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Antioxidant therapy prevents ethylene glycol-induced renal calcium oxalate crystal deposition in Wistar rats

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Abstract
Purpose Renal epithelial cell injury by reactive oxygen species is a prerequisite step in the pathogenesis of urolithiasis, and there is increasing evidence that reactive oxygen species is produced and oxidative stress (OS) is developed during idiopathic calcium oxalate nephrolithiasis. It appears that the administration of natural antioxidants has been used to protect against nephrolithiasis in human and experimental animals.
Methods Calcium oxalate urolithiasis was induced experimentally by administration of 0.75 % v/v ethylene glycol in drinking water of male Wistar rats weighing 150–200 g. Study was conducted in 4- and 8-week periods. In the 4-week period, Group 1 (control) was fed a standard commercial diet. Group 2 received the same diet with the addition of 0.75 % of ethylene glycol (EG). Group 3 received EG plus the diet, and water with additional antioxidant nutrients, and lemon juice as the dietary source of citrate (EG + AX). Group 4 was the same as Group 3, but with no EG in water. In the 8-week study protocol, Group 5 was fed the standard diet with EG in water for the first 28 days, followed with no EG. Group 6 (curative group) received the diet with EG for the first 28 days, followed by discontinuation of EG plus the addition of antioxidant nutrients. Group 7 was provided the diet with antioxidant nutrients for 8 weeks. Group 8 (preventive group) received the diet with antioxidant nutrients for 4 weeks, followed by antioxidant nutrients with EG for the next 4 weeks. Lime juice was given along the antioxidants. After treatment period, kidneys were removed and used for histopathological examination.
Results In the 4-week study, the mean number of crystal deposits in Group 2 was significantly higher than that of animals in Group 3. After 8 weeks, animals given curative antioxidant supplementation within the second 4-week period developed fewer deposits in Group 6 as compared to Group 5 animals. In the other preventive AX loading Group 8, the number of crystal deposits was substantially less than that of either Group 2 or Group 5 animals (EG-treated rats).
Conclusion Results showed a beneficial effect on treating and superior renal protection for preventing stone deposition in the rat kidney. These results provide a scientific rationale for preventive and treatment roles of antioxidant nutrient complex in human kidney stone disease.

Keywords Ethylene glycol · Calcium oxalate urolithiasis · Oxidative stress · Antioxidants · Wistar rats

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Introduction

Stone disease is an increasingly common form of renal disease that is associated with crystal deposition in the

renal medulla in all cases studied so far. One suggested mechanism for the formation of stones, especially calcium stones, is increased urinary supersaturation of stone-forming salts, which leads to homogeneous nucleation in the lumen of the nephron, followed by crystal growth and consequent obstruction in the distal nephron. It is a complex process that is a consequence of an imbalance between promoters and inhibitors in the kidney. Therapy to prevent stones is based on lowering supersaturation, using both diet and medication. To reduce the incidence, it must be understood that urine composition is directly related to diet [1, 2]. Treatment for symptomatic stone passage is based on high oral fluid intake in all stone formers. It has been shown that increasing water intake to ensure a urinary volume of approximately 2.5 l/day was associated with reduced urinary supersaturation with calcium oxalate and a significant reduction in stone recurrence [3]. Fluid intake as fruit juice, specifically orange juice, is also effective in reducing urinary calcium oxalate saturation and increasing urinary citrate excretion [4]. Pharmacological treatment is needed in most recurrent calcium kidney stone formers as well as in specific stone-forming populations [5].

Although a complete picture of the pathophysiological mechanisms involved is still unclear, there is increasing evidence that reactive oxygen species is produced and oxidative stress is developed during idiopathic calcium oxalate nephrolithiasis. It is reported that oxidative stress, renal epithelial injury and inflammation are also engaged in idiopathic stone formation that is indicated by the urinary excretion of reactive oxygen species, products of lipid peroxidation, enzymes indicative of renal epithelial injury as well as many markers of chronic kidney disease and suggested that stone formation can lead to hypertension, diabetes, chronic kidney disease and myocardial infarction [6]. It also appears that several systemic diseases such as hypertension, diabetes mellitus, hypercholesterolemia and infection; use of antibiotics, chemotherapeutics, and radiocontrast agents; and exposure to environmental toxins, occupational chemicals, radiation, and smoking; as well as alcohol consumption induce oxidative stress in kidney [7].

Moreover, the administration of antioxidants has been used to protect against nephrotoxicity in human and experimental animals. In the kidney, these treatments are reported to diminish the increase in malondialdehyde (MDA) and the decrease in protective enzyme activity that are induced by chemical and pharmacological agents [8]. As experimental and clinical studies have demonstrated, the most frequently studied natural antioxidant free radical scavengers known to provide superior renal protection are vitamin A and carotenoids, E, C, B6 and antioxidant trace elements selenium and zinc [8–15]. They can be easily and safety increased in tissues by supplementation. Medical treatment of stone-forming patients using pyridoxine is considered as an effective first-line therapy to decrease hyperoxaluria in patients who form stones [16]. Zinc is believed to have an inhibitory effect on calcium oxalate stone formation [17] and is a constituent of the enzymatic antioxidant superoxide dismutase (SOD) [18]. The enhanced activity of antioxidant enzyme SOD can contribute to nephrolithiasis prevention via direct effects on renal epithelial cells [19]. Among dietary factors, evidence exists that boron may have antioxidant and anti-inflammatory properties [20–22] and seems to have an impact on kidney stone removal, dissolving or passing out with pain alleviation and cease of hematuria [23].

Overall, it appears that the kidney is a highly vulnerable organ to damage caused by reactive oxygen species, due to the abundance of long-chain polyunsaturated fatty acids. Antioxidant and reactive oxygen scavengers have been shown to be effective in animals for protecting kidney. Therefore, the aim of the current study was to test the effects of a combination of natural antioxidant vitamins A, C, E and B6; zinc and selenium as two essential constituent trace elements associated with an adequate amount of antioxidant enzyme production; and boron with a proposed antioxidant capacity against ethylene glycol (EG)-induced nephrolithiasis or kidney calculi.

Materials and methods

Male Wistar rats weighing 150–200 g were obtained from the Animal House of Physiology Group, Baqiyatallah University of Medical Sciences. Seven rats in each group (control vs. seven treatment groups) were weighed and randomly kept in plastic cages in a controlled environment with a 12-h light/dark cycle and a constant temperature (22–25 °C) and humidity (55–65 %), with free access to food and water. They had access to a normal rat chow diet and water for 7 days before the beginning of experimental protocols. Animals were provided clean cages weekly, for either 4 or 8 weeks. All experiments were performed in accordance with the guidelines of the care of animals and approved by the University's Research and Ethics Committee.

Diets and animal treatments

Rats in all groups were fed with standard rat chow from Pars Animal Food Co. (Tehran, Iran) and water ad libitum throughout study. According to the manufacturer, it

contained 9.0 IU vitamin A, 18.0 μg vitamin E, 3.0 μg vitamin B6, 0.20 μg selenium and 85.0 μg zinc per g of dry food, with no added vitamin C and boron. The daily supplementation of the nutrients in the diet or the water consumed by the animals was calculated given that a 150–200 g animal's food intake is approximately 20.0 g/day, and it drinks water at the rate of 10–12 ml/100 g body weight/day. Therefore, the supplementation rate was considered to provide the amounts of antioxidant nutrients approximately five to seven times of the natural daily intake. Accordingly, in the first part of the study (4-week study), Group 1 (control) was fed a standard commercial diet (rat chow). Group 2 received the same diet with 0.75 % of ethylene glycol (EG) (Sigma-Aldrich Co., St. Louis, MO, USA) in drinking water for 28 days ad libitum to induce the kidney stone formation. Group 3 received the standard diet enriched with 4,000.0 μg vitamin E and 1,500.0 IU vitamin A for each rat per day added to the diet once per week, and 5.0 mg vitamin C, 400.0 μg vitamin B6, 20.0 μg selenium, 12.0 mg zinc and 2.0 mg boron for each rat per day were provided in their drinking water, plus 0.75 % of EG. Group 4 is same as Group 3 with no EG in water. Commercial lemon juice as the dietary source of citrate was added to the water at the level of 1 ml/rat/day for all the groups receiving antioxidant nutrients.

The second part of the study lasted for 8 weeks. Group 5 was fed the standard diet with EG in water for the first 28 days, followed by the standard diet with no EG. Group 6 received the diet with EG in water for the first 28 days, followed by discontinuation of EG and addition of antioxidant nutrients. Group 7 was provided the diet with antioxidant nutrients for 8 weeks. Group 8 received the diet with antioxidant nutrients for 4 weeks, followed by antioxidant nutrients with EG for the next 4 weeks. The antioxidant protocol was same as Group 3.

To provide the antioxidant nutrients, 300.0 IU (200.0 mg) of a softgel capsule of vitamin E as dl-alpha tocopheryl acetate (Vitane Pharmaceutical Inc., Costa Mesa, CA, USA) and 75,000.0 IU of a softgel capsule of vitamin A as palmitate (Daana Pharma. Co., Tabriz, Iran) was dissolved in 3.0 ml of corn oil and added to 85.0 g of standard diet ($\sim$12.0 g/rat) and given once a week to provide vitamin E and vitamin A at doses same as those mentioned above. The non-antioxidant groups were given 3.0 ml of corn oil alone added to 85.0 g of standard diet once a week.

Pharmaceutical vitamin C (ascorbic acid) 250.0 mg tablets (Osvah Pharmaceutical Co., Tehran, Iran), pyridoxine HCL (vitamin B6) 40.0 mg tablets (Ramopharmin Pharmaceutical Lab., Tehran, Iran), selenium (selenium amino acid chelate) 200.0 μg dietary supplement capsules (Alfa Vitamins Lab., Inc., Doral, FL, USA), zinc (zinc sulfate) 50.0 mg capsules (Alhavi Pharmaceutical Co., Tehran, Iran) and boric acid (Merck,Germany) as the source of boron were used and added to their drinking water to provide the above-mentioned doses/rat/day, respectively. Lime juice (Mahram Co. Group, Shiraz, Iran) was purchased from local market. Fresh food and water containing the above chemicals was provided three times per week, and the consumption was monitored and recorded.

The experimental procedure adopted for the study is detailed as follows.

The first part of the study (4-week study):

Group 1 or control (CONT): the standard diet and drinking water
Group 2 (EG): same as Group 1 + 0.75 % of ethylene glycol (EG) in drinking water
Group 3 or treatment (EG + AX): same as Group 2 + antioxidant nutrients, boron and lime juice
Group 4 or antioxidants (AX): same as Group 3 with no EG in water.

The second part of the study (8-week study):

Group 5 (EG/−EG): the standard diet + EG in water for the first 4 weeks, followed for 4 weeks with no EG
Group 6 or post EG treatment with AX (EG/−EG + AX): the diet with EG in water for the first 4 weeks, followed by discontinuation of EG and addition of antioxidant nutrients, boron and lime juice
Group 7 or antioxidants (AX/AX): the diet and water + antioxidant nutrients, boron and lime juice for 8 weeks
Group 8 or prevention with AX loading (AX/AX + EG): same as Group 7, added with EG after 4 weeks.

Four and eight weeks after the experimental periods, rats from all groups were anesthetized for the collection of blood by cardiac puncture with a syringe and needle for further biochemical studies. The rats were then killed and both kidneys were excised. The left kidney from each animal was placed and fixed in 10 % formalin and dehydrated in a gradient of ethanol, embedded in paraffin, and then cut into 5-μ serial sections. Serial sections were cut and stained by hematoxylin and eosin and von Kossa (for phosphate detection). Nine slides from lateral, middle and medial sections were selected randomly. The histopathological changes and calcium oxalate crystal deposition at 40× magnifications under light microscope were studied in six fields of each section, composed of three fields in medulla and three fields in cortex. Aggregations of crystal deposits in the renal tubules were counted in 54 microscopic fields and an average was calculated. The size of crystal deposition was measured using Motic system Image analyzer (Motic Image 2000 1.2 Micro-Optic Industrial

Table 1 Size and the number of stone formation and crystal deposit in the cortex and medulla kidney section of the rats [$n = 7$] in different groups

Size and numbers						
Kidney segment	Cortex			Medulla		
Size	Small	Medium	Large	Small	Medium	Large
Groups: 4 weeks						
G1 [control]	0	0	0	0	0	0
G2 [EG]	26	36	12	105	89	47
G3 [EG + AX]	6	1	0	10	1	1
G4 [AX]	0	0	0	0	0	0
Groups: 8 weeks						
G5 [EG +/EG−]	33	15	4	72	42	7
G6 [EG +/EG−AX +]	8	7	3	11	7	2
G7 [AX/AX]	0	0	0	0	0	0
G8 [AX/AX + EG]	29	9	3	30	8	1

Small, crystal size: 0–10 μg; medium, crystal size: >0–20 μg; large, crystal size: > 20 μg

Group Co. Ltd.) and categorized as zero (no crystal), small (1–10 μg), medium (>10–20 μg) and large (>20 μg)].

Furthermore, to conduct statistical analysis, each kidney was scored for crystal deposition using the scoring system as following: 0—no crystal; 1—small size crystals; 2—medium size crystals and 3—large size crystals.

Statistical analysis

Data are expressed as mean ± SD and a Statistical Package for the Social Sciences ([SPSS 18.0], New York: McGraw-Hill) was used to perform all comparisons, and independent sample *t* test was used for the comparison of the mean of the score of crystal depositions. A *P* value of less than 0.05 was considered significant for the differences.

Results

The mean number of crystal deposits in the microscopic fields in the kidney specimens in different groups is shown in Table 1. In the 4-week study, the mean number of deposits in EG group (G2) was higher than the EG + AX group (G3). Crystal deposition was completely absent in kidneys in the control group (G1) and antioxidant group (G4).

Table 2 Estimation of the amount of stone formation in the kidney section of the rats [$n = 7$] in different groups calculated by scoring system

Score of stone formation			
Kidney segment	Cortex	Medulla	Kidney
Groups: 4 weeks			
G1 [control]	0	0	0
G2 [EG]	134	424	558
G3 [EG + AX]	8	15	23
G4 [AX]	0	0	0
Groups: 8 weeks			
G5 [EG +/EG−]	75	178	253
G6 [EG +/EG−AX +]	31	32	63
G7 [AX/AX]	0	0	0
G8 [AX/AX + EG]	55	49	104

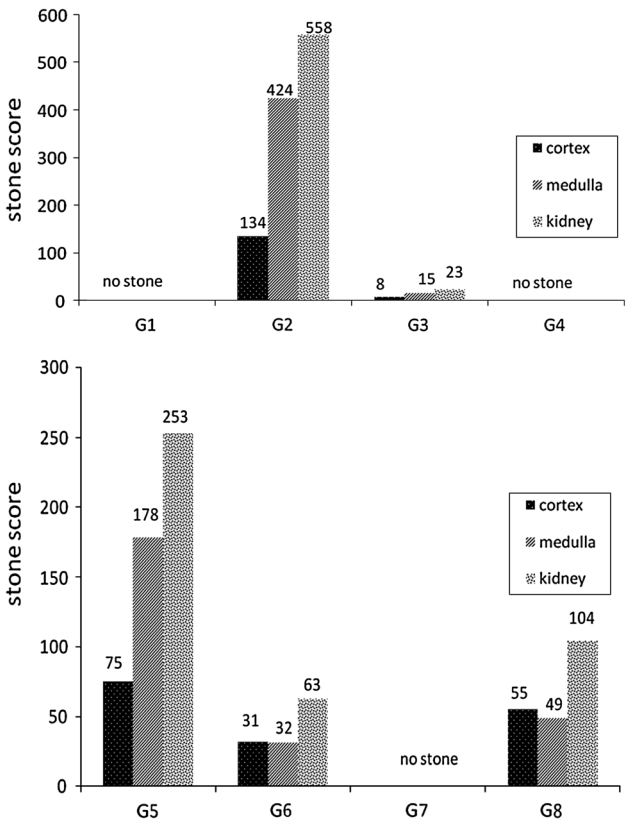
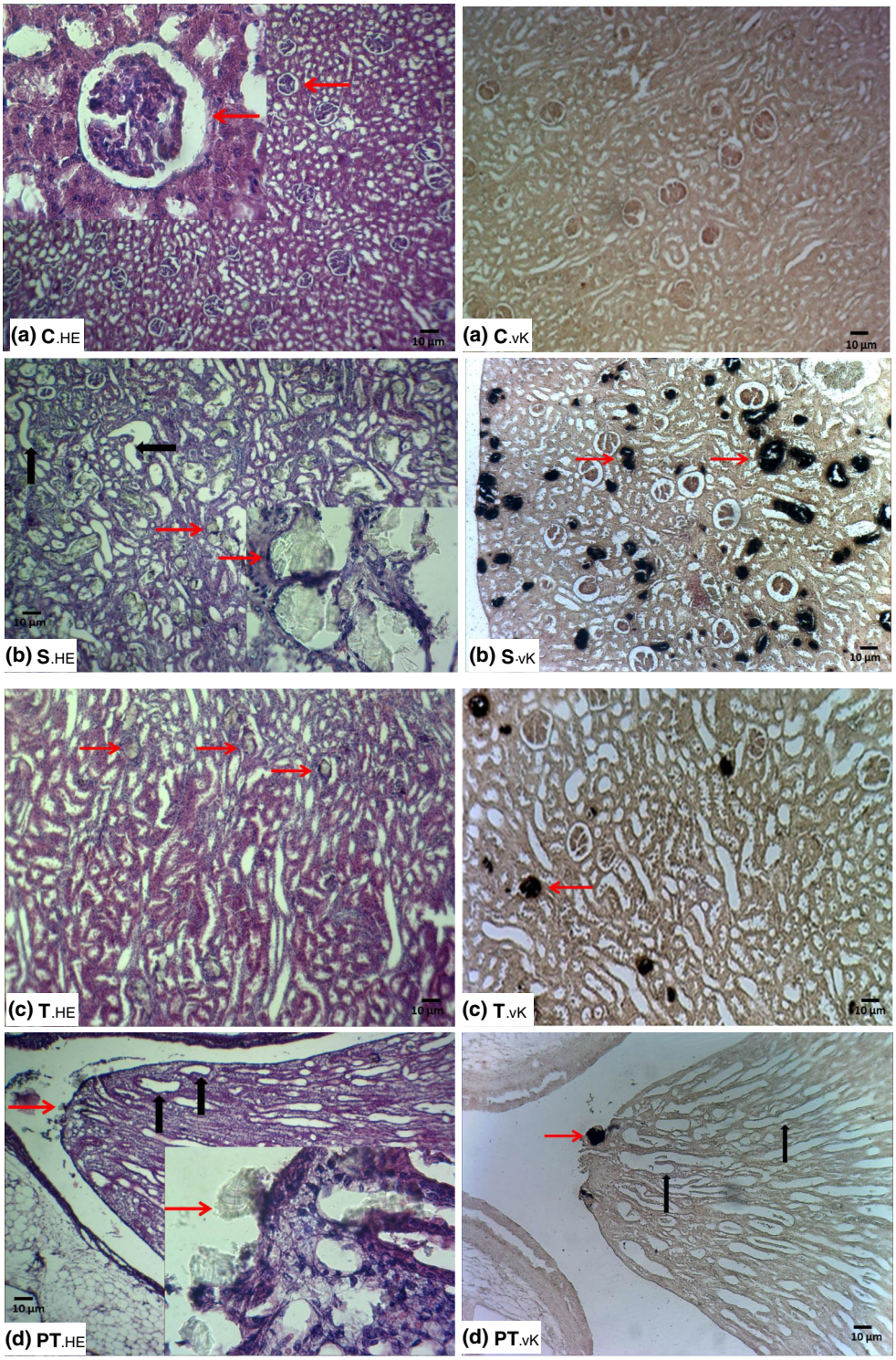


Fig. 1 Comparison of the score estimation of crystal deposits in the kidney of the rats [$n = 7$] in different groups at **a** 4 [G2 statistically significant with G3, *P*(0.000)] and **b** 8 weeks [G5 statistically significant with G6, *P*(0.002), and higher than G8, P(0.07)]

In the 8-week study, the EG-treated rats (G5) showed substantial persistent crystal deposition, even after 4-week cessation of EG consumption, whereas rats given antioxidant supplementation as a curative measurement within the second 4-week period developed fewer deposits in Group 6


(a) C.HE
10 µm
(a) C.vK
10 µm
10 µm
(b) S.HE
(b) S.vK
10 µm
(c) T.HE
10 µm
(c) T.vK
10 µm
10 µm
(d) PT.HE
(d) PT.vK
10 µm

◀**Fig. 2** Microscopic images of kidney sections from **a** vehicle control animals, **b** lithiatic group, **c** group treated with supplements and **d** crystal deposit on the surface of the papillary tip in group treated with supplements. **a** Normal tubules and collecting ducts are shown in a rat's kidney in negative control group (C.H&E (hematoxylin–eosin), C.vK (von Kossa (for phosphate detection))). **b** Multiple tubular calculi (*red arrows*) in renal tubules (S.H&E, S.vK) with marked histological changes including tubular dilation (*black arrows*), with epithelial damage secondary to multiple calculi accompanied with inflammatory infiltration (red arrows) in peritubular space from ethylene glycol-treated rat. **c** Significant reduction and less number of crystal deposits (*red arrows*) in rats treated with ethylene glycol and supplements (T.H&E, T.vK). **d** Fewer crystal deposit on the surface of the papillary tips (*red arrows*) with renal tubular dilation (*black arrows*) in a kidney of rat treated with supplements (PT.H&E, PT.vK). Crystals observed by light microscope (H&E, von Kossa staining, x40–x400)

(G6). However, the number of deposits in the EG-treated rats (G5) after 4 weeks of EG abstinence stage or deprivation was lower than that in 4 weeks EG-treated rats (G2). In the other preventive AX loading group (G8), the number of crystal deposits was substantially less than that of EG-treated rats (compared to G2 or G5).

To round up the results and to provide better evidence indicating that supplementation has a protective role against crystal formation and deposition, the number of crystals determined in each kidney segment or region (cortex and medulla) multiplied by its relevant score was calculated, and the mean of scores was recorded in Table 2.

The estimated score of crystal formation was significantly higher in the EG-treated rats (G2) compared to the EG + AX group (G3) at 4 weeks ($P = 0.000$); at 8 weeks, a significant higher score of crystals was estimated for the EG-treated rats followed for 4 weeks with no EG (G5) in comparison with Group 6, which is treated for 4 weeks with antioxidants (G6) ($P = 0.02$), and in comparison with preventive AX loading group (G8) ($P = 0.07$), as shown in Fig. 1.

Moreover, antioxidant loading in Group 8 resulted in lower score of crystals compared with Group 2 ($P = 0.001$).

The results of histopathological examinations are presented in Fig. 2.

Discussion

The incidence of urinary stone formation and crystal deposits in rats treated with 0.75 % EG implicated that the majority of stones were evenly distributed in the renal parenchyma. No stone or crystal deposition was found either in the control group or in the Group 4 receiving antioxidants. The results indicate that antioxidant stone preventive agents in Group 3 significantly decreased calcium oxalate stone formation resulting from free drinking of EG. Also, calcium oxalate crystals in different parts of the renal tubules in Group 3 were clearly smaller in comparison with EG group. It is clearly shown that a combination of excess dietary antioxidants may have a protective effect against free radical injury in stone formation. The kidney is a highly vulnerable organ to damage caused by reactive oxygen substances, due to the abundance of long-chain polyunsaturated fatty acids. Lipid peroxidation and antioxidant depletion are associated with several pathophysiological conditions, including urinary stone formation [12, 24]. Antioxidant and reactive oxygen scavengers have been shown to be effective in animals for protecting kidney.

It is confirmed that vitamin E acts as an excellent antioxidant for the kidney, which is greatly susceptible to oxalate-induced free radical damage, and supplementation with vitamin E maintains the optimal antioxidant enzyme levels necessary to protect renal tubes from peroxidative injury [14, 25]. *Rubus idaeus*, a medicinal plant having a significantly high content of vitamin E, has an impressive prophylactic effect on calcium oxalate stones in nephrolithic mice [26]. In the study of free radical-mediated damage to biological systems, the coadministration of vitamin E and selenium to alleviate lung oxidative damage induced by an organophosphorus compound [27] and its cardiotoxicity [28] and in lithogenic rats to decrease the levels of lipid peroxides and the activities of oxalate synthesizing enzymes, with a concomitant increase in the activities of enzymatic antioxidants and increased levels of non-enzymatic antioxidants [11], confirmed the potential protective effects of selenium and vitamin E.

To evaluate the association between serum antioxidant levels and the prevalence of kidney stones, the likely role of oxidative tissue damage in the pathophysiology of stone disease is demonstrated. Lower levels of α-carotene, β-carotene, β-cryptoxanthin and vitamin A deficiency are associated with a history of kidney stones and may indicate a role for these antioxidants in prevention [10, 11, 29]. The activities of hepatic glycolate oxidase and glycolate dehydrogenase were markedly enhanced in vitamin A- and vitamin B6-deficient rats [30], and pyridoxine is known to be an effective first-line therapy to decrease hyperoxaluria in patients who form stones [16]. Ascorbic acid as well as other radical scavengers and the antioxidant enzymes decreased in urolithic condition, suggestive of the active involvement of free radical-mediated lipid peroxidation-induced membrane damage in kidney [31]. Vitamin C is a major water-soluble antioxidant and acts as the first defense against reactive oxygen species in whole blood and plasma. At 24 h after extracorporeal shock wave lithotripsy (ESWL), patients given antioxidants had significantly reduced mean serum concentration of malondialdehyde

and higher levels of serum ascorbic acid which protect these patients from short-term renal injury [32]. However, high vitamin C supplementation either 1 or 2 g for 3 days may increase urinary oxalate excretion and the risk of calcium oxalate crystallization in calcium stone-forming patients [33]. The efficacy of antioxidant itamins A, E and C alone and in combination is well known [15, 34, 35] and has been shown to be effective in animals for protecting kidney [7].

It is reported that zinc and magnesium have inhibitory effect on calcium oxalate stone formation [17], and low concentrations of Zn, Mg and Mn in stones appear to make them resistant to shockwave lithotripsy (SWL) fragmentation [36]. After zinc supplementation, plasma zinc and antioxidant power increased; lipid peroxidation products decreased in the zinc-supplemented patients, compared with the placebo group [37]. This antioxidant power seems to be effective for protecting the kidney. It is speculated that alterations in steroids by boron and the reduction in cytokines with a possible change in urine calcium concentration may have an impact on the prevention of stone formation or removal [23]. Therefore, it was decided to add boron to the supplemented complex used in this study. Other recently proposed properties of boron such as anticarcinogenic properties [20, 38, 39], antioxidant capacity [21, 22, 40] and against brain oxidative stress [41] support a protective role for boron in the treatment of urolithiasis.

In addition, findings suggest that oral antioxidant therapy prior to lithotripsy may reduce the severity of long-term renal injury caused by the shock waves [42].

Our results demonstrate that nutrient supplementation decreases EG-induced calcium oxalate crystal deposition in the kidney by enhancing the antioxidant defense mechanism; therefore, our complex would be considered in the treatment of kidney stone formation and may benefit individuals with current kidney stone disease. In most experimental protocols, crystal-inducing drug and the therapeutic agents are administered simultaneously. In real life, patients actually seek medical treatment after appearance of common initial symptom of nephrolithiasis such as pain and hematuria or after medical confirmation of renal calculi diagnosis.

Therefore, in the 8-week study, antioxidant supplementation as a curative measurement after 4-week calculi induction represents a design as a curative protocol which developed fewer kidney stone deposits and size in Group 6 (G6) compared to the EG-treated rats (G5).

Moreover, in the other preventive AX loading group (G8) followed by 4 weeks of EG administration, the number of crystal deposits was substantially less than corresponding groups receiving EG (e.g., EG-treated rats (G2) or (G5)).

Results showed a beneficial effect on curing and preventing stone deposition in the rat kidney. These results provide a scientific rationale for preventive and treatment roles of antioxidant nutrients complex in human kidney stone disease.

Conclusion

Oxidative stress and active involvement of free radical-mediated lipid peroxidation-induced membrane damage associated with antioxidant imbalances are considered as important mechanisms involved in many pathological conditions and have been implicated in the pathogenesis of several systemic diseases such as crystal attachment and aggregation, resulting in nephrolithiasis. Restoration of antioxidant levels is capable of preventing calcium oxalate crystal deposition by reducing accumulation of the lipid peroxidation products and to prevent free radical-induced renal injuries.

Overall, the current study data indicated that administration of a combination of natural antioxidants showed beneficial effects on prevention and elimination of calcium oxalate calculi in the rat kidney. It seems that the effect of the selected nutrients on prevention and disruption of the kidney stones may be, at least, in part due to its antioxidant effects.

Moreover, it may be concluded that supplementation of the antioxidants contributes to the total antioxidative capability that could be considered as a healthier option and an effective strategy to protect against the deposition of calcium oxalate stones in the kidney.

Conflict of interest None.

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