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Protective Effect of Oral Ascorbic Acid (Vitamin C) on Acetaminophen-Induced Renal Injury in Rats

[a,*]Adeneye A. A. and [b]Olagunju, J. O

Departments of [a]Pharmacology, [b]Medical Biochemistry,
Lagos State University College of Medicine, Ikeja, Lagos State, Nigeria

ABSTRACT: The incidence of acetaminophen-induced nephropathy is reported to be increasing, with no available prophylactic or curative regimen. The present study is an experimental animal study designed to evaluate the protective effects of graded oral doses of ascorbic acid (ASC) in acetaminophen (APAP)-induced nephrotoxic rats for 14 days. A total of thirty, young adult Wistar rats were randomly divided into five groups (I – V) of six rats in each group. Group I rats were administered 10 ml/kg/day of normal saline via the oral and intraperitoneal routes, respectively, while group II rats were pretreated with 10 ml/kg of normal saline one hour before administration of 200 mg/kg/day of intraperitoneal APAP. Groups III – V rats were administered single, daily, oral 100 - 500 mg/kg of ASC 1 hour before 200 mg/kg/day of intraperitoneal APAP for 14 days. On the 15th day, blood samples for serum urea and creatinine and full blood count were collected via cardiac puncture under inhaled diethyl ether. The rat kidneys were also harvested for histopathological examination. Results showed that repeated, single daily intraperitoneal 200 mg/kg of APAP for 14 days, reliably induced a significant ($p<0.05$) increase in the serum urea and creatinine while causing a significant ($p<0.05$) decrease in PCV, TLC and MCHC values along with acute tubular nephritis on histopathology in group II rats. However, these effects were significantly ($p<0.05$) reversed in rats pre-treated with ASC in dose related fashion. The nephroprotection of ASC could be due to its inherent antioxidant effect.

Keywords: Acetaminophen-induced nephrotoxicity; Ascorbic acid; renal function parameters; Rats

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INTRODUCTION

Acetaminophen (marketed as Panadol® in Great Britain, Tyelonol® in the US) is an effective, well-tolerated, widely used over-the-counter analgesic-antipyretic alternative to aspirin (Jalan *et al.*, 2006), with more than 1 billion tablets sold annually in the United States alone (Nourjah and Wiley, 2002). Due to its high tolerance and its availability over-the-counter, overdose on acute or chronic use of the drug, causing serious and debilitating renal damage is common, particularly, at high daily doses greater than 4 g in adults (Ostapowicz *et al.*, 2002). It is estimated that over 56,000 emergency visits and nearly 500 deaths occur in the US annually, resulting from acetaminophen toxicity, owing to either intentional or accidental overdoses (Nourjal and Wiley, 2002). However, these figures continue to rise (Bernal, 2003). In recent time, the safety of acetaminophen, even, at therapeutic doses has generated considerable debate (Jalan *et al.*, 2006). Results of recent study by Watkins and co-workers as well as of other studies have reopened the issue of the actual safety of therapeutic doses of acetaminophen on long term continuous treatment (Watkins *et al.*, 2006; Yin *et al.*, 2001; Kwan *et al.*, 1995). Acetaminophen has been implicated in drug-induced renal injuries (Marsh, 1985). Acetaminophen-induced renal damage is reported to be mediated through an increased lipoperoxidation in renal tissues (Schnellman, 2001; Bessems and Vermeulen, 2001). Thus, the search for

Manuscript received: September 2008; Revision Accepted December 2008

*Address for correspondence: adeneye2001@yahoo.com
Phone: +234-803-583-5589

chemoprophylactic agents for acetaminophen-associated renal complications becomes imperative. Literature has shown effective antioxidants in the body to include vitamins A, C and E, zinc, selenium, and cysteine (Murray and Pizzorno, 1998). Of these, vitamin C is considered the most abundant and essential in plasma, offering most effective protection in humans (Frei *et al.*, 1989). Vitamin C is a water-soluble antioxidant with diverse biological functions. It enzymatic actions include acting as a cofactor for the enzymes involved in collagen hydroxylation, biosynthesis of carnitine and norepinephrine, tyrosine metabolism and peptide hormone amidation (Padayatty and Levine, 2001). It also works along with antioxidant enzymes such as glutathione peroxidase, catalase and superoxide dismutase. It is responsible for regenerating oxidized vitamin E in the body and increases the antioxidant benefits of vitamin E (Freit *et al.*, 1989). Vitamin C's non-enzymatic actions include offering protection against oxidative damage to low density lipoprotein cholesterol (Abbey *et al.*, 1993; Howard and Meyer, 1995), enhancing immunity, endothelial functions, bone and tissue formation (Murray and Pizzorno, 1998), and promoting iron absorption (Carr and Frei, 1999; Padayatty *et al*., 2003). In view of the intrinsic antioxidant activity of vitamin C, the present study was designed to investigate protective effects of 100 – 500 mg/kg of single, daily oral doses of vitamin C in acetaminophen-induced nephrotoxic rats for 14 days.

MATERIALS AND METHODS

Experimental Animals

All experimental procedures were conducted in strict compliance with the United States National Institutes of Health guidelines for Care and Use of Laboratory Animals in Biomedical Research (1985). Young adult Wistar rats, weighting 110 – 140 g were obtained from the Animal House of the College of Medicine Of the University of Lagos, Idi-Araba, Lagos, Nigeria, in the month of June, 2007. They were housed in polyethylene cages in the Animal House of the Lagos State University College of Medicine, Ikeja, Nigeria, and allowed to acclimatize for 14 days before use. The rats were maintained under standard laboratory conditions with free access to standard rat chow (Livestock Feed, Ikeja, Lagos State, Nigeria) and tap water made available *ad libitum.*

Drugs

The drugs used in the experiment include ascorbic acid salt (Sigma Chemical Co. St. Louis, U.S.A.), acetaminophen injections (Juhel Paracetamol®, Lona Pharmaceuticals, New Delhi, India), normal saline (Unique Pharmaceuticals, Sango-Otta, Nigeria). All other reagents used in this study were of analytical grade.

Oral administration of drugs

Before the experiment began, the rats were fasted overnight but tap water was made available *ad libitum.* The rats were randomly divided into 5 groups of 6 rats per group. Groups I and II, which served as the negative and positive controls were intraperitoneally and orally administered 10 ml/kg of body/day of normal saline, respectively, except that the latter was administered 200 mg/kg of daily, single intraperitoneal acetaminophen 1 hour after oral administration of normal saline. Groups III - V rats were orally dosed with single, daily 100 – 500 mg/kg of ascorbic acid 1 hour before single, daily intraperitoneal injection of 200 mg/kg acetaminophen for 14 days.

Weekly body weight measurement

On days 1, 7, and 15 of the experiment, the rat weights were measured using Mettler weighing balance (Mettler Toledo Type BD6000, Mettler-Toledo GmbH, Greifensee, Switzerland) and the difference in weight in reference to the initial weight per group was calculated on each occasion.

Blood Collection and Measurement of serum urea and creatinine

Following termination of the experiment on the day 14, the rats were fasted overnight for 14 hours. Blood samples for serum urea and creatinine were obtained by cardiac puncture with 21G needle mounted on 5 ml syringe (Becton Dickinson S.A., Fraga, Spain) under diethyl ether anaesthesia (Sigma Chemical Co., St. Louis, U.S.A.). The blood samples obtained were collected into plain sample bottles and centrifuged at 3000 rev/min. for 30 minutes to separate sera. Serum urea and creatinine were all assayed using Randox Diagnostic kits (Randox Laboratories Ltd., Crumlin, U.K.).

Measurement of full blood count parameters

Blood samples for full blood count were collected into Ethylene Diamine Tetra-acetic Acid (EDTA)-coated sample bottles for FBC, which included PCV, TLC, DLC, MCV, MCH and MCHC. The collected blood samples were analyzed using Automated Haematology System (Sysmex Haematology-Coagulation Systems®, Model KX-21N, Sysmex Incorporation, Kobe, Japan).

Histopathological studies of rat kidneys
After the animals were sacrificed, postmortem examination was performed and the rat kidneys were identified and carefully dissected out *en bloc* for histopathological examination. After rinsing the dissected kidneys in normal saline, sections were taken from each organ. The tissue was fixed in 10% formo-saline, dehydrated with 100% ethanol solution and embedded in paraffin. It was then processed into 4-5 µm thick sections stained with hematoxylin-eosin and observed under a photomicroscope (Model N - 400ME, CEL-TECH Diagnostics, Hamburg, Germany).

Statistical Analysis: Data were presented as mean ± S.E.M. of six observations. Statistical analysis was done using two-way analysis of variance followed by post-hoc test, Student-Newman-Keuls test on SYSTAT 10.6. Statistical significance was considered at $p<0.05$, $p<0.01$ and $p<0.001$.

RESULTS

Effects of daily oral 100 – 500 mg/kg of ascorbic acid on serum urea and creatinine in acetaminophen induced nephrotoxic rats: Table 1 shows effect of graded oral doses of ascorbic acid on serum urea and creatinine concentrations in acetaminophen-induced nephrotoxic rats for 14 days. Acetaminophen induced significant ($p<0.05$) elevations in the serum concentrations of urea and creatinine in group II. These elevations were significantly ($p<0.05$, $p<0.01$, $p<0.001$) attenuated in groups III – V rats in dose related fashion, returning their values to normal.

Effects of daily oral 100 – 500 mg/kg of ascorbic acid on full blood count parameters in acetaminophen-induced nephrotoxic rats for 14 days: As shown in table 2, daily, high dose intraperitoneal acetaminophen induced significant ($p<0.05$) reduction in the PCV, TLC and MCHC values while having no significant ($p>0.05$) effect in DLC, MCV and MCH values in group II rats. These alterations were significantly ($p<0.05$) attenuated by graded oral doses of ascorbic acid in dose related fashion.

Effects of daily oral 100 – 500 mg/kg of ascorbic acid on the average body weight of acetaminophen induced nephrotoxic rats: Figure 1 depicts effects of single, daily intraperitoneal administration of APAP and consequent graded oral 100 – 500 mg/kg of ASC in acetaminophen-induced nephrotoxic rats for 14 days. As shown in the table, intraperitoneal APAP administration induced significant ($p<0.05$) weight loss in the APAP-treated rats. The weight loss was significantly ($p<0.001$) enhanced by graded oral ASC, in dose related fashion.

Effect of daily, graded oral doses of ascorbic acid on histological changes in acetaminophen-induced nephrotoxic rats for 14 days: Figures 2, 3 and 4 depict histopathological changes in kidneys for normal, acetaminophen-induced nephrotoxic rat and acetaminophen-induced nephrotoxic rat pre-treated with 500 mg/kg of ASC, respectively. As shown in figure 3, daily high dose intraperitoneal acetaminophen induced focal tubulonephritis with lymphocytic infiltration. These changes were ameliorated in rats pre-treated with graded oral doses of ASC, with the most profound amelioration seen in group treated with the highest dose of ASC.

Table 1: Effects of single, daily oral 100 - 500 mg/kg of ascorbic acid on renal function test in acetaminophen-induced nephrotoxic rat for 14 days

Group	*Treatment*	*Serum urea (mg/dl)*	*Serum creatinine(mg/dl)*
I	10 mg/kg/i.p. NS	18.7 ± 1.8	0.8 ± 0.1
II	200 mg/kg/i.p. APAP	49.0 ± 2.3[c]	1.5 ± 0.1[c]
III	100 mg/kg/oral ASC + 200 mg/kg/i.p. APAP	37.7 ± 1.3[d]	0.8 ± 0.1[d]
IV	200 mg/kg/oral ASC + 200 mg/kg/i.p. APAP	35.7 ± 0.9[d]	0.7 ± 0.1[e]
V	500 mg/kg/oral ASC + 200 mg/kg/i.p. APAP	28.0 ± 2.3[e]	0.2 ± 0.1[f]

[c] represents significant increase at p<0.001 when compared to group I values
[d, e, f] represent significant decrease at p<0.05, p<0.01 and p<0.001, respectively, when compared to group II values

Table 2:
Effects of single, daily oral 100 – 500 mg/kg of ascorbic acid on full blood count parameters in acetaminophen-induced nephrotoxic rats for 14 days

Blood Indices		***Treatment Groups***				
		I	II	III	IV	V
PCV (%)		40.9 ± 0.9	33.6 ± 1.8[d]	39.9 ± 0.3[a]	40.9 ± 1.3[a]	41.0 ± 1.5[a]
TLC		19.2 ± 0.4	13.7 ± 2.6[d]	18.7 ± 1.3	20.9 ± 1.9[a]	20.6 ± 0.4[a]
DLC	Lymph	78.8 ± 1.3	77.0 ± 1.9	78.3 ± 1.8	77.7 ± 2.4	76.4 ± 1.1
	Neutro	14.5 ± 1.0	16.3 ± 1.3	15.1 ± 1.3	10.9 ± 0.6	14.6 ± 0.8
	Granul.	06.6 ± 0.4	06.7 ± 0.6	06.7 ± 0.6	08.1 ± 1.1	09.0 ± 0.8
MCV (fL)		61.8 ± 0.4	59.3 ± 1.4	61.2 ± 0.6	63.9 ± 1.4	63.7 ± 0.5
MCH (pg)		19.9 ± 0.4	19.1 ± 0.4	21.1 ± 0.3	21.1 ± 0.8	21.1 ± 0.6
MCHC (g/dL)		31.8 ± 0.4	28.0 ± 1.0[d]	32.6 ± 0.7[a]	31.8 ± 0.7[a]	31.8 ± 1.0[a]

[a] represents significant increase ($p<0.05$) when compared to group II
[d] represents significant decrease ($p<0.05$) when compared to group I

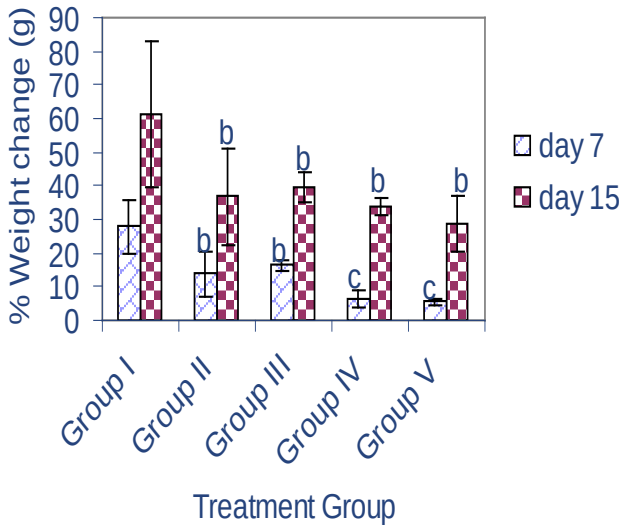


Figure 1:
Effect of graded oral doses of ascorbic acid on percentage weight changes in acetaminophen hepatotoxic rats on days 7 and 15

DISCUSSION

As shown in figure 1, intraperitoneal acetaminophen injection induced significant ($p<0.01$) and progressive weight loss in group II rats compared to group I rats. The weight loss was further enhanced by ASC in dose related fashion, with the most significant ($p<0.001$) recorded for group V. The weight loss recorded for group II could be related to the greater urinary volume recorded for this group which necessitated frequent changing of the beddings, indicating the renal lesion induced by APAP. This observation is in accord with that reported by Tomson and Plant (1997). The diuresis recorded for group II was absent in groups III - V. The weight loss recorded for groups III-V could be related to the weight losing effect of ascorbic acid. This hypothesis requires validation.

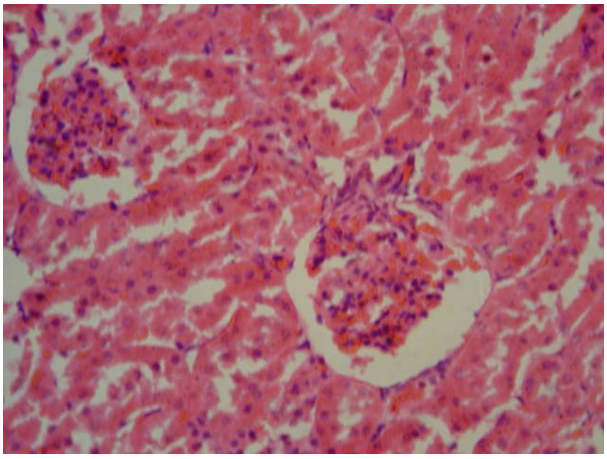


Figure 2:
A representative section of a normal kidney showing normal tubular brush-borders and intact glomeruli (x 640)

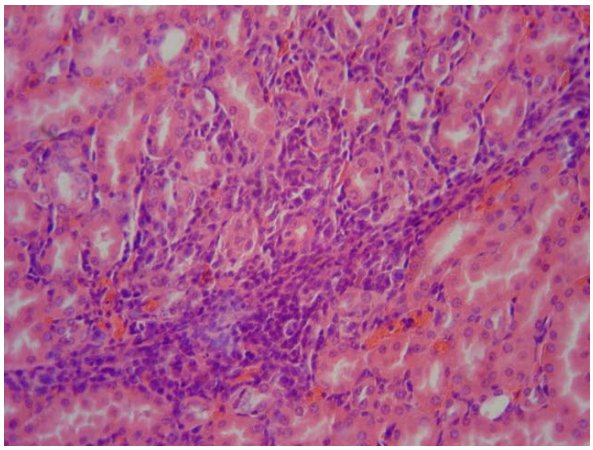


Figure 3:
Representative of 200 mg/kg/day of intraperitoneal acetaminophen-treated rat kidney showing multiple focal tubulo-nephritis with marked lymphocytic infiltration (H&E, X400)

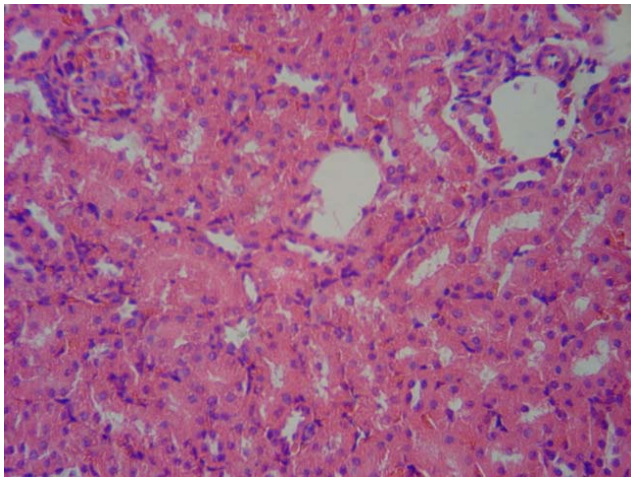


Figure 4
A representative of oral 500 mg/kg/day of ascorbic acid-treated acetaminophen-induced nephrotoxic rat kidney showing mild tubulo-nephritis with mild lymphocytic infiltration (H&E, X400)

DISCUSSION

As shown in figure 1, intraperitoneal acetaminophen injection induced significant ($p<0.01$) and progressive weight loss in group II rats compared to group I rats. The weight loss was further enhanced by ASC in dose related fashion, with the most significant ($p<0.001$) recorded for group V. The weight loss recorded for group II could be related to the greater urinary volume recorded for this group which necessitated frequent changing of the beddings, indicating the renal lesion induced by APAP. This observation is in accord with that reported by Tomson and Plant (1997). The diuresis recorded for group II was absent in groups III - V. The weight loss recorded for groups III-V could be related to the weight losing effect of ascorbic acid. This hypothesis requires validation.

Different models of experimental induction of drug-induced nephrotoxicity have been established (Weijl *et al.*, 1997). In the present study, acetaminophen-induced renal injuries were induced by the repeated intraperitoneal injection of 200 mg/kg of acetaminophen for 14 days. Mechanisms of acetaminophen toxicity have been extensively documented (Mitchell *et al.*, 1974; Bessems and Vermeulen, 2001; James *et al.*, 2003). Excessive formation of a highly reactive intermediate metabolite, N-acetyl-para-benzoquinone-imine (NAPQI), occurs when large doses of the drug are ingested. In the absence of glutathione, NAPQI arylates proteins (selenium-binding protein and glutamine synthetase) in the proximal tubule of the kidneys and initiates cell death (Emeigh Hart *et al.*, 1994). Acetaminophen nephrotoxicity is characterized by marked elevation of serum urea and creatinine as well as proximal tubular necrosis (Schellman, 2001). Results of present study showed that acetaminophen induced significant ($p<0.05$) serum elevation of markers of renal functions in group II rats. These elevations were significantly ($p<0.01$) attenuated by ASC pre-treatment in dose related fashion, with the most significant ($p<0.001$) ameliorating effect recorded at 500 mg/kg/day of ASC. These elevations were significantly ($p<0.01$) attenuated by ASC pre-treatment in dose related fashion, with the most significant ($p<0.001$) ameliorating effect recorded at 500 mg/kg/day of ASC. These elevations in the serum renal function parameters were corroborated by the histological findings which showed multiple focal tubulonephritis characterized by oedematous tubular cells with vacuolar degeneration and marked lymphocytic infiltration (Figure 3) in group II rats. These histological changes were also improved by oral administration of ASC, with the most significant amelioration recorded in group V rats (figure 4). Vitamin C, as an antioxidant agent, may have inhibited the chain reactions of acetaminophen-generated free radicals or scavenged the reactive oxygen species before reaching its renal targets. Both animal (Odigie *et al.*, 2007) and human (Idogun and Ajala, 2005) studies have shown ascorbic acid to be a potent antioxidant which mediates its antioxidant effect by scavenging free reactive oxygen radicals (ROS). Thus, the results of the present study suggests vitamin C ameliorating effects to be likely mediated via

inhibition of free radicals generation and/or free radical scavenging activity.

The hematological effect of ASC is also significant. Repeated, daily, high dose intraperitoneal APAP induced significant ($p<0.05$) reduction in the PCV, TLC and MCHC values in the treated rats but had no significant ($p>0.05$) effect on other hematological parameters. The reduction in these measured hematological parameters were also significantly ($p<0.05$) ameliorated by graded oral doses of ASC in dose related fashion. Literature has shown 80 – 90% of all erythropoietin to be synthesized by the macula densa of the renal tissues and injuries to the kidneys are associated with reduced erythropoietin synthesis and consequent anemia (Guyton, 1991). Again, the haematoprotective effect of ASC could be due to inhibition of lipoperoxidation associated with high dose of APAP. It may also be due to the inherent haematopoietic effect of ASC because ASC has been documented to be essential for blood, blood vessels and bone tissue formation and maintenance (Ofuya *et al.*, 1996; Osilesi *et al.*, 1997). Several experimental and clinical studies have reported immune-enhancing effects (including enhancing white blood cell response and function (Bendich, 1991; Hemila, 1995; Hemila and Herman, 1995). Results of the present study showing significant amelioration of APAP-induced leucopenia and elevation in the TLC in ASC-pretreated rats is in accord with those of previous studies.

In conclusion, the overall results showed ASC to be protective against acetaminophen-induced renal lesions. In the near future, ASC may be found useful as prophylactic agent against drug-induced nephrotoxicity.

Acknowledgements

The authors appreciate the technical supports of Dr. A.S. Benebo, a Consultant Anatomy Pathologist in the Department of Pathology and Forensic Medicine, and Mr. M.O. Arogundade of the Department of Haematology and Blood Transfusion, LASUCOM, Ikeja, Nigeria.

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