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K Shravankumar
Department of Veterinary Pathology, College of Veterinary Science, Rajendranagar, Telangana, India

D Madhuri
Department of Veterinary Pathology, College of Veterinary Science, Korutla, Jagtial district, Telangana, India

M Lakshman
Department of Veterinary Pathology, College of Veterinary Science, Rajendranagar, Telangana, India

B Kalakumar
Department of Veterinary Pharmacology & Toxicology, College of Veterinary Science, Mamnoor, Telangana, India

Corresponding Author:
D Madhuri
Department of Veterinary Pathology, College of Veterinary Science, Korutla, Jagtial district, Telangana, India

Evaluation of hemato-biochemical changes Induced by glyphosate toxicity and its amelioration with ascorbic acid in wistar rats

K Shravankumar, D Madhuri, M Lakshman and B Kalakumar

Abstract
Glyphosate (GLP) mediated toxicity is due to excessive production of reactive oxygen species and ascorbic acid (AA) is a naturally occurring primary antioxidant which can scavenge or inhibit ROS production. A total of forty- eight (48) male rats were divided into four groups *viz*. group 1 served as control, group 2 served as GLP toxic control (500 mg/kg b.wt, orally), groups 3 and 4 rats were administered with AA (250 mg/kg b.wt, orally) and GLP (500 mg/kg b.wt) + AA (250 mg/kg b.wt) respectively for 21 days. Results showed significant (p<0.05) reduction in the body weights of rats treated with glyphosate alone. Hematological examination revealed a significant (p<0.05) decrease in the Total Erythrocyte Count (TEC), Total Leukocyte Count (TLC), Hemoglobin (Hb) concentration and Packed Cell Volume (PCV) whereas erythrocyte indices revealed a significant (p<0.05) increase in the Mean corpuscular volume and Mean corpuscular hemoglobin and in significant (p<0.05) decrease in Mean corpuscular hemoglobin concentration in rats treated with GLP alone. The serum biochemical assays revealed a significant (p<0.05) increase in the Aspartate Transaminase (AST), Alanine Transaminase (ALT), Alkaline Phosphatase (ALP), Total Proteins (TP), Blood Urea Nitrogen (BUN) and serum creatinine in GLP alone treated rats. A significant improvement in all the parameters was observed in rats treated with both ascorbic acid and GLP (group 4). The results concluded that supplementation of ascorbic acid ameliorates glyphosate induced hemato-biochemical alterations.

Keywords: Ascorbic acid, hemato-biochemical, glyphosate, toxicity

1. Introduction
Weed infestation leads to great reductions in the productivity of crops as well as deteriorate the quality of the production. Herbicide are effective means of approach for controlling weeds; however, overuse of these herbicides causes numerous morphological, physiological and biochemical disturbances in cells and organisms of animals, including mammals [1].

Glyphosate, a broad-spectrum organophosphorus herbicide is abundantly used worldwide in agriculture, commercial and residential settings. In recent years, major concerns have arisen due to the intensive use of glyphosate worldwide [2, 3] and its accumulation in the environment and edible products resulting in harmful side effects on plant, animal and human health. Glyphosate and its metabolite AMPA were also found frequently in the urine and feces of farm animals, dogs and cats reflecting the high GLP residues in food chain [4-6]. Previous *in vitro* studies have reported that AMPA the metabolite of Glyphosate affects human red blood cells and can lead to chromosomal aberrations in fish. Exposure to glyphosate causes significant alteration with reductions of RBC, hematocrit, and hemoglobin, in both sexes of mice [7]. Besides Cxavusxog'lum [8] observed significant increase in the serum BUN, and creatinine levels in mice treated with glyphosate.

Studies have shown that main mechanism of glyphosate toxicity to biological system is the generation of ROS causing oxidative stress [9, 10] which leads to lipid peroxidation [11]. Pesticides exposure [12, 13] significantly depletes the levels of both the antioxidant enzymes and the non-enzymatic antioxidants vitamins like A, E and C including GSH due to increased cytotoxicity caused by overproduction of ROS.

Ascorbic acid (vitamin C) is a primary antioxidant present in biological system to counter oxidative stress [14]. It is easily available and widely used to mitigate toxicity evoked by various pesticides [15]. It has been reported that vitamin C ameliorates organophosphate pesticide-induced hematological and biochemical alterations in humans and animals[16]. Hematology and biochemical data play a major role in determining the toxicity induced by any substance [17]. The present work was aimed to study the growth performance and hemato-

Biochemical changes induced by glyphosate toxicity and its amelioration by ascorbic acid in Wistar rats.

2. Materials and Methods

2.1 Drugs and Chemicals

Glyphosate (Roundup ®-41% w/w solution) was procured from Seed Research and Technology Center, (SRTC) Professor Jayashankar Telangana State Agriculture University, Rajendranagar, Hyderabad, which was manufactured by Monsanto India Ltd. Mumbai. Ascorbic acid (Vitamin C) as L-Ascorbic acid was obtained from S.D. Fine-Chem. Ltd., Mumbai, India. All the chemicals (for preparation of reagents and buffers) were procured from Qualigens Pvt. Ltd. Mumbai, HI media Pvt. Ltd., and SRL Pvt. Ltd., Mumbai.

2.2 Experimental Design

A total of forty- eight (48) male Wistar rats were divided into four groups consisting of twelve (12) in each. The group 1 served as control, group 2 served as GLP toxic control (500 mg/kg b.wt, orally). Groups 3 and 4 rats were administered with AA (250 mg/kg b.wt, orally) and GLP (500 mg/kg b.wt) + AA (250 mg/kg b.wt) respectively and the experiment was carried out for 21 days. All the experimental animals were closely observed thrice daily for clinical signs and mortality if any, during the entire period of study. The experiment was carried out according to the guidelines and prior approval of Institutional Animals Ethics Committee (IAEC-No.03-2019).

2.3 Body Weights

Individual body weights of all the rats were recorded by using electronic balance soon after arrival and subsequently on 7th, 14th and 21st day of experiment to study body weight gains.

2.4 Hemato-biochemical Parameters

Six (6) rats from each group were sacrificed on 7th and 21st day of experiment. On the day of sacrifice, two to three milliliters of blood was collected from retro- orbital plexus of rats, with the help of a capillary tube into an anticoagulant-coated vaccutainers {K2-EDTA tube, 13 mm × 75 mm, 4 mL (Rapid Diagnostics Pvt. Ltd., Delhi)} to carry out all hematological parameters. Prior to blood collection, the selected experimental rats were put to fast for 12 h. The blood samples were used for estimation of total erythrocyte count (TEC), total leukocyte count (TLC), hemoglobin (Hb) concentration, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), by using an automatic whole blood analyzer (BC-2800Vet).

For serum samples, approximately 2 ml of blood was collected separately in a sterile serum vials and stored at -20^0 C. The stored samples were used for serum biochemistry using semi- automatic biochemical analyser (Star 21 plus – Aspen diagnostics Pvt. Ltd., Delhi) by using Aspen biochemical kits (Rapid Diagnostics Pvt. Ltd., Delhi). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), Alkaline phosphatase (ALP) were estimated as per modified International Federation of Clinical Chemistry (IFCC) method [18], serum creatinine was measured by modified Jaffes reaction as per alkaline picrate techniques [19], serum total protein (TP) as per the standard Biuret procedure [20], blood urea nitrogen (BUN) as per Glutamate dehydrogenase (GLDH) – Urease method [21].

2.5 Statistical Analysis

Data obtained were subjected to statistical analysis by applying one-way ANOVA using statistical package for social sciences (SPSS) version 25.0. Differences between the means were tested using Duncan's multiple comparison tests and significance level was set at $p < 0.05$ [22].

3. Results

The mean body weights were significantly (p<0.05) lower in group 2 when compared to group 1 and group 3. In group 4 significant increase in the mean values of body weights was noticed when compared to group 2 on 7th, 14th and 21st day of experiment (Table 1).

Table 1: Weekly body weights (g) in different groups

Group	Day 7	Day 14	Day 21
Group 1	276.17 ± 2.65^c	277.17 ± 3.79^c	283.00 ± 2.56^c
Group 2	256.00 ±1.52^a	255.83 ± 2.73^a	253.17 ± 3.93^a
Group 3	277.83 ± 2.15^c	278.67 ± 4.95^c	285.33 ± 6.69^c
Group 4	264.33 ± 2.53^b	266.67 ± 1.33^b	262.67 ± 2.41^b

Values are Mean ± SE (n=6); One way ANOVA
Means with different superscripts in a column differ significantly at p<0.05 (*)

The mean hematological values of rats recorded at the 7th and 21st interval of experiment are presented in Table (2, 3 &4). The mean values of TEC, Hb, PCV and TLC were significantly (p<0.05) decreased in group 2 when compared to group 1, group 3 and group 4 on 7th and 21st day of experiment. The erythrocyte indices showed significant (p<0.05) increase in MCV, MCH and in significant decrease in MCHC in group 2 when compared to all other groups (1, 3 & 4) on 21st day of the experiment.

Table 2: Hematological values in different groups on day 7 of the experiment

Group	Tec (10^6/µl)	Pcv (%)	Hb (g %)	Tlc (10^3/µl)
Group1	10.21 ± 0.08^c	53.68±0.87^c	17.55±0.18^b	18.28±0.12^c
Group 2	8.86 ± 0.10^a	46.75±0.57^a	14.98±0.21^a	13.95±0.19^a
Group 3	10.20 ± 0.07^c	53.46±0.87^c	17.48 ± 0.20^b	17.98±0.19^c
Group 4	9.55 ± 0.13^b	49.95±1.17^b	15.71±0.39^a	15.40±0.15^b

Values are Mean ± SE (n=6); One way ANOVA Means with different superscripts in a column differ significantly at p<0.05 (*)

Table 3: Hematological values in different groups on day 21 of the experiment

Group	Tec (10^6/µl)	Pcv (%)	Hb (g %)	Tlc (10^3/µl)
Group1	10.73±0.4c	55.35 ± 1.10^c	17.76 ± 0.32^c	18.53±0.11^c
Group 2	7.61 ±0.33^a	42.35 ±2.51^a	13.25 ± 0.50^a	11.76±0.38^a
Group 3	10.40±0.1^c	53.95 ±0.97^c	17.23 ± 0.24^c	18.23±0.23^c
Group 4	9.31 ±0.07^b	48.78 ±0.82^b	15.55 ± 0.27^b	14.33±0.19^b

Values are Mean ± SE (n=6); One way ANOVA Means with different superscripts in a column differ significantly at p<0.05 (*)

Table 4: Erythrocyte indices in different groups on day 21 of the experiment

Group	(Mcv-fL)	(Mch-pg)	Mchc-g/dL
Group1	51.43 ± 0.39^a	16.53 ± 0.17^a	32.10 ± 0.36
Group 2	55.11 ± 1.09^b	17.30 ± 0.11^b	31.46 ± 0.69
Group 3	51.76 ± 0.78^a	16.56 ± 0.26^a	31.96 ± 0.70
Group 4	52.01 ± 0.68^a	16.60 ± 0.25^a	31.88 ± 0.14

Values are Mean ± SE (n=6); One way ANOVA Means with different superscripts in a column differ significantly at p<0.05 (*)

The mean biochemical values of different parameters showed significant (p<0.05) increase in AST, ALT, ALP, BUN, Creatinine and total protein in group 2 when compared to other groups (Table 5 &6). There was significant improvement of above parameters in group 4 as compared to group 2.

Table 5: Biochemical parameters in different groups on day 7 of the experiment

Parameters	Group 1	Group 2	Group 3	Group 4
AST (IU/L)	36.24 ±.22^a	59.66 ±.18^c	34.20 ±.85^a	50.16 ± 1.85^b
ALT (IU/L)	35.08 ±.28^a	53.74 ±.88^b	35.66 ±.29^a	48.81 ±3.11^b
ALP (IU/L)	83.33 ±.54^a	122.00 ±.52^c	81.33 ±.37^a	106.00 ± 2.14^b
TP (g/dL)	4.40 ±.09^a	5.76 ± 0.31^b	4.55 ± 0.08^a	5.45 ± 0.42^b
BUN (mg/dL)	37.83± 30^a	56.67 ±.49^b	38.33 ±.11^a	53.17 ± 0.79^b
Creatinine (mg/dL)	0.56 ±.05^a	1.21 ± 0.08^c	0.73 ± 0.06^a	0.98 ± 0.06^b

Values are Mean ± SE (n=6); One way ANOVA
Means with different superscripts in a column differ significantly at p<0.05 (*)

Table 6: Biochemical parameters in different groups on day 21 of the experiment

Parameters	Group 1	Group 2	Group 3	Group 4
AST (IU/L)	38.87 ± 2.12^a	71.83 ± 1.86^c	38.03 ± 2.94^a	63.50 ± 1.52^b
ALT (IU/L)	34.99 ± 2.93^a	63.92 ± 3.21^b	34.33 ± 3.63^a	56.00 ± 2.84^b
ALP (IU/L)	88.50 ± 1.94^a	135.50 ± 1.97^c	89.66 ± 1.28^a	115.50 ± 2.64^b
TP (g/dL)	4.61 ± 0.07^a	6.75 ± 0.04^c	4.46 ± 0.04^a	6.48 ± 0.11^b
BUN (mg/dL)	38.67 ± 0.91^a	64.83 ± 2.08^c	40.50 ± 0.92^a	56.17 ± 3.12^b
Creatinine(mg/dL)	0.80 ± 0.03^a	1.75 ± 0.08^c	0.83 ± 0.03^a	1.03 ± 0.07^b

Values are Mean ± SE (n=6); One way ANOVA
Means with different superscripts in a column differ significantly at p<0.05 (*)

4. Discussion

In the present study, significant reduction in body weights were recorded in GLP treated rats (group 2) which might be due to decreased feed and water intake on the account of toxic action of GLP on GIT and also due to oxidative stress at sub cellular level in liver and kidneys. This is in agreement with earlier workers [23, 26]. Significantly increased body weights in group 4 rats when compared to group 2 could be due to the ameliorative action of Vitamin C against GLP induced cytotoxicity.

A significant alteration in hematological values in group 2 rats as compared to other groups indicated glyphosate toxicity causes anemia and lowers immunity. A significant decrease in the RBC count could be attributed to impairment of rbcs and hemoglobin synthesis. The anemia observed might have been due to the ability of the herbicide to cause extravascular hemolysis or it might be due to its ability to cause oxidative stress [27]. The RBC is susceptible to lipoperoxidative changes because of its direct association with molecular oxygen, high content of metal ions catalyzing oxidative reactions, and availability of high amount of PUFAs, which are susceptible to lipid peroxidation. The reduction in hemoglobin values might be due to increased destruction of rbcs in blood forming system and disturbance in synthesizing of iron [28]. Glyphosate induces oxidative stress leading to increased breakdown of membranes of rbcs causing reduction in packed cell volume. Thus decreased mean values of TEC, PCV and Hb concentration could be due to free radical induced hemolysis of erythrocytes and similar opinion was expressed by earlier worker [29]. The increased MCV in group 2 indicate presence of immature rbcs in peripheral blood reflecting body compensatory mechanism in response to GLP induced rbcs destruction. The significant increase in MCH and non-significant decrease in MCHC in group 2 indicates normal Hb concentration. Thus GLP toxicity induces macrocytic anemia. The decrease in TLC in the present study may be attributed to the toxic effects of GLP on leucopoiesis *in vitro* studies showed that glyphosate presented some toxicity to human mononuclear peripheral blood cells [30]. Many pesticides have been shown to induce immunotoxicity either via induction of apoptosis or necrosis [31]. The OPs induced immunotoxicity has been associated with either inhibition of serine hydrolases or esterases in components of the immune system, through oxidative damage to immune organs, or by modulation of signal transduction pathways controlling immune functions [32]. Therefore probable mechanism of immunotoxicity caused by GLP may be oxidative damage of immune organs.

In group 4 (GLP + Vit. C), significant improvement in all the hematological parameters was observed in comparison to group 2 (GLP) rats. These changes could be due to antioxidant defensive action of Vitamin C against free radical induced oxidative stress in different tissues including blood cells. Vitamin C in its reduced form, has been shown to improve the absorption of iron from the gut [33-34], thereby increasing its serum concentration of iron essential for heme synthesis. Earlier studies have shown vitamin C to be helpful in the management of anemia induced by pesticides toxicity[35]. Ascorbic acid (Vitamin C) was able to ameliorate GLP induced immunotoxicity via inducing lymphocytic proliferation and inhibiting apoptosis [36, 37].

A significantly (p<0.05) increased mean values of AST, ALT and ALP were recorded in group 2 rats as compared to control rats which is indicative of hepatic damage due to GLP induced lipid peroxidation causing damage to lipid membrane bilayers of hepatocytes leading to the release of membrane bound enzymes from the damaged cell cytoplasm into circulation. The results are in accordance with earlier findings [38, 39]. In the present experiment, a steady raise in transaminases is indicative of moderate to severe damage of hepatocytes at 500 mg/kg b.wt dose of GLP. ALP is a membrane bound enzyme present in tissues in greatest amount in intestinal mucosa and in lesser but substantial amount in kidneys, placenta, liver and bone. In liver ALP isoenzyme is derived from bile canaliculi membrane. The elevated levels of ALP in present study may be attributed to both biliary damage and intestinal mucosal which was severely affected with GLP exposure. Contrary to this, Caglar and Kolankaya[40] observed a decrease in serum levels of AST

and ALT in rats exposed to GLP (56 and 560 mg/kg b.wt.). A significant reduction in the mean values of AST, ALT and ALP in group 4 rats when compared to Group 2 rats indicated ascorbic acid had protective effect against GLP induced liver toxicity. GLP induces its major toxic effects through oxidative damage to the hepatocytes resulting in elevation in MDA and decrease in antioxidant levels. Ascorbate has been demonstrated to be an effective antioxidant. It can act both directly, by reaction with aqueous peroxyl radicals, and indirectly, by restoring the antioxidant properties of fat-soluble vitamin E. The overall consequence of these antioxidant activities is the beneficial control of lipid peroxidation of cellular membranes including those surrounding as well as within intracellular organelles [39].

Significantly increased mean values of TP were recorded in groups 2 and 4 as compared to group 1 and could be due to impaired protein synthesis by liver. The opinion was supported by earlier workers [41, 42].

A significant increase in mean values of BUN and serum creatinine were recorded in group 2 as compared to group 1 and the findings are in harmony with the earlier observations [41, 43]. The elevated values of BUN and serum creatinine are due to renal tubular damage and glomerular filtration indicating GLP is nephrotoxic. The primary mechanism of glyphosate-induced nephrotoxicity is uncoupling of oxidative phosphorylation causing ATP depletion and cell death[44]. Putatively, this could also be due to over production of ROS. The reduction in BUN and serum creatinine in group 4 showed that antioxidant property of Vitamin C protected the kidneys from the lipid peroxidation changes caused by GLP.

5. Conclusion

The present study has shown that GLP exposure induced hemotoxicity and elevated biochemical parameters are indicative of hepatic and renal damage. Ascorbic acid (Vitamin C) mitigates the toxic effect of GLP through its free radical scavenging activity. A further detailed study at molecular level is needed to know the mechanism of glyphosate toxicity and the protective mechanism of ascorbic acid could be explored.

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7. Conflicts of interest

The authors declare that they have no conflicts of interest

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