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ORIGINAL PAPER

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Renal tubular cell damage and oxidative stress in renal stone patients and the effect of potassium citrate treatment

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Abstract Our objective was to evaluate the oxidative stress and renal tubular cell damage in patients who have renal stones compared to normal subjects. The patients were re-evaluated after 1-months supplementation with potassium citrate. We recruited 30 patients (11 males and 19 females) diagnosed with kidney stones and scheduled for surgical stone removal the following month, and 30 healthy non-stone formers (14 males and 16 females). Two 24-h urine samples and one heparinized blood sample were collected from each subject. Plasma was separated from the erythrocytes and assayed for creatinine, potassium, sodium, calcium, magnesium, phosphate, malondialdehyde (MDA, a lipid peroxidation product) (P-MDA), protein thiol as an indicator of protein oxidation, and vitamin E. Erythrocytes were analysed for MDA (E-MDA), reduced glutathione (GSH) and cellular glutathione peroxidase (cGPx) activity. The urine was analyzed for pH, creatinine, potassium, sodium, calcium, magnesium, phosphate, oxalate, citrate, MDA (U-MDA), total protein (U-protein) and N-acetyl-β-glucosaminidase (NAG) activity. For the stone patients, urine and blood samples were re-evaluated after supplementation with potassium citrate (60 mEq/day) for 1 month. Renal stone patients had higher plasma creatinine and lower plasma potassium, urinary pH, potassium, magnesium, phosphate and citrate than the controls. The patients had higher P-MDA, E-MDA, U-MDA, U-protein and NAG activity, but lower GSH, cGPx activity, protein thiol and vitamin E, when compared with controls. After potassium citrate supplementation, P-MDA and E-MDA decreased while plasma vitamin E, urinary NAG activity and citrate increased. Renal stone disease is associated with high oxidative stress and damage to renal tubular cells. These abnormalities are coincident with an increase in blood lipid peroxidation products and a decrease in antioxidant status. Although supplementation with potassium citrate improved urinary citrate levels and oxidative stress, it neither reduced urinary lipid peroxidation products nor remedied the damage to renal tubular cells, probably due to the existence of kidney stones.

Keywords Renal tubular cell damage · Oxidative stress · Antioxidants · Lipid peroxidation · Potassium citrate

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Introduction

Renal stones is a kidney disease with varying worldwide prevalence. Its primary and contributing pathogenic factors are genetic, nutritional and environmental, as well as personal habits [1]. Among several mechanisms postulated for the formation of stones in the urinary tract, the most tenable given the evidence is that tubular cell injury facilitates crystal attachment and subsequent accretion [2, 3]. It has been demonstrated, both in cultured cells and animal models, that renal tubular cell

injury is itself caused by oxalate and calcium oxalate crystals [4, 5].

Oxalate-induced membrane injury is mediated by lipid peroxidation (LPO), a degradative process of polyunsaturated fatty acids, through the generation of oxygen free radicals [3, 4, 6]. Direct evidence for the increased production of free radicals has been observed in the LLC-PK_1 and MDCK cells incubated with oxalate [4, 6]. These reactive oxygen species then mediate membrane damage by promoting the LPO reaction. A similar course of events has been demonstrated in vivo in rats with induced hyperoxaluria [7].

A positive correlation has been observed between the level of urinary oxalate and N-acetyl β-glucosaminidase (NAG) activity (an indicator of tubular cell injury) both in animals [8, 9] and renal stone patients [10, 11]. By contrast, a negative correlation has been observed between the degree of LPO and the level of antioxidants (i.e. vitamin C, vitamin E and reduced glutathione-GSH) [3, 12]. This LPO-mediated tissue injury can be normalized by supplementation with antioxidants [4, 5].

Since renal stone disease is endemic in rural areas of northeastern Thailand [13, 14], and oxalate crystalluria is common among such patients [15], our purpose was to evaluate the oxidative stress and damage to renal tubular cells in renal stone patients, both before and after supplementation with potassium citrate.

Materials and methods

Experimental subjects

A total of 30 patients (11 males and 19 females) diagnosed with renal calcium oxalate stone(s) in one kidney, without hydronephrosis and hydroureter as determined by renal ultrasonography, comprised the renal stone patient group. The patients' ages ranged between 30 and 55 years. They were appointed to have surgical removal of the stones at Khon Kaen Regional Hospital within the next month. Those patients with renal diseases, urinary tract infection, renal dysfunction, or plasma creatinine higher than 2 mg/dl were excluded. The patients were from rural communities in northeastern Thailand, an area endemic for renal stone disease [13, 14]. The comparative control group comprised 30 healthy, age-matched, non-stone-forming subjects (14 males and 16 females), residing in Khon Kaen city. Written informed consent was obtained from all participating subjects.

Blood analysis

Blood samples were treated with the anticoagulant heparin. Within 30 min after sample collection, the heparinized samples were centrifuged at 2,500 rpm to separate the plasma and erythrocytes. Measurements of malondialdehyde (MDA) (P-MDA), protein thiol and vitamin E were performed on the plasma fraction. The P-MDA was measured using the thiobarbituric acid (TBA) test, in which the MDA reacts with TBA in the presence of butylated hydroxytoluene and ferric chloride to form a colored complex with a maximum absorbance of 532 nm [16]. The protein thiol level in the plasma was determined by 5,5′-dithiobis (2-nitrobenzoic acid) as per Hu et al. [17]. Plasma vitamin E concentration was measured by a modification of the Emmerie-Engel method [18]; after extraction with xylene, the plasma vitamin E reacted with bathophenanthroline to form a colored product, which was measured spectrophotometically at 520 nm.

After the separation of plasma, the erythrocytes were washed three times with cold 0.9% sodium chloride solution before measuring the MDA (E-MDA), GSH and hemoglobin (Hb) levels and cellular glutathione peroxidase (cGPx) activity. The GSH concentration was determined according to the method described by Inal et al. [19]. The GPx activity was assayed as per Gunzler et al. [20], wherein enzyme activity is measured spectrophotometrically from the oxidation of NADPH in the presence of glutathione and H_2O_2. The concentration of Hb was determined by the cyanmethemoglobin method.

Urine analysis

Two consecutive days of 24-h urine samples were collected using thymol as the preservative. Urinary volume and pH were measured prior to biochemical analysis. After centrifugation, the urine supernatants were separated for determination of creatinine by the modified Jaffe's reaction, citrate by a new citrate lyase method [21], oxalate by atomic absorption spectrophotometry after binding to calcium [22], and phosphate by the phosphomolybdate method. Urinary calcium and magnesium were measured using atomic absorption spectrophotometry while sodium and potassium were measured by flame photometry. The urine supernatants were also analyzed for MDA, total protein (U-protein) and NAG (U-NAG) activity. The urinary MDA (U-MDA) level was measured by a method similar to that used for P-MDA. U-protein content was measured by a standard method [23]. The U-NAG activity was assayed by the spectrophotometric method established by Horak et al. [24].

One month before the date appointed for stone surgery, all 30 renal stone patients were supplemented with potassium citrate (60 mEq/day in two oral doses) for a period of 1 month. Trained village health volunteers, living in the same villages as the patients, were employed to do the supplementation. During this period, the patients were allowed to perform their daily activities and eat as they chose, but all other medications were withheld. Heparinized blood and 24-h urine samples were collected for evaluation before and after supplementation.

Data are expressed as the means ± SD. The statistical significance of the values was determined using SPSS to calculate paired *t*-tests and multiple-regression correla-

tion analysis. A P value <0.05 indicated statistical significance.

Results

Table 1 shows the relevant clinical data for the subjects. Compared to the normal controls, stone patients had higher plasma creatinine ($P<0.05$) but lower potassium ($P<0.01$) and lower urinary pH and excretion of potassium ($P<0.01$), sodium ($P<0.01$), magnesium ($P<0.05$), phosphate ($P<0.05$) and citrate ($P<0.001$). Data on various biochemical parameters as indices of oxidative stress and tubular cell damage are shown in Table 2. The stone patients exhibited a higher degree of LPO than the control subjects i.e., higher P-MDA ($P<0.01$), E-MDA ($P<0.0001$) and U-MDA ($P<0.001$).

Levels of free radical scavengers in the stone patients, i.e., protein thiol, vitamin E, GSH and the antioxidant enzyme activity in erythrocytes (GPx), were significantly reduced. Moreover, stone patients had a higher degree of damage to renal tubular cells, as indicated by an increase in U-protein ($P<0.0001$) and U-NAG activity ($P<0.01$). The level of U-MDA correlated positively ($r=0.555$, $P<0.001$) with the activity of U-NAG (Fig. 1) and U-protein ($r=0.796$, $P<0.0001$) (Fig. 2).

The effects of potassium citrate supplementation on the plasma, erythrocyte and urine parameters of the patients are presented in Table 3. As expected, potassium citrate caused an increase in urinary citrate excretion ($P<0.001$). It also caused a significant reduction of P-MDA ($P<0.01$) and E-MDA ($P<0.001$) only, but not U-MDA. Although treatment with potassium citrate had no effect on free radical scavengers in the erythrocytes (GSH and GPx activity), it resulted in a significant increase in plasma vitamin E ($P<0.0001$). Treatment with potassium citrate worsened injury to tubular cells as evidenced by a significant increase in U-NAG activity ($P<0.02$).

Table 1 Baseline clinical characteristics of renal stone patients and normal control subjects. BMI indicates body mass index

	Renal stone patient	Control	P
Subject number (male/female)	30 (11/19)	30 (14/16)	0.297
BMI (kg/m^2)	23.2 ± 3.3	24.6 ± 4.4	< 0.05
Plasma (mmol/l)	0.13 ± 0.07	0.09 ± 0.01	< 0.01
Creatinine	3.68 ± 0.43	4.27 ± 0.26	0.441
Potassium	139 ± 2.80	142 ± 2.10	NS
Sodium	2.45 ± 0.15	2.38 ± 0.11	0.183
Calcium	0.92 ± 0.11	0.96 ± 0.04	0.633
Magnesium	1.04 ± 0.22	1.13 ± 0.33	0.325
Phosphate		1137 ± 296	< 0.050
24-h urine			
Volume (ml)	1103 ± 360	6.29 ± 0.22	0.092
pH	5.76 ± 2.1	11.4 ± 2.1	< 0.010
Creatinine (mmol/day)	9.8 ± 2.7	43.8 ± 19.3	< 0.010
Potassium (mmol/day)	20.4 ± 8.6	129 ± 54	0.119
Sodium (mmol/day)	53.1 ± 27	3.75 ± 1.7	< 0.050
Calcium (mmol/day)	4.02 ± 2.1	3.5 ± 0.7	< 0.050
Magnesium (mmol/day)	2.61 ± 0.7	18.5 ± 5.5	0.955
Phosphate (mmol/day)	12.6 ± 4.3	0.29 ± 0.1	< 0.001
Oxalate (mmol/day)	0.36 ± 0.1	2.44 ± 0.5	
Citrate (mmol/day)	1.46 ± 1.0		

Table 2 Biochemical parameters as indices of oxidative stress and damage to renal tubular cells, in plasma, erythrocytes and 24-h urine of renal stone patients and normal control subjects

	Renal stone patient	Control	P
Plasma			
P-MDA (μM)	3.75 ± 1.2	2.77 ± 1.18	< 0.01
Protein thiol (μM)	396 ± 57	520 ± 53	< 0.01
Vitamin E (μM)	8.05 ± 3.04	18.8 ± 5.02	< 0.0001
Erythrocytes			
E-MDA (μM)	21.1 ± 3.6	7.6 ± 1.0	< 0.0001
GSH (μmol/gHb)	5.74 ± 1.1	7.42 ± 1.5	< 0.002
GPx (μmol/gHb)	8.11 ± 3.83	22.4 ± 4.21	< 0.0001
24-h urine			
U-MDA (μM/gCr)	5.79 ± 2.63	3.7 ± 1.8	< 0.001
U-protein (mg/day)	309 ± 130	80 ± 39	< 0.0001
NAG (U/gCr)	5.91 ± 4.58	2.20 ± 1.35	< 0.01

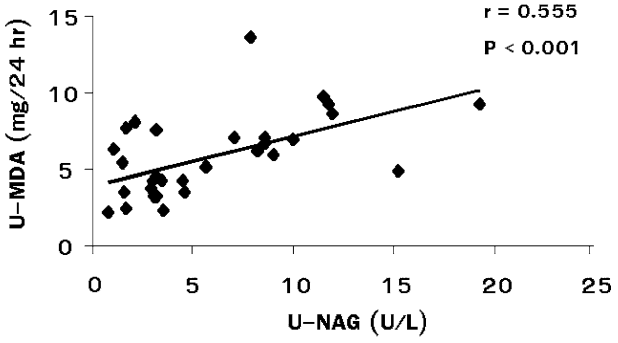

Fig. 1 The correlation between 24-h urinary malondialdehyde (*U-MDA*) and N-acetyl-β-glucosaminidase (*U-NAG*) activity in renal stone patients

Discussion

Oxidative stress is a term used to describe the imbalance between pro-oxidants and antioxidants that favors either an increase in the former or a decrease in the latter. To

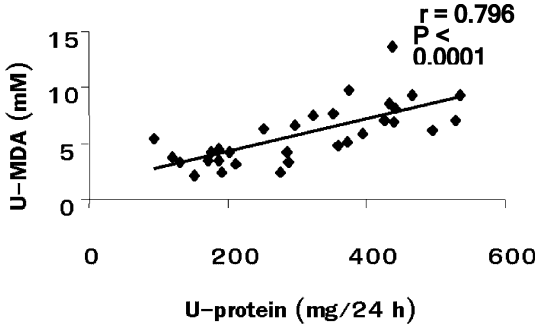

Fig. 2 The correlation between 24-h urinary malondialdehyde (*U-MDA*) and total protein (*U-protein*) in renal stone patients

Table 3 Levels of plasma MDA (P-MDA), vitamin E and protein thiol, erythrocyte MDA (E-MDA), GSH and GPx activity, and urinary MDA (U-MDA), U-protein, citrate and NAG activity before and after potassium citrate supplementation

	Potassium citrate supplementation		
	Before	After	*P*
Plasama			
P-MDA (μM)	3.75 ± 1.2**	2.86 ± 0.97	0.01
Protein thiol (μM)	396 ± 57**	399 ± 32**	NS
Vitamin E (μM)	8.05 ± 3.04***	13.10 ± 3.02**	0.0001
Erythrocytes			
E-MDA (μM)	21.1 ± 3.6***	11.0 ± 2.2**	0.001
GSH (μmol/gHb)	5.74 ± 1.15	5.87 ± 0.95	NS
GPx (U/gHb)	8.11 ± 3.83***	8.04 ± 1.67***	NS
Urine			
U-MDA (μM/gCr)	5.5 ± 4.8**	6.42 ± 4.04**	NS
U-protein (mg/day)	281 ± 58	350 ± 93	NS
Citrate (mM)	1.24 ± 0.4***	2.46 ± 0.55	0.001
NAG (U/gCr)	3.03 ± 1.4**	6.39 ± 4.7***	0.02

** $P < 0.01$ compared to the control (Table 2), *** $P < 0.0001$ compared to the control (Table 2)

optimize oxidative stress, cells are equipped with various antioxidant systems including enzymes, vitamins and minerals. Free radicals, normally produced by biochemical redox reactions of cellular metabolism, are pro-oxidants that cause injury to cells through the LPO reaction. An increase of MDA in plasma, erythrocytes and urine, indicated that our stone patients were in a state of greater oxidative stress than the normal control subjects. Furthermore, the observed significant correlations between the levels of U-MDA and U-protein and the activity of U-NAG support the occurrence of tubular cell damage from oxidative stress in these patients. Our results support a previous study by Huang et al. vis-à-vis the correlation between U-MDA and U-NAG [25].

Various studies have shown that hyperoxaluria and/or calcium oxalate crystals directly induce renal tubular injury through the LPO pathway [2, 3, 7]. Decreased urinary excretion of citrate, a crystallization inhibitor, might facilitate the formation of calcium oxalate crystals even though urinary excretion of oxalate and calcium in the stone patients were within normal limits (and not different from that of the controls).

Hypocitraturia is the main metabolic abnormality among renal stone patients in this region [26], and these patients have a high prevalence of oxalate crystalluria [15]. The culturally endemic high carbohydrate and low fat diet among these stone subjects [27, 28] might also contribute to the observed hypocitraturia and low vitamin E status. A low fat diet can also stimulate cellular fat synthesis [29], a process requiring acetyl-CoA from the breakdown of citrate as the precursor and NADPH as the reducer. This process may lead to the reduction of intracellular citrate content and eventually stimulates tubular citrate reabsorption, which causes hypocitraturia. Evidence of increased fat synthesis among the stone patients is adduced from an increase in the activity of ATP-citrate lyase, a citrate-break down enzyme, in the leucocytes [28]. Such an increase in fat synthesis may reduce the ratio of $NADPH/NADP^+$, which would directly reduce the levels of GSH and GPx activity. Before treatment with potassium citrate, the levels of measured antioxidants i.e., vitamin E in plasma and GSH and GPx activity in the erythrocytes, were significantly reduced in the stone patients when compared with controls.

In addition to having a high prevalence of hypocitraturia and oxalate crystalluria, stone patients in northeastern Thailand are potassium depressed [26, 30]. The kidneys of experimental rats fed a potassium-deficient diet showed tubulointerstitial injury [31]. One study has shown that oxalate exposure resulted in a significant increased in tubular injury [5]. Thus, the significant increase in markers of tubular injury, i.e. U-MDA, U-protein and NAG activity in urine of stone patients, suggests a state exacerbated by oxalate crystalluria, potassium depletion and a paucity of free radical scavengers (i.e. vitamin E, GSH and GPx activity).

Our results suggest that although potassium citrate treatment might prevent stone formation from the increase in urinary citrate excretion, its effects on parameters of oxidative stress and tubular damage are uncertain. In this study, the drug caused the reduction of radical-mediated reactions in plasma and erythrocytes, as seen from the decrease in P-MDA and E-MDA, and increase in plasma vitamin E. Huang et al. also reported the decrease of free-radical generation after the treatment of urolithic rats with vitamin E and potassium citrate [8]. Therefore, the significant increase of vitamin E after treatment was due to an indirect effect of potassium citrate in reducing reactive oxidants.

However, potassium citrate could not correct tubular damage, as indicated by the unchanged levels of U-MDA and U-protein and the significant increase in NAG activity in the urine. On the other hand, work on cultured cells by Khan and Byer have shown the potential power of potassium citrate in protecting renal epithelial cells from further injury by reducing free radical production and preventing lipid peroxidation [32]. Surprisingly, Thamilselvan et al. showed that after treating cells with oxalate together with calcium oxalate monohydrate (COM) crystals and vitamin E, the level of LDH (a marker indicating renal tubular damage) was increased [5]. From our findings on renal tubular damage, although vitamin E in plasma was increased, the significant increase in NAG activity was probably due to the presence of stones in the patients' kidneys, similar to the presence of COM crystals in cells with a hyperoxalate condition [5].

These findings are similar to a previous study using experimental urolithic rats by Selvam and Bijikurien [33]. Moreover, Anbazhagan et al. showed that vitamin E administration had hypercitraturic, hypocalciuric, and hypooxaluric effects on stone patients who have undergone surgical stone removal [34]. In addition, in stone patients after extracorporal shock wave lithotripsy, Golovanov et al. found a decrease in plasma MDA after treatment with vitamin E [35]. Thus, to achieve the best therapeutic results among these renal stone patients, the

existing stones must be removed, followed by long-term treatment with potassium citrate plus antioxidants to prevent stone recurrence.

In conclusion, renal stone patients in rural, north-eastern Thailand were in a state of high oxidative stress plus hypocitraturia, hypokaliuria and hypokalemia. Injury to renal tubular cells was apparent after potassium citrate treatment. Therefore, coincident treatment of stones and oxidative stress is advised.

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