Effect of curcumin in the prevention of experimentally induced nephrolithiasis in rats by ethylene glycol and Vitamin D3

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SUMMARY

Curcumin (CMN) is known to have beneficial role in anorexia, coryza, cough, diabetic wounds, and hepatic disorders apart from its inherent antioxidant effects. Therefore, the present study was aimed to evaluate antioxidant effect of CMN in prevention of nephrolithiasis in rats-induced by ethylene glycol (EG) and Vitamin D3 (Vit. D3). Male Wistar rats (175 - 200 g) were randomized in groups like control, EG + Vit. D3 induced nephrolithiatic rats, CMN treated rats, CMN + EG + Vit. D3 treated rats, Vit. E + EG + Vit. D3 treated rats. Urine was collected weekly throughout the experimental protocol and estimated for calcium oxalate (CaO) count. After completion of experimental protocol serum was estimated for blood urea nitrogen and creatinine. Both the kidneys were excised and used to evaluate levels of biomarkers of oxidative stress and calcium oxalate crystal deposition by histopathological studies. Administration of EG and Vit. D3 to rats resulted in increased oxidative stress, hyperoxaluria and renal deposition of CaO crystals. Supplementation with CMN improves kidney function, reduces elevated oxidative stress, urinary oxalate level and renal deposition of CaO which shows its protective action in nephrolithiasis. The increased deposition of stone in the kidney and stone forming constituents of nephrolithiatic rats were effectively lowered by treatment of CMN.

Key words: Antioxidant; Calcium oxalate; Ethylene glycol; Hyperoxaluria; Nephrolithiasis

INTRODUCTION

The incidence of urolithiasis in adults increases with age affecting up to 12% of men and 5% of women by the age of 70 years (Hoppe and Hesse, 1999). It is worthwhile to point out that more than 100 - 400 out of 100,000 people per year worldwide suffer from kidney stones owing to the consumption

of diet rich in proteins and nucleic acids (Shukkur *et al.*, 2005). The vast majority of subjects are idiopathic calcium oxalate (CaO) stone formers (Fredric *et al.*, 2005).

Acute and chronic production of CaO and crystal deposition induces lipid peroxidation. The generation of lipid peroxidation due to reactive oxygen species (ROS) cause renal epithelial cells injury, produce favorable condition for deposition of crystals (Huang *et al.*, 2002). Therefore ROS may play an important role in CaO stone formation.

Nephrolithiasis in rats may be induced by ethylene glycol (EG) alone, or in combination with

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other crystal-inducing drugs such as ammonium chloride, Vit. D3 (Halabea *et al.*, 2003; Giuseppe *et al.*, 2004), gentamicin or a magnesium deficient diet (Jie *et al.*, 1999). EG is a metabolic precursor of oxalate. (Bruijn *et al.*, 1994; Jie *et al.*, 1999; Yamaguchi *et al.*, 2005) Oxalate is produced primarily from glycolate or glyoxylate in the liver and it has been demonstrated that glycolic acid oxidase, xanthine oxidase, and lactate dehydrogenase can catalyze the oxidation of glyoxylate to oxalate in mammalian systems (Shahid, 1982; Thamilselvan *et al.*, 1997). Vitamin D3, through its active metabolite, 1,25 dihydroxy vitamin D3 (Calcitriol), plays a major role in calcium and phosphorus homeostasis. Calcitriol increases oxalate absorption as well as its urinary excretion by promoting calcium absorption (Xiao *et al.*, 1993; Fredric *et al.*, 2005).

So in the present study, nephrolithiasis was induced by administration of EG and Vit. D3 as previously reported for the evaluation of potential therapies for kidney stones (Naveen *et al.*, 2005).

Biological compounds with an antioxidant properties and renal membrane-regenerating potential may be a boon in alleviating nephrolithiasis. The significance of *Curcuma longa* Linn (Turmeric) in health and nutrition has changed considerably due to the discovery of the antioxidant properties of naturally occurring phenolic compounds (Balasubramanyam *et al.*, 2003). The present study was undertaken to determine, whether free radical scavenger, CMN a major active phytoconstituent of turmeric can provide protection against the oxalate associated free radical injury and nephrolithiasis.

MATERIALS AND METHODS

Drugs and chemicals

Curcumin (crystalline, purity $\geq 98.5\%$) was obtained from Himedia laboratories, Mumbai - 400086; other chemicals used in experiment were of laboratory grade.

Animals

Male Wistar rats, weighing between 175 - 200 g were utilized for experimental purpose. The animals were maintained under standard conditions of humidity, temperature ($25 \pm 2^\circ\text{C}$) and light (12 h light/12 h dark). They were fed with standard rat pelleted diet (M/s Pranav Agro Industries Ltd., India) and were allowed free access to water ad libitum. Experimental animals were handled with human care according to the University and Institutional legislation, regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Animals were divided into 5 groups containing six animals in each group. Group1 (GP1) served as control and received regular rat food and drinking water ad libitum and 0.05 w/v % sodium carboxy methyl cellulose (p.o). Group 2 (GP2) served as nephrolithiasis induced group received EG (0.75 v/v %, daily, p.o) Vit. D3 (5 $\mu\text{g/kg}$, alternate day, p.o). Group 3 (GP3) served as treated group, received EG (0.75 v/v %, daily, p.o) Vit. D3 (5 $\mu\text{g/kg}$, alternate day, p.o) and CMN (100 mg/kg/day, p.o) (Shukla *et al.*, 2008), Group 4 (GP4) served as standard drug treated group, received EG (0.75 v/v %, daily, p.o) Vit. D3 (5 mg/kg, alternate day, p.o) and Vit.E (100 mg/kg/day, p.o) (Sharma *et al.*, 2000). Treatment is continued for 28 days in each group respectively.

Index of renal functions (Serum creatinine and BUN)

Assessment of renal function: After treatment schedule, blood was collected from all the animals. Serum samples were assayed for blood urea nitrogen (BUN) and serum creatinine by using standard diagnostic kits (Span Diagnostics, Gujarat, India) according to Jaffe's (Harry and Abraham, 1968) and DAM (Ayantika and Parames, 2007) method respectively.

Urine collection and analysis

Urine samples were collected using a metabolic

cage from each rat on day 1, 7, 14, 21 and 28th days of the experiment to determine the presence of CaO crystals. A 24 h urine sample was collected in 50 ml graduated centrifuge tubes containing 1% glacial acetic acid attached to urine collection funnel for crystalluria analysis (Metabolic cage, Dolphin instruments, Mumbai). For microscopy, 1 ml of the urine sample was centrifuged at 3,000 rpm for 10 min, and then 950 μ l of the supernatant was discarded, and 10 μ l of the vortex-mixed sediment was then transferred to a hemocytometer. The type and number of the crystals were identified and counted per high power field (HPF) using an Olympus light microscope (Tawashi *et al.*, 1982).

Histopathology

At the end of study, rats were sacrificed with spinal dislocation method and both kidneys were removed and placed in crushed ice immediately. The left kidney was used for measure various biomarkers of oxidative stress level, like Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GSH), and Melondi aldehyde (MDA). The right kidney was fixed with 10% buffered formalin, embedded in paraffin, cut to 5 mm sections and stained with hematoxylin and eosin for histopathological studies. The slides were examined with a BIOXL light microscope. The sections were observed under 100 $\times$ magnification.

Markers of oxidative stress level

GSH was estimated by the method of Moran *et al.* (1979). MDA was estimated by the method of Slater and sawyer (1971). SOD was estimated by the method of Misra and Fridovich (1972). CAT was estimated by the method of Hugo (1983) (Chintan *et al.*, 2008)

RESULTS

In the present study, chronic administration of EG 0.75 v/v % daily and Vit. D3, 5 μ g/kg alternate day to male wistar rats resulted in development of

Fig. 1. Index of renal functions. (a) Effect of CMN on serum creatinine levels in hyperoxaluric rats. Unit: mg/dl. Values are expressed as mean $\pm$ S.D. for 6 animals in the group. Comparisons are made between: GP 1 and 2, 3, 4. The symbols represent statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; (b) Effect of CMN on serum BUN levels in hyperoxaluric rats. Unit: mg/dL. Values are expressed as mean $\pm$ S.D. for 6 animals in the group. Comparisons are made between: GP 1 and 2, 3, 4. The symbols represent statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

hyperoxaluria, nephrolithiasis and oxidative insult. (GP 2 in Figs. 1 - 4).

Index of renal functions

The serum creatinine was significantly increased by 45.13% (from 0.616 ± 0.038 to 0.895 ± 0.022) in

Fig. 2. Urinary oxalate excretion. (a) Counting of CaO crystals in urine samples of Group1 (control) rats on the day 7, 14, 21 and 28. Values are expressed as mean $\pm$ S.D. for 6 animals in the group. Comparisons are made between: CaO count in urine on the day 7 and 14, 21, 28. The symbols represent statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; (b) Counting of CaO crystals in urine samples of Group 2 (EG + Vit. D3) rats on the day 7, 14, 21 and 28. Values are expressed as mean $\pm$ S.D. for 6 animals in the group. Comparisons are made between: CaO count in urine on the day 7 and 14, 21, 28. The symbols represent statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; (c) Counting of CaO crystals in urine samples of Group 3 (EG + Vit. D3 + CMN) rats on the day 7, 14, 21 and 28. Values are expressed as mean $\pm$ S.D. for 6 animals in the group. Comparisons are made between: CaO count in urine on the day 7 and 14, 21, 28. The symbols represent statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; (d) Counting of CaO crystals in urine sample of Group 4 (EG + Vit. D3 + Vit. E) rats on the day 7, 14, 21 and 28. Values are expressed as mean $\pm$ S.D. for 6 animals in the group. Comparisons are made between: CaO count in urine on the day 7 and 14, 21, 28. The symbols represent statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

nephrolithiatic animals (Fig. 1a, GP 2) as compared with the control group, indicating marked renal damage. However, serum BUN was not significantly elevated in nephrolithiatic rats (Fig. 1b, GP2). There was a significant reduction in serum creatinine (0.700 ± 0.028 , $P < 0.001$) and BUN (24.33 ± 0.71 , $P < 0.05$) due to CMN treatment (GP3) in nephrolithiatic rats (Figs. 1a and b) as compared to GP2 animals. Treatment with Vit. E improved renal function but not as

effective as CMN.

Urinary oxalate excretion

There was a 3-fold (Fig. 2b, $P < 0.001$) increased in urinary CaO excretion at the end of 4th week in rats treated with Vit. D3 and EG as compared to control rats, whereas urinary CaO excretion was significantly (Fig. 2c, $P < 0.001$) reduced in rats receiving CMN. Treatment with Vit. E also showed reduction in

Fig. 3. Verification of urinary stone formation. Microscopic observations of kidneys tissue sections with BIOXL light microscope showing accumulation of CaOx crystals. Images were taken under light microscopy using hematoxylin and eosin (100 ×). (a) Group 1 (control); (b) Group 2 (nephrolithiatic animals received EG + Vit. D3); (c) Group 3 (EG + Vit. D3 + CMN); (d) Group 4 (EG + Vit. D3 + Vit. E).

CaO excretion but not as effective as CMN.

Histopathology (Verification of urinary stone formation)

Histopathology of kidney from control rats did not show urinary stone formation. While rats treated with Vit. D3 and EG (GP2) showed deposition of the crystalline components in the renal tissue, namely CaO when observed under 100 ×. After treatment with curcumin there was reduction in the level of crystal deposition and urinary stone formation as compared to diseased group. Treatment with Vit. E also showed reduction in urinary stone formation but not as effective as CMN.

Biomarkers of oxidative stress

Treatment with EG and Vit. D3 significantly ($P <$

0.001) decrease the levels of GSH by 32.06% (from 409.7 ± 7.72 to 278.3 ± 4.24), SOD by 40.63% (from 95.17 ± 3.53 to 56.50 ± 2.59) and CAT by 20.01% (from 1426 ± 9.91 to 1141 ± 21.08) (Figs. 4a,b,c). This reduction was significantly improved by treatment with CMN.

Melondialdehyde level was significantly (from 87.00 ± 4.05 to 147.8 ± 4.71 , $P < 0.001$) increased up to 1.7-fold in GP2 hyperoxaluric animals as compared with control animals (Fig. 4d). The increase in melondialdehyde level highlights the oxidative stress associated with hyperoxaluria. There was a significant ($P < 0.01$) reduction of 24.57% in the MDA levels in GP3 animals, as compared to GP2 animals. Treatment with Vit. E also showed reduction in oxidative stress level but not as effective as CMN.

Fig. 4. Biomarkers of oxidative stress. (a) Effect of CMN on kidneys GSH levels in hyperoxaluric rats. Unit: $\mu\text{g/g}$ of tissue. Values are expressed as mean $\pm$ S.D. for 6 animals in each group. Comparisons are made between: Group 1 and 2, 3, 4. The symbols represent statistical significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; (b) Effect of CMN on kidneys SOD activity in hyperoxaluric rats. Unit: SOD U/g of tissue. Values are expressed as mean $\pm$ S.D. for 6 animals in each group. Comparisons are made between: Group 1 and 2, 3, 4. The symbols represent statistical significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; (c) Effect of CMN on kidney CAT level in hyperoxaluric rats. Unit: mM H_2O_2 Consumed/gm tissue. Values are expressed as mean $\pm$ S.D. for 6 animals in each group. Comparisons are made between: Group 1 and 2, 3, 4. The symbols represent statistical significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; (d) Effect of CMN on kidney LPO level in hyperoxaluric rats. Unit: nM of MDA/g tissue. Values are expressed as mean $\pm$ S.D. for 6 animals in each group. Comparisons are made between: Group 1 and 2, 3, 4. The symbols represent statistical significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

DISCUSSION

Interesting advances in the understanding of the pathophysiology and molecular mechanisms of urolithiasis have been made during the last few

years. Previous studies demonstrated that hyperoxaluria and CaO crystalluria are accompanied by enzymuria and membranuria, a finding shows severe damage to renal tubular cells. Moreover, these changes were observed even in the unexpected absence of

crystalluria, suggesting that the Oxalate induced damage was not solely on account of the injury produced by CaO crystals (Thamilselvan *et al.*, 1997). Previous studies also indicate aggravation of nephrolithiasis by oxidative stress (Sivagnanam *et al.*, 2000; Hayretin *et al.*, 2006). However, the role of free radicals in nephrolithiasis has not been fully established (Khand *et al.*, 2002).

At this point it is worthwhile to mention CMN, widely used for its rich nutritional and medicinal values. Various in-vitro and in-vivo studies have established the antioxidant properties of CMN. It is well documented that CMN scavenges superoxide anions, peroxynitrite radicals, and quenches singlet oxygen. CMN has also been shown to inhibit hydrogenperoxide-induced cell damage (Balasubramanyam *et al.*, 2003; Naveen *et al.*, 2005).

In the present study increase in calcium oxalate crystals started to appear in rats treated with Vit. D3 and EG on day 7 and showed progressive increase in nephrolithiatic animals (GP 2) till day 28th. Urinary calcium oxalate (CaO) crystals and crystal agglomerates are normally harmlessly excreted, but in nephrolithiasis they are retained by tubular epithelial cells and shifted into the renal interstitium (Water *et al.*, 1999). However, CMN administration lowers the levels of CaO count in urine.

Exposure of oxalate at a concentration of greater than or equal to 175 μ M produced damage to renal proximal tubular epithelial cell line derived from the human kidney and this effect was inhibited by superoxide dismutase mimetic, suggesting role of oxalate in renal membranes damage via a process dependent on reactive oxygen intermediates (Jonassen *et al.*, 1999). Studies proved that ethylene glycol produces renal tubular dilatation. Moreover, it enhances the influx of leukocytes into the kidney and change phagocytes, especially macrophages and monocytes, which produce various reactive oxygen metabolites by activating the complement system in the kidney (Ho-shiang, 2002).

Increased oxidative stress in nephrolithiatic rats

(GP2) associated with oxalate toxicity might be the result of an increased concentration of free oxalate ions/insoluble calcium oxalate interacting directly with renal tubules (Thamilselvan, 1997; Khand *et al.*, 2002).

In our study, after 4 weeks of Vit. D3 and EG treatment, the endogenous free radical scavengers SOD, GSH, CAT were significantly decreased in the renal tissues, which ultimately lead to increase MDA formation and cell lyses. LPO, a degenerative pathway of the membrane components mediated through the free radicals, is a hallmark feature of oxidative stress (Coothan *et al.*, 2007). Supplementation with CMN was found to increase the activity of the antioxidant enzymes. Moreover, low molecular weight heparin and green tea supplementation protect against nephrotoxicity as a result of reduction in oxidative stress (Itoh *et al.*, 2005, Palaninathan and Arjunan, 2006). Thus oxidative stress may play the vital roles in aggravation of nephrolithiasis.

Studies showed oxidative stress can promote the formation of a variety of vasoactive mediators that can affect renal function directly by causing renal vasoconstriction or decreasing the glomerular capillary ultrafiltration coefficient; and thus reducing glomerular filtration rate (Khan, 1982; John *et al.*, 2004; Veena *et al.*, 2006).

In urolithiasis, the glomerular filtration rate decreases in consequence of the obstruction to the outflow of urine by stones in the urinary system. As a result of this, the waste products, particularly nitrogenous substances such as urea, creatinine and uric acid get accumulated in blood. In nephrolithiatic rats (GP2), marked decrease in kidney function is seen as indicated by increase in serum BUN and creatinine (Khand *et al.*, 2002). The significant decrease in BUN and creatinine levels in CMN + EG + Vit. D3 treated animals (GP3) may indicate the fact that CMN via anti-oxidant effect noticeably boost the renal excretory system by increasing the elimination of these metabolite wastes.

From day 7 the gradual attenuation of renal antioxidant activities may make the kidney unable

to cope with persistently elevated reactive oxygen species, and so the proximal and distal renal tubules were damaged (Ho-Shiang, 2002).

Histopathologic observations of hyperoxaluric renal sections showed deposition of crystals.

This abnormal CaO crystal formation and deposition in kidney of CMN + EG + Vit. D3 treated animals (GP3) was corrected to near normalcy, demonstrating the protective effect of the CMN in maintaining the integrity of the renal membrane and is supported by the fact that CMN administration prevented enzyme leakage from renal tissues in oxalate toxicity. CMN known to be beneficial in the management of wound healing suggesting their membrane-regenerative potential.

From the present findings, it can be concluded that CMN can reduce the toxic effects of oxalate on renal cells through its antioxidant property. CMN can prevent the development of urinary stones formation. The mechanism underlying this effect is still unknown. However, we demonstrate ability of CMN to protect against solid phase development, which may be due to CaO crystals in urine, apparently related to decrease in oxidative stress and lowering of urinary concentrations of stone forming constituents.

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