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Protective effect of vitamin C supplementation on oxonate-induced hyperuricemia and renal injury in rats

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Recent studies have suggested a potential direct role of mild hyperuricemia in development of chronic kidney disease independent of urate crystal formation. The present study was designed to investigate the possible anti-hyperuricemic and renoprotective effect of vitamin C, as a natural antioxidant with uricosuric property on a rat model with chronic mild hyperuricemia-induced nephropathy. A model of mild hyperuricemia was induced in male Wistar rats with an uricase inhibitor, oxonic acid (OA) (750 mg/kg per day for 4 weeks by gastric gavage). Rats were divided into four groups: (1) control; (2) OA only; (3) OA + vitamin C (200 mg/kg for 4 weeks by gastric gavage); and (4) vitamin C only. At the end of the study, rats were sacrificed under diethyl ether general anesthesia and serum levels of uric acid, creatinine and blood nitrogen urea (BUN) as well as glutathione (GSH) levels and activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S-transferase (GST) were measured as indices of oxidative stress and anti-oxidative status in kidney tissues. Also, histopathological examination of isolated kidney tissues was performed. The administration of OA resulted in 2.4 fold increase in serum uric acid levels, and was associated with development of kidney damage characterized by a significant increase in serum levels of creatinine and BUN, and significant decreases in renal GSH levels and activities of SOD, GPx and GST. By contrast, simultaneous administration of vitamin C significantly ameliorated all these biochemical changes induced by OA. The histopathological findings supported these biochemical observations, whereby vitamin C supplementation remarkably reduced OA-induced tubulointerstitial damage and cellular infiltration in rat kidneys. These results indicate that vitamin C therapy significantly attenuated the biochemical indices, histopathological findings and oxidative stress parameters of OA-induced hyperuricemia and nephrotoxicity in rats. This may provide insight into the possible potential renoprotective effect of vitamin C supplementation against hyperuricemia nephropathy.

Key words: Vitamin C, oxonic acid, hyperuricemia, nephrotoxicity, oxidative stress.

INTRODUCTION

Vitamin C (Ascorbic acid) is the most important vitamin in fruits and vegetables, and has been regarded as the most potent natural antioxidant. Although most of the higher animals can synthesize vitamin C in their liver or kidneys, in humans; the terminal enzyme in its synthetic pathway is absent and thus, vitamin C has become an essential dietary component for human survival (Vissers et al., 2011). Vitamin C is required for the prevention of scurvy, and in addition to its powerful antioxidant activity, it plays an important role as a cofactor in enzymes activation and immune function (Schlueter and Johnston, 2011). Vitamin C has anti-inflammatory effects, prevents endothelial dysfunction and apoptosis, and reduces the risk of arteriosclerosis, cardiovascular disease and some

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forms of cancer (Ozkanlar and Akcay, 2012). In concordance with the aforementioned research findings, beneficial effects of vitamin C in kidneys have been reported by several other researchers. For example vitamin C treatments improved kidney function in renal allograft recipients (Williams et al., 2001), decreased renal inflammation and improved impaired renal function in salt-sensitive hypertensive rats (Tian et al., 2007), and inhibited oxidative damage and renal injury in experimental modalities of chemical-induced nephrotoxicity (Atasayar et al., 2009).

Hyperuricemia is a common metabolic disorder and a well-established causative factor for development of gouty arthritis, tophi formation, uric acid kidney stones and acute kidney failure (Edwards, 2008). However, recent epidemiologic studies have suggested the potential direct role of chronic mild hyperuricemia in development of interstitial nephritis and progressive renal failure (Mok et al., 2012). Hyperuricemia as an independent risk factor, has also been associated with metabolic syndrome, cardiovascular disease, hypertension, obesity, obstructive sleep apnea, stroke, vascular dementia, and preeclampsia (Feig et al., 2008). More importantly, it should be emphasized that the currently used anti-hyperuricemic medications, such as allopurinol or probenecid, carry significant side effect profiles and are not indicated in patients with kidney or heart disease. The use of these agents is supported only in the treatment of gout, the treatment of uric acid kidney stones, and the prevention of tumor lysis syndrome (Juraschek et al., 2011). Recommendations to use dietary approaches to lower uric acid have been suggested (Gelber, 2008). In this aspect, supplementation with vitamin C has recently attracted a great deal of attention as an alternative dietary anti-hyperuricemic approach (Gao et al., 2008; Lagowska-Lenard et al., 2010). Despite these promising trials, future studies are needed to precisely determine whether vitamin C treatment can effectively prevent hyperuricemia and its associated co-morbidities (Juraschek et al., 2011). An animal model of chronic mild hyperuricemia induced by an uricase inhibitor, oxonic acid (OA), has recently shed new light on a potential mechanism of elevated uric acid in inducing renal tissue injury and microvascular changes (Sanchez-Lozada et al., 2008). Therefore, this study was aimed to investigate whether vitamin C supplementation could attenuate the development and outcome of chronic hyperuricemia nephropathy by using this experimental model of OA-induced mild hyperuricemia and renal injury in rats.

MATERIALS AND METHODS

Animals and experimental approach

A total of 50 adult male Wistar albino rats weighing 200 to 250 g obtained from Animal Center Laboratory, Umm Al-Qura University, Saudi Arabia, were housed in metabolic cages with a 12:12-h light:dark cycle at a constant temperature of 23 to 25°C, and had free access to standard commercial rat food (pellet form) and tap water. To induce the experimental rat model with mild hyperuricemia, an uricase inhibitor, OA was given orally by gastric gavage at a dose of 750 mg/kg/day for 4 consecutive weeks as described previously (Sanchez-Lozada et al., 2008). The animals were randomized into four groups as follow: Group 1 (n = 10) served as normal controls; Group 2 (n = 15) received only OA and served as disease untreated group; Group 3 (n = 15) received OA (as in group 2) and simultaneously treated with vitamin C (200 mg/kg/day by gastric gavage for 4 consecutive weeks), and Group 4 (n = 10) received only vitamin C (as in group 3). The selected dose of vitamin C was based on our preliminary study and on those tested previously. All experiments were performed in accordance with the Standards Regulations and Guidelines Relating to the Care and Management of Animal Experimentation, and all standard reagents were purchased from Sigma (St. Louis, Mo., USA), unless indicated otherwise.

Sacrificing and sampling

At the end of experimental period of 4 weeks, the animals were fasted overnight but still allowed free access to water, weighed, and then sacrificed under ether anesthesia. Blood samples were drawn from the inferior vena cava and allowed to clot and retract at room temperature for 1-h before centrifugation at 3,000 ×g for 10 min. Supernatants (that is, serum samples) were separated and stored at -20°C until used. Both kidneys of each rat were immediately excised, weighed, and rinsed with a phosphate buffered saline (PBS) solution, pH 7.4, to remove any blood cells and clots. The right kidney was used for histological examination, while the left one was cut into small pieces, homogenized in 5 volume of ice-cold Tris HCl buffer (50 mM, pH 7.4), and centrifuged at 10,000 ×g for 10 min. The volume of the resultant supernatant was measured and stored at –20°C until used.

Uric acid measurement and biochemical assays

Serum levels of uric acid (UA), creatinine (Cr), blood urea nitrogen (BUN), total cholesterol (TC) and triglycerides (TG) were measured using commercial assay kits (BioAssay Systems, Hayward, CA) and following the manufacturers' instructions.

Histological examination

Isolated kidney specimens were fixed in 10% neutral formalin, paraffinized and were processed for histological examination. Paraffin sections, at 4 µm thickness, were prepared from each kidney, stained with hematoxylin and eosin (H&E) and then, microscopically examined for the existence of renal tubular and glomerular damage, tubulointerstitial nephritis as well as cellular infiltration. All histological analyses were performed blinded by a pathologist unaware of the type of treatment so as to avoid biased results, and the scoring was done as none (-), mild (+), moderate (++) and severe (+++).

Assessment of renal antioxidant defense elements

Levels of glutathione (GSH) and enzymatic activity of superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione S-transferase (GST); as strong indices of tissue antioxidant mechanisms, were spectrophotometrically assayed in the prepared supernatants of kidney tissue homogenates using commercial enzyme-linked immunosorbent assay (ELISA) kits (Sigma-Aldrich, St. Louis, Mo.,

USA), and according to the manufacturers' instructions.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Science (SPSS) version 16 (SPSS Inc., Chicago, IL, USA.). All data are presented as means ± SD. Differences in the various parameters in more than two groups were evaluated by a one-way analysis of variance (ANOVA). Continuous variables between two groups were analyzed using Student's *t*-test. Differences between groups were considered significant at $p < 0.05$.

RESULTS

Vitamin C improved OA-induced hyperuricemia and renal injury

This study was designed on the hypothesis that persistent mild hyperuricemia at concentrations that do not lead to intrarenal urate crystal deposition can induce chronic renal injury and dysfunction, and vitamin C supplementation could produce anti-hyperuricemic effect and improve hyperuricemia associated nephropathy. To test this hypothesis, an experimental model of mild hyperuricemia in rats with OA were induced, which is a urate oxidase inhibitor, and the animals were simultaneously treated or not treated with vitamin C. As shown in Table 1, after four weeks of OA administration, there was a significant increase in serum uric acid level compared to normal control rats (3.1 ± 0.5 versus 1.3 ± 0.1 mg/dl, $p < 0.01$); while co-administration of vitamin C significantly ($p < 0.05$) reduced this elevation (Table 1). In comparison to normal controls, OA also significantly increased kidney weight as well as serum levels of creatinine and BUN, as biomarkers of renal dysfunction, and significantly decreased serum albumin concentration. However, simultaneous treatment with vitamin C significantly ameliorated these alterations on kidney weight and serum levels of creatinine, BUN and albumin that were induced by OA (Table 1). At the end of the study, there were no significant differences (P = NS) in the values of body weights and serum concentrations of sodium and potassium ions among all experimental animal groups, suggesting that OA and/or vitamin C administration did not affect food consumption during the experimental period (Table 1).

Histopathological observations strongly supported the aforementioned biochemical findings, whereby OA administration (Figure 1B) caused prominent damage in the kidney interstitial tissues compared with the control rats (Figure 1A). The kidneys of rats administered with OA alone showed marked deleterious tubulointerstitial inflammation, sloughing of the tubular epithelial cells and predominant interstitial cellular infiltration (Figure 1B). In contrast, simultaneous treatment of these OA-administered rats with vitamin C almost preserved normal histoarchitecture of the kidney (Figure 1C). The renal histopathological changes among all animal groups were graded and summarized in Table 1.

Vitamin C restored OA-induced depletion of renal antioxidant defence elements

The research hypothesis suggested that hyperuricemia could induce renal injury due to induction of oxidative stress. To confirm this hypothesis, the levels of total GSH and the activities of antioxidant enzymes, SOD, GPx and GST were measured in the renal tissue homogenates of all experimental groups at the end of the study. As shown in Table 2, OA administration significantly decreased ($p < 0.05$) GSH levels as well as activities of SOD, GST and GPx, and this is suggestive of induction of oxidative stress phenomenon in OA administered rats; however, simultaneous therapy with vitamin C significantly ($p < 0.05$) elevated the decreased GSH, and also significantly increased ($p < 0.05$) the reduced antioxidant enzyme activities towards almost their normal values (Table 2). The kidneys of normal rats treated with vitamin C alone also showed significant increases in the levels of GSH and in the activities of SOD, GPx and GST antioxidant enzymes in comparison of normal control groups, indicating the natural antioxidant property of vitamin C (Table 2).

DISCUSSION

The present study demonstrates the attenuating effects of vitamin C supplementation on experimental rat model induced with chronic mild hyperuricemia resulting to remarkable intrarenal oxidative damage and renal injury. These data support the notion that an elevation in circulating uric acid, rather than being a surrogate biomarker of kidney dysfunction, is actually an active player in the pathogenesis of renal disease (Inaba et al., 2013). It also support the hypothesis that vitamin C might be an effective dietary approach in the prevention and management of hyperuricemia and its related diseases (Choi et al., 2009; Juraschek et al., 2011), particularly in patients with nephropathy.

In the present study, the hypothesis that mild elevation of serum uric acid may have a direct causal role in the development of renal disease was tested by development of a rat model of mild hyperuricemia induced by OA, which is an uricase inhibitor and, thus, prevents the breakdown of endogenous uric acid (Mazzali et al., 2001). After four weeks of OA administration, there was a significant (2.4 fold) increase in serum uric acid level, as well as in kidney weights and serum levels of creatinine and BUN (as indicators of impaired kidney function) (Table 1). The histopathological observations were consistent with these biochemical changes, whereby a marked tubulointerstitial inflammation and a striped pattern of interstitial cellular infiltration were observed in

Table 1. Effects of oxonic acid (OA) and vitamin C on body and kidney weights; serum levels of uric acid, creatinine, blood urea nitrogen (BUN), albumin, sodium and potassium ions; and scores of renal injury and cellular infiltration.

Parameter	Group			
	Control (n = 10)	OA (n = 15)	OA + Vitamin C (n = 15)	Vitamin C (n = 10)
Body weights (g)	274 ± 5.9	272 ± 7.3^{c}	273 ± 4.5^{c}	276 ± 6.5^{c}
Kidney weights (g)	0.98 ± 0.13	1.32 ± 0.27^{a}	1.05 ± 0.11^{d,c}	0.98 ± 0.19^{c}
Uric acid (mg/dl)	1.3 ± 0.1	3.1 ± 0.5^{b}	1.6 ± 0.2^{d,c}	1.1 ± 0.1^{c}
Creatinine (mg/dl)	0.63 ± 0.03	1.31 ± 0.09^{a}	0.89 ± 0.08^{d,c}	0.64 ± 0.04^{c}
BUN (mg/dl)	32.5 ± 1.6	79.4 ± 5.5^{a}	39.0 ± 1.7^{d,c}	31.7 ± 1.5^{c}
Albumin (g/dl)	1.2 ± 0.2	0.67 ± 0.06^{a}	0.90 ± 0.06^{c,e}	1.2 ± 0.4^{c}
Na^{+} (mmol/L)	142 ± 9.7	139 ± 13.5^{c}	143 ± 5.6^{c,e}	141 ±8.3^{c}
K^{+} (mmol/L)	5.80 ± 0.30	5.56 ± 0.5^{c}	5.67 ± 0.33^{c,e}	5.9 ± 0.5^{c}
Tubulointerstitial nephritis	–	+++	+	-
Renal cellular infiltration	–	+++	+	-

Values are expressed as the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$ and ^{c}P = NS versus normal control group; $^{d}P < 0.05$ and ^{e}P = NS versus oxonic acid (OA) alone received group. Severity of renal histological changes and levels of intrarenal cellular infiltration were scored on a scale of none (–), mild (+), moderate (++) and severe (+++) degree as described in part of the methodology.

Table 2. Changes in the antioxidant defense elements in kidney homogenates of experimental animal groups.

Parameter	Group			
	Control (n = 10)	OA (n = 15)	OA + Vitamin C (n = 15)	Vitamin C (n = 10)
Glutathione content (µmol/ml)	5.37 ± 1.0	2.3 ± 0.3^{a}	5.40 ± 0.9^{c}	7.3 ± 1.1^{a}
Superoxide Dismutase activity (Units/ml)	0.20 ± 0.01	0.09 ± 0.01^{b}	0.19 ± 0.03^{d,c}	0.24 ± 0.02^{a}
Glutathione peroxidase activity (Units/ml)	33.3 ± 5.27	17.34 ± 2.5^{a}	31.4 ± 6.2^{d,c}	40.0 ± 5.1^{a}
Glutathione S-transferase activity (µmol/ml/min)	2.63 ± 0. 3	0.85 ± 0.09^{b}	2.29 ± 0.08^{d,c}	3.14 ± 0.84^{a}

Values are expressed as the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$ and ^{c}P = NS versus normal control group; $^{d}P < 0.05$ and ^{e}P = NS versus oxonic acid (OA) alone received group.

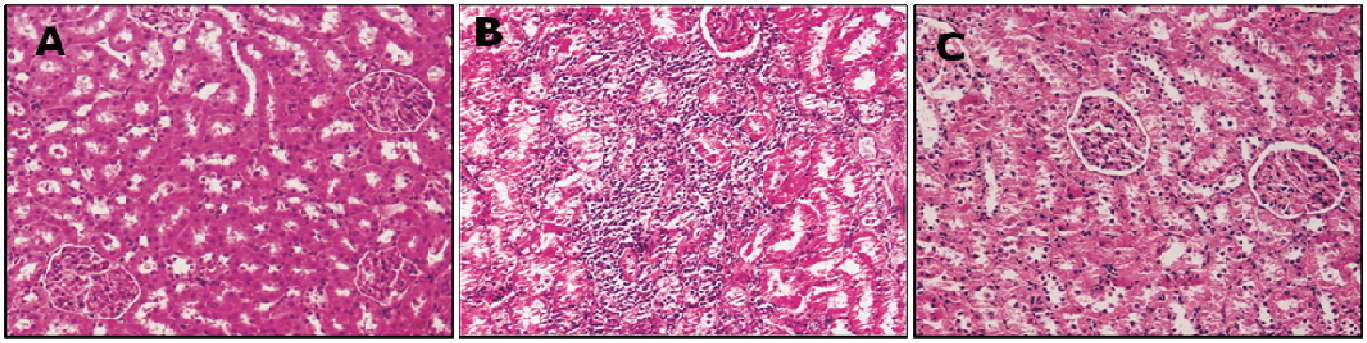

Figure 1. Histopathological findings. (A) Kidneys of normal rats showing normal glomerular and tubular structure. **(B)** Kidneys of rats that received oxonic acid (OA) alone; demonstrating clear hallmarks of tubulointerstitial nephritis with excessive number of interstitial cellular infiltration. **(C)** Kidneys of rats that received a combination of OA plus vitamin C; revealing significant improvements in all renal histomorphological structures.

the kidneys of hyperuricemic rats (Figure 1). These biochemical and histopathological findings have also been previously reported by Sanchez-Lozada et al. (2008), and support the direct involvement of hyperuricemia

in induction of renal disease. In agreement, Nakagawa et al. (2003) showed that hyperuricemia has direct proinflammatory effects and causes glomerular hyper-trophy in rat kidneys independent of intrarenal crystal formation. Moreover, soluble uric acid has been found to stimulate monocyte chemotaxis and the release of proinflammatory mediators from vascular cells (Kanellis et al., 2003). Virtually, chemotaxis and recruitment of inflammatory cells into kidney tissues play pivotal roles in development of renal injury and progressive renal diseases through the spillover of proinflammatory mediators, modulate extracellular matrix synthesis, and promote oxidative stress (Sean and Cockwell, 2005).

In this study, co-administration of vitamin C with OA interestingly resulted in significant anti-hyperuricemic and renoprotective effects that were reflected by reducing the elevations in serum levels of uric acid, creatinine and BUN, and almost preserved the normal histoarchitecture of the kidney (Table 1 and Figure 1). Previously, it has been reported that vitamin C can lower serum uric acid via a direct uricosuric effect, which is likely due to a competition for renal reabsorption of uric acid via an anion-exchange transport system at the proximal tubule (Choi et al., 2009), or by increasing glomerular filtration rate, and thus providing another potential mechanism for the uricosuric effect of vitamin C intake (Huang et al., 2005). Moreover, effective antioxidant vitamin C decreases free radical-induced damage to body cells, thereby reducing production and ultimately serum concentration of uric acid (Gao et al., 2008). Human observational studies have confirmed the uricosuric effect of vitamin C and an inverse association between vitamin C intake and serum uric acid concentrations has been reported (Gao et al., 2008). Vitamin C supplementation has been shown to lower serum uric acid in haemodialysis patients and during exhaustive exercise (Tauler et al., 2003). Moreover, a recent randomized trial of daily intravenous infusion of 500 mg of vitamin C for 10 days in patients with acute ischemic stroke resulted in a significant reduction in serum uric acid compared to placebo infusion (Lagowska-Lenard et al., 2010). Moreover, the specific renoprotective effects of vitamin C have also been reported in other modalities of kidney diseases at both the clinical and experimental levels (Tian et al., 2007; Atasayar et al., 2009).

It remains speculative whether the antioxidant action of vitamin C may have a protective effect against hyperuricemia-induced nephropathy. There is a strong body of evidence that increased oxidative damage due to an excess of free radicals, or generation of reactive oxygen species (ROS), is one of the most important pathogenic mechanisms in the development of many diseases and pathophysiological problems, including renal disease (Wilcox and Gutterman, 2005; Sanchez- Lozada et al., 2008). Generation of ROS can lead to various forms of cellular injury, such as inflammation, cell death, necrosis, and DNA damage or fragmentation (Passos and Von Zglinicki, 2006). The kidney tissues are rich sources of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase-derived ROS, which under pathological conditions can contribute to renal dysfunction and damage (Gill and Wilcox, 2006). On the other hand, ROS can be detoxified by a battery of antioxidant cellular defences, including scavenging action of glutathione (GSH) as well as antioxidant activities of SOD, GST, GPx and other antioxidant enzymatic systems. Modulations of these enzymes and concentrations of GSH appear to be sensitive indicators of the overall health of a cell and its ability to resist toxic challenges (Matés, 2000). The findings of the present study are in harmony with these collective facts, whereby OA-induced hyperuricemia and renal injury was significantly associated with a significant decrease in GSH levels and activities of SOD, GST and GPx (Table 2), suggesting that OA-induced hyperuricemia in rats was associated with oxidative damage of their renal tissues. By contrast, simultaneous therapy with vitamin C significantly restored the renal content of GSH as well as activities of SOD, GST and GPx towards almost their normal values (Table 2). Similar observations that OA induced hyperuricemia and renal injury triggered intrarenal oxidative stress have also been reported by Sanchez-Lozada et al. (2008). Moreover, in an *in vitro* study, uric acid was able to stimulate oxidant generation and reduction in antioxidant levels in cultured cells (Sautin et al., 2007).

Conclusion

These findings show that vitamin C supplementation exhibits anti-hyperuricemic, anti-oxidation and nephroprotective activity in rat model of hyperuricemia-induced oxidative damage and renal injury. This study may provide an evidence to support clinical therapeutic value of vitamin C in treatment of hyperuricemia with renal dysfunction.

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