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ORIGINAL PAPER

A traditional Chinese herbal antilithic formula, Wulingsan, effectively prevents the renal deposition of calcium oxalate crystal in ethylene glycol-fed rats

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Abstract We investigated the effects of a traditional Chinese herbal formula, Wulingsan (WLS), on renal stone prevention using an ethylene glycol-induced nephrocalcinosis rat model. Forty-one male Sprague-Dawley (SD) rats were divided into four groups. Group 1 ($n = 8$) was the normal control; group 2 ($n = 11$) served as the placebo group, and received a gastric gavage of starch and 0.75% ethylene glycol (EG) as a stone inducer; group 3 received EG and a low dose of WLS (375 mg/kg); and group 4 received EG and a high dose of WLS (1,125 mg/kg). Baseline and final 24 h urine samples were collected individually; biochemical data of urine and serum were also obtained at the beginning and at the end of the experiment. After 4 weeks, animals were killed and kidneys were harvested. The kidney specimens were examined by polarized light microscopy and the crystal deposits were evaluated by a semi-quantitative scoring method using computer software (*ImageScoring)*. The results revealed that the rats of placebo group gained the least significant body weight; in contrast, the rats of WLS-fed groups could effectively reverse it. The placebo group exhibited lower levels of free calcium ($p = 0.059$) and significantly lower serum phosphorus ($p = 0.015$) in urine than WLS-fed rats. Histological findings of kidneys revealed tubular destruction, damage and inflammatory reactions in the EG-water rats. The crystal deposit scores dropped significantly in the WLS groups, from 1.40 to 0.46 in the low-dose group and from 1.40 to 0.45 in the high-dose group. Overall, WLS effectively inhibited the deposition of calcium oxalate (CaOx) crystal and lowered the incidence of stones in rats ($p = 0.035$). In conclusion, WLS significantly reduced the severity of calcium oxalate crystal deposits in rat kidneys,

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indicating that Wulingsan may be an effective antilithic herbal formula.

Keywords Calcium oxalate · Ethylene glycol · Traditional Chinese medicine · Urinary stone · Wulingsan

Introduction

In Taiwan, urinary stone is a common disease; moreover, the cost of stone treatment is high [1–3]. The overall prevalence of upper urinary calculi in Taiwan is 9.6% (14.5% in men and 4.3% in women), which is higher than in neighboring countries, such as Korea (3.5%) and Japan [4–7]. From the medical resources point of view, although the population of Taiwan was estimated at 22.9 million, in an area of 35,801 square kilometers, Taiwan has more than 130 extracorporeal shockwave lithotriptors [3, 8, 9]. In other words, there are six lithotriptors per million population [2]. Furthermore, the yearly medical expenditure for treating urinary stones is over 100 million US dollars, which is more than 4% of the national health insurance budget [1, 3]. In order to reduce the medical cost, we are looking for an alternative treatment for stone prevention.

The components of Wulingsan (WLS) have been shown to prevent the formation of urinary stones. Also, medicinal herbs are widely accepted by many people in Taiwan and in Chinese societies elsewhere [10–13]. We previously reported that WLS effectively inhibited the process of CaOx nucleation, crystallization and aggregation in vitro [14]. These findings inspired us to clarify the antilithic effects of WLS in an animal model.

Wulingsan is composed of five dried herbs: *Alisma orientalis* (Sam.) Juzep. (澤瀉), *Polyporus umbellatus* (Pers) Fries (豬苓), *Atractylodes macrocephala* Koidez (白朮), *Poria cocos* (Schw.) Wolf (茯苓), and *Cinnamomon cassia* Presl (桂枝). Urinary stone disease is called "stone urinate (石淋)" in TCM references. Symptoms of this disease include stone in urine, painful urination, and lower abdominal pain with radiation to the umbilical region. In ancient China, many herbal preparations, including WLS, were used to treat stone diseases.

The indications for administering WLS have varied over the centuries. WLS formula was first mentioned in the TCM book *Shang han lun* (傷寒論 / *Treatise of Cold-induced Disorders*) written by *Zhang Zhong-Jing* (張仲景) in the third century. This book is said to be the true ancestor of many TCM formulae [13]. The original indications for this formula were symptoms of headache, fever, irritability, strong thirst with vomiting immediately after drinking, urinary difficulty and a floating pulse [13]. Treating "stone urinate" by Wulingsan was first recorded in the late sixteenth century (*Ming* dynasty) in the book *Zheng zhi zhun sheng* (證治準繩 / *Standards of Patterns and Treatment*) compiled by *Wang Ken-Tang* (王肯堂).

In brief, our previous study indicated that the WLS formula effectively inhibited CaOx crystal growth, aggregation, and formation in vitro [14]. In the present study, we focus on the in vivo effect of WLS. Male SD rats fed EG-added drinking water and a gastric gavage of WLS solution were used as experimental models. We analyzed the changes in body weight and the biochemical parameters of urine and serum. A polarized light microscope and a computer-assisted image scoring system were used to evaluate the severity of CaOx deposition in rat kidneys.

Materials and methods

Chromatographic fingerprint of Wulingsan

Commercial WLS powder was kindly provided by the Koda pharmaceutical company (Taichung, Taiwan). The WLS powder was examined and documented by the Medical and Pharmaceutical Industry Technology and Development Center (Taiwan); the HPLC fingerprint is shown in Fig. 1. Two grams of Wulingsan powder was extracted two times consecutively with 8 ml of 70% methanol. The extract was sonicated (15 min, 40°C, Cole-Parmer 8893), centrifuged (10,000g for 10 min, 25°C, HERMLE Z323K), and the supernatant was collected. Next, 70% methanol was added (final volume 20 ml) and the extract solution was filtrated (0.45 μm filter) and chromatographed by reversed phase high performance liquid chromatography (RP–HPLC). The HPLC system (Agilent 1100 series) consisted of a vacuum degresser (G1379A), a quaternary pump (G1311A), an autosampler (G1329A), a 37°C thermostated column compartment (G1316A), a diode array detector (G1315A), a LiChrospher 100 RP-18e (5 μm, MERCK), and a reverse-phase C18 column (Cosmosil 5C18-MS-II Waters 4.6 × 250 mm, 5 μm, NACALAI). A linear gradient of 0.1% H_3PO_4 and acetonitrile at a flow rate of 1 ml/min was used (0 min at 5% acetonitrile, reaching 48and 100% acetonitrile in 70 and 120 min, respectively, total 130 min). Chromatographed samples were monitored at UV 203 nm. The metal contents of WLS powder were analyzed; the result revealed Cd 0.237 ± 0.001 μg/g and Pb 1.658 ± 0.031 μg/g.

Preparation of Wulingsan for gastric feeding

Wulingsan formula is composed of five dried herbs: *Alisma orientalis* (Sam.) Juzep. (澤瀉), *Polyporus umbellatus* (Pers) Fries (豬苓), *Atractylodes macrocephala* Koidez (白朮), *Poria cocos* (Schw.) Wolf (茯苓), and

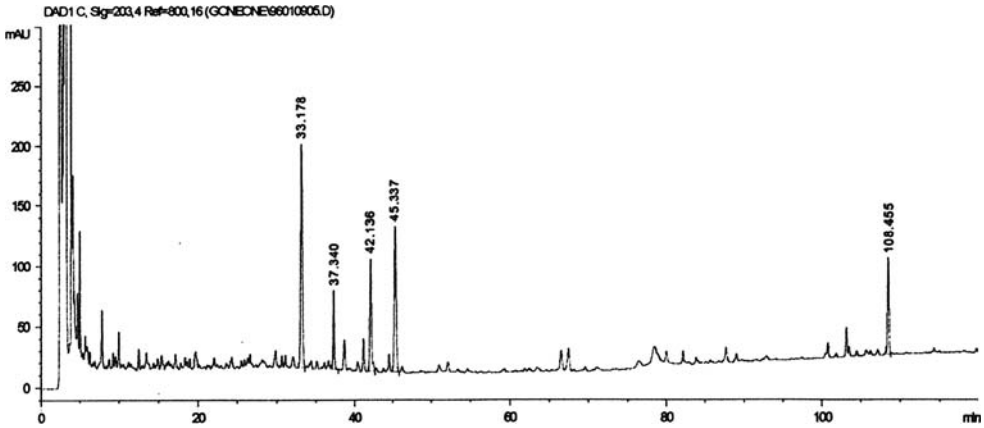

Fig. 1 Typical HPLC chromatogram profile of methanol extract from Wulingsan. Diode array detector set at absorbance UV 203 nm

Cinnamomon cassia Presl (桂枝), at a ratio of 4:3:3:3:2, respectively. The voucher specimens were identified by the manufacturer Koda pharmaceutical company (Taichung, Taiwan). In order to prepare a solution for gastric gavages, 100 g of finely powdered WLS and 500 ml of doubly distilled (dd) water were heated in an autoclave for 15 min at 121°C. After the boiling and sterilization procedure, the WLS powder turned into a jelly like paste. Next, the product was dissolved in dd water to reach a final volume of 750 ml. Then, the solution was stored at 4°C for 7 days. Subsequently, the solution was poured out and centrifuged at a rate of 1,500 rpm for 10 min. Finally, the concentration of WLS feeding solution was determined by the procedure below. One milliliter of supernatant was put into a tube and kept in an oven at 60°C overnight. The tube was weighed to measure the dry weight of WLS. Double distilled water was then added to the supernatant to arrive at a final concentration of 80 mg WLS powder per milliliter of solution. In other words, the feeding solution is containing 80 mg of WLS per milliliter.

Experimental animals and treatments

Forty-one male Sprague-Dawley rats, 4 weeks of age, weighing 180–225 g, were purchased from BioLASCO Co. (Taiwan). They were then acclimated to a room temperature of 22°C with a 12 h light, 12 h dark cycle, and housed in the animal breeding center of the China Medical University (Taiwan). This experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC, No: CMU 95-38-N).

All of the rats were fed standard commercial rat chow during the entire course of the study. They were randomly divided into four groups. Groups 2, 3 and 4 received 0.75% EG-added drinking water as a stone inducer throughout the entire experimental period. The WLS solution and starch were fed by gastric gavage.

Group 1 (control group, $n = 8$) was provided free access to food and normal drinking water. Group 2 (placebo group; $n = 11$ rats) received EG-added drinking water and fed with normal chow plus 1.5 ml starch. Group 3 (low dose WLS; $n = 11$ rats) received EG- added drinking water and fed with normal chow plus 1.5 ml (375 mg/kg) WLS. Group 4 (high dose WLS, $n = 11$ rats) received EG- added drinking water and fed with normal chow plus 4.5 ml (1,125 mg/kg) WLS. If used in mankind, the WLS powder is taken in 3–6 grams doses 1–3 times a day [13]. In our experiment, the high-dose group received a 10-fold dosage as the human does.

The experimental period was 4 weeks. At the end of the experiment, all rats were anesthetized by ether inhalation, blood samples were drawn from heart, and the rats were killed by carbon dioxide. Both kidneys were harvested and weighed. Right kidneys were fixed in formalin, embedded in paraffin and stained with hematoxylin and eosion solution. Histological sections of kidneys were examined with a polarized light microscope for the presence of crystal deposits.

Analysis of serum and urine

Twenty-four-hour urine samples were collected at the beginning and at the end of the experiment. Rats were kept in a metabolic cage individually for 24 h for urine collection. Serum specimens were obtained for biochemical analysis at baseline and at the fourth week. Blood was collected from the tail vein at the beginning of the experiment and from the heart at the end of the experiment. The following urine and serum risk parameters were measured using a Hitachi-7150 and a Roche-Omnic analyzer: calcium, free calcium, phosphorus, and pH value.

Evaluation of the severity of renal crystal deposition

A polarized light microscope was used to highlight the birefringent crystal of CaOx at a magnification factor of 100. The extent of crystal deposition was semi-quantitatively evaluated using a subjective scoring system graded

from 0 to 3+ (where 0 = none, 1+ = few, 2+ = several and 3+ = many crystal deposits) [15–17].

Computer-assisted image scoring system

In order to increase the objectivity of our scoring method, we designed software entitled *ImageScoring* to assist us (Fig. 2b). This software offers randomized and blinding selection of digital images spontaneously. We used the *Borland Delphi*® version 6.0 (Borland software Co., CA) programming language to compose the program, dBASEIII for data structure, and the jpeg format for image files storage.

Digital images of all of the pathological sections were stored in a database. Before doing so, a sagittal section of each renal specimen was divided into eight equal-sized regions by four virtual lines (Fig. 2a). We then randomly took a digital image of one of the eight regions under a polarized light microscope (100×) using a digital camera mounted onto the microscope. Each picture was stored as a JPEG image with a resolution of 4,080 × 3,072 pixels. A total of 328 image files, representing all of the kidney sections of 41 rats, were stored and saved to a DVD. After all of the images had been captured and stored, six independent coworkers used this computer-assisted system to score every individual picture in the database. The program randomly selects and displays an image allowing the user to make a subjective score. This randomized image selection and scoring process continued until all the image files had been selected and scored. Next, these scores were saved as dBASEIII data type, and then recorded on the user's personal score files, titled with the user's name plus the date-time stamp to avoid mismatching the results. All of the coworkers worked independently of one another and all were blinded to the results obtained by their counterparts. Finally, all of the personal score files were collected, merged, and sent for statistical analysis.

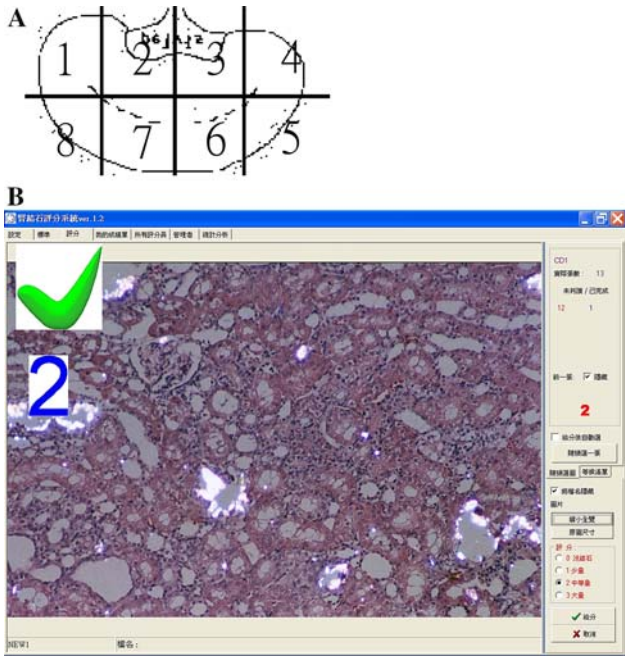

Fig. 2 **a** Each sagittal section of rat kidneys was divided into eight regions by four virtual lines. We randomly took a picture from each region under a polarized light microscope with magnification of 100; then, this image was stored as a digital image file for the scoring work-up later. **b** A snapshot of computer assisted image scoring system, the *"ImageScoring"*

Statistical methods

The data regarding body weights and parameters of urine and serum are expressed as mean ± SD, and were analyzed using the one-way ANOVA test. The severity of renal crystal deposition was expressed on an ordinal scale using the semi-quantitative scoring method described above. Statistical analysis of these crystal deposit scores was performed by the non-parametric Kruskal–Wallis test for inter-group comparison, and by the Wilcoxon rank sum test (Mann–Whitney *U*) for pair wise comparison. A P value of <0.05 denotes the presence of statistical significance. The software used for analysis was *the Statistical Package for the Social Science* (SPSS® for Windows, release 15.0, SPSS Inc., Chicago, IL).

Results

There were no differences in baseline biochemical data among the four groups (Table 1). No significant differences in urine phosphorus, urine free calcium, urine pH value, serum calcium or serum phosphorous at the beginning of the study were noted between the groups. After the experiment, rats in group 2 (EG + starch) gained the least significant body weight ($P = 0.007$). During the entire course, the body weights in placebo group only increased 130.63 ± 23.06 g compared with the normal rats, which had increased 179.63 ± 13.41 g (Table 2). In contrast, groups 3 and 4 (EG + WLS) could effectively reverse this body weight lost effect, the average increase were 158.13 g (group 3) and 156.25 g (group 4) compared with the limited increase in body weight in the placebo group (130.63 g).

There were no significant differences in kidney weights among the four groups.

Table 3 shows the effect of WLS on urine and serum biochemical data. After the experiment, the placebo group exhibited lower serum phosphorus (9.45 ± 2.26) than normal rats (12.54 ± 1.25; $P = 0.015$). Groups 3 and 4, which received low or high dosages of WLS, exhibited significantly lower serum free calcium (group 3, 0.91 ± 0.07 and

Table 1 Biochemical data of rats at the baseline

Baseline data	Group 1 Normal control	Group 2 EG + starch (placebo)	Group 3 EG + WLS (low dose)	Group 4 EG + WLS (high dose)
Urine phosphorus (mg/dl)	22.16 ± 0.84	22.64 ± 0.40	21.90 ± 0.45	22.07 ± 0.60
Urine free calcium (mmol/L)	0.79 ± 0.39	1.05 ± 0.40	0.84 ± 0.24	0.62 ± 0.31
Urine pH	6.48 ± 0.53	6.50 ± 0.24	6.50 ± 0.14	6.53 ± 0.45
Serum calcium (mg/dl)	6.92 ± 1.41	6.88 ± 1.14	7.01 ± 1.12	6.73 ± 3.32
Serum phosphorus (mg/dl)	21.96 ± 2.08	21.43 ± 2.03	22.26 ± 5.40	20.77 ± 1.46

Values are expressed as mean ± SD. Statistical method is one-way ANOVA test

The biochemical data at the baseline revealed that there were no significant differences between these four groups

EG ethylene glycol, *WLS* Wulingsan

Table 2 Effect of Wulingsan on the body weight, kidney weight of rats

	Group 1 Normal control	Group 2 EG + starch (placebo)	Group 3 EG + WLS (low dose)	Group 4 EG + WLS (high dose)
Body-weight-gain (gm)	179.63 ± 13.41	130.63 ± 23.06 [a]*	158.13 ± 30.58	156.25 ± 37.77
Kidney weight, left (gm)	1.83 ± 0.34	1.83 ± 0.33	1.64 ± 0.47	1.68 ± 0.07
Kidney weight, right (gm)	1.91 ± 0.50	1.74 ± 0.21	1.73 ± 0.49	1.66 ± 0.18

Values are expressed as mean ± SD. Statistical method is one-way ANOVA test

EG ethylene glycol, *WLS* Wulingsan

Body-weight-gain is computed as the final body weight of each rat minus the baseline body weight of same rat

Group 2 rats gained least body weight significantly, on the contrast, group 3 and 4 which received WLS can effectively reverse this body weight lost effect

[a] Values are revealed statistical significance in compared with normal group * $P < 0.05$

Table 3 Effect of Wulingsan on urine and serum biochemical data in rats

	Group 1 Normal control	Group 2 EG + starch (placebo)	Group 3 EG + WLS (low dose)	Group 4 EG + WLS (high dose)
Urine phosphorus (mg/dl)	20.98 ± 1.12	19.52 ± 0.68	20.40 ± 0.82	19.36 ± 1.59[a]*
Urine free calcium (mmol/L)	0.35 ± 0.21	0.19 ± 0.06	0.40 ± 0.12	0.28 ± 0.08
Urine pH	6.92 ± 0.45	6.74 ± 0.32	6.46 ± 0.36	6.57 ± 0.12
Serum calcium (mg/dl)	11.24 ± 0.26	10.69 ± 0.59	11.11 ± 0.92	10.69 ± 0.59
Serum phosphorus (mg/dl)	12.54 ± 1.25	9.45 ± 2.26[a]*	10.84 ± 1.68	11.10 ± 1.66
Serum free calcium (mmol/L)	1.09 ± 0.07	1.10 ± 0.06	0.91 ± 0.07[a]*[b]*	0.94 ± 0.17[a]*[b]*
Serum pH	7.11 ± 0.16	7.13 ± 0.09	6.96 ± 0.07[b]*	6.96 ± 0.17[b]*

Values are expressed as Mean ± SD. Statistical method is one-way ANOVA test

EG ethylene glycol, *WLS* Wulingsan

[a] Values are significantly different compared to normal group * $P < 0.05$

[b] Values are significantly different compared to placebo group * $P < 0.05$

group 4, 0.94 ± 0.17; $P = 0.001$), and lower serum pH values (group 3, 6.96 ± 0.07 and group 4, 6.96 ± 0.17; $P = 0.006$) than the placebo group. Urine phosphorus decreased significantly in the high-dose WLS group (19.36 ± 2.59; $P = 0.021$).

In addition, urine free calcium, urine pH value and serum calcium were similar among the four groups. It is worthwhile to mention that the urine free calcium in the placebo group (0.19 ± 0.06) was lower than in the other groups; however, the difference was not significant ($P = 0.059$).

Histological studies revealed that there was no calcification in the kidneys of normal control rats. As expected, several crystal deposits were found in the renal cortex and

medulla of EG-fed rats (groups 2, 3, and 4). Under polarized light microscopy, the crystal exhibited a birefringent appearance; an example is shown in Fig. 3a, in which multiple crystals were found both in the renal cortex and medulla. Pathological changes were also noticed, including intratubular crystals, collecting duct crystal plugs, tubular epithelial damage, interstitial inflammation and crystals being digested by giant cells (Fig. 3 b, c, d). Analysis of the crystal deposit scores revealed that the scores of the two WLS-fed groups were significantly lower than the placebo group. Namely, the score dropped from 1.40 to 0.46 in group 3 and from 1.40 to 0.45 in group 4 (Mann–Whitney U test, $P = 0.019$ and $P = 0.035$) (Table 4). Overall, WLS seemed to have inhibited calcium oxalate crystal deposition in the kidneys of rats (Kruskal–Wallis test, $P = 0.034$). The stone incidence, computed as the number of rats that had renal crystals divided by the number of rats in that group, also declined from 90.9 to 81.8% in the low-dose group and from 90.9 to 72.7% in the high-dose group (Table 5). The relative risk, compared with the placebo group respectively, also dropped from 1 to 0.90 in group 3 and from 1 to 0.80 in group 4.

Discussion

Ethylene glycol is an effective method for inducing calcium oxalate renal crystals in rats, and our present study successfully induced crystal formation using this model [16–18]. EG is broken down in vivo into four organic acids: glycoaldehyde, glycolic acid, glyoxylic acid and oxalic acid; in addition, oxalate precipitates as calcium oxalate crystals in the kidneys [19]. But, this model has been criticized because of the side effect of metabolic acidosis induced by EG. However, Green et al. [20] stated that metabolic acidosis does not always if renal function is preserved. Our data indicate that rats that received EG-added water encountered some growth retardation due to a slowdown in body weight gain (Table 2). Interestingly, WLS exhibited a protective effect on body weight gain in groups 3 and 4 as well as in the normal group. The WLS-fed rats (groups 3 and 4) exhibited lower serum free calcium and lower serum pH values than rats in the placebo group. Although the serum pH values slightly decreased, rats in the WLS groups (groups 3 and 4) had weight gains similar to those in

Table 4 Analysis of crystal deposit scores in experimental rats

Group	Number of rats	Treatment	Mean of scores	P value Mann–Whitney U test
Group 2	11	EG + starch	1.40 ± 1.20	
Group 3	11	EG + WLS (low dose)	0.46 ± 0.72[a]*	0.019[a]*
Group 4	11	EG + WLS (high dose)	0.45 ± 0.88[a]*	0.035[a]*

Values are expressed as mean ± SD

Kruskal–Wallis test for comparison of three groups, $P = 0.034$

* $P < 0.05$

[a] Values are significantly different in comparison with placebo group (group 2) by Mann–Whitney U test

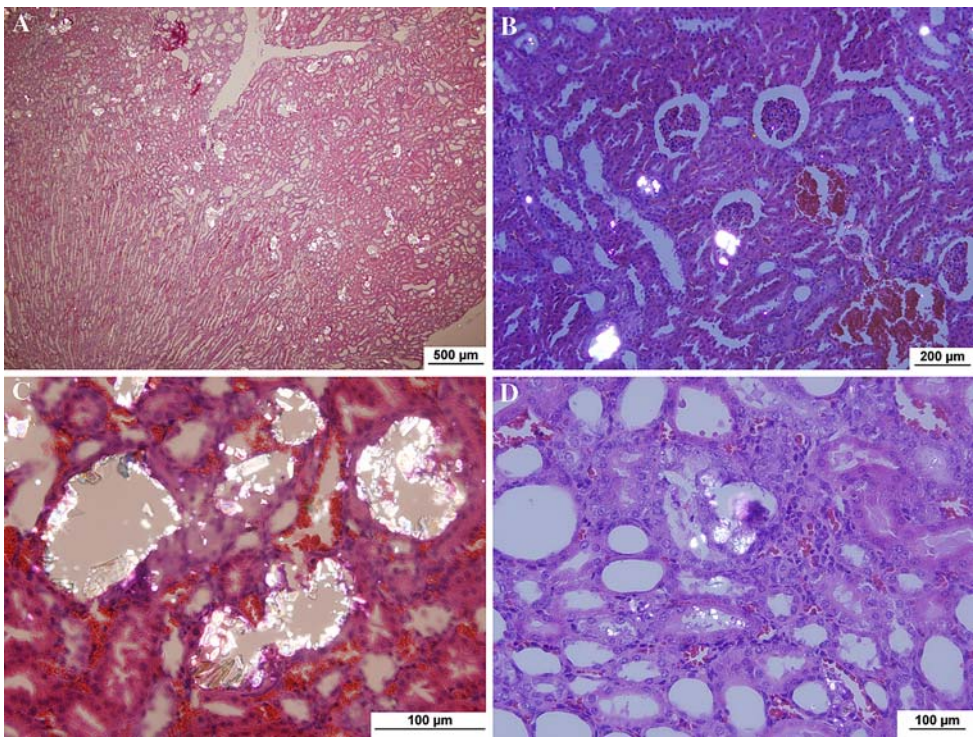

Fig. 3 (H&E stain) Histological appearance of rat kidneys under polarized light microscopy. **a** Multiple CaOx crystals with birefringent appearances were noted both in the renal cortex and medulla. Magnification: ×40. **b** Crystals plug the proximal renal tubule (*arrow*). Magnification: ×100. **c** Damages of renal tubule epithelial appeared as thin and destructed lining cells. Magnification: ×400. **d** Foreign body reaction with interstitial inflammation and giant cells digestion of crystals. Magnification: ×200

Table 5 Incidence of crystal formation in rats kidney

Group	Treatment	Rat number	Number of urolithic rats	Number of rats without stone	Incidence rate of crystal formation (%)	Relative risk
1. Normal	dd water, normal chow	8	0	8	0	
2. Placebo	EG water, starch gavage	11	10	1	90.9	1
3. Low dose	EG water, low dose WLS gavage	11	9	2	81.8	0.90
4. High dose	EG water, high dose WLS gavage	11	8	3	72.7	0.80

The relative risk is compared to the placebo group, respectively

normal rats (Tables 2, 3). Whether WLS affected metabolism is unclear. Not enough data were obtained to be able to clarify the mechanism of Wulingsan's effect on serum pH and renal function.

Low levels of free calcium in urine were noted in placebo rats; this could be because most of the urine calcium had bound with oxalate to form crystals. Therefore, very little free calcium was detected. On the other hand, the free urine calcium levels in the WLS groups were still within the normal range, indicating that fewer crystals had formed.

The software (*ImageScoring*) we created has proved to be a helpful tool because it can easily reduce the time-consuming process of reviewing a multitude of images. Furthermore, the automatically randomized selection process diminishes the interference between different coworkers and offers a reliable method for blindness. Moreover, the format of scoring files is compatible with the statistical package; thus no data transfer is needed, which prevents the possibility of transfer errors.

To the best of author's knowledge, the antilithic mechanism of WLS is unclear. Some studies have suggested that macromolecules may be involved in the antilithic mechanism of WLS. Many medicinal plants are used to treat urolithiasis, WLS is one of those [21–24]. Two of the ingredients of WLS, *Alisma orientalis* and *Poria cocos* Wolf, have been shown to inhibit the stone formation process [25–30]. Suzuki et al. [26] reported that *Alisma orientalis* (also known as Takusha) strongly suppressed each step of crystal formation, growth and aggregation of CaOx crystals, in vitro. Yin et al. [27] reported that *Alisma* inhibited the growth and aggregation of CaOx crystals in vitro; furthermore, the renal calcium content decreased in *Alisma*-treated rats. Wang et al. [28] also stated that *Alisma* could downward regulate mRNA expression of OPN in a glyoxylic acid-induced renal stone rat model. In addition, several studies have shown that this herb significantly decreased the formation of CaOx deposits, and downregulated the expression of inter-alpha-trypsin inhibitor, bikunin and osteopontin [10, 25, 31]. The extract of *Poria cocos* Wolf, a natural diuretic agent, can effectively treat original-type anti-GBM nephritis in rats; Hattori et al. [12] stated that this mechanism might be due to the inhibition of C3 deposition in the glomeruli. In addition to reducing CaOx crystals, WLS also suppressed the development of hydroxylapatite renal calcinosis in rats fed a high phosphorus diet [11]. An in vitro study noted that WLS significantly inhibited CaOx crystallization in human urine; our previous study also concluded that WLS effectively inhibits the process of CaOx nucleation, crystallization and aggregation [14, 30]. All of these reports suggest that WLS may be a useful drug for preventing renal stones.

Using modern molecular techniques, researchers have identified many macromolecules that act as modulators of stone formation, such as osteopontin (OPN), Tamm-Horsfall protein, urinary prothrombin fragment-1, and bikunin [32, 33]. The most well known is the osteopontin. In one study, in vivo evidence has revealed that the oxalate-induced upregulation of OPN is most likely mediated via the renal renin-angiotensin system, where the kidneys of hyperoxaluric rats on angiontensin II type I receptor blocker (ARB) had fewer CaOx crystals, fewer ED-1-positive cells, and reduced expression of OPN mRNA [34, 35]. *Alisma*, one ingredient of WLS, has also been shown to reduce crystal deposition via the down-regulation of OPN [28]. Proteins may either bond with surface calcium atoms to prevent crystal growth or prevent small particles from aggregating into larger ones [36]. Overall, researches of proteins contributing to the pathogenesis of stone formation are presently of great interest.

In conclusion, we successfully induced renal CaOx crystallization in an EG rat model. Moreover, Wulingsan significantly reduced the severity of CaOx crystal deposition in rat kidney, indicating that this formula is an effective antilithic medicine. Although the mechanism of WLS on crystal inhibition is still unclear, it may involve urinary macromolecules.

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References

1. http://210.69.214.131:/webdata/AttachFiles/Attach_8327_2_94_css.pdf
2. http://210.69.214.131:/webdata/AttachFiles/Attach_2493_1_2-2-4.pdf
3. Ho SZ, Kuo HC (2002) Pathogenesis and epidemiology of urolithiasis. Tzu Chin Med J 14:337–346

4. Menon M, Resnick MI (2002) Urinary lithiasis: etiology, diagnosis, and medical management. In: Walsh PC, Retik AB, Vaughan ED Jr., Wein AJ (eds) Campbell's urology. 8th ed. WB Saunders, Philadelphia
5. Lee YH, Huang WC, Tsai JY (2002) Epidemiological studies on the prevalence of upper urinary calculi in Taiwan. Urol Int 68:172–177
6. Kim HH, Jo MK, Kwak C, Park SK, Yoo KY, Kang D, Lee C (2002) Prevalence and epidemiologic characteristics of urolithiasis in Seoul, Korea. Urology 59:517–521
7. Yoshida O, Terai A, Ohkawa T, Okada Y (1999) National trend of the incidence of urolithiasis in Japan from 1965 to 1995. Kidney Int 56:1899–1904
8. http://en.wikipedia.org/wiki/Taiwan#Environment_and_pollution
9. http://en.wikipedia.org/wiki/List_of_countries_by_population_density#_note-cia
10. Cao ZG, Liu JH, Zhou SW (2004) Effect of alisma orientalis extract on renal stone formation and the expression of inter-alpha-trypsin inhibitor in rat urolithiasis model. Chin J Exp Surg 21:295–297
11. Liu QL, Sato S, Kishikawa T (2001) Effectiveness of a traditional Chinese medicine, Wulingsan, in suppressing the development of nephrocalcinosis induced by a high phosphorus diet in young rats. Med Electron Microsc 34: 103–114
12. Hattori T, Hayashi K, Nagao T (1992) Studies on antinephritic effects of plant components(3): effect of pachyman, a main component of *Poria cocos* Wolf on original-type anti-GBM nephritis in rats and its mechanisms. Jpn J Pharmacol 59:89–96
13. Bensky D, Barolet R (eds) (1990) Chinese herbal medicine: Formulas & Strategies. Eastland, Seatle, Washington
14. Chen YC, Ho CY, Chen LD, Hsu SF, Chen WC (2007) Wu-Ling-San formula inhibits the crystallization of calcium oxalate in vitro. Am J Chin Med 35:533–541
15. Nelde HJ, Bichler KH, Strohmaier WL (1988) Nephrocalcinosis in the kidney of the rat on atherogenic diet and the efffect of calcium antagonists (nifedipine). In: Bichler KN, Strohmaier WL (eds) Nephrocalcinosis calcium antagonists and kidney, Springer-Verlag, Berlin pp 113–125
16. Lee YH, Chang LS, Chen MT (1991) Characterization of ethylene glycol induced urolithiasis modle in rats. J Urol ROC 2:518–524
17. Lee YH, Huang WC, Chiang H (1992) Determinant role of testosterone in the pathogenesis of urolithiasis in rats. J Urol 147:1134–1138
18. Lee YH, Tsai JY, Huang JK (2000) Combined use of 30% lactose rich diet and 1% ethylene glycol: a new animal model for study of urolithiasis. J Urol ROC 11:149–154
19. Leth PM, Gregersen M (2005) Ethylene glycol poisoing. Forensic Sci Int 155:179–184
20. Green ML, Hatch M, Freel RW (2005) Ethylene glycol induces hyperoxaluria without metabolic acidosis in rats. Am J Physiol Renal Physiol 289:F536–F543
21. Selvam R, Kalaiselvi P, Govindaraj A (2001) Effect of *A. Lanata* leaf extract and *Vediuppu chunnam* on the urinary risk factors of calcium oxalate urolithiasis during experimental hyperoxaluria. Pharmacol Res 43:89–92
22. Das I, Gupta SK, Ansari SA (2005) In vitro inhibition and dissolution of calcium oxalate by edible plant *Trianthema monogyna* and pulse *Macrotyloma uniflorum* extracts. J Cryst Growth 273:546–554
23. Viel TA, Domingos CD, Monteriro APS (1999) Evaluation of the antiurolithiatic activity of the extract of *Costus spiralis* Roscoe in rats. J Ethnopharmacol 66:193–198
24. Gohel MDI, Wong SP (2006) Chinese herbal medicines and their efficacy in treating renal stones. Urol Res 34:365–372
25. Cao ZG, Liu JH, Zhou SW (2004) The effect of the active constituents of *Alisma orientalis* on renal stone formation and bikunin expression in rat urolithiasis model. Matl Med J China 84:1276–1279
26. Suzuki K, Kawamura K, Tsugawa R (1999) Formation and growth inhibition of calcium oxalate crystals by Takusha (*Alismatis rhizoma*). Scanning Microsc 13:183–189
27. Yin CP, Liu JH, Zhang YS (1997) Effects of *Alisma orientalis* Juzep on calcium oxalate crystallization in vitro and calcium oxalate renal stone in rats. Acta Univ Med Tongji 26:99–101
28. Wang SY, Deng CQ, Shi ZL (2003) Influcence of *Rhizoma alismatis* on inhibition of renal stone formation. J Guangzhou Univ Tradit Chin Med 20:294–296
29. Yasui T, Fujita K, Sato M (1999) The effect of takusha, a kampo medicine, on renal stone formation and osteopontin expression in a rat urolithiasis model. Urol Res 27:194–199
30. Yashimura K, Miake O, Okuyama A (1998) Effect of chorei-to and gorei-san on calcium oxalate crystallization in human urine. Hinyokika Kiyo 44:13–16
31. Wesson JA, Johnson RJ, Mazzali M (2003) Osteopontin is a critical inhibitor of Calcium oxalate crystal formation and retension in renal tubules. J Am Soc Nephrol 14:139–147
32. Grover PK, Dogra SC, Davidson BP (2000) The prothrombin gene is expressed in the rat kidney: implications for urolithiasis research. Eur J Biochem 267:61–67
33. Kumar V, Lieske JC (2006) Protein regulation of intrarenal crystallization. Curr Opin Nephrol Hypertens 15:374–380
34. Umekawa T, Chegini N, Khan SR (2003) Increase expression of monocyte chemoattractant protein-1(MCP-1) by renal epithelial cells in culture on exposure to calcium oxalate, phosphate and uric acid crystals. Nephrol Dial Transplant 18:664–669
35. Umekawa T, Hatanaka Y, Kurita T (2004) Effect of angiotensinII receptor blocakge on osteoponton expression and calcium oxalate crystal deposition in rat kidneys. J Am Soc Nephrol 15:653–644
36. Coe FC, Evan A, Worcester E (2005) Kidney stone disease. J Clin Invest 115:2598–2608