

The preventive effect of vitamin C on the cellular and functional integrity of kidney cells in rats following repeated exposure to paraquat

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Abstract

Paraquat (PQ) is a bipyridylium herbicide that is applied around trees in orchards and between crop rows to control broad-leaved and grassy weeds. Its oxidation results in the formation of superoxides which causes damage to cellular components. In this study, we determined the antioxidant effect vitamin C has on the cellular integrity of kidney function in rats following repeated exposure to PQ. Ninety-six male rats, grouped twelve rats per subgroup (A, $A_{vit.c}$, B, $B_{vit.c}$, C, $C_{vit.c}$, D and $D_{vit.c}$) were intraperitoneally injected with different sublethal increasing doses (0, 0, 2, 2, 4, 4, 6 and 6 mg/kg body weight) of PO respectively on biweekly (14 days) intervals over a period of three months (84 days). Subsequently, the subgrouped animals (Avit.c, Bvit.c, Cvit.c and Dvit.c) were maintained orally with 1 g/L vitamin C, while the other subgrouped animals (A, B, C and D) received drinking water with negligible vitamin content throughout the study period. At the end of each monthly (28 days) treatment, four animals per subgroup were selected. Urine samples were collected from each of the selected rats, after which each of the animals were anaesthetized with gaseous isoflurane and 5 mL of blood samples were collected using cardiac puncture procedure. The animals were later decapitated and their kidneys harvested. The samples collected were analyzed for urine [specific gravity (SG), pH, protein and glucose], blood (urea, creatinine, total protein and glucose), and the histological studies on kidney slides. The dose and exposure-time dependent PQ toxicity resulted in the reduction in urinary pH, elevation in urinary SG, and the detectable presence of protein and glucose in urine. It also caused

marked elevation in serum urea and creatinine levels with reduction in serum protein and glucose levels and alterations in the cellular integrity of the renal architecture, especially the glomeruli and tubular tissues. Treatments on the PQ insulted animals with vitamin C resulted in the significant presentation of biochemical and histological values that were a complete reversal or near normalization in the parameters assayed, except the urinary pH which presented with more acidic values (vitamin C is acidic in solution). Vitamin C treatment significantly brought down the urine SG level to normal or near normal. It caused near or total disappearance of protein and glucose in urine, reduction in serum urea and creatinine concentration and elevation or normalization of serum total protein and glucose values with restoration of the renal function of treated rats. These findings clearly show that vitamin C has an important role to play in the body's antioxidant defense system. Its use caused normalization of the biochemical and histological parameters studied and restored the health status of the affected animals.

Introduction

Paraquat (PQ) a bipyridylium herbicide that is effective as a non-selective synthetic herbicide when applied around leaves.1 Because PQ has a redox potential of 446 mV, any reducing agent with sufficient energy can donate an electron to the bipyridylium divalent cation (PQ²⁺) to form a free radical (PQ⁺). The oxidation of the bipyridylium radical to form the original PQ²⁺ results in the transfer of the electron to oxygen and the formation of superoxides called reactive oxygen species (ROS).2,3 Subsequent Haber-Weiss and Fenton reactions yield toxic hydroxyl radicals³. Thus the herbicide (PQ) functions as a catalyst to transfer reducing equivalent to oxygen. These ROS so formed may escape the electron transport chain and when in excess causes damage to cellular components.4

The kidneys being one of the organs involved in the excretion of waste products of metabolism in the body is composed of nephrons and collecting tubules. The functions of the kidney include maintenance of water and electrolyte balance, maintenance of the blood pH and the excretion of unwanted substances from the body.⁵ To perform these functions the kidney utilizes the processes of glomerular filtration, tubular reabsorption and tubular secretion.⁶ Renal functions are easily affected by toxins.⁷ During PQ poisoning, normal renal processes actively secrete PQ into the urine, efficiently clearing it from the blood. But toxic doses overwhelms the secretory Correspondence: Benjamin Nnamdi Okolonkwo, Department of Medical Laboratory Sciences, Rivers State University of Science and Technology, Nkolu-Orowurokwo, Port-Harcourt, Rivers State, Nigeria. E-mail: benbruceph@yahoo.com

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Contributions: BNO, lead author, research and study design, specimens analysis and overall supervision; EON, manuscript revising; PCE, preparation of kidney histological slides; CUO, animals handling for sample production, sample analysis and statistical computation of results; GOA, histological procedures and tissue slide microscopic examination, data proofreading.

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capability of the nephrons thereby causing destruction of the cellular integrity of the glomeruli and tubular cells leading to the release of reabsorbable substances which collects in the bladder for excretion. The voided urine presents with proteinuria, hematuria, ketonuria, glycosuria, etc. this situation if not arrested progresses to renal failure and azotemia.8-11 Some of the metabolic indicators of kidney function (urea, creatinine, glucose and protein) are of diagnostic importance in the establishment of renal injuries or diseases, because they respond favorably to the smallest insult or injury on the renal tubules and can be detected early. Also the architectural status of the nephrons helps in confirming the degree of injury and site of localization of the injury.8

Previous studies have shown that PQ toxicity causes acute oliguric renal failure and in less severe cases lead to impaired renal function with manifestations like proteinuria with hyaline casts, glycosuria, aminoaciduria and excessive leakage of phosphorus, sodium and uric acid. Sometimes necrosis of the proximal tubules is found together with extreme dilatation of the distal tubules of the kidney. Wershana¹² in his study on the influence of vitamin C or selenium on PQ - induced toxicity in Guinea pigs showed that animals injected (ip) with sublethal dose [1 mg/kg body weight (bw)] of PQ for 7 days presented with abnormal hematocrit values, abnormal liver functions, impaired renal function and electrolyte imbalance as compared to those previously administered daily (ip) injection of vitamin C (100 mg/kg bw) and selenium (20 mol/kg bw) before the PQ insult, which showed little or no abnormalities on the parameters evaluated.

Antioxidants are classified into two broad divisions depending on their solubility in water (hydrophilic) or in lipids (hydrophobic /lipophilic). Water soluble antioxidants react with oxidants in the cell cytosol and the blood plasma, while the lipid soluble ones protect cell membranes from lipid peroxidation.13 Though various antioxidants behave synergistically, vitamin C can degrade or reactivate other antioxidants.13 Vitamin C can directly scavenge oxygen free radicals with and without enzyme catalyst, and can indirectly scavenge them by recycling others to their reduced form.^{14,15} By reacting with activated oxygen more readily than any other aqueous components, vitamin C protects critical macromolecules from oxidative damage.¹⁴ In general, vitamin C antioxidant system either prevents these ROS from being formed or removes them before they can become injurious to vital components of the cell.^{16,17}

Because of this potent functionality of vitamin C as an antioxidant, this study was geared towards assessing the acceptability of vitamin C as an adjunct in the treatment and management of PQ toxicity cases.

Materials and Methods

Paraquat

20% W/V Dizmazone[®] (paraquat dichloride solution) from Dizengoff W.A. Ltd. (Lagos, Nigeria) sealed in an opaque plastic container with a shelf-life of two years was employed.

Vitamin C

Pure vitamin C (1000 mg) caplets from Mason Natural[®] (Mason Vitamins, Inc., Miami Lakes, FL, USA) were employed.

Urinalysis strips

Urine reagent strips (9-parameters) for urinalysis, product of Atlas Medical Ltd., Cambridge, UK (http://atlas-medical.com/index. php) were employed.

Animals and treatments

A total of 96 white male albino rats (Rattus

norvegicus), weighing between 180-220 g (average bw of 0.2 ± 0.02 kg), obtained from the animal house of the Department of Pharmacology and Toxicology, College of Health Sciences, University of Port-Harcourt, Choba, Rivers Statem Nigeria, were fed *ad libitum* with pelletized animal finisher feed with negligible vitamin C (vit. C) content and allowed to acclimatize for two weeks in metabolic cages before the commencement of the study.

Following acute toxicity study by Dede et al., 18 where 50% lethal dose of PQ was calculated to be 0.35 g per kg bw of rat (35 mg/kg). A selection of four sublethal doses of PQ was made (0, 2, 4)and 6 mg/kg) to be used in this study [it was generally accepted that at these dose range PQ fatality to the animals will be greatly eliminated since it was a sub-chronic study], and the four selected doses were grouped (A, B, C and D) respectively. Each of the group was subdivided into two no vitamin C- and vitamin C-subgroups (A: A_{vit.c}, B: B_{vit.c}, C: C_{vit.c} and D: D_{vit.c}), with each subgroup containing 12 animals. Avit.c is a positive control (note: a subgroup of control A to be treated with vitamin C to check if vitamin C at the dose given was injurious to the animals).

Two mL of the respective sublethal doses of the toxicant (PQ) was ip administered to the animals under anesthetics19 every two weeks (14 days) for up to three months (84 days) in the presence or absence of vitamin C medicated water (1 g/L). [Note: the animals were anaesthetized before PQ treatment to avoid injection site injury on the animal during struggle (i.e. if given without anesthesia), and also the route and frequency of PO treatment was chosen in simulation of environmental contamination of animals/man from polluted feeds, air or water bodies]. The animals were made to drink water or medicated vitamin C water (1 g/L) from suspended inverted water bottles that have glass-sipper tubes. The liquid content bottles were exchanged for new ones,



aseptically, trice daily irrespective of the volume of the remnant liquid content (Table 1).

Urine collection and analysis

This method was designed to obtain urine from a large number of animals (rats) for qualitative and semi-quantitative analysis, e.g. to detect the presence or absence of glucose, protein or ketone in conditions presenting with glycosuria, proteinuria or ketonuria.^{20,21} It is simple, reliable and efficient. A 5 mL polystyrene beaker (Dispo Beaker Polystyrene) was attached to the perineal wall using tape that is adherent on both sides. This allowed the collection beaker to be attached and removed with ease. The rat was then held by the tail, after the beaker was attached and the back of the animal was stimulated tactilely using the fingers of the opposite hand. It was observed that 80% of the animals voided between 0.1 and 0.8 mL of the urine within a few seconds, while it took 5-10 min for the remaining 20% of the animal to void urine. The specimens collected were analyzed using urine qualitative/semi-quantitative test strips. The parameters examined were pH, specific gravity (SG), glucose and protein.

Blood sample collection and analysis

The blood sample collection was done using cardiac puncture procedure.²² At the end of each treatment month (28^{th} , 56^{th} and 84^{th} days), four animals per subgroup were selected, anaesthetized with gaseous isoflurane and 5 mL of blood sample were collected using cardiac puncture procedure.¹⁹ The blood sample collected were dispensed into a centrifuge glass-test tubes, allowed to clot and retract, centrifuged at 2000 r/min for 5 min, after which the serum is separated from the red cells and transferred into a plain sample container, labeled and stored frozen at 0°C until needed for analysis of the parameters - urea,

Table 1. Chart of the treatment method used.

Group A: Control group given 0 g PQ/kg bw and divided into two subgroups
A: Maintained on non-vit.c medicated water and feed (negative control) $A_{il.c}$: Maintained on vit.c medicated water (1 g/L) and feed (positive control)
Group B: Test group given 2 g PQ/kg bw and divided into two subgroups
B: Subsequently maintained on non-vit.c medicated water and feed $B_{vit.c}$: Subsequently maintained on vit.c medicated water (1 g/L) and feed
Group C: Test group given 4 g PO/kg hw and divided into two subgroups
C: Subsequently maintained on non-vitic medicated water and feed

C_{vitc}: Subsequently maintained on vit.c medicated water (1 g/L) and feed

Group D: Test group given 6 g PQ/kg bw and divided into two subgroups

D: Subsequently maintained on non-vit.c medicated water and feed $D_{vit.c}$: Subsequently maintained on vit.c medicated water (1 g/L) and feed PQ, paraquat; bw, body weight.





creatinine and total protein. The serum samples were estimated using methods for glucose,²³ urea,²⁴ creatinine²⁵ and total protein.²⁵

Histopathology

After collection of blood samples the animals were euthanized and the kidneys removed, washed thoroughly in ice-cold physiological saline (0.9% W/V sodium chloride) and fixed in 10% formaldehyde solution for 24 h.26 The tissues were sliced and dehydrated with different increasing concentrations (50, 70, 95 and 100%) of ethanol for about 24 h. After then, they were cleared with xylene to remove the alcohol and improve their refractive index. It was then embedded in the molten paraffin wax, allowed to solidify inside the wax. The resulting blocks were sectioned with a Shandon AS 325 microtome, and later slides were prepared with the best of the sections. The slides were stained with hematoxylin/eosin solution,26 and the stained slides were carefully studied for any histopathological lesions as a result of the toxicant (PQ) and the antioxidant (vitamin C). Photomicrographs were made using a Leitz Wetzlar (Model Dialux 20) microscope at 100, 200 and 400 magnification depending on the underlying lesions observed or studied. The remaining carcasses were incinerated to avoid environmental pollution.

Statistical analysis

The results or data produced were analyzed

using Excel 2007 Microsoft package and results expressed as (X=mean±standard error mean), and the statistical comparison were done with two-way analysis of variance (2-WAY ANOVA) system, with the level of significance calculated at (P \leq 0.01) depending on the values obtained and how the interaction correlated.

Animal care

We do affirm that in carring out this research The Nigerian Institutional and National Guide for the care of laboratory animals were followed.

Results

Paraquat treatment on rats produced changes in their behavioral pattern which were dose and exposure-time dependent. As the ip dose were increased from 0 to 6 mg/kg bw of rat, symptoms such as respiratory distress, Irritation or redness of the eye, sedation or restiveness became more pronounced, even as the duration of treatment lasted, especially on the PQ only treated subgroups (B, C and D). Also their feeding and fluid intake habit significantly reduced as the dose and duration of treatment increased. This was observed more on the animal subgroups dosed 4 and 6 mg/kg PQ without vitamin C medicated water intake. Intraperitoneally administered sublethal increasing doses of PQ (0, 2, 4 and 6 mg/kg bw) to rats subgroups produced significant changes in biochemical parameters assayed, which were both dose and exposure-time dependent. The subsequent treatments of the differently dosed subgroups ($A_{vit.c}$, $B_{vit.c}$, $C_{vit.c}$ and $D_{vit.c}$) with vitamin C medicated water (1 g/L) within the study period was seen to have restored the biochemical markers assayed to near-control or control levels.

The results obtained in Table 2 and Figure 1 indicated that the urine SG values of the test subgroups were significantly higher when compared to the control subgroups (A and Avitc) at P≤01. The increase in values was found to be dose and exposure-time dependent. The control subgroups had no statistical difference between them, meaning that vitamin C (1 g/L) used in medicating the drinking water for subgroup A_{vite} was not toxic. This confirms that the changes observed on the test subgroups were subject to only PO toxicity. Similarly, significant decreases in SG values were observed in PO treated subgroups maintained with vitamin C medicated water ($B_{vit,c}$, $C_{vit,c}$ and $D_{vit,c}$) than the subgroups on PQ treatment only (B, C and D), especially in samples collected at the end of months 2 and 3, and mainly on rats subgroups dosed 4 mg and 6 mg/kg PQ. Also within each test subgroup, significant increases (P≤0.01)

Month	Rat no.	Subgroup	SG (unit)	pH (unit)	Glucose	Protein
1	4	Δ	1 008+0 01	7 13+0 05	NII.	NII.
1	4	Α.	1.000 ± 0.01 1.010 ± 0.01	6.18 ± 0.00	NIL.	NIL.
	1	R _{vit.c}	1.010 ± 0.01 1.015 ± 0.02^{a}	6.02 ± 0.02^{a}	NIL	NII
	1	B	1.015±0.02	5 85±0 03a,b	NIL	NIL
	1	D _{vit.c}	1.015 ± 0.05 $1.020\pm0.06a$	5 57 + 0.06	NIL	
	4	C	$1.020 \pm 0.00^{\circ}$	5.57 ±0.00	NIL	Τ NII c
	4	D	1.020 ± 0.04 $1.020\pm0.09a$	5.00±0.04°	INIL	
	4	D	$1.020 \pm 0.02^{\circ}$ 1.020 + 0.07a	5 25 + 0 02ab	+ 20	т <u>а</u>
_	4	D _{vit.c}	1.020±0.07ª	5.23 ± 0.03	Ŧ	±"
2	4	A	1.010 ± 0.05	6.52 ± 0.06	NIL	NIL
	4	A _{vit.c}	1.010 ± 0.06	6.54 ± 0.03	NIL	NIL
	4	В	$1.010 \pm 0.02^{a,b}$	$5.15{\pm}0.05^{a,b}$	$\pm^{a,b}$	NIL
	4	B _{vit.c}	$1.020{\pm}0.03^{a,b,a}$	$5.35 \pm 0.05^{a,b,b}$	NIL ^c	NIL
	4	С	$1.030{\pm}0.02^{\rm a,b}$	$5.09 \pm 0.06^{a,b}$	+ ^{a,b}	\pm^{a}
	4	C _{vit.c}	$1.020{\pm}0.03^{a,a}$	$5.03 {\pm} 0.07^{\mathrm{a,b,b}}$	$\pm^{a,b,c}$	$\pm^{a,b}$
	4	D	$1.030 \pm 0.04^{a,b}$	$5.08 \pm 0.02^{a,b}$	++ ^{a,b}	+ ^{a,b}
	4	D _{vit.c}	$1.025 {\pm} 0.03^{a,b,a}$	$4.05{\pm}0.08^{\mathrm{a,b,b}}$	+ ^{a,b,c}	+ ^{a,b}
3	4	А	1.010 ± 0.03	7.01 ± 0.06	NIL	NIL
	4	A _{vit c}	1.015 ± 0.02	6.02 ± 0.05	NIL	NIL
	4	B	$1.020{\pm}0.06^{\mathrm{a,b}}$	$5.52{\pm}0.01^{a,b}$	+ ^{a,b}	$\pm^{a,b}$
	4	B _{uit c}	$1.020{\pm}0.07^{\mathrm{a,b}}$	$5.04 \pm 0.02^{a,b,b}$	$\pm^{a,b,c}$	$\pm^{a,b}$
	4	C	$1.030 \pm 0.04^{a,b}$	$5.05 \pm 0.04^{a,b}$	++ ^{a,b}	$+^{a,b}$
	4	Cuite	$1.025{\pm}0.05^{\rm a,b,a}$	$5.25 \pm 0.08^{a,b,b}$	+ ^{a,b,c}	$\pm^{a,b,c}$
	4	D	$1.035 {\pm} 0.05^{\mathrm{a,b}}$	$5.04 \pm 0.05^{a,b}$	+++ ^{a,b}	$+^{a,b}$
	4	D _{vit.c}	$1.030 {\pm} 0.02^{a,b,a}$	$4.28{\pm}0.03^{\mathrm{a,b,b}}$	$++^{a,b,c}$	+ ^{a,b}

Table 2. Mean urine specific gravity, pH, glucose and protein results obtained during study period.

SG, specific gravity. X=mean±standard error mean; statistical significance: P=0.01. 'Statistically significant difference between the test subgroups and the control subgroups at each treatment month; ^bstatistically significant difference in the exposure time effect from months (1 to 3) of each subgroups (*i.e.* month 1 *vs* month 2 and month 1 *vs* month 3); 'statistically significant difference observed within each subgroup (*i.e.* Group B: B *vs* B_w.) at each month.



were observed in the exposure time comparison between months 2 and 3 against month 1 (*e.g.* B_{vite} : month 1 *vs* month 2 or 3). This confirmed that dose effects were increasing proportionately with exposure time of the study.

The data in Table 2 and Figure 2, indicated that the urine pH of the test animal's subgroups were dose and exposure time significantly lower (P≤0.01) than that of the control subgroups. The test subgroups on vitamin C medicated water ($B_{vit,c}$, $C_{vit,c}$ and $D_{vit,c}$) had values in pH that were significantly much lower than that of the test subgroups on PQ treatment only (B, C and D) at P≤0.01. Even the vitamin C treated control subgroup ($A_{vit,c}$) was having a significantly lower pH values when compared to the control subgroup without vitamin C (A) at P≤0.01. This result has shown that vitamin C also contributed to the acidic pH nature of the test animals urine samples analyzed.

Tables 2-4 illustrated the findings in the semi-quantitative analysis of the presence of glucose and protein in the urine samples of test animals. The significant increases in urinary glucose and protein excretion were more on the test subgroups on PQ treatment only (B, C and D) than on the subgroups that were maintained with vitamin C medicated water during PQ treatments (B_{vit.c}, C_{vit.c} and D_{vit.c}) at $P \le 0.01$, which were also dose and exposure time dependent. It was also evident that the established glycosuria was semi-quantitatively more than the proteinuria. This could suggest that the renal injuries created by PQ intoxication allowed the leakage of more micro molecular substances than macromolecules.

Table 5 and Figures 3-6 below illustrated that PQ exposure caused significant increases in serum levels of urea and creatinine and reductions in serum levels of glucose and total protein concentrations ($P \le 0.01$) in rats treated with (2, 4 and 6 mg/kg PQ) at month 1 (28th day), month 2 (56th day) and month 3 (84th day) compared to those of the respective monthly control subgroups, and the comparison was found to be dependent on the dose given and the exposure-time of treatment. The control subgroups of each parametric treatment (Figures 3-6) showed no observable significant changes when the subgroups on vitamin C medicated water maintenance (A_{vitc}) were compared to the subgroup maintained on ordinary drinking water with negligible vitamin C content (A). But on comparing the test subgroups (PQ only vs PQ + vit. C), a significant decrease in values were observed on the vitamin C medicated subgroups at each dosed subgroup and at each month in serum urea and creatinine, while a significant increase were similarly observed on the same subgroups in serum total protein and glucose levels. When an exposuretime comparison was done on the test subgroups (e.g. subgroup B: month 1 vs months 2 or 3) in all the parameters assayed. There were



Figure 1. The mean urine specific gravity values of the different subgroups during the three months study period.



Figure 2. The mean urine pH values of the different subgroups during the three months study period.

Table 3. Mean urine glucose qualitative detection in treated rats.

Subgroups	Month 1	Month 2	Month 3
A	NIL	NIL	NIL
A _{vit.c}	NIL	NIL	NIL
В	NIL	$\pm^{a,b}$	+ ^{a,b}
B _{vit.c}	NIL	NIL ^c	$\pm^{a,b,c}$
С	NIL	+ ^{a,b}	++ ^{a,b}
C _{vit.c}	NIL	$\pm^{a,b,c}$	+ ^{a,b,c}
D	+ ^a	$++^{a,b}$	+++ ^{a,b}
D _{vit.c}	$\pm^{a,c}$	+ ^{a,b,c}	++ ^{a,b,c}

X=mean \pm standard error mean; statistical significance: P=0.01. *Significant difference between the test subgroups and the control subgroups at each treatment month; ^bstatistically significant difference in the exposure time effect from months (1 to 3) of each subgroups (*i.e.* month 1 us month 2 and month 1 us month 3); "statistically significant difference observed within each subgroup (*i.e.* Group B: B us B_{utc}) at each month.

Table 4. Mean urine protein qualitative detection in treated	rats
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Subgroups	Month 1	Month 2	Month 3
A	NIL	NIL	NIL
A _{vit.c}	NIL	NIL	NIL
В	NIL	NIL	$\pm^{a,b}$
B _{vit.c}	NIL	NIL	$\pm^{a,b}$
С	\pm^{a}	\pm^{a}	$+^{a,b}$
C _{vit.c}	NIL ^c	$\pm^{a,b}$	$\pm^{a,b,c}$
D	\pm^a	+ ^{a,b}	+ ^{a,b}
D _{vit.c}	\pm^{a}	+ ^{a,b}	+ ^{a,b}

X=mean \pm standard error mean; statistical significance: P \leq 0.01. ^aSignificant difference between the test subgroups and the control subgroups at each treatment month; ^bstatistically significant difference in the exposure time effect from months (1 to 3) of each subgroups (*i.e.* month 1 us month 2 and month 1 us month 3); ^cstatistically significant difference observed within each subgroup (*i.e.* Group B: B us B_{int}) at each month.



significant increases for urea and creatinine serum level while significant decreases for total protein and glucose serum levels were observed in the data obtained.

In all the serum parameters assayed, the vitamin C maintained subgroups had values that were near-control or at control levels all through the study period. This shows that vitamin C given caused an improvement on the affected parameters in PQ intoxicated rats.

Histological results

Histological examination of the kidney sections (Figure 7) of the control subgroups A and A_{vitc} revealed normal cortex with normal renal corpuscles, proximal and distal tubules as represented at months (1), (2) and (3) in the slide pictures. These pictures are showing clearly the glomeruli tuft (G) with its surrounding Bowman's capsules and the renal tubules, lined by simple cuboidal or columnar epithelium and the renal vein running all through the tubular cells, emptying at the tuft of the glomerulus as capillaries.

Animals treated with 2 mg/kg PQ only (Figure 8) showed pictures of mild congested renal veins and tubules, progressing to shrunken Bowman's capsule and G as the exposure-time extended to months (2) and (3). The other PQ only dosed subgroups (Figures 9 and 10) showed dose and exposure-







Figure 4. The mean serum creatinine $(\mu mol/L)$ values of the different subgroups during the three months study period.

Table 5. Mean serum urea, creatinine, glucose and total protein results obtained during study period.

Month	Rat no.	Subgroup	Urea (mmol/L)	Creatinine (µmol/L)	Glucose (mmol/L)	Protein (mg/dL)
1	4	A G	3.10±0.02	1.07 ± 0.01	6.18 ± 0.22	7.52 ± 0.08
	4	A _{vit.c}	4.15 ± 0.03	1.45±0.02	0.00±0.20	1.54±0.09
	4	B	4.05 ± 0.05	$21.52 \pm 0.03^{\circ}$	$4.75 \pm 0.55^{\circ}$	$0.05 \pm 0.07^{\circ}$
	4	D _{vit.c}	4.10±0.00	$17.50 \pm 0.04^{a,0}$	$0.10 \pm 0.01^{a,c}$	$0.42 \pm 0.00^{\circ}$
	4	C	4.97 ± 0.05	51.95±0.08ª	4.05±0.50°	$0.20 \pm 0.09^{\circ}$
	4	C _{vit.c}	4.13 ± 0.07	$19.01 \pm 0.03^{a,b}$	$5.08 \pm 0.09^{a,c}$	6.60±0.07°
	4	D	5.33 ± 0.12	98.22±0.19ª	4.02±0.49 ^a	6.54±0.05 ^a
	4	D _{vit.c}	4.18±0.06	$26.17 \pm 0.10^{a,o}$	$4.25 \pm 0.24^{a,c}$	6.25 ± 0.04^{a}
2	4	А	2.40 ± 0.05	2.44 ± 0.06	5.78 ± 0.23	7.24 ± 0.03
	4	A _{vit.c}	2.78 ± 0.09	3.04 ± 0.07	5.93 ± 0.18	7.27 ± 0.10
	4	В	$8.53 {\pm} 0.15^{a,b}$	$58.95 {\pm} 0.08^{\mathrm{a,b}}$	$4.08 \pm 0.68^{a,b}$	$5.08{\pm}0.09^{\mathrm{a,b}}$
	4	B _{vit c}	$7.55 {\pm} 0.06^{\rm a,b,c}$	$36.65 \pm 0.14^{a,b,c}$	$4.60 {\pm} 0.25^{\mathrm{a,b,c}}$	$5.19 {\pm} 0.05^{\rm a,b,c}$
	4	C	$11.60 \pm 0.12^{a,b}$	$154.43 \pm 1.05^{a,b}$	$3.25 \pm 0.27^{a,b}$	$5.00{\pm}0.10^{\rm a,b}$
	4	C _{vit c}	$9.00 \pm 0.13^{a,b,c}$	$25.33 \pm 0.19^{a,b,c}$	$4.10 \pm 0.04^{a,b,c}$	$5.28 \pm 0.20^{a,b,c}$
	4	D	$13.78 \pm 0.12^{a,b}$	$180.75 \pm 2.75^{a,b}$	$2.65 {\pm} 0.09^{\rm a,b}$	$4.69 \pm 0.13^{a,b}$
	4	D _{vit.c}	$7.83{\pm}0.06^{\rm a,b,c}$	$70.89 {\pm} 1.93^{\rm a,b,c}$	$3.58 {\pm} 0.23^{\rm a,b,c}$	$5.13 {\pm} 0.09^{\rm a,b,c}$
3	4	А	5.45 ± 0.04	4.15 ± 0.04	5.85 ± 0.55	7.83 ± 0.13
	4	A _{vit c}	3.65 ± 0.06	7.98 ± 0.05	6.05 ± 0.65	8.44 ± 0.21
	4	B	$13.60 \pm 0.09^{a,b}$	$97.21 \pm 0.19^{a,b}$	$3.25{\pm}0.35^{a,b}$	$4.85 \pm 0.08^{a,b}$
	4	B _{vit}	$10.45 \pm 0.05^{a,b,c}$	$81.13 \pm 0.25^{a,b,c}$	$4.15 \pm 0.35^{a,b,c}$	$5.15 \pm 0.07^{a,b,c}$
	4	C	$17.95 \pm 0.06^{a,b}$	$145.51 \pm 0.46^{a,b}$	$2.55 {\pm} 0.05^{a,b}$	$4.36 {\pm} 0.04^{\mathrm{a,b}}$
	4	C _{vit c}	$13.00 \pm 0.05^{a,b,c}$	$83.41 \pm 1.27^{a,b,c}$	$3.05{\pm}0.05^{\rm a,b,c}$	$4.85 \pm 0.10^{a,b,c}$
	4	D	$30.00 \pm 0.08^{a,b}$	$231.05 \pm 1.26^{a,b}$	$2.45{\pm}0.25^{a,b}$	$4.03 {\pm} 0.05^{\mathrm{a,b}}$
	4	D _{vit.c}	$26.15 {\pm} 0.13^{\rm a,b,c}$	$84.06 \pm 1.95^{a,b,c}$	$3.30{\pm}0.20^{\mathrm{a,b,c}}$	$4.31{\pm}0.09^{\rm a,b,c}$

X=mean±standard error mean; statistical significance: P≤0.01. *Significant difference between the test subgroups and the control subgroups at each treatment month; *statistically significant difference in the exposure time effect from months (1 to 3) of each subgroups (*i.e.* month 1 us month 1 us month 3); *statistically significant difference observed within each subgroup (*i.e.* Group B: B us B_{vic}) at each month.

time dependent structural abnormalities in the cortex and medulla regions. The changes were more marked at month (3) in Figures 9 and 10. The main findings were: i) narrowed appearance of Bowman's space; ii) degeneration of tubular epithelial lining; iii) swollen or edematous lumen and congestion of the renal tissues. These changes increased in a dose and exposure-time dependent manner.

These structural abnormalities were also observed in a dose and exposure-time dependent manner in the test subgroups medicated with vitamin C (B_{vite} , C_{vite} and D_{vite}), but its severity were somewhat mild when compared to the PQ only treated test subgroups (B, C and D) as shown in Figures 11-13. Most of the renal tubules were damaged and lost their characteristic appearance [Figures 8 (month 3), 9 (month 3) and 10 (months 2 and 3), 11 (month 2)]. The epithelial cells linings became undistinguishable and their contents lost. The walls of the Bowman's capsules were eroded and the glomeruli atrophied [Figure 12 (month 2)] and in some sections appeared as empty spaces [Figure 10 (month 3) containing amorphous debris.

Discussion

Humans are exposed to a mixture of environmental pollutants, such as pesticides, herbicides, fungicides, etc., by various routes: air, food and water.27 These compounds have the potential to disrupt metabolic processes, resulting in various metabolic abnormalities, reproductive problems, cancers and other effects.²⁸ PQ is a synthetic bipyridylium herbicide, widely used in weed control, which when accidentally ingested causes damage to cellular component as a result of its highly oxidative properties that produce superoxides. Following the findings from the acute toxicity study,¹⁸ where it was observed that toxicity effects existed with increasing dose and exposure-time of PQ ip insult on the kidney cells. A chronic study was designed to assess the toxicity of PQ at longer period of treatment (3 months), and also to find the possibility of ameliorating the toxic injury using the antioxidant, vitamin C.

In this study, the biochemical and histological changes in the rat's kidney were examined, *in vivo*, after 3 months exposure to sublethal doses of PQ. Dose and exposure-time dependent PQ toxicity resulted in significant reduction in urine pH, elevation of urine SG, proteinuria, glycosuria, reduction in serum glucose and total protein, elevation of serum urea and creatinine concentrations and progression of glomerular and tubular injuries. The results were in agreement with the findings of other studies.^{29,30} Abdel-Mageid³¹ observed that kidney tissues of PQ intoxicated rats, revealed degeneration of renal tubules, shrunken glomeruli and congested dilated blood vessels. Damin, et al.³² emphasized that PQ poisoning caused renal damage. Laurence and Bennett³³ reported that toxicity of PQ caused renal tubular necrosis followed by kidney failure. Hassina²⁷ in his work on biochemical and hietopathological changes in the kidney and adrenal gland of rats following repeated exposure to lambda-cyhalothrin (LCT) observed that the kidneys of treated rats showed narrowed appearance of Bowman's space, degeneration of tubular epithelial lining, widened lumen and tissue congestions. Fetoui et al.34 also observed similar lesions in the kidneys of rats treated with LCT. Other published works also accented to the fact that PQ is highly toxic to the kidney cells and that inflammatory cells were found in the interstitial tissues of the kidneys of PO treated animals.^{18,35-39} The subsequent maintenance of PO intoxicated rats with vitamin C medicated water (1 g/L) ameliorated the toxic insult by rejuvenating the injured cells and restoring the normal metabolic processes in vivo. Asada14 confirmed that vitamin C acted as a substrate for the antioxidant



enzyme ascorbate peroxidase, a function that is particularly important in stress resistance⁴⁰ thereby maintaining and repairing cellular integrity and functions of affected organs.^{12,41,42}

From a practical perspective, it is useful to apply a tiered strategy in analyses of urine to gain different levels of knowledge on kidney function. First, urine osmolality, urine volume and urine pH provides basic insight into overall fluid homeostatic and acid-base state. These parameters are highly dependent upon intakes and metabolic state, and treatmentrelated changes are most easily detected by comparison with untreated controls or appropriate historical controls. While urine osmolarity or SG is the ratio of excreted osmotically active solutes to excreted water, and is susceptible to changes in either water or solute excretion. Urine pH is an indicator of relative total body acid (or base) load. From the results of this study, it is evident that, the pH of the samples of the test subgroups became more acidic as the dose and exposure-time of PO toxicity increased. The acidic nature of the test subgroups were more on the PQ dosed subgroups maintained with vitamin C medicated water than on the subgroups on only PQ toxic-



Figure 5. The mean serum total protein (mg/dL) values of the different subgroups during the three months study period.



Figure 6. The mean serum glucose (mmol/L) values of the different subgroups during the three months study period.



ity throughout the study period. This result was corroborated by the findings of Lee et al.,³⁰ which the pH of urine is a reflection of the kidneys ability to maintain normal hydrogen ion concentration in plasma and extracellular fluid, and that the xenobiotic-induced metabolic activities produces non-volatile acids that cannot be extracted by the lungs which when in excess renders the urine acid in reaction. Schumann and Schweitzer⁴³ also supported the findings by stating that the capacity to exchange hydrogen ions for cation and the formation of ammonia is decreased when tubular function is impaired, resulting in systemic acidosis. Thus, it appears that both PQ toxicity effect and vitamin C medication lead to the increase in urinary pH of the test animals.

It could also be suggested that the increase in urinary SG of the test subgroups could either be as a result of PQ toxicity effect or by the reduction in fluid intake by the test animals with increase in dose and exposure time of treatment. It is expected that the test-animal's urine became more concentrated with reduction in urine volume leading to raised excretable solutes, which in-turn raised the urine SG. These increases were directly proportional to the increase in dose and exposuretime of treatment. Lee et al.30 and Schumann and Schweitzer⁴³ corroborated this findings with their various reports summed up that the volume of excreted urine and its concentration of solute are varied by the kidney to maintain homeostasis of the body fluid and electrolyte. To achieve this, they stated that with reduction in fluid intake, the kidneys produce urine that are more concentrated than in solutes than the plasma from which it was derived, thereby riding itself of unwanted solutes that would have been injurious to the system. Paraguat is known to be filtered at the glomerulus and actively secreted by tubular cells unchanged.44 In addition, it has been reported that the kidney is next to the lungs in having the highest concentration of PQ in animals.⁴⁵ The damage done to the renal tubules as a result of high PQ toxicity effects could result in leakage of the reabsorbable solutes causing the establishment of raised urinary SG.

The semi-quantitative established presence of glucose and protein in the urine specimens of treated animals can also be explained by the findings of the supporting literatures above.^{30,43-45} It was also observed in our study that the established proteinuria and glycosuria were more in the PQ only treated subgroups than in the subgroups that were maintained with vitamin C medicated water. The degrees of glycosuria in the result were more than the proteinuria observed. Sastry *et al.*⁴⁶ and Das and Mukherjee⁴⁷ in support, observed that exposure of fish for a long time to most toxicants, including pesticides, interfered with protein metabolism. Depletion of total protein



Figure 7. Hematoxylin and eosin staining after administration of 0 mg/kg of paraquat representing control subgroups maintained with no vitamin C and vitamin C medicated (1 g/L) water (A and Avitc) respectively for a period of 3 months (original magnification 400X). The slides represent results obtained at months 1, 2 and 3. The results are showing normal architecture. The renal corpuscles, Bowman's capsules, glomeruli (G) and convoluted tubules presented normal features.



Figure 8. Hematoxylin and eosin staining after administration of 2 mg/kg of paraquat representing test subgroups maintained with ordinary drinking water with negligible vitamin C content for a period of 3 months (original magnification 400X). The slide at month (1) is showing a picture of congested renal veins and renal tubules (CR), progressing to shrunken Bowman's capsules and glomerular (G) and renal vascular congestion (R) at month (2). At month (3) the picture indicated advanced renal tubular atrophy (R) with glomerular (G) and tubular degeneration. Changes increased in an exposure time dependent manner.



Figure 9. Hematoxylin and eosin staining after administration of 4 mg/kg of paraquat representing test subgroups maintained with no vitamin C medicated drinking water for a period of 3 months (original magnification 400X). The slide at month (1) is showing a picture of shrunken Bowman's capsules and swollen glomeruli (G), leucocytes infiltration and vascular congestion (DCT) progressing glomerular congestion, intralobular vacuolation and renal vascular congestion (R) at month (2). At month (3) the picture indicated advanced renal tubular hypertrophy (R), swollen Bowman's capsules (BC). Changes increased in an exposure time dependent manner.



in plasma or serum of fish may be due to impaired synthesis of protein or enhanced loss via excretion through the kidney. Jee et al.48 suggested that toxic insult were responsible for the severe proteinuria and glycosuria observed in their study. The progress report of International Programme on Chemical Safety (IPCS)³⁶ confirmed that the resulting proteinuria was as a result of kidney failure occasioned by PO insult in the renal tubular cells. The case of a Korean woman who presented upon admission with generalized proximal tubular dysfunction with aminoaciduria, phosphaturia and glycosuria after accidental PO intoxication also gave support to the findings.⁴⁹ It was also observed in this report that the level of glycosuria and proteinuria were much lower in the vitamin C medicated subgroups than the PQ only treated subgroups.

The dose and exposure-time dependent increases in serum urea and creatinine levels as observed in Table 5 and Figures 3 and 4, dosed 4 and 6 mg/kg PQ were considered as significant markers of renal dysfunction.^{50,51} Also, the PO treated vitamin C medicated subgroups had increased values that were significantly lower than the PQ only treated subgroups. The controls showed no observable significant difference when a comparison was placed between subgroup on vitamin C and the subgroup on no vitamin C or PQ treatments. This is also confirming that the concentration of vitamin C used was non-toxic to the animals treated. Urea levels can be increased by many factors such as dehydration, diet and antidiuretic drugs, while creatinine is more specific to the kidney. Raised creatinine concentration is an index of kidney dysfunction.52 The significant increases in serum urea and creatinine in this study were classical signs of adverse affects of PQ toxicity on the kidney cells. Akinloye et al.,39 asserted that the increased level of blood creatinine with decreased level of blood protein may indicate protein catabolism and/or dysfunction. Cobe53 confirmed that increased blood urea and creatinine values were as a result of PQ poisoning. Hassina²⁷ and Garba et al.52 in their works with a pyrethroid insecticide LCT also confirmed the establishment of increased levels of urea and creatinine in plasma of toxic insulted animals. The positive lowering effect vitamin C medication has on the urea and creatinine concentrations in PQ intoxicated rats is corroborated by the report of Wershana¹² which stated that vitamin C succeeded partially in preventing the impairment in renal function as seen from the significantly reduced urea and creatinine concentrations when compared to the findings in the test subgroups on only PQ insult. Also McCall and Balz⁵⁴ confirmed that antioxidant nutrients as vitamin C, vitamin E, B-carotene, etc. are considered to give protection against oxidative damage induced by different toxicants and reduce the activity of free radicalinduced reactions.

The significant findings of a dose and exposure-time dependent decreases in serum total protein and glucose levels in PQ treated test subgroups when compared to the controls are considered as markers of renal dysfunction. The test animal subgroups maintained with vitamin C medication had better improved serum glucose and total protein levels (P \leq 0.01) than the test subgroups on PQ only treatment. The control subgroups comparison also showed no observable effect between the vitamin C medicated control (Avitc) and the null (A) subgroups. Hypoglycemia has been reported in a wide variety of acquired hepatic and renal diseases, it could also be caused by excessive metabolic loss, by inadequate production or by a combination of both mechanisms.¹² Excessive insulin secretion occasioned by toxic insult could also result in hypoglycemia.⁵⁵ PQ significantly decreased plasma levels of total protein and albumin.³⁹ Post treatments with vitamin C resulted in marked protection against PQ-induced liver, kidney and lungs injuries with observable improvement in



Figure 10. Hematoxylin and eosin staining after administration of 6 mg/kg of paraquat representing test subgroups maintained with no vitamin C medicated drinking water for a period of 3 months (original magnification 400X). The slide at month (1) is showing a picture of shrunken Bowman's capsules (BC) and swollen glomeruli (G) progressing to massive renal vascular congestion (R) and degeneration (DCT) at month (2). At month (3) the picture indicated advanced renal tubular atrophy (VC), interlobular vaculation with glomerular (G) and tubular degeneration. Changes increased in an exposure time dependent manner.



Figure 11. Hematoxylin and eosin staining after administration of 2 mg/kg of paraquat representing test subgroups maintained with vitamin C medicated drinking water (1 g/L) for a period of 3 months (original magnification 400X). The slide at month (1) is showing a picture of shrunken Bowman's capsules (BC) and swollen glomeruli progressing to renal vascular congestion (R) and leucocyte infiltrations (LF) at month (2). At month (3) the picture indicated advanced renal tubular hypertrophy (R), leucocytes infiltrations (LF), interlobular vacuolation with glomerular and tubular degeneration. Changes increased in an exposure time dependent manner.



the levels of plasma protein.^{39,56} Seifi *et al.*⁵⁷ observed in their study that rats treated with Deoxycorticostrone acetate salt for 4 weeks exhibited a marked increase in urinary protein excretion, hypoproteinemia and structural abnormalities in the kidney. When the rats were treated with antioxidants (vitamin C and/or vitamin E), an observable decreases in urinary protein excretion, and improvement in plasma protein levels and improvement in renal architecture were significantly noted. In corroboration, the findings that the co-administration of vitamin E and C to a mouse under

PQ insult completely restored the liver and kidney functions with normalization of the liver enzyme activities, plasma glucose, urea, protein and creatinine levels within 28 days of treatment.^{58,59} Wershana¹² gave a contrary opinion in his findings that administration of vitamin C or selenium to PQ treated animals induced more hypoglycemic effect than that caused by PO insult.

So far, paraquat is widely used for recreational, agricultural and industrial purposes. Thus residues of PQ and its metabolites could remain as pollutants in water, air and food,⁶⁰



Figure 12. Hematoxylin and eosin staining after administration of 4 mg/kg of paraquat representing test subgroups maintained with vitamin C medicated drinking water (1 g/L) for a period of 3 months (original magnification 400X). The slide at month (1) is showing a picture of shrunken Bowman's capsules and congested glomeruli (G) and leucocyte infiltrations (LF), progressing to renal vascular congestion (CR) and leucocyte infiltrations (LF) with shrunken glomeruli at month (2). At month (3) the picture indicated renal tubular (R) desquamated from the underlying basement membranes (arrow), interlobular vacuolation with glomerular (G) and tubular degeneration. Changes increased in an exposure time dependent manner but much better than Figure 10.



Figure 13. Hematoxylin and eosin staining after administration of 6 mg/kg of paraquat representing test subgroups maintained with vitamin C medicated drinking water (1 g/L) for a period of 3 months (original magnification 400X). The slide at month (1) is showing a picture of shrunken Bowman's capsules and glomeruli (G) with leucocytes infiltration (LF), progressing to renal vascular congestion (R) and leucocyte infiltrations (LF) at month (2). At month (3) the picture indicated advanced renal tubular hypertrophy (R), leucocyte infiltrations (LF), interlobular vasculation with glomerular (G) and tubular degeneration. Changes increased in an exposure time dependent manner.

when accidentally inhaled, contacted or ingested at toxic doses could lead to major organ toxicity effect resulting in altered metabolic processes which could finally culminate in death.^{18,61-63} The present study therefore clearly shows that PQ has a harmful influence on the renal tissues and caused alterations in the levels of metabolic markers of kidney functions. Vitamin C has also been shown to offer protection against toxic insults^{64,65} as indicated in the findings of the present study.

Conclusions

Our data has shown that vitamin C administration in PQ intoxicated animals improved the values of the biochemical parameters used to assess toxicity. The decreases in urinary protein and glucose excretion, the restoration of serum protein, glucose, urea and creatinine levels and the improvement in renal and function architecture in vitamin C treated subgroups suggests a major role of antioxidant, vitamin C, in the treatment of toxic insult. It will be imperative that vitamin C medication should be given priority attention in cases of PQ poisoning as a first-line treatment.

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