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Glyphosate induced renal toxicity and its amelioration with vitamin C in rats

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Abstract
The main clinical effects of consuming glyphosate are hepatotoxicity and renal toxicity. Vitamin C, a well-known antioxidant, helps to prevent cellular damage brought on by free radicals. The goal was to assess the impact of glyphosate on renal tissues in male albino Wistar rats at a dose of 500 mg/kg body weight (1/10 LD_{50}) and its amelioration by vitamin C at a level of 250 mg/kg body weight. For three weeks, the animals received daily oral treatments. The histology and ultrastructure pathologies of renal tissues, tissue oxidative stress parameters, and serum biochemical assays were investigated. In the treated group, there was a significant increase in the levels of the blood enzymes urea and creatinine. Malondialdehyde concentration, reduced glutathione, and superoxide dismutase levels were measured to assess oxidative stress in renal tissues. Kidney segment histopathology and ultrastructure pathology showed significant alterations. However, vitamin C treatment had a slight to moderately ameliorative effect on the examined parameters.

Keywords: Glyphosate induced renal toxicity, amelioration, vitamin C, rats

Introduction
Herbicides have been an integral part of the expanding agrochemical pesticide industry over the past 20 years. Water-soluble, non-selective herbicide glyphosate [N-(phosphomonomethyl) glycine] (GLP) is applied to foliage and causes the weeds to die (Kremer and Means, 2009). One of the most widely used and environmentally harmful herbicides is glyphosate, which is sold under the brand name Roundup®. 15% polyoxyethylene amine (POEA) and other unidentified surfactants are combined in Roundup® (Howe *et al*. 2004) [14]. According to studies, this mixture is more harmful than glyphosate by itself (Howe *et al*. 2004; Williams *et al*. 2000 and Santos *et al*. 2005) [14, 38, 31]. Because of its widespread use, glyphosate is now more prevalent in the environment, surface water, and groundwater (Benbrook, 2016) [6]. Cytochrome P450 and two additional enzymes (G-6-P dehydrogenases and glutathione-S-transferases), which are critical to the body's detoxification of toxins, were negatively impacted by Roundup (Acquavella *et al*. 2004) [1]. GLP disrupts the body's normal DNA repair machinery and damages DNA in human cells as an endocrine disruptor, which leads to genomic instability and the spread of cancer. The main cause of GLP's toxicity is the decoupling of oxidative phosphorylation (Ikpeme *et al*. 2012) [15].
Numerous contaminants can cause harm to biological systems by producing reactive oxygen species (ROS) like hydroxyl radicals (OH), superoxide anions, and hydrogen peroxide (H2O2) (Harish and Murugan, 2011; Ahmad *et al*. 2000) [12, 2]. Animals have an antioxidant defence system that includes non-enzymatic antioxidants including non-protein thiols, particularly glutathione, as well as enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) (GSH). One of the most harmful effects of oxidative stress is membrane lipid peroxidation, which happens when the balance between pro-oxidants and antioxidants shifts (LPO) (Scandalios, 2005) [32].
The objective of the current study is to examine the damaging effects of glyphosate on the male Wistar albino rats' renal tissues by evaluating the serum biochemistry tests, oxidative stress indices, histopathology, and ultrastructure pathology due to the dearth of literature on glyphosate toxicity. A powerful antioxidant known as vitamin C was employed to mitigate the damage caused by free radicals brought on by glyphosate.

Materials and Methods
Chemicals: A commercial glyphosate formulation called Roundup® was purchased from Hyderabad-30's Professor Jayashankar Telangana State Agriculture University (PJTSAU)

Seed Research and Technology Centre (SRTC), and vitamin C was purchased from Mumbai, India's S.D. Fine-Chem Ltd. Glyphosate is the primary ingredient in Roundup® at a 41 percent concentration.

Experimental animals

Wistar albino adult male rats, raised at Jeeva Life Sciences (an ISO 9001:2015-certified company), Hyderabad, weighed 200–240 g for the study. Animals underwent one week of acclimatization. A 12-hour cycle of light and dark was employed in the lighting cycle, and the temperature was kept at 222 °C. Throughout the trial, all of the rats received a regular pellet meal and unlimited access to deionized water.

Animal ethics: In accordance with the criteria established by the Institutional Animal Ethics Committee, all of the animals received humane treatment (IAEC). The study was conducted after receiving IAEC's previous clearance (01/2019).

Treatment

We randomly divided 48 adult male rats into four groups (G1-G4), each having twelve (12) individuals. This was preferred since the normal sample size for scientific experiments with inbred rodents is between 5 and 7 (Kubota and Wakana, 2011) [19]. Group 1 was regarded as control and was given distilled water, Group 2 was given GLP (500 mg/kg body weight), Group 3 was given vitamin C (250 mg/kg body weight) and Group 4 was given GLP + vitamin C (500 mg/kg body weight + 250 mg/kg body weight), daily orally for three weeks. After one week and three weeks of therapy, six rats from each group were sacrificed by cervical dislocation. Quickly separated, weighed, and placed in a cold bath were the kidneys.

Biochemical evaluation

Without the application of anesthesia, 1-1.5 mL of blood samples were taken from the rats using ocular puncture at the conclusion of the first and third weeks. Animal blood samples were drawn into simple tubes, allowed to clot, and then centrifuged to extract the serum. The serum was then stored at 4 0C until it was needed. Blood urea nitrogen (BUN) and creatinine were two biochemical parameters that were examined using Erba Mannheim Diagnostic kits and an automated serum analyzer (Star 21 Plus, Rapid Diagnostic Pvt. Ltd.).

Oxidative stress indices

To get 10% homogenate for all the tissue antioxidant profiles, one gramme of kidney tissue was placed into a tissue homogenizer along with 10 mL of 0.2 M Tris HCl buffer (pH 7.2). Malondialdehyde and other lipid peroxidation end products were used to measure the tissue oxidation (Balasubramanian *et al*. 1988) [5]. Using procedures, tissue protein was estimated (Lowry *et al*. 1951) [24], kidney SOD activity was assayed by the method of Madesh and Balasubramanian, (1998) [5] and reduced glutathione (GSH) (Moron *et al*. 1979) [27] to know the antioxidant status.

Histopathological studies

Following euthanasia, samples of kidney tissue were removed surgically from the animals. All samples were fixed in Neutral Buffered Formalin (NBF) at a concentration of 10%, then washed, dehydrated in alcohol, clarified in xylene, and mounted in paraffin blocks. Following the normal approach (Luna, 1968) [25], the tissues were cut into 5 m slices, stained with hematoxylin and eosin (H&E), mounted in neutral Dibutyl phthalate Xylene (DPX) media, and examined under a light microscope.

Ultrastructure Pathology

As soon as the kidneys were sacrificed, they were cut into thin slices, fixed in 2.5 percent glutaraldehyde (PBS-based), and prepared for TEM and SEM analysis according to procedure (Lakshman, 2017 and 2019) [20-21].

Statistical analysis

The Statistical Package for Social Sciences (SPSS) version 16.0 was used to do a one-way analysis of variance (ANOVA) on the data gathered from the experimental animals. The significance level was set at P 0.05, and the results were presented as mean + SE (Snedecor and Cochran, 1994) [33].

Results

When compared to the control group, the BUN and serum creatinine levels in the rats exposed to GLP for one week and three weeks significantly increased. BUN and creatinine levels were somewhat to moderately reduced by co-administration of vitamin C (G4) (Table 1)

When compared to the control rats, the MDA levels in the kidney homogenates of the rats given GLP for one and three weeks were significantly (p<0.05) higher. Following treatment, there was a discernible decrease in vitamin C. (Table 2)

When compared to the control group, there was a significant (p<0.05) decrease in the mean values of SOD and GSH in renal homogenates of rats treated with glyphosate for one and three weeks. Only half of the enzyme activity were restored when vitamin C was added. The enzymes' activities were partially recovered when vitamin C was also provided. The rats given vitamin C alone (G3) displayed results comparable to the control group. (Table 2)

The kidneys of rats given glyphosate treatment for a week revealed hyaline casts, enlarged Bowman's gap, glomerular atrophy, and damaged tubules. After three weeks of treatment, there was congestion, fibrous tissue proliferation, glomerulus engorgement, intratubular hemorrhages, significant round cell infiltration, and tubular necrosis with pyknotic nuclei. In kidney sections of rats treated with glyphosate and vitamin C, mild tubular regeneration was seen coupled with lesser intensity lesions. (Figure 1)

Anisocytosis and poikilocytosis of mitochondria with loss of cristae were seen in kidney sections from rats given glyphosate treatment for a week, as well as epithelial cells with pyknotic and enlarged nuclei. After three weeks of treatment, a thin portion of the kidney revealed deformed tubules, enlarged microcapillary walls, and increased basement membrane tubule thickness. Co-administration of vitamin C resulted in modest tubule and cytosol reconstruction, a large number of vesicular mitochondria, the loss of epithelial junctions, and increased tubular membrane thickness. (Figure 2)

One week after glyphosate treatment, SEM of kidney sections revealed engorged glomeruli and tubular necrosis. After three weeks of treatment, mononuclear cell infiltration, tubular necrosis, and fibrous tissue proliferation were evident. (Figure 3)

Table 1: Serum biochemical parameters in different groups at different time intervals

Group	BUN (mg/dL)		Creatinine (mg/dL)	
	Day 7	Day 21	Day 7	Day 21
Group 1	37.83±1.30[a]	38.67±0.91[a]	0.56±0.05[a]	0.8±0.03[a]
Group 2	56.67±1.49[b]	64.83±2.08[c]	1.21±0.08[c]	1.75±0.08[c]
Group 3	38.33±1.11[a]	40.5±0.92[a]	0.73±0.06[a]	0.83±0.03[a]
Group 4	53.17±0.79[b]	56.17±3.12[b]	0.98±0.06[b]	1.03±0.07[b]

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

Table 2: Oxidative stress indices in different groups at different time intervals

Parameter	Group 1		Group 2		Group 3		Group 4	
	Day 7	Day 21	Day 7	Day 21	Day 7	Day 21	Day 7	Day 21
MDA(µM/ mg of protein)	3.70±0.31[a]	3.80±0.39[b]	7.35±0.18[c]	8.30±0.18[d]	3.43±0.77[a]	3.35±0.76[a]	6.46±0.11[b]	7.35±0.14[c]
GSH (µM/mg protein)	10.35±0.08[c]	10.57±0.05[c]	8.45±0.11[a]	7.35±0.06[a]	10.49±0.04[c]	10.61±0.07[c]	8.85±0.03[b]	8.63±0.14[b]
SOD (U/mg protein)	11.45±0.13[c]	11.59±0.13[c]	9.68±0.14[a]	9.68±0.14[a]	11.66±0.14[c]	11.82±0.12[c]	10.53±0.19[b]	9.35±0.27[b]

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

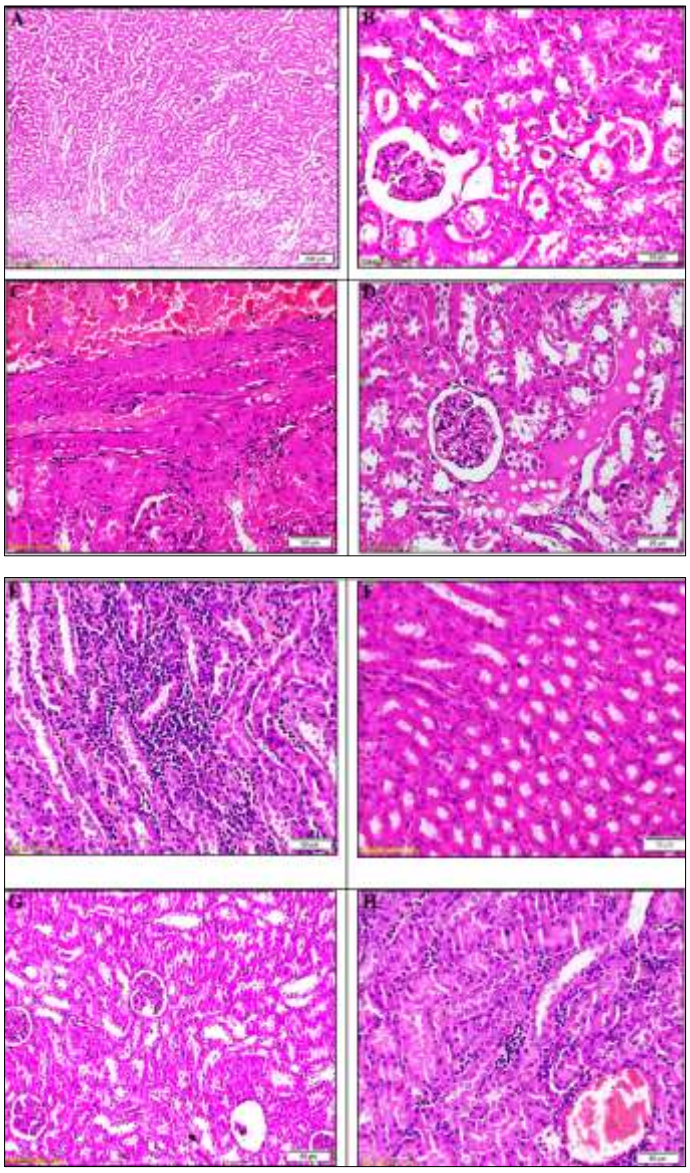

Fig 1: Histological analysis of kidneys of different groups (H&E). A: Group 1: Kidney showing normal glomeruli and tubules (x40), B: Group 2, Day 7: Kidney section showing glomerular atrophy, increased Bowman's space, degenerated tubules and hyaline casts (x200), C: Group 2, Day 21: Kidney section showing congestion, fibrous tissue proliferation, engorgement of glomerulus and tubular necrosis (x100), D: Group 2, Day 21: Kidney section showing edema, vacuolar degeneration in glomerulus, sloughing of tubular epithelium and mild fibrous tissue proliferation (x200), E: Group 2, Day 21: Kidney section showing intertubular hemorrhages, marked round cell infiltration and tubular necrosis with pyknotic nuclei (x200), F: Group 3, Day 21: Kidney section showing normal appearance of the tubules (x200), G: Group 4, Day 7: Kidney section showing mild regeneration of tubules and glomerular atrophy (x200), H: Group 4, Day 21: Kidney section showing congestion, mild round cell infiltration and tubular necrosis with pyknotic nuclei (x200)

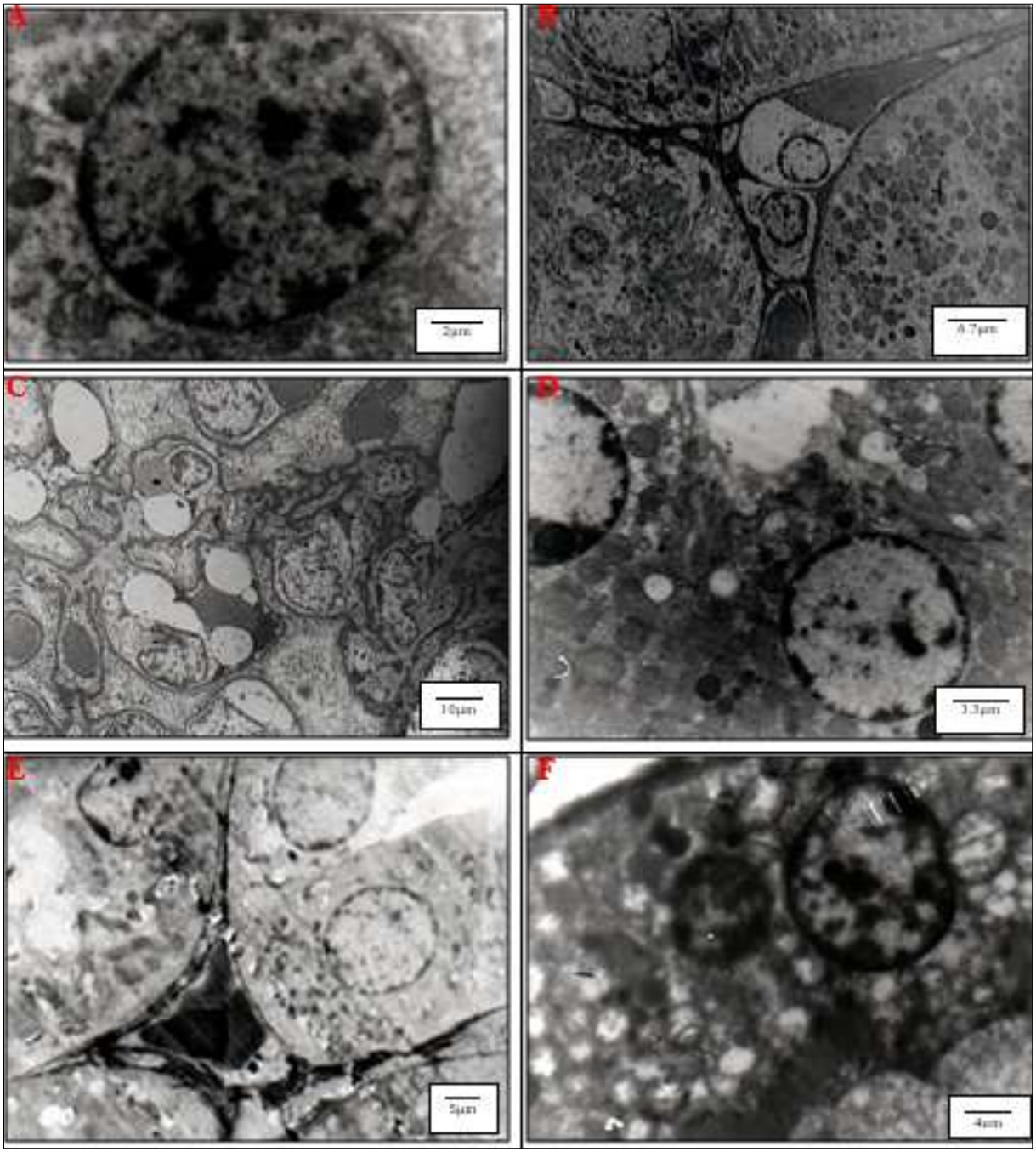

Fig 2: Transmission electron micrograph of kidney sections from different groups (UA&LC), A: Group 1, Day 21: A thin slice of the kidney displaying mitochondria in a normal state (9650x), B: Group 2, Day 7: A thin section of the kidney reveals dilated intratubular space with blood cells, thickened capillary walls, enlarged and dilated RER cisternae, anisocytosis and poikilocytosis of mitochondria with lack of cristae, and epithelial cells with pyknotic and swollen nuclei (2895x), C: Group 2, Day 21: Renal ultrathin slice demonstrating deformed tubules, thickening microcapillary wall, and increased thickness of tubule basement membrane (1930x), D: Group 3, Day 21: a thin segment of kidney epithelial cells displaying a normal nucleus and uniformly sized mitochondria (5790x), E: Group 4, Day 7: Kidney ultrathin section demonstrating dilated intertubular junctions, tubular epithelial cell necrosis, and kidney deterioration (3860x), F: Group 4, Day 21: Kidney ultrathin section demonstrating epithelial junction loss, modest reconstruction of tubules, cytosol, pyknotic nuclei, many vesicular mitochondria, and thickening of the tubular membrane (4825x)

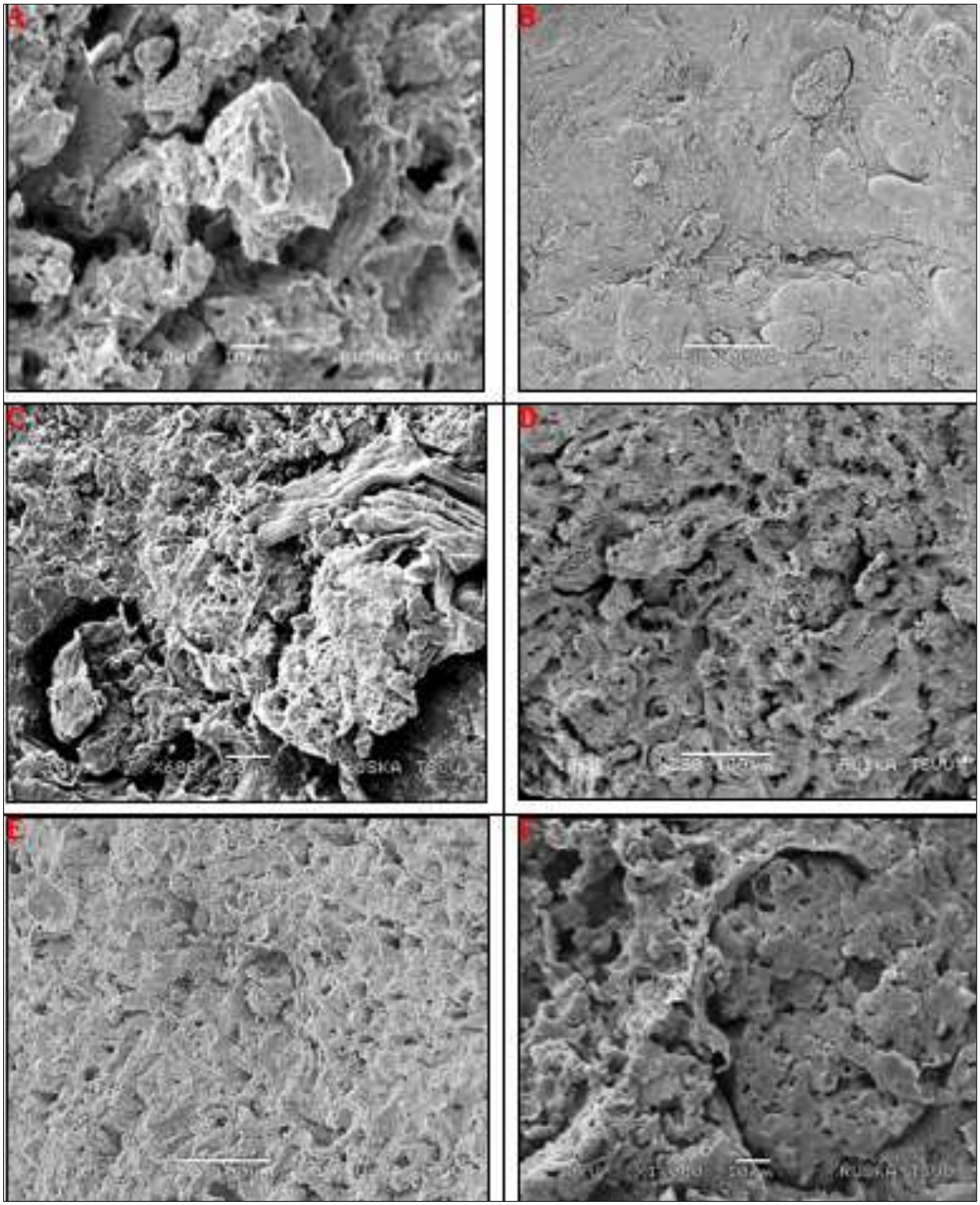

Fig 3: Scanning electron micrograph of kidney sections from different groups, A: Group 1, Day 21: Ultrathin section of kidney showing normal glomerulus and intact Bowman's capsule, B: Group 2, Day 7: Ultrathin section of kidney showing engorged glomerulus and tubular necrosis, C: Group 2, Day 21: Ultrathin section of kidney showing infiltration of MNCs, tubular necrosis and fibrous tissue proliferation, D: Group 3, Day 21: Ultrathin section of kidney showing normal intact tubules, E: Group 4, Day 7: Ultrathin section of kidney showing atrophy of glomerulus and narrow tubules, F: Group 4, Day 21: Ultrathin section of kidney showing glomerular engorgement with dilated tufts

Discussion

Notably, farmers use pesticides frequently to eradicate weeds all around the world. During application and container disposal, both humans and animals are directly and indirectly exposed, which causes mortality. The results of the current investigation showed that kidney function in rats exposed to glyphosate was severely impaired, as evidenced by the obvious histological and ultrastructural changes. Anorexia, decreased water intake, lethargy, moderate watery diarrhea, and weakness were some of the clinical symptoms that rats exposed to glyphosate displayed. However, no fatalities were recorded during the course of the investigation.

To monitor nephrotoxicity, serum creatinine and BUN levels are employed as an early and sensitive biomarker (Lall *et al.* 1997) [22]. Cavusoglu *et al.* (2011) [7] argued that glomerular filtration impairment and renal tubular injury are to blame for the rise in BUN and serum creatinine levels. El-Shenawy (2009) [11] stated that when tissues are disturbed, the enzyme

xanthine dehydrogenase is converted to xanthine oxidase by the oxidation of essential "SH" groups, which results in kidney damage due to an enormous increase in free radical production and ATP depletion as a result of tissue hypoxia. Weir *et al.* (2003) [37] the conversion of hypoxanthine to uric acid, xanthine, and superoxide is catalyzed by xanthine oxidase, it has been reported. This might be one of the causes of the increased uric acid levels in the rats treated with glyphosate and Roundup. Considered to be a powerful antioxidant molecule is uric acid (Nieto *et al.* 2000) [29]. The increased serum uric acid levels in glyphosate-treated animals may be a result of a protective mechanism to offset the increased oxidative stress. According to the results of the current study, rats given glyphosate treatment exhibited significantly higher levels of BUN and serum creatinine. These findings can be linked to histopathological findings, specifically tubular degeneration, necrosis, along with fibrosis and glomerulus atrophy, which may explain the rise in serum enzyme levels. Supposedly, hypoxia and excessive ROS generation are also to blame for this. (Jasper *et al.* 2012; Cavusoglu *et al.* 2011 and Youness *et al.* 2016) [16, 7, 40] also published similar results. The ameliorative group's lower BUN and serum creatinine levels are a sign of improved cell respiration and vitamin C's antioxidant properties.

Oxidative stress is the term for the *in vivo* imbalance of oxidation and anti-oxidation (Hou *et al.* 2013) [13]. Oxidative damage can occur when defensive mechanisms are insufficient to neutralize ROS; membrane LPO is one of the more severe forms (Ahmad *et al.* 2004) [3]. The MDA is a biomarker for LPO and the stable metabolite of LPO products (Sun *et al.* 2001) [34]. The results of the current study showed a significant rise in the levels of MDA in the kidney tissues of glyphosate-treated rats. This rise in MDA levels may be attributable to increased LPO, which in turn causes an increase in the intracellular accumulation of ROS. MDA, a byproduct of lipid peroxidation, is known to be produced by ROS acting on the unsaturated fatty acids of the phospholipids that make up membrane components. The components of the herbicide directly interact with the cytoplasmic membrane of the kidney cells, causing a disruption in the structure of the membrane, which is the mechanism underlying this peroxidation (Dedeke *et al.* 2018) [10]. Vitamin C (G4) co-administration in the current study significantly reduced MDA levels, demonstrating its antioxidant properties. Vitamins C and E and N-acetyl-L-cysteine (NAC) are antioxidants that may lessen the harmful effects of GLP (Youness *et al.*, 2016) [40]. In addition to acting as an antioxidant, vitamin C also acts as a pro-oxidant (Pehlivan, 2017) [30]. Vitamin C securely interacts with free radicals and breaks down chain reactions before damaging essential components. Additionally, it prevents other oxidative processes and removes free radical intermediates (Ikpeme *et al.*, 2012) [15].

Additionally, a significant amount of the renal disease is linked to the drop in intracellular GSH content (Atef, 2010) [4]. Therefore, GSH content is crucial for cell survival. In addition, glutathione peroxidase uses it as a substrate. Tripeptide glutathione is one of the most significant modulatory mechanisms for neutralizing free radicals and preventing the attack of electrophilic xenobiotics on cellular macromolecules (Cnubben *et al.*, 2001) [9]. In the current investigation, there was a noticeable decrease in GSH levels after glyphosate treatment is in accordance with the observations of El-Shenawy (2009) [11], Cavusoglu *et al.* (2011) [7] and Tang *et al.* (2017) [35]. It's possible to suppose that Roundup increased cell death by increasing oxidative processes and creating an oxidative imbalance by lowering GSH levels (El-Shenawy, 2009) [11]. Due to its capacity to cause mitochondrial damage and oxidative stress in kidney homogenates, the present study's considerable drop in the mean values of GSH and SOD can be explained. The theory is supported by the ultrastructural alterations in the mitochondria of tubular epithelial cells in the current investigation. When compared to group 2, the rats in group 4 had a little to moderate improvement, demonstrating the preventive effect of vitamin C against GLP-induced nephrotoxicity.

Namratha *et al.* (2019) [28] revealed that kidney weights in rats given glyphosate (500 mg/kg body weight, orally) orally for three weeks showed a substantial drop, followed by marked congestion and an atrophied look. The multiple histological impairments to the kidney tissues could have been caused by the increased LPO, decreased antioxidant defenses, increased blood urea, and creatinine, which were all seen in rats treated to the Roundup formulation in this investigation. Rats exposed to Roundup showed kidney sections with distorted renal-cortical histoarchitecture, engorged glomeruli, intratubular fibrous tissue proliferation, noticeable round cell infiltration, glomerular atrophy with vacuolar degeneration, and severity that was directly inversely proportional to the number of days of exposure. Cavusoglu *et al.* (2011) [7]; Karimi *et al.* (2014) [17]; Tizhe *et al.* (2014) [36] and Tang *et al.* (2017) [35] also reported similar findings. Wunnapuk *et al.* (2014) [39] confirmed that during the acute stages of Roundup poisoning, renal cell death mechanisms occurred in tubules and glomeruli. Therefore, taking the herbicide Roundup by mouth has the potential to not only harm the kidney but also to result in chronic renal disease and eventual kidney death. The modifications in the current study may have resulted from excessive ROS production, which outweighed endogenous antioxidants produced by the body and had stronger effects on calcium influx and the Na+/K+ ion transport system. The ATP depletion seen as a result of the cytotoxic effects of GLP, presumably through increased ROS or oxidative stress, may have been the cause of the tubular necrosis. When compared to group 2, these glyphosate-induced nephrotoxic effects were least pronounced in group 4. Despite vitamin C's antioxidant qualities, these lesions may be a result of the GLP's propensity for toxicity. These histological changes are related to the ultrastructure observations. According to Langeswaran *et al.* (2012) [23] In addition to controlling membrane permeability and ion transport across the cellular membrane at the expense of ATP through hydrolysis, ATPases are crucial enzymes that fuel metabolic energy for life processes. These ion-dependent ATPases are inhibited, which causes abnormalities in ion homeostasis that disrupt signal transduction, affect cellular metabolism, alter cell membrane integrity and permeability, increase membrane fluidity, and impede essential processes (Chodon *et al.* 2008) [8]. The herbicide Roundup has the ability to decrease the activities of membrane-bound enzymes (Ca-ATPase, Mg-ATPase, total ATPase, and Na/K-ATPase) in the kidney of the exposed animal, suggesting disruptions in the cellular metabolism of the kidney and changes in the kidney cell membrane and integrity brought on by the herbicide compositions (Dedeke *et al.* 2018) [10].

Overproduction of ROS, which may have caused mitochondrial malfunction, can be blamed for the

ultrastructural alterations observed in glyphosate-treated rats. We can draw the conclusion that vitamin C alone was unable to entirely counteract the damaging effects of GLP caused by ROS and other molecular mechanisms, such as apoptosis.

Conclusion

The findings of the current study demonstrated that exposure to a commercial formulation of the herbicide Roundup containing glyphosate at different time points led to severe renal damage brought on by increased oxidative stress, which increased serum urea and creatinine as well as histopathological and ultrastructural alterations. It's not yet apparent if glyphosate's presence in the Roundup formulation has a synergistic effect or whether another chemical in the formulation is primarily to blame for the documented nephrotoxicity. Despite vitamin C's antioxidant abilities, co-administration of the vitamin (250 mg/kg body weight) showed a minor improvement in biochemical and oxidative stress markers as well as a modest improvement in histological and ultrastructural alterations, indicating the possible toxicity of GLP. Although vitamin C has potent antioxidant properties, supplementation by itself has not completely corrected the negative effects caused by GLP. Research is still needed to determine the most effective ameliorative agent or combination of agents that can reduce the negative effects of GLP.

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