



Changes of Klotho protein and Klotho mRNA expression in a hydroxy-L-proline induced hyperoxaluric rat model

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ABSTRACT. Klotho protein is recognized as having a renoprotective effect and is used as a biomarker for kidney injury. We investigated the level of Klotho protein in hyperoxaluria-induced kidney injury and the effects of vitamin E (Vit E) and vitamin C (Vit C) supplementation. Hyperoxaluria was induced by feeding 2% (w/v) Hydroxy-L-proline (HLP) in the drinking water for 21 days. Rats were divided into 5 groups; control (Group 1, n=7), HLP treated rats that received nothing else (Group 2, n=7), Vit E (Group 3, n=6), Vit C (Group 4, n=6) and both Vit E and Vit C (Group 5, n=7). Vit E (200 mg/kg) was injected on days 1, 6, 11 and 16, while Vit C (500 mg/kg) was given intravenously on days 1 and 11. The Klotho protein levels and oxidative status were measured. The expression level of kidney Klotho protein expression was significantly reduced by HLP-treatment, while the mRNA expression was higher ($P<0.05$), the plasma and kidney malondialdehyde and kidney superoxide dismutase activities were increased, and the kidney reduced glutathione and urinary total antioxidant status were decreased ($P<0.05$). All of these changes were ameliorated by administration of Vit E, Vit C or especially the co-administration of both. In conclusion, HLP-induced hyperoxaluria reduced the kidney Klotho protein level, which could be restored by Vit E and/or Vit C.

KEY WORDS: hyperoxaluria, Klotho protein, oxidative stress, vitamin C, vitamin E

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Urolithiasis, the formation of mineral stones in the kidney, bladder or urinary tract, is a common disease found in humans and other mammals. The most common type of stone is calcium oxalate (CaOx) [35]. Hypercalciuria and hyperoxaluria are important risk factors of CaOx stone formation. In addition, kidney injury was observed in stone forming patients and in experimental animals with hyperoxaluria [40, 45]. The mechanism of kidney injury and subsequently chronic kidney disease in kidney stone is mainly due to inflammation and oxidative stress. Supplementation of antioxidants is one of many strategies for reducing kidney injury and prevention of stone formation. The therapeutic role of vitamin E (Vit E) in renoprophylaxis has been reported in both hyperoxaluric rat models and human patients [38, 40]. However, the use of vitamin C (Vit C) is controversial due to the fact that it is a potential source of intrinsic oxalate production [6]. Previous studies demonstrated that the combination of Vit E and Vit C exerted a renoprotective effect against oxalate in both *in vitro* and *in vivo* studies [19, 43].

Klotho, a single-pass transmembrane protein, was discovered in 1997 [27]. It plays a central role in both acute and chronic kidney diseases, as revealed by many previous studies [2, 14, 26, 33, 49, 50]. Klotho protein is found as the three types of full-length membrane, soluble and secreted forms [47]. The functions of each form of Klotho proteins are not yet clearly understood, but the evidence supports that Klotho proteins play a role in homeostasis in the renal handling of electrolytes, including calcium and phosphate [15], fibrogenesis and the de-repression of the Wnt-beta catenin signaling pathway [49] and the regulation of lipid raft formation [8]. Recently, Klotho protein level has been suggested as a biomarker for kidney injury. The level of soluble Klotho protein was reduced rapidly after kidney injury [12, 13]. An injection of the naked plasmid encoding secreted Klotho (pV5-sKlotho) could protect injury lesions in the kidney tissue of the CKD rat model [50], suggesting the essential role of this protein in kidney function. Furthermore, the single nucleotide polymorphism G395A in the Klotho gene was linked to the risk of CaOx stone

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formation [39]. These suggest that the Klotho protein plays a part in stone formation and kidney injury of urolithiasis. However, the association between the Klotho protein level and oxidative stress in stone forming patients has not yet been elucidated. Therefore, the aims of this study were to evaluate the correlation between Klotho protein levels and the oxidative stress status in the hydroxyl-L-proline (HLP)-induced hyperoxaluric rat model, and the renoprotective effects of Vit E and/or Vit C on Klotho protein levels and oxidative stress status.

MATERIALS AND METHODS

Approvals

The present study was approved by the Chulalongkorn University Animal Care and Use Committee, Faculty of Veterinary Science, Chulalongkorn University following protocol review number 1431056.

Animals and experimental protocol

Male Sprague Dawley rats, weighing between 250–350 g, were obtained from the National Laboratory Animal Center, Mahidol University. The rats were divided into five groups as follows. Rats were either untreated (Group 1; control rats; n=7) or were treated with HLP only (Group 2; n=7), or with HLP and supplemented with either Vit E (Group 3; n=6), Vit C (Group 4; n=6) or a combination of Vit E and Vit C (Group 5; n=7). The administration of HLP, Vit E and Vit C was performed as described previously (19). In brief, the control rats received regular drinking water, while the other four groups received 2% (w/v) HLP (ACROS ORGANICS, Morris Plains, NJ, U.S.A.) added into their drinking water throughout the experimental period (21 days). The intravenous (i.v.) injection of Vit C (Atlantic Laboratories Corporation Ltd., Bangkok, Thailand) at 500 mg/kg body weight (BW) in 200 μ l/100 g BW isotonic saline was given on days 1 and 11 in Groups 4 and 5 under anesthesia with intraperitoneal (i.p.) injection of 60 mg/kg BW of pentobarbital sodium. The Vit E (DURVET, Inc., Blue Springs, MO, U.S.A.) was administered at 200 mg/kg BW in 200 μ l/100 g BW olive oil (VIDHYASOM Co., Ltd., Bangkok, Thailand) by i.p. injection in rats in Groups 3 and 5 on days 1, 6, 11 and 16. Olive oil alone was given to Groups 1, 2 and 4 as a placebo of Vit E vehicle, and isotonic saline was given in Groups 1–3 as a placebo of Vit C vehicle.

All rats were subjected to this experimental study for 21 days. The BW, food intake and water intake were recorded daily in all rats throughout the experiment. Each rat was placed in a metabolic cage on day 20. The 24 hr urine collected from the metabolic cage at day 21 was used to determine the concentration of urinary malondialdehyde (UMDA), total antioxidant status (UTAS), creatinine (UCr) and Klotho level. The plasma was collected at the end of experiment by cardiac puncture to determine the concentration of MDA (PMDA) and Klotho level. The right kidney was removed for subsequent determination of the kidney MDA and activities of catalase (CAT) and superoxide dismutase (SOD), reduced glutathione (GSH) level and mRNA expression of Klotho. After the right kidney had been removed, the left kidney vasculature was perfused and fixed with cold sodium phosphate buffer and 4% (w/v) paraformaldehyde for immunohistochemistry based evaluation of the Klotho protein expression and for histopathologic evaluation as previously described [4].

Analytical procedure

The measurement of Cr, MDA and TAS was performed as described previously [19]. The SOD and catalase activity was determined as reported previously by McCord and Fridovich [29] and Aebi [1], respectively. The levels of GSH in kidney tissues were measured by the method of Beutler [7].

Soluble Klotho protein

The level of Klotho protein (shedding ectodomain of full length transmembrane and Klotho from alternatively splicing) was evaluated by a commercial sandwich ELISA assay (SEH757Ra, Cloud-Clone Corp., Houston, TX, U.S.A.). Briefly, urine and plasma samples were incubated on a pre-coated and blocked microplate of biotin-conjugated antibody specific to Klotho (rat). Then, avidin conjugated to Horseradish Peroxidase (HRP) was added to each well of the microplate and incubated. After washing, the substrate solution was added and exhibited a change in color of the biotin-conjugated antibody and enzyme-conjugated avidin-substrate complex. The stop solution, sulfuric acid, was added to terminate the reaction. The color was measured at a wavelength of 450 nm. The Klotho protein levels in the samples were determined by comparison of the optical density of the samples to a prepared Klotho protein standard curve. The detection range is 0.312–20 ng/ml with the minimum detectable dose of Klotho less than 0.118 ng/ml. The urinary Klotho protein was expressed as the urine Klotho and Cr ratio (Urine Klotho/UCr).

Klotho protein expression

The procedure for Klotho protein expression in renal tissue was performed by immunohistochemistry (IHC) as previously described with a modification [31]. Paraffin embedded kidney tissue was deparaffinized and rehydrated. 4–6 μ m renal tissue slides were antigen retrieved which were pre-treated with citrate buffer (pH 6.0) and heated for 20 min. Then, 3% (v/v) hydrogen peroxide (H₂O₂) was used to block endogenous peroxidase activity. Non-specific binding was blocked by incubation with 3% (w/v) bovine serum albumin for 45 min. Sections were incubated with rabbit anti-Klotho primary antibody (5 μ g/ml, Abcam ab154163, Cambridge, MA, U.S.A.) at 4°C overnight. Detection used the EnVision® System HRP labeled polyclonal anti-rabbit antisera (Dako, Glostrup, Denmark) at room temperature for 45 min followed by 3,3'-diaminobenzidine chromogen (DAB solution, Dako). All sections were counterstained with Mayer's hematoxylin and examined under a light microscope. The score was estimated as the

Table 1. Effects of Vit E, Vit C and its combination on soluble Klotho protein in plasma (ng/ml) and urine (ng/mg Cr)

Soluble Klotho	Group 1	Group 2	Group 3	Group 4	Group 5
Plasma Klotho	5.033 ± 2.224	1.091 ± 0.156 ^{a)}	5.698 ± 2.095	1.447 ± 0.225	3.163 ± 1.254
Urine Klotho/UCr	0.603 ± 0.088	0.373 ± 0.122	0.592 ± 0.073	0.909 ± 0.264	4.451 ± 1.918 ^{b)}

Data are shown as mean ± SEM. a) $P < 0.05$ compared with group 1; b) $P < 0.05$ compared with group 2 using One-way ANOVA. UCr=urinary creatinine.

percentage of Klotho positive area using the IMT *i*-Solution program (Vancouver, BC, Canada).

Kidney Klotho mRNA expression

The Klotho mRNA expression was determined using real-time PCR (qPCR). In brief, total RNA was extracted from the kidney with an RNeasy[®] Mini Kit (QIAGEN[®], Hilden, Germany) according to manufacturer's instructions. The concentration of total RNA obtained was determined using a NANODROP 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.). In the first stage of qPCR, first strand cDNA was synthesized from 100 ng/ μ l of total RNA with Thermo Scientific RevertAid Reverse Transcriptase (Lot 00317047). Then, in the second stage qPCR, QPCR Green Master Mix HRox, 2^x(Biotech rabbit, Hennigsdorf, Germany) was used for specific amplification of the cDNA. The qPCR products were detected by StepOnePlus[™] Real-Time PCR System and software (Applied Biosystems, Foster city, CA, U.S.A.).

Gene activity was normalized using GAPDH as a housekeeping gene for hyperoxaluric rats, as previously described [10]. The PCR primer sequences were selected as previously shown [11] and are shown below:

Rat Klotho	Forward 5'-CGTGAATGAGGCTCTGAAAGC-3'
	Reverse 5'-GAGCGGTCCTAAGCGAATACG-3'
Rat GAPDH	Forward 5'-TCCCTCAAGATTGTCAGCAA-3'
	Reverse 5'-AGATCCACAACGGATACATT-3'

The Relative change of Klotho mRNA expression level was calculated by the comparative C_T method ($2^{-\Delta\Delta C_T}$ method).

Histopathology

The embedded kidney tissues in paraffin were cut at 5 μ m thickness. The sections were deparaffinized, rehydrated and stained with Hematoxylin and Eosin (H&E). Histopathological lesions in the cortex and medulla regions of the renal tissues were examined and scored under a light microscope by a Veterinary pathologist as previously described [46].

Statistical analysis

All data are expressed as mean ± standard error of mean (SEM). The difference between groups was compared with the control (Group 1) and the HLP-treated (Group 2) rats using One-way ANOVA followed by Dunn's post-hoc method. A simple linear regression was determined, while the correlations between each parameter were performed using Pearson's correlation. A significant difference was considered at the $P < 0.05$ level.

RESULTS

Body weight, food and water intake

On day 21, the percentage increase in the average body weight of the rats was numerically different among the groups (10.1 ± 3.3, 14.9 ± 3.3, 15.2 ± 2.8, 21.1 ± 4.1 and 7.8 ± 2.6%, for Groups 1–5, respectively), but none of these differences was significant. There was slight numerical decrease in the average food intake on day 19 in rats subjected to the HLP-treatment, which was not reversed by Vit E and/or Vit C administration (25.1 ± 1.2, 22.5 ± 0.9, 22.8 ± 2.2, 21.9 ± 0.7 and 20.5 ± 0.7 g, for Groups 1–5, respectively), but these were not significant. In contrast, significant higher water intakes were found on day 20 in all groups receiving HLP (22.0 ± 6.1, 51.9 ± 4.8, 41.0 ± 6.2, 43.8 ± 2.3 and 31.2 ± 1.9 ml/d, for Groups 1–5, respectively), but the numerically reduced average water intake following Vit E and/or Vit C was only significant for the combined Vit E plus Vit C treatment.

Plasma and urinary Klotho proteins

The level of plasma Klotho was significantly reduced in the HLP-treated rats compared to the control group, and administration of Vit E, but not Vit C, could restore the plasma Klotho levels (Table 1). The urine Klotho/UCr ratio was decreased by HLP-treatment, but increased by Vit E or Vit C administration and especially by the combined Vit E plus Vit C, which was significantly higher.

Kidney Klotho protein expression

The Klotho proteins expression level in kidney tissues in each group of rats is shown in Fig. 1, as representative immunohistochemistry images of the cortex and medulla regions along with the quantitative data of the average positive area. The expression of membrane Klotho protein in either the cortex or medulla was mostly identified in the distal convoluted tubule and collecting duct. Klotho protein expression in the HLP-treated rats was markedly reduced compared to the control rats, while this

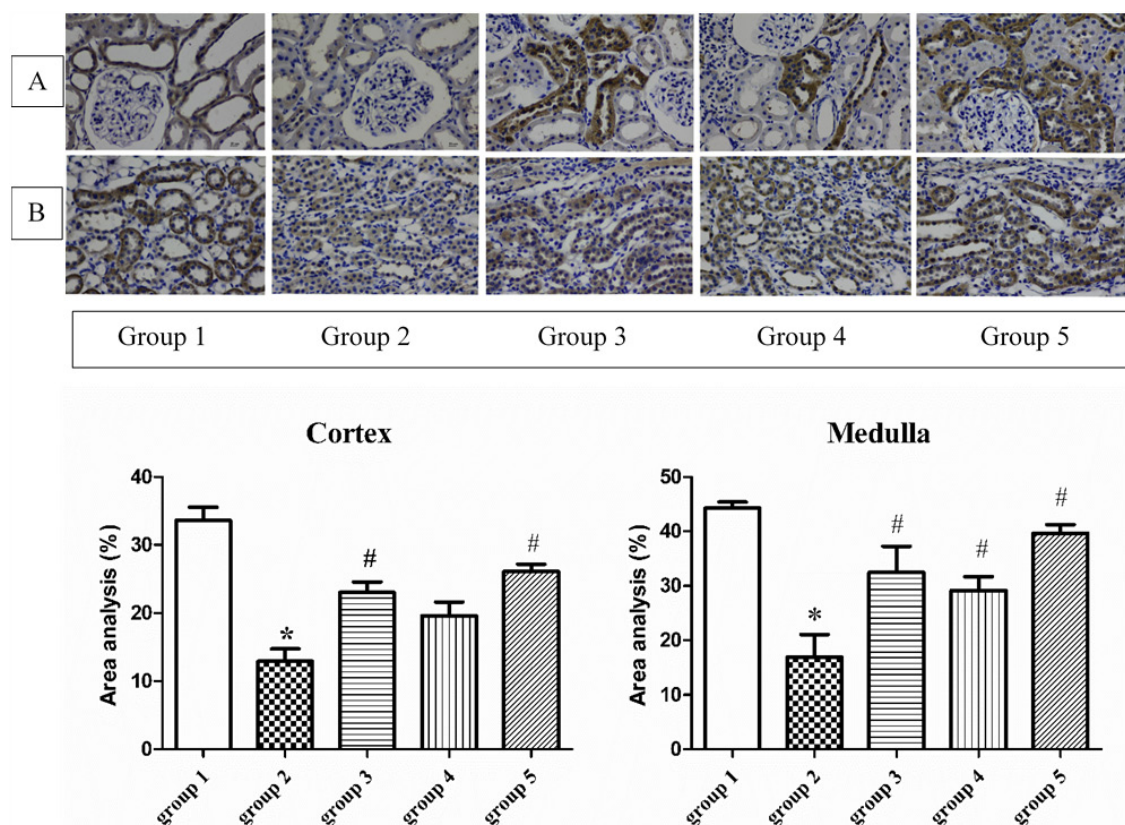


Fig. 1. The (Above) histopathology of kidney tissue showing Klotho protein expression using immunohistochemistry from the (upper panel, A) cortex and (lower panel, B) medulla regions and (Below) the percentage positive area analysis in all rat groups. * $P < 0.05$ compared with the control (Group 1); # $P < 0.05$ compared to HLP-treated rats (Group 2) using One-way ANOVA. (40 \times mag, IHC)

Table 2. Effects of Vit E, Vit C and its combination on PMDA (nmol/m), UMDA/UCr (nmol/mg Cr) and UTAS (%)

	Group 1	Group 2	Group 3	Group 4	Group 5
PMDA	2.028 \pm 0.229	3.783 \pm 0.182 ^{a)}	2.193 \pm 0.381 ^{b)}	2.063 \pm 0.552 ^{b)}	2.400 \pm 0.416 ^{b)}
UMDA/UCr	0.127 \pm 0.017	0.245 \pm 0.047	0.221 \pm 0.026	0.230 \pm 0.040	0.191 \pm 0.037
UTAS	84 \pm 1	34 \pm 3 ^{a)}	72 \pm 6 ^{b)}	78 \pm 3 ^{b)}	75 \pm 3 ^{b)}

Data are shown as mean \pm SEM. a) $P < 0.05$ compared with group 1; b) $P < 0.05$ compared with group 2 using One-way ANOVA. PMDA=plasma malondialdehyde, UMDA=urinary malondialdehyde. UCr=urinary creatinine, UTAS=urinary total antioxidant status.

HLP-mediated reduction was mostly negated by administration of Vit E and/or Vit C, especially in the medulla region.

Kidney Klotho protein mRNA expression

The expression level of Klotho mRNA was significantly higher in the HLP-treated rats than the control rats (10.20 \pm 3.98 vs 1.00 \pm 0) ($P < 0.05$). The elevated mRNA level was partially reduced by administration of Vit E and/or Vit C (4.02 \pm 1.92, 1.56 \pm 0.36 and 5.56 \pm 2.27 in Groups 3–5, respectively), with the Klotho mRNA level being reduced in the HLP plus Vit C rats to close to the control level.

Oxidative stress status

The PMDA level was increased in the HLP-treated rats, while Vit E and/or Vit C supplementation significantly reduced the PMDA level of the HLP-treated rats, causing it to revert to almost that of the control rats (Table 2). A similar numerical trend was found for the UMDA/UCr, but these changes were not significant. Likewise, the UTAS of the HLP-treated rats was significantly lower than the control rats, while it was rescued after receiving the Vit E and/or Vit C supplementation.

For kidney antioxidant status, the kidney MDA was significantly increased in the HLP-treated rats compared to the control rats and was partially reduced by Vit E, but not by Vit C (or both Vit E and Vit C) administration (Fig. 2). The activity of SOD in kidney tissue was significantly increased by HLP-treatment, but this increase was ameliorated by treatment with Vit E and/or Vit C (Fig. 2). The average CAT enzyme activity did not differ between the treatment groups and the control rats, while the GSH level

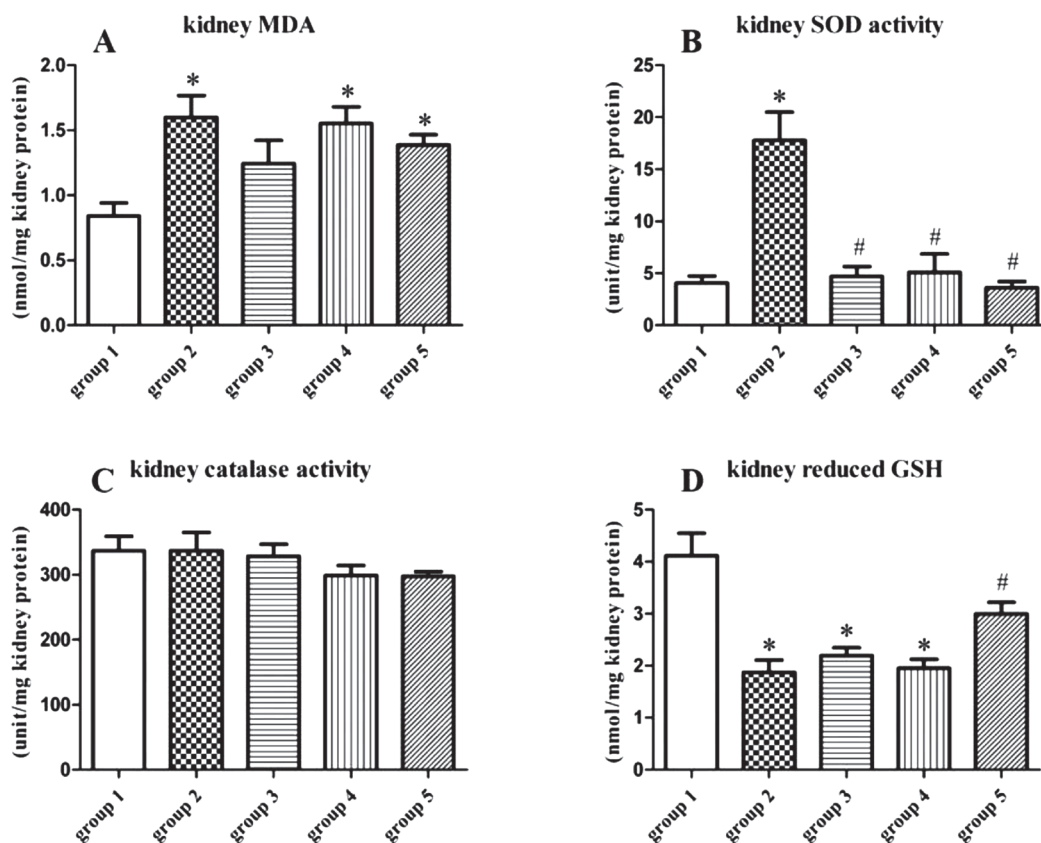


Fig. 2. The oxidative stress, in terms of the (A) MDA level, (B) SOD activity, (C) CAT activity and (D) GSH level in kidney tissues of all rat groups. Data are shown as the mean \pm SEM, * P <0.05 compared with the control (Group 1); # P <0.05 compared to HLP-treated rats (Group 2) using One-way ANOVA.

in kidney tissues was drastically reduced by HLP-treatment and was modulated by Vit E and /or Vit C administration, where the combination of both Vit E and Vit C significantly increased the GSH levels (Fig. 2).

Histopathology results

Representative histopathologic images and the quantitative lesion score of kidney tissue from the cortex and medulla are shown in Fig. 3. There was no remarkable lesion of tubular cells in either the cortex or the medulla regions in the control rats, whereas the HLP-treated rats developed moderate to severe flattened tubular epithelial cells in proximal, distal and collecting duct regions with a significantly higher score than the control rats (P <0.05). Antioxidant treatment, in terms of administration of Vit E and /or Vit C alleviated the tubular injury, where milder tubular epithelial lesions with significantly reduced lesion scores were observed compared to the HLP-treated rats.

Correlation between kidney Klotho protein expression and kidney oxidative stress

The quantitative area analysis of membrane Klotho protein expression level in both the cortex and medulla regions revealed significant positive correlations with the level of kidney reduced GSH (cortex: $Klotho = 11.004 + (4.566 * \text{kidney reduced GSH})$, $r = 0.608$, $n = 33$ and medulla: $Klotho = 15.117 + (6.576 * \text{kidney reduced GSH})$ ($P < 0.01$), $r = 0.583$, $n = 33$ (Fig. 4). The Klotho protein in the cortex and medulla also had significant negative correlations with the level of kidney MDA (cortex: $Klotho = 38.803 - (11.853 * \text{kidney MDA})$, $r = -0.617$, $n = 33$, ($P < 0.01$) and medulla: $Klotho = 51.723 - (14.475 * \text{kidney MDA})$, $r = -0.502$, $n = 33$) ($P < 0.01$), and kidney SOD activity (cortex: $Klotho = 28.068 - (0.685 * \text{kidney SOD activity})$, $r = -0.565$, $n = 33$ ($P < 0.01$) and medulla: $Klotho = 39.181 - (0.916 * \text{kidney SOD activity})$, $r = -0.504$, $n = 33$) ($P < 0.01$).

DISCUSSION

The HLP-induced hyperoxaluria rat model has been used in many previous studies on the pathophysiology of CaOx stone [20, 22, 23]. The BW of rats receiving HLP were not significantly changed, although HLP caused a slightly reduced food intake with a significantly increased water intake, similar to previous studies of hyperoxaluria rats induced by either ethylene glycol [48] or HLP [19]. This reflects the deleterious effect of kidney stone on the well-being of rats.

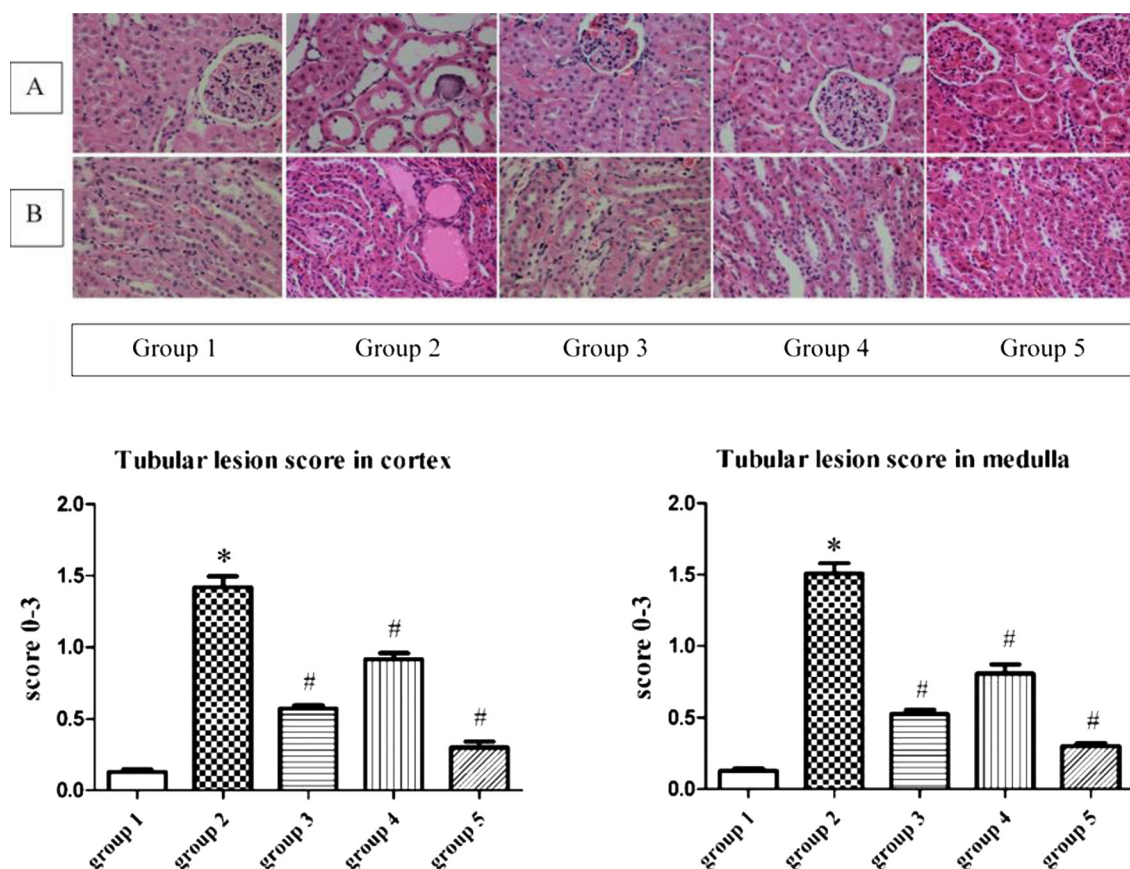


Fig. 3. The (Above) histopathology of kidney tissues stained with H&E from the (upper panel, A) cortex and (lower panel, B) medulla in all rat groups and (Below) the tubular lesion score, shown as the mean ± SEM, * $P < 0.05$ compared with the control (Group 1); # $P < 0.05$ compared to HLP-treated rats (Group 2) using One-way ANOVA.

Although it has previously been demonstrated that reduced renal function may limit the clearance of Klotho [16], the expression of Klotho was found to be associated with chronic kidney disease, but not in acute kidney injury patients [36]. The reduction in plasma Klotho protein levels in the HLP-treated rats seen in this study may reflect the low production of Klotho in relation to tubulo-interstitial injury rather than to changes in the clearance rate.

The Klotho protein expression in the kidney tissues of rats in the present study was drastically reduced in the HLP-induced hyperoxaluric rats, while the mRNA expression was increased. These hyperoxaluric rats may have enhanced gene activity, as previously reported in CDDP-induced kidney injury, although it was decreased in the volume depletion model [25]. In the sepsis model, acute inflammation suppressed FGF23 expression, which in turn upregulated Klotho synthesis [9]. It is plausible that the elevated Klotho mRNA expression was caused by a lack of negative feedback due to reduced FGF23 or Klotho protein production, and the failure in mRNA translation, such as endoplasmic reticulum (ER) stress induced by ROS and inflammation, inhibited Klotho protein production. It has previously been reported that a Klotho protein supplement ameliorated ER stress [5] as well as apoptosis in injured kidneys [28] and heart [37], which in turn suggests that inadequate Klotho protein level may be a risk factor for ER stress and apoptosis.

The role of Klotho protein may be closely related to oxidative stress. Aging related syndrome was found following mutation of the Klotho gene [27], while aging and senescence were also related to overproduction of reactive oxygen species [34], and Klotho gene expression was suppressed in a dose-dependent manner by H_2O_2 -treatment of cells [30]. Accordingly, Klotho gene transcription/translation could be regulated by oxidative stress. An enhanced oxidative stress level was indicated by the increased PMDA level, which has been reported previously in the hyperoxaluric rat model [19, 44] and *in vitro* studies [41, 42] as well as in CaOx stone patients [18]. In addition, in this present study, kidney oxidative stress was likely to have been increased since an elevated level of kidney MDA and SOD activity levels, and a diminished level of GSH was found. This was likely to be because the HLP-induced hyperoxaluria triggered an elevation in superoxide anion and free radical families in the kidney via NADPH oxidase [24, 51]. Accordingly, as the correlations between the Klotho protein expression level and the MDA, SOD and GSH levels in the kidney were also seen, we hypothesized that hyperoxaluria-induced oxidative stress is the major cause of decreased Klotho expression levels in CaOx rats. Therefore, Klotho protein level may potentially be used as an indicator for CaOx stone-induced kidney injury.

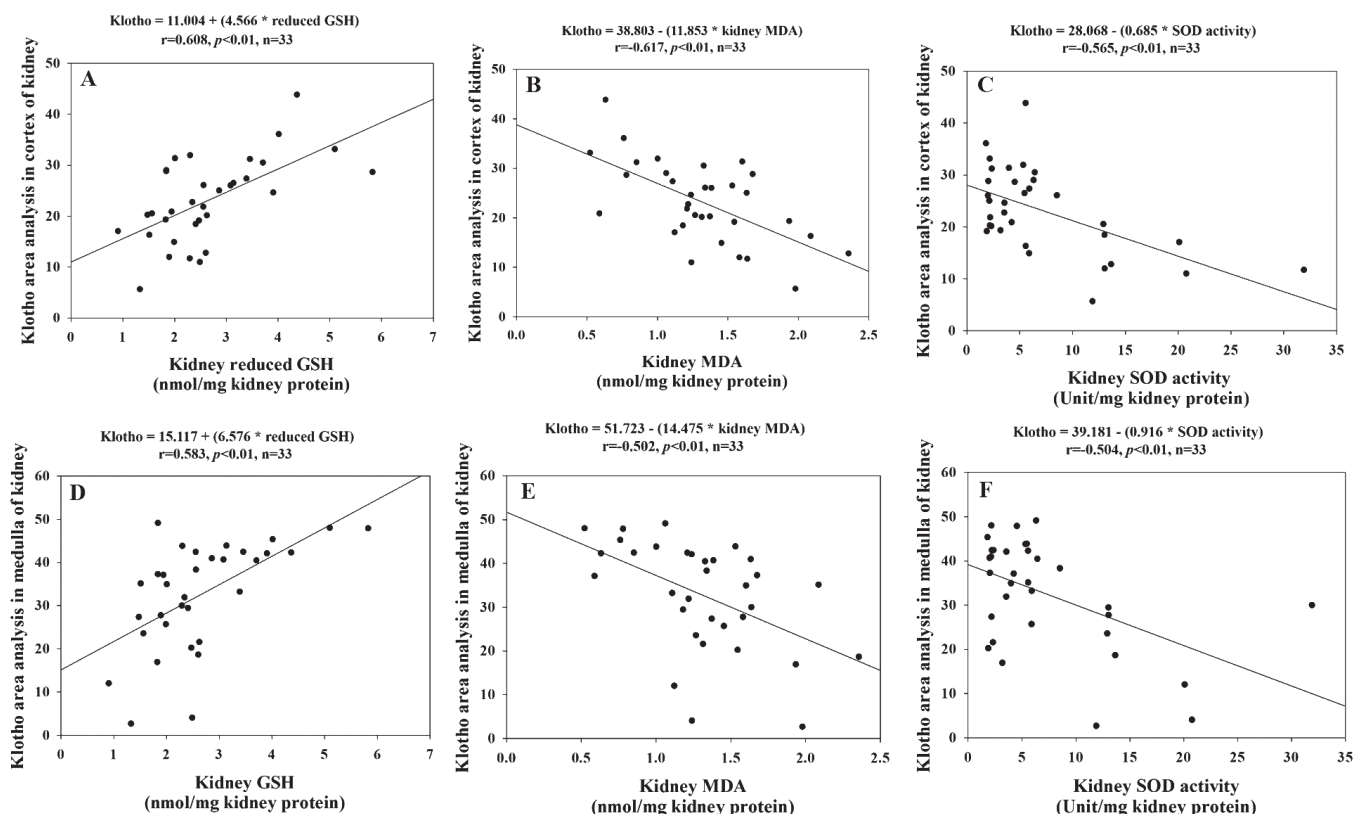


Fig. 4. Linear regression analysis showing the relationships between the kidney Klotho protein expression level and the oxidative stress parameters of (A, D) GSH, (B, E) MDA and (C, F) SOD activity in the (A–C) cortex and (D–F) medulla regions.

Alpha-tocopherol (Vit E) plays an important role in preventing kidney injury from free radical. In urolithiasis, a diminished plasma concentration of Vit E with increased PMDA levels was observed [21]. Administration of Vit E in CaOx stone patients improved their oxidative status and increased citrate excretion with a possible reduction in stone formation [3]. In drug-induced hyperoxaluric rats, supplementation with Vit E could restore the normal renal antioxidant activity and inhibit CaOx deposition in kidney tissues [40]. Moreover, Vit E reduced the renal tubular cell death and enhanced the activity of CaOx inhibitor molecules, including osteopontin and Tamm-Horsfall protein [17]. The results of the present study confirmed the beneficial effects of Vit E as previously reported in hyperoxaluria rats [19]. Additionally, the administration of Vit C or a combination of Vit E and Vit C showed renoprotective effects. This then raises the question whether changes in Klotho protein expression are consistent with renal damage.

In HLP-treated rats supplemented with Vit E, the plasma Klotho protein level was preserved, while the kidney Klotho protein expression level was increased in both the cortex and medulla regions. However, the kidney Klotho mRNA expression level in HLP/Vit E treated rats was lower compared to the HLP-treated rats. The increased Klotho protein expression suggested a lower degree of kidney injury, which is consistent with a previous study where Vit E ameliorated renal hemodynamics, tubular function at the proximal tubule and crystalluria [19]. A lower oxidative stress level was detected, in term of a lower PMDA level and higher urine TAS level, which could have been caused by either elevation of Klotho protein or inhibition of crystal formation. The kidney SOD activity was reduced following Vit E treatment of HLP-treated rats, while the GSH levels remained low, which might be due to inadequate antioxidant supplement or a lack of NADPH. Therefore, it is plausible that calcium oxalate crystal-induced oxidative stress, which suppressed Klotho protein production, could be restored by Vit E supplement. The kidney morphology of HLP-treated rats also given Vit E treatment appeared to be nearly normal.

In the present study, Vit C treatment resulted in unchanged plasma Klotho protein levels, but relatively high urine Klotho protein levels compared with rats receiving HLP alone. HLP-treated rats receiving Vit C had slightly higher kidney MDA levels compared with those receiving Vit E. Previous data showed that Vit C caused improved renal hemodynamics to the greater extent than Vit E did, although the level of crytalluria was higher [19]. Our study found that Vit C had a lower effectiveness than Vit E did on preserving the Klotho protein expression level in kidneys in CaOx crystalluria. However, this may depend upon many variable factors including the dose, stability, route of administration and lipid solubility, as well as whether the situation was related to oxidative damage.

The combination of Vit E and Vit C had synergistic antioxidant activities, resulting in maintaining the Vit E level in biological membrane [32]. Because of these important roles, a combination of Vit E and Vit C could be more effective in preventing renal damage and be more beneficial than using them independently [19]. The present study showed that the combination of Vit E and

Vit C gave the highest urine Klotho/UCr ratio and kidney Klotho protein expression level than the HLP-treated rats with Vit E or Vit C alone or with no supplementation. The kidney SOD activity in HLP-treated rats with Vit E plus Vit C administration was lower, while the kidney GSH level was higher than the HLP-treated rats receiving Vit E or Vit C alone. The higher level of GSH suggests lower level of reactive oxygen species, due to the high efficiency of these two antioxidants when given together.

In summary, this study demonstrated that the Klotho protein expressions were lower in rats receiving HLP-treatment, which also resulted in an enhanced oxidative stress and renal impairment. Giving Vit E, Vit C or a combination of both could limit the HLP-induced kidney tubular damage and restore the kidney Klotho protein level and alleviate oxidative stress, while a combination of both Vit C and Vit E was the most effective. The improved renal hemodynamic and reduced oxidative stress when giving both Vit E and Vit C could be beneficial in preventing the kidney damage caused by hyperoxaluria.

CONFLICT OF INTEREST. The authors had no conflict of interest.

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