ and $p<0.001$, respectively).
Conclusion: This study identified a deregulation of the immune system characterized by a progressive immunosuppression of CD4 T cell counts, indicating a down regulation of antibody-mediated (humoral) immune responses alongside an up regulation of cytotoxic T cells. This phenomenon aligns with the animals' increasing exposure to the chemicals, showing no significant difference in the changes due to dosage across both sexes. The chronic effects of these cytotoxic actions could potentially lead to tissue damage or trauma, resulting in subsequent chronic inflammation.

Keywords: Glyphosate, Aluminum Phosphide, Humoral immune Response, Cellular Immune Response, Albino Rat, Chemicals, Toxicity

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INTRODUCTION

The Environmental Protection Agency (EPA) defines pesticides as a category of chemical compounds used to control and deter pest populations [1, 2]. Pesticide residues can be found in a variety of common foods and beverages, including processed fruit juices, wine, water, snacks, poultry feeds, and other meals, leading to potential exposure to chemical toxins in humans. In this study, we examine the possible immunoregulatory effects of Glyphosate (a herbicide) and Aluminum Phosphide (an insecticide) toxicity in animals, as these effects may relate to human health.

Glyphosate (N-[phosphonomethyl]-glycine) was first synthesized in 1950 by Swiss scientist Dr. Henri Martin, and it has become the most widely used non-selective herbicide since its commercialization by Monsanto in the 1970s [3, 4]. Owing to its desirable characteristics—such as high efficacy, broad-spectrum effectiveness, low cost, and a favorable toxicological profile—along with the introduction of genetically modified herbicide-tolerant crops, glyphosate is regarded as the most vital herbicide for weed management [5]. Its mode of action in humans involves the inhibition of the enzyme acetylcholinesterase, allowing glyphosate to function as a neurotoxin that alters androgen and estrogen receptors [6].

On the other hand, aluminum phosphide (ALP) is a cost-effective and widely used insecticide. Regrettably, it has also become one of the most common causes of poisoning among agricultural pesticides. Aluminum phosphide releases lethal phosphine gas upon contact with atmospheric moisture or hydrochloric acid in the stomach. The estimated fatal dose for an average-sized individual is believed to be between 150 and 500 milligrams, indicating that even less than half a pill can be sufficient to kill an adult, especially if it is ingested from a newly opened container. The mechanism of toxicity involves cellular hypoxia resulting from its effects on mitochondria,

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the inhibition of cytochrome C oxidase, and the generation of highly reactive hydroxyl radicals. These adverse physiological effects raise concerns regarding the potential extended or progressive impact that chemical-induced injuries may have on humoral and cellular adaptive immune responses due to pesticide exposure. The influence of these pesticides on T cells remains inadequately understood. This study investigated the up regulation and down regulation of T helper cells and cytotoxic T cells in albino rats.

METHODS

The Chemicals

Glyphosate-based herbicide (Glyphosate 1) and aluminum phosphide insecticide (Celphos) were obtained from a common market (Onitsha Main Market). A 30g aluminum phosphide tablet was pulverized, with 4.5mg being measured out for the high dose group and 1.0mg for the low dose group. For the Glyphosate treatment, 6.6ml/kg was mixed into the water for the high dose group, while 1.9ml/kg was added for the low dose group.

Animals

Fifty adult Albino rats (both male and female), averaging a weight of 160±10g, were acquired from the animal house of the Faculty of Pharmaceutical Sciences at Nnamdi Azikiwe University, Awka. The animals were subsequently transported to the pharmacology and toxicology laboratory of the same institution, where they were randomized into groups of 10 (n=5). They were housed in plastic cages and provided with unrestricted access to a suitable diet and water ad libitum. The rats were maintained at a temperature of 23±2°C with a 12-hour dark/light cycle, allowing them to acclimatize for two weeks [7]. before the commencement of the study. The animal handling and experimental protocols received approval from the Ethical Committee on Animal Handling at Nnamdi Azikiwe University, Awka, Anambra State, Nigeria (NAU/AREC/2023/00095).

Experimental Approach

The animal groups were treated daily according to the following groups: Group 1: male control (administered oral distilled water and food without pesticide); Group 2: female control (administered oral distilled water and food without pesticide); Group 3: male low-dose Glyphosate (1000 mg/kg/day); Group 4: female low-dose Glyphosate (1000 mg/kg/day); Group 5: male high-dose Glyphosate (3500 mg/kg/day); Group 6: female high-dose Glyphosate (3500 mg/kg/day); Group 7: male low-dose ALP (1.1 mg/kg/day); Group 8: female low-dose ALP (1.1 mg/kg/day); Group 9: male high-dose ALP (5.0 mg/kg/day); Group 10: female high-dose ALP (5.0 mg/kg/day).

The exposure doses for these pesticides were selected based on the LD50 and no observed adverse effect level (NOAEL) was recorded in previous studies [8, 9]. The high dose of Glyphosate (3500 mg/kg body weight) corresponds to half of the LD50 (½ of 7000 mg/kg body weight) [10], while the low dose (1000 mg/kg body weight) is the maximum amount at which no adverse effects were reported [9]. Similarly, the high dose of aluminum phosphide (5.0 mg/kg body weight) represents half of its LD50 (½ of 10 mg/kg body weight) [11], with the low dose (1.1 mg/kg body weight) being the highest dose without observed adverse effects [12]. Food and water were provided ad libitum, and any remnants were discarded daily. The animals were monitored each day for general health and signs of toxicity. Individual body weights and cage-based feed consumption were recorded daily. This study adhered to the National Institutes of Health guidelines for laboratory animal care and use.

Sample Preparation

Up to 0.5 ml of blood was collected from the rats via ocular puncture at the beginning of the study for pre-analysis and was repeated every four weeks until the conclusion of the study, which lasted for a total of 90 days. The collected blood was placed in an EDTA container, gently mixed, and promptly transported to the laboratory for CD4+ and CD8+ counts using Flow Cytometry (Sysmex Cyflow Counter, Germany).

RESULTS

Mean Levels of CD4+ and CD8+ at Pre-exposure Stage

Pre-exposure stage showed mean levels of CD4+ with normal 2:1 ratio with CD8+ cells. The mean levels of CD4+ helper T cell counts of both male and female rats at this stage showed no significant difference within the variables (p=0.372). the same development was observed on mean levels of CD8+ cytotoxic T cell counts of both male and female rats (p=0.322) (table 1).

Table 1. Mean Levels of CD4+ and CD8+, at Pre-exposure Stage

	CD4 (cells/µl)	CD8 (cells/µl)
GP 1	19.8±9.1	10.2±4.5
GP 2	30.4±8.1	14.8±4.0
GP 3	22.6±17.3	10.6±8.3
GP 4	21.0±6.2	10.2±2.8
GP 5	58.2±40.4	28.2±19.1
GP 6	22.8±10.9	10.0±4.5
GP 7	33.8±11.3	14.8±4.0
GP 8	26.0±5.7	12.6±3.0
GP 9	27.8±16.2	14.6±7.3
GP 10	31.0±11.7	15.6±5.5
F-value	1.2	1.3
P-value	0.372	0.322
	Post Hoc	
1 vs 7	0.542	0.764
1 vs 8	0.927	0.984
1 vs 10	0.778	0.772
2 vs 7	1.000	1.000
2 vs 8	0.983	0.985
2 vs 9	1.000	1.000
2 vs 10	1.000	1.000

Key: GP1 and GP2=Male and female control Group; GP3 and GP4=Male and female Rats under low dose of Glyphosate; GP5 and GP6=Male and female Rats under high dose of Glyphosate; GP7 and GP8=Male and female Rats under low dose of Aluminum phosphide; GP9 and GP10=Male and female Rats under high dose of Aluminum phosphide

Mean Levels of CD4+ and CD8+, at 1 Month Post exposure Stage

At the one-month post-exposure stage, statistical analysis revealed a reduction in the mean levels of CD4+ T cell counts compared to the pre-exposure stage. However, no significant differences were observed between the variables in both male and female groups (p=0.156). In contrast, the mean levels of CD8+ cytotoxic T cell counts in both male and female rats showed a significant increase at this stage (p=0.000). The Howell post hoc analysis indicated that the significant increase in the mean levels of CD8+ T cells in male and female rats exposed to GLP (low dose: p=0.009 and p=0.044, respectively; high dose: p=0.027 and p=0.025, respectively) was compared to the mean levels in the male control group that did not experience chemical exposure. Additionally, a significant increase in mean CD8+ T cell counts was observed in female rats exposed to ALP (low dose: p=0.007), while no significant increase was found in male rats under the same exposure (low dose: p=0.472) compared to the male control. Moreover, both male and female rats exposed to ALP (high dose) exhibited significantly elevated levels of CD8+ T cells (p=0.017 for both groups (Table 2).

Mean Levels of CD4+ and CD8+ T Cells at the Second Month Post-Exposure Stage

During the second month post-exposure, the statistical analysis revealed a significant decrease in the mean levels of CD4+ T cell counts when compared to male and female control groups. Notably, significant differences were observed within both male and female rat groups (p=0.001). The Game Howell post hoc analysis indicated that the substantial decrease was evident in female rats exposed to GLP (low dose, P=0.002) when compared to the female control group that had no chemical exposure. Conversely, the decrease in the mean level of CD4+ T cell counts in male rats exposed to the same low dose of the chemical was not statistically significant (P=0.051). Additionally, a statistically significant decrease in CD4+ T cell counts was observed in both male and female rats exposed to GLP at high doses (P=0.038 and 0.021, respectively). In contrast, only male and female rats exposed to ALP (low dose) exhibited significant differences in mean levels of CD4+ T cells compared to the female control group. There were no significant differences in the decreased levels of CD4+ T cells among any of the groups when compared to the male control group.

The mean levels of CD8+ cytotoxic T cell counts in both male and female rats at this stage exhibited a significant increase across the variables (p<0.001). A Game Howell post hoc analysis revealed that this significant increase in CD8+ cell counts was observed in both male and female rats exposed to GLP, with low doses yielding p-values of <0.001 and <0.001, respectively, and high doses also showing p-values of <0.001 and <0.001, respectively, when compared to the mean CD8+ counts in the male control group without chemical exposure. Conversely, a statistically significant increase in mean CD8+ counts was noted in both male and female rats exposed to ALP, with low doses resulting in p-values of 0.000 and 0.000, respectively, and high doses yielding p-values of <0.001and 0.001, respectively (Table 3).

Mean Levels of CD4+ and CD8+ T Cells at the 3rd Month Post-Exposure Stage

At the third post-exposure stage, statistical analysis revealed a further significant decrease in mean CD4+ T cell counts in rats exposed to GLP and ALP chemicals compared to the mean levels in both male and female control groups (p<0.001). Subsequent Game Howell post hoc analysis indicated a significant reduction in CD4+ counts between male and female rats exposed to GLP (low dose: 5.0±1.0, p=0.030 and 7.4±2.3, p=0.023, respectively) when compared to the female control group without chemical exposure (28.4±7.7). Similarly, a statistically significant decrease in CD4+ T cell counts was observed in both male and female rats exposed to GLP (high dose: 5.6±1.1, p=0.025 and 5.8±1.8, p=0.029) when measured against the female control group without exposure to chemicals.

The mean levels of CD8+ cytotoxic T cell counts in both

Table 2. Mean Levels of CD4+ and CD8+, at 1st Month Post exposure Stage

	CD4 (cells/µl)	CD8 (cells/µl)
GP 1	22.8±8.7	11.0±4.6
GP 2	30.8±7.3	14.8±4.1
GP 3	22.6±6.0	70.6±16.4
GP 4	19.2±2.0	70.6±23.7
GP 5	21.6±9.6	61.6±18.0
GP 6	19.4±2.7	61.6±17.7
GP 7	17.0±5.8	110.6±92.0
GP 8	23.0±3.3	71.4±15.7
GP 9	22.6±4.7	83.2±22.3
GP 10	17.6±4.6	77.8±20.9
F/P-value	1.8/0.156	23.7/0.000
	Post Hoc	
1 vs 3	1.000	0.009
1 vs 4	0.776	0.044
1 vs 5	1.000	0.027
1 vs 6	0.860	0.025
1 vs 7	0.995	0.472
1 vs 8	1.000	0.007
1 vs 9	1.000	0.017
1 vs 10	0.698	0.017
2 vs 3	1.000	0.013
2 vs 4	0.198	0.056
2 vs 5	1.000	0.038
2 vs 6	0.974	0.035
2 vs 7	0.995	0.506
2 vs 8	0.999	0.010
2 vs 9	1.000	0.021
2 vs 10	0.852	0.022

Table 3. Mean Levels of CD4+ and CD8+, at 2nd Month Post exposure Stage

	CD4 (cells/µl)	CD8 (cells/µl)
GP 1	19.4±8.5	13.0±5.1
GP 2	29.4±7.6	15.4±5.2
GP 3	11.8±3.7	54.0±5.5
GP 4	7.6±2.2	77.4±7.7
GP 5	9.4±1.5	88.0±6.9
GP 6	6.6±1.9	60.0±3.2
GP 7	8.8±2.6	87.4±10.6
GP 8	9.4±4.3	83.2±11.5
GP 9	15.2±4.1	66.8±5.6
GP 10	13.4±4.6	79.2±11.8
F/P-value	5.9/0.001	75.6/0.000
	Post Hoc	
1 vs 3	0.707	0.000
1 vs 4	0.283	0.000
1 vs 5	0.408	0.000
1 vs 6	0.227	0.000
1 vs 7	0.371	0.000
1 vs 8	0.468	0.000
1 vs 9	0.981	0.000
1 vs 10	0.894	0.001
2 vs 3	0.051	0.000
2 vs 4	0.025	0.000
2 vs 5	0.038	0.000
2 vs 6	0.021	0.000
2 vs 7	0.029	0.000
2 vs 8	0.028	0.001
2 vs 9	0.123	0.000
2 vs 10	0.079	0.001

Table 4. Mean Levels of CD4+ and CD8+, at 3rd Month Post exposure Stage

	CD4 (cells/µl)	CD8 (cells/µl)
GP 1	18.9±1.4	11.8±2.9
GP 2	28.4±7.7	16.4±4.4
GP 3	7.4±2.3	53.4±6.4
GP 4	5.0±1.0	87.0±3.7
GP 5	5.6±1.1	91.2±11.4
GP 6	5.8±1.8	67.8±8.4
GP 7	10.8±1.6	91.4±8.2
GP 8	9.8±1.6	85.2±14.2
GP 9	11.0±1.6	73.-6±11.1
GP 10	14.2±2.9	83.8±5.4
F- value	14.3	177.2
P-value	0.000	0.000
	Post Hoc	
1 vs 3	0.410	0.000
1 vs 4	0.259	0.000
1 vs 5	0.289	0.001
1 vs 6	0.363	0.000
1 vs 7	0.073	0.000
1 vs 8	0.612	0.003
1 vs 9	0.729	0.002
1 vs 10	0.975	0.000
2 vs 3	0.030	0.000
2 vs 4	0.023	0.000
2 vs 5	0.025	0.000
2 vs 6	0.029	0.000
2 vs 7	0.062	0.000
2 vs 8	0.051	0.002
2 vs 9	0.065	0.001
2 vs 10	0.124	0.000

male and female rats at this stage demonstrated a significant increase across the variables ($p<0.001$). When compared to the mean CD8+ count (11.8±2.9) in the male control group without chemical exposure, the Game Howell post hoc analysis indicated a notable increase in the mean CD8+ counts for both male and female rats exposed to GLP (low dose: 53.4±6.4, $p<0.001$and 87.0±3.7, $p<0.001$, respectively; high dose: 91.2±11.4 and 67.8±8.4, $p=0.001$ and 0.000, respectively). Moreover, a statistically significant increase in mean CD8+ counts was observed in male and female rats treated with ALP (low dose: 91.4±8.2, $p<0.001$ and 85.2±14.2, $p=0.003$, respectively; high dose: 73.6±11.1, $p=0.002$ and 83.8±5.4, $p<0.001$, respectively).

Conversely, in comparison with the mean level of CD8+ count (16.4±4.4) in female control group without chemical exposure, the post hoc analysis revealed a significant increase in mean CD8+ counts for both male and female rats exposed to GLP (low dose: 53.4±6.4, $p<0.001$ and 87.0±3.7, $p<0.001$, respectively; high dose: 91.2±11.4 and 67.8±8.4, $p<0.001$ respectively). Similarly, a statistically significant increase in mean CD8+ counts was observed in male and female rats exposed to ALP (low dose: 91.4±8.2, $p<0.001$ and 85.2±14.2, $p=0.002$ respectively; high dose: 73.6±11.1, $p=0.001$ and 83.8±5.4, $p=0.000$, respectively) (table 4).

DISCUSSION

Pesticides play a significant role in global agricultural production, ensuring safer storage of food commodities and enhancing public health security—an undeniable advantage. However, the increasing use of pesticides for crop protection raises the risk of food contamination. When applied, these chemicals can contaminate the environment and accumulate within the food chain [13], posing potential threats to both human health and the ecosystem. Prior to this study, our field surveys revealed that sellers often sell chemically treated grains either immediately or when they require funds for purchasing farm inputs, while some even feed these grains to

animals. A majority of farmers and traders reported storing these treated grains in their residential homes, exposing vulnerable family members, including children, to pesticide hazards. Alarmingly, many are unaware that aluminum phosphide, the most commonly used insecticide, is a restricted pesticide that has been banned in various countries. The findings indicate a concerning lack of knowledge regarding pesticide application and regulatory policies in the study areas, suggesting that such policies are not effectively implemented.

Our previous research findings showed that many foods consumed in Nigeria, such as apples, peppers, beans, and rice, are contaminated with pesticide residues, including organophosphates, organochlorines, and carbamates [14]. Although the levels of these chemicals in food crops were found to be low, it is crucial to recognize that these staples are frequently consumed, which raises concerns about the potential for chronic exposure to these chemicals and subsequent low-grade contamination within the body. This accumulation poses significant questions regarding the clinical implications for human health and longevity due to ongoing chemical exposure. Consequently, the primary aim of this study is to evaluate the effects of two commonly used agricultural pesticides, namely Aluminum phosphide and Glyphosate, by analyzing systemically expressed immune cells before and after exposure, utilizing high-throughput techniques. This work aimed to pre-clinically investigate the immune-regulatory effects of these chemicals, focusing on both antibody-mediated and cytotoxic immune responses.

At the pre-exposure stage, the ratio of CD4+ to CD8+ T cells was approximately 2:1. One month after the initial exposure, a decrease in the level of T helper cells (CD4+) was observed. Although this decline was not statistically significant, the depletion of this crucial immunological cell became more pronounced after the second exposure, which showed a significant reduction in cell count. The depletion of CD4+ T cells was noted in both male and female albino rats; however, a more detailed statistical analysis revealed that the adverse effects were more pronounced in female rats, suggesting potential sex-related vulnerabilities to the chemicals. Furthermore, the impact of the chemical on the CD4+ T cell count intensified with continued exposure, as evidenced after the third exposure, which resulted in a more significant decrease in cell count. This finding suggests a trend of humoral adaptive immunosuppression under prolonged exposure in rats. While the implications for humans are not directly applicable, a study referenced in [15] investigated whether Glyphosate could influence immune cells by using peripheral blood mononuclear cells (PBMC) from healthy human donors. The study found a reduction in the T helper 1/T helper 2 (Th1/Th2) ratio, primarily due to a decrease in Th1 cells following Glyphosate exposure. However, there is limited data regarding the effects of aluminum phosphide on CD4+ T cells and the immune system as a whole.

The initial exposure led to an increase in the ratio of CD8+ T cells, reaching a maximum of 1:4, which underscores the critical role these cytotoxic T cells play in this condition. While the elevation of cytotoxic (CD8+) T cells indicates a robust immune response, it also raises concerns about potential harm to host cells due to the excessive cytotoxic activity stemming from the ongoing presence of these chemicals. Both glyphosate (GLP) and aluminum phosphide (ALP) were identified as potent stimulators of the CD8+ response, showing significant increases in both male and female rats, with a more pronounced increase observed in those exposed to high doses of ALP. The significance of this research is underscored by the fact that these chemicals may appear harmless with short-term use, yet there are indications of long-term exposure toxicity. This justifies the prolonged exposure of our test animals to these substances in order to assess their long-term effects. The long-term consequences of glyphosate and aluminum phosphide have been underreported, and various factors contribute to prolonged human exposure, including the continued use of these chemicals. Moreover, glyphosate's resistance to complete degradation—attributed to the stable C-P linkage in its molecular structure—results in a slow breakdown process in dead plant material, soil, and water by various microorganisms [16]. This persistence raises concerns about potential long-term exposure when in contact with humans, highlighting the need for further investigation into the chronic effects of these chemicals.

In this study, the increase in CD8+ levels was associated with the progression of exposure, with the highest levels recorded at the third month. The findings indicate a progressive depletion of CD4+ cells and an elevation of CD8+ cells in albino rats when exposed to GLP and ALP. These substances are therefore identified as potent immune suppressors and stimulators, suggesting a significant suppression of CD4+ counts—crucial for antibody generation and the antibody-mediated immune response (humoral immune response)—while CD8+, which plays a vital role in mediating cytotoxic immune response (cellular immune response), is stimulated. This may lead to adverse effects, including normal cell cytotoxicity. Additionally, the animals exposed to glyphosate exhibited significantly lower CD4+ counts compared to those exposed to ALP, indicating that glyphosate has a more substantial impact on CD4+ cells than ALP. This aligns with findings reported in previous studies [15], which suggested that glyphosate affects the immune system by altering lymphocyte responses and increasing the production of pro-inflammatory cytokines, impacting lymphocyte functions and their interactions with microorganisms [17, 18]. Although many outcomes are still under discussion, existing evidence underscores the need for further research to better understand the risks associated with glyphosate and to advocate for more stringent regulations regarding its global use.

CONCLUSION

This study found that the adaptive humoral and cellular immune systems were deregulated, with a progressive immunosuppression of CD4 T cells, suggesting a down regulation of antibody-mediated immune response and an up regulation of cytotoxic T cells. This phenomenon was associated with persistent exposure of the animals to the chemicals, without significant difference in the increase due

to dosage in both sexes, indicating that even persistent low-grade exposure to these chemicals could be detrimental to an adequate immune response. The chronic effect of these cytotoxic actions could also induce tissue damage or trauma and subsequent chronic inflammation, which is detrimental to normal physiological homeostasis and possible exposure to tumorigenesis.

LIMITATIONS

This study has several limitations. First, the animals were kept in a controlled environment that may not reflect real-world conditions, such as ambient chemical exposures and other environmental factors. Second, different animal species and strains may yield varying results, making comparisons and generalizations difficult. Third, ethical considerations limit the extent of suffering and conditions that animals can be exposed to, potentially reducing the relevance of the study.

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Ethical Approval: This study followed the National Institutes of Health's guidelines for the care and use of laboratory animals (NIH Publications No. 8023, revised 1996).

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