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Effectiveness of a traditional Chinese medicine, Wulingsan, in suppressing the development of nephrocalcinosis induced by a high phosphorus diet in young rats

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Abstract The development of nephrocalcinosis in rats fed a high phosphorus diet, and the effectiveness of the Chinese traditional medicine, Wulingsan, and its components (Poria, Alismatis Rhizoma, Atractylodis Rhizoma, Cinnamomi Ramus, Polyporus) in suppressing the development of calcinosis were studied. The rats were fed a high phosphorus diet (1.5% P) supplemented with Wulingsan or its individual components (0.5 g/kg body weight) as separate experimental groups for a 2-week period. Upon histological observation by light microscopy and electron microscopy, signs of nephrocalcinosis were observed in almost all areas of the kidney. Calculi, consisting mainly of needle-shaped crystals of hydroxyapatite, were observed in the proximal tubules, in the collecting ductal lumina, and in the mitochondria of the proximal tubular cells and the interstitial cells. X-ray microanalysis revealed that the calculi were composed of hydroxyapatite (Ca and P). In the group fed the diet supplemented with Wulingsan, the severity of calcinosis in the corticomedullary junction was only slight. In all groups fed individual components of Wulingsan, the severity of calcinosis was almost the same as that in the group fed the high phosphorus diet (1.5% P). Wulingsan suppressed the development of calcinosis in rats fed the high phosphorus diet supplemented with this Chinese medicine, whereas its individual components alone had no effect. The process of calcinosis and the mechanism responsible for the activity of this Chinese medicine in the suppression of calcinosis are discussed.

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Introduction

Nephrocalcinosis is generally observed as bilateral diffuse calcification deposits in the renal parenchyma, and is demonstrable by X-ray examination. In nephrocalcinosis, renal function is disturbed, and it is usually associated with hyperchloremic acidosis.[1] Clinically, nephrocalcinosis is reported to develop in children with X-linked familial hypophosphatemic rickets when they have been treated with phosphate or vitamin D[2,3] administered orally. In other patients, nephrocalcinosis occurs as a secondary effect of hyperparathyroidism,[4,5] endstage kidney disease,[6] chronic glomerulonephritis,[7] hypervitaminosis or milk-alkali syndrome,[8,9] sarcoidosis,[10] idiopathic hypercalcemia in infancy,[11] sulfonamide toxicity,[1] spongy kidney,[12] toxicity affecting the thyroid (tertroxin),[13] bone tumors,[14] or primary hyperoxaluria.[15]

In experiments using rats, nephrocalcinosis has been found to be induced by sodium phosphate,[16,17] calcium gluconate,[18] vitamin D_2,[19] acetazolamide,[20] parathyroid hormone,[21] oxamide,[15] magnesium,[22–25] and magnesium and vitamin B_6.[26] In these studies of nephrocalcinosis, the biochemical and histochemical changes[26–29] have been well characterized, whereas the ultrastructural changes and the mechanism of progression of this disease have not been much studied.[18,30–32] There have been few studies on the prevention of nephrocalcinosis, except for those in which the development of nephrocalcinosis induced by a high phosphorus diet was suppressed by magnesium intake.[33] In uremic patients, the degree of calcification was less in those undergoing dialysis, than in those not undergoing dialysis.[6]

In the present study, the ultrastructural changes in rats with nephrocalcinosis induced by a high phosphorus diet were studied by electron microscopy to assess the mechanism of disease progression. In addition, experiments were

performed to assess whether Wulingsan, a Chinese medicine widely used for the treatment of nephrosis, dropsy, and uremia, was effective in suppressing the development of nephrocalcinosis induced by the high phosphorus diet. The effectiveness of its individual components (Poria, Alismatis Rhizoma, Atractylodis Rhizoma, Cinnamomi Ramus, Polyporus) was examined as well.

Materials and methods

All experiments were performed in accordance with the Nippon Medical School Animal Ethics Committee regulations and recommendations.

Preparation of diets

The high phosphorus diet was prepared as described by Matsuzaki et al.,[28] by slight modification of the American Institute of Nutrition (AIN)-76 diet. The concentration of phosphorus in the experimental diets was adjusted to 0.5% (normal phosphorus diet) or 1.5% (high-phosphorus diet), using potassium tripolyphosphate (Nacalai Tesque, Kyoto, Japan). The concentrations of calcium and magnesium in the two experimental diets were adjusted to 0.5% and 0.05%, using calcium carbonate (Kanto Chemical, Tokyo, Japan) and magnesium oxide (Wako Pure Chemical Industries, Osaka, Japan), respectively. The mineral mixture used was a modification of the AIN-76 mineral mixture,[34] without sources of calcium, magnesium and phosphorus, while the AIN-76A vitamin mixture[35] was used intact. The purified diets were stored at 4°C until use.

Chinese medicine

The components of the Chinese medicine tested were purchased as powders from Peking Tong Rei Tang (Beijing, China) Poria was from Hubei, China; Alismatis Rhizoma was from Fujian, China; Atractylodis Rhizoma was from Zhejiang, China; Cinnamomi Ramus was from Guangxi, China; and Polyporus was from Shanxi, China.

Wulingsan was prepared by mixing the components in the same proportion as that of commercial Wulingsan: Poria, 4.5; Alismatis Rhizoma, 6.0; Atractylodis Rhizoma, 4.5; Cinnamomi Ramus, 2.0; and Polyporus, 4.5. Each component or Wulingsan was added to the high phosphorus diet described above at a final concentration of 0.25%. The dose of Wulingsan and that of each of the individual components was set at 0.5 g/kg body weight, in consideration of the usual dose for humans (0.1 g/kg) and the dynamics of metabolism in rats.

Animals and experimental design

Forty-two male Wistar rats (5 weeks old, body weight around 100 g; Clea Japan, Tokyo, Japan) were individually housed in stainless-steel cages. During the experiment, the cages were located in a room with controlled lighting, on a 12-h light (0800–2000 h): dark (2000 h–0800 h) cycle, at a temperature of 22 ± 1°C and a relative humidity of 60%–65%.

All of the rats were given free access to a normal phosphorus diet and demineralized water for a 1-week pre-experimental period before initiation of the study. After the pre-experimental period, the rats were divided into seven experimental groups (six rats/group) at random. Each group was assigned one of the experimental diets supplemented with Chinese medicine (Wulingsan, Poria, Alismatis Rhizoma, Atractylodis Rhizoma, Cinnamomi Ramus, Polyporus) or the diet without Chinese medicine (control group). Rats fed the diet with a normal level of phosphorus were given an amount of food equivalent to that consumed by the rats fed the high-phosphorus diet throughout the 14-day period of the experiment. The rats were given free access to demineralized water throughout the experiment. Food intake and body weight were recorded daily.

The rats were killed under ether anesthesia and the kidneys were removed for histological analysis.

Histological examination of the kidney

Light microscopy

Immediately after collection and decapsulation, the kidney was fixed in a 10% neutral formalin phosphate buffer. The tissue samples were embedded in paraffin wax and cut into sections 2-μm-thick; the sections were stained with hematoxylin-eosin. Calcium deposits in tissues were demonstrated by von Kossa's reaction.[36]

Electron microscopy

After decapsulation, the tissue was cut into 1-mm cubes, fixed in 2.5% glutaraldehyde, and postfixed in 1% osmium tetroxide. The tissue samples were dehydrated through a graded alcohol series and embedded in Epok 812. Ultrathin sections (60-nm) were cut with a diamond knife and stained with uranyl acetate and lead citrate. The sections were examined in a Hitachi H-800 (75 kV) transmission electron microscope (Hitachi, Tokyo, Japan).

X-ray microprobe analysis

Ultrathin sections were cut, placed upon nickel grids and examined.

The elemental composition of needle-like crystal calcium deposits observed by transmission electron microscope (TEM) was examined by energy dispersion X-ray microanalysis;[37] the TEM used was the EDX (JEOL Oxford, Tokyo, Japan) with JEM 2010 Link ISIS. Mainly mitochondria and calcified spherules, the trilamellar bodies, were examined.

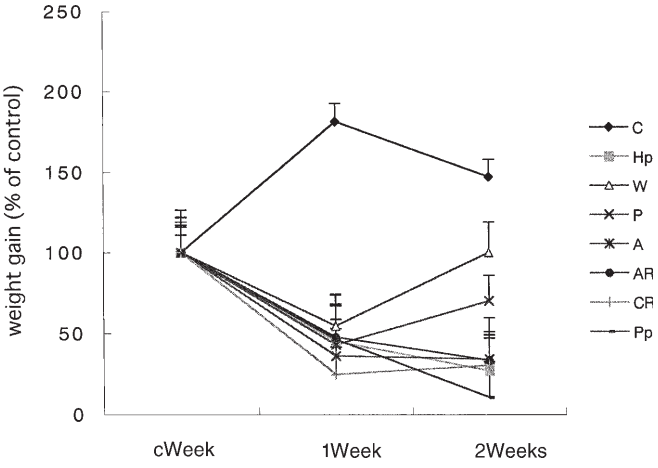


Fig. 1. Weight gain of rats fed the 0.5% or 1.5% phosphorus diet, and weight gain of rats fed the 1.5% phosphorus diet supplemented with traditional Chinese medicine. Values are means ± SE. *C*, control; *Hp*, high phosphorus (1.5% P); *W*, Wulingsan; *P*, Poria; *A*, Alismatis Rhizoma; *AR*, Atractylodis Rhizoma; *CR*, Cinnamomi Ramus; *Pp*, Polyporus

Results

Body weight

In the control group, the body weight increased during the course of the experiment, whereas in the high phosphorus diet group, and in each of the groups given the high-phosphorus diet plus an individual component of Wulingsan, the body weight decreased. In the group given the high-phosphorus diet plus Wulingsan, the body weight increased in a manner similar to that in the control group (Fig. 1).

Light microscopic observation of nephrocalcinosis

When tissue specimens were examined by von Kossa's staining, nephrocalcinosis was evident after 1 week of the high phosphorus diet. The calcinosis was observed mainly in the corticomedullary junction and not in the cortex. After 2 weeks of feeding with this diet, the calcinosis (calcium deposits) had developed in almost all areas of the cortex and the medulla (Fig. 2A). The glomerulus in the cortex appeared normal, without any calcinosis. In the proximal tubules, calcinosis (Fig. 2B) was conspicuously evident, whereas calcium deposits were not observed in the distal tubules. In the area of the corticomedullary junction, the results of calcium staining were positive, especially in the collecting tubules, where there were many particles. In the collecting tubules, some areas were destroyed, seemingly after the severe deposition of calcium phosphate (Fig. 2C). In addition, interstitial infiltration of these areas by inflammatory cells was observed.

Electron microscopic observations

After 2 weeks of the high phosphorus diet, the glomerulus appeared normal ultrastructurally and no increase in mesangium cells was evident. In the peripheral loops of the nephron, no hypertrophy of the basement membrane was observed, and there was no evidence of degradation of endothelial cells or epithelial cells (Fig. 3). In the proximal tubules, dilatation of the rough endoplasmic reticulum in epithelial cells was seen, and deposition of small hydroxyapatite-like needle-shaped crystals in mitochondria was evident (Fig. 4A insert). Calcified spherules, trilamellar bodies containing hydroxyapatite-like crystals, were observed in the proximal tubules (Fig. 4B). The same trilamellar bodies were observed in the collecting duct (Fig. 4C). The small hydroxyapatite-like needle-shaped crystals were also observed in the mitochondria of interstitial cells in the cortex (Fig. 4D). In the distal tubules, such deposits of needle-shaped crystals were not seen.

X-ray microprobe analysis

Two types of crystals were observed: one type was needle-shaped crystalline deposits, seen in the mitochondria of proximal epithelial cells (Fig. 5A), in interstitial cells, and in the inner and middle layers of the trilamellar bodies. The other type was large hydroxyapatite-like needle-shaped crystals, seen only in the outer layer of the trilamellar bodies (Fig. 5B).

In the control epithelial cells of renal tubules, no phosphorus or calcium was detected (Fig. 6A). In the mitochondria, aluminum and sulfur were also detected (Fig. 6B). In the high phosphorus diet group, calcium and phosphorus were found in both the larger (Fig. 6C) and smaller types of crystals (Fig. 6D) in the trilamellar bodies by X-ray microprobe analysis, and the Ca/P ratios of the signals were almost the same in all crystals; chlorine and sodium signals were also evident in the background. In the preparation of the material, at the second fixation, osmium tetraoxide (Os) was used, and at observation in X-ray microprobe analysis, nickel (Ni) grids were used. Therefore, in the charts, signals of Os and Ni appeared in the background. In the X-ray microprobe analysis, the signals of phosphorus and osmium tetraoxide appear in the same position. Therefore, the actual value of phosphorus could be smaller than the apparent value.

Effectiveness of Wulingsan and each of its components in suppressing the development of calcinosis

Wulingsan markedly suppressed the development of renal calcinosis, and only slight calcium deposits in the corticomedullary junction were evident (Fig. 7A). Electron microscopic observations showed RER dilatation and slight deposits of needle-shaped crystals in the mitochondria of epithelial cells in the proximal tubules after 2 weeks of feeding (Fig. 8; arrows). Necrosis of tubular epithelial cells and degeneration of microvilli in the proximal tubules were also observed. However, trilamellar bodies containing needle-shaped crystalline deposits, such as those observed in the group given the high phosphorus diet alone, were not observed.

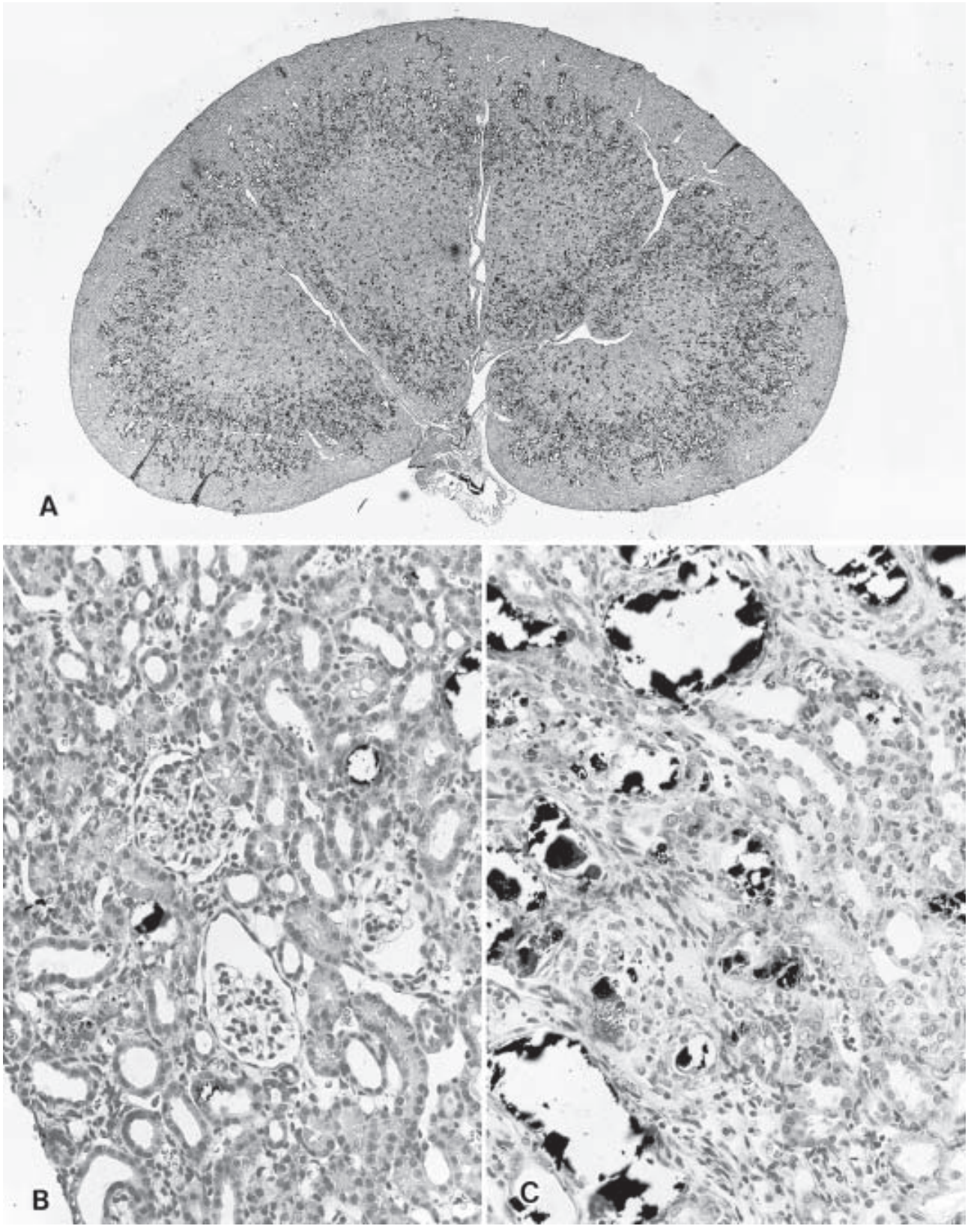


Fig. 2A–C. Light micrographs of a kidney from a rat fed the high phosphorus diet (1.5% P), 2 weeks after the start of feeding. **A** Nephrocalcinosis in the cortex, corticomedullary junction, and medulla. Severe calcinosis in the corticomedullary junction is evident (grade 4; see Table 1). von Kossa's stain, ×20. **B** Calcinosis in the proximal tubule. von Kossa's stain, ×200. **C** Calcinosis in a dilated collecting tubule of the corticomedullary junction, and inflammatory cell infiltration in the interstitium, are evident. von Kossa's stain, ×200

Poria slightly suppressed the development of calcinosis in the corticomedullary junction, compared with findings in the group given the high phosphorus diet alone (Fig. 7B). However, the other components, Alismatis Rhizoma, Atractylodis Rhizoma, Polyporus, and Cinnamomi Ramus, were not effective in suppressing nephrocalcinosis (Fig. 6C,D,E,F). Furthermore, the group given the diet supplemented with Cinnamomi Ramus showed more severe renal calcinosis than the group given the high phosphorus diet alone. The scores for these effects are shown in Table 1. The degree of nephrocalcinosis was evaluated as described in the report of Matsuzaki et al.[33]

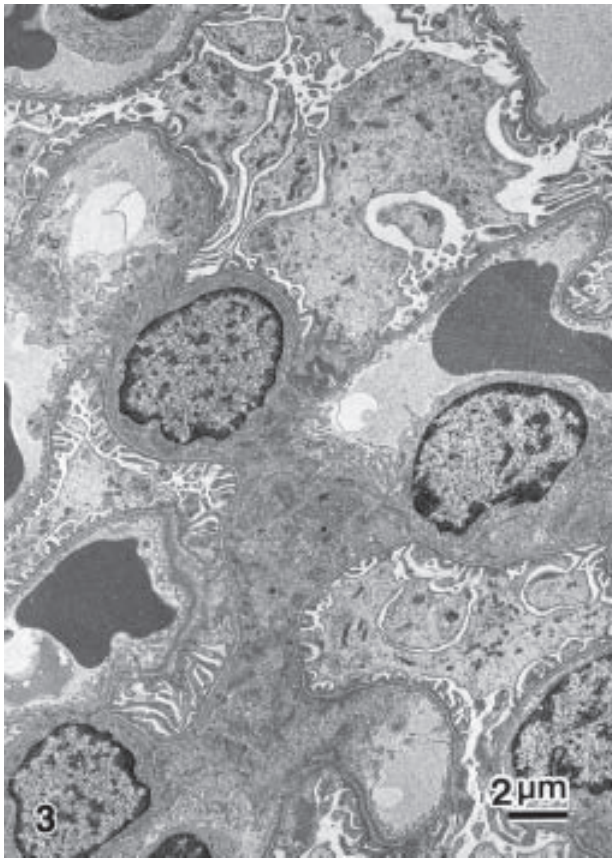


Fig. 3. Electron micrograph of the glomerulus of a kidney from a rat fed the 1.5% phosphorus diet, 2 weeks after the start of feeding. No damage is evident in the glomerulus

Discussion

Calcinosis induced by a high phosphorus diet

A high phosphorus diet has been reported to induce renal calcinosis, mainly in the collecting duct of the corticomedullary junction, early after the start of feeding.[30] In the present study, after rats had been fed a high phosphorus diet for 1 week, abundant calcium deposits were observed in the collecting duct of the corticomedullary junction. After 2 weeks of feeding, severe calcium deposits were observed by light microscopy, not only in the corticomedullary junction but also in the cortex and the medulla. The calcium deposits were hydroxyapatite-like needle-shaped crystals, which consisted of calcium and phosphorus, as determined by X-ray microprobe analysis. In a study in which 91 patients with nephrocalcinosis were examined, 75% of the patients had idiopathic hyperparathyroidism, hyperchloremic acidosis, or chronic pyelonephritis, and all had calcium deposits in the collecting duct in the renal medulla.[38] These calcium deposits were reported to consist of calcium and phosphorus.[39] In this context, the calcinosis in the rats fed the high phosphorus diet in the present study can be considered to serve as a disease model that shows the same pathological status as rephrocalcinosis in humans. In an animal model of hypervitaminosis D-related renal calcinosis associated with hyperparathyroidism, calcinosis was reported to occur in the basement membrane of Bowman's capsule and in the tubules in the renal cortex, whereas, in patients with hypoparathyroidism, calcinosis did not occur in the cortex, but in the collecting duct of the corticomedullary junction.[13] In the present study, in spite of the calcium deposits that occurred in the proximal tubules after the animals were fed the high phosphorus diet, the glomerulus remained normal. Matsuzaki

Table 1. Comparison of the effect of the high phosphorus (HP) diet alone and the effect of the HP diet supplemented with traditional Chinese medicine

	Time of feeding (weeks)	Degree of nephrocalcinosis[33]						
		HP	W	P	A	AR	Pp	CR
Cortex	2	+	−	−	+	+	+	++
Corticomedullary junction	2	++ +	+	++	++ +	++ +	++ +	++ +
Medulla	2	++	−	+	+	++	++	++

−, Grade 0, no nephrocalcinosis was evident (photomicrograph not shown in the Figs.); ±, grade 1, showing slight nephrocalcinosis; +, grade 2, showing mild nephrocalcinosis; ++, grade 3, showing moderate nephrocalcinosis; +++, grade 4, showing severe nephrocalcinosis. W, Wulingsan; P, Poria; A, Alismatis Rhizoma; AR, Atractylodis Rhizoma; Pp, Polyporus; CR, Cinnamomi Ramus

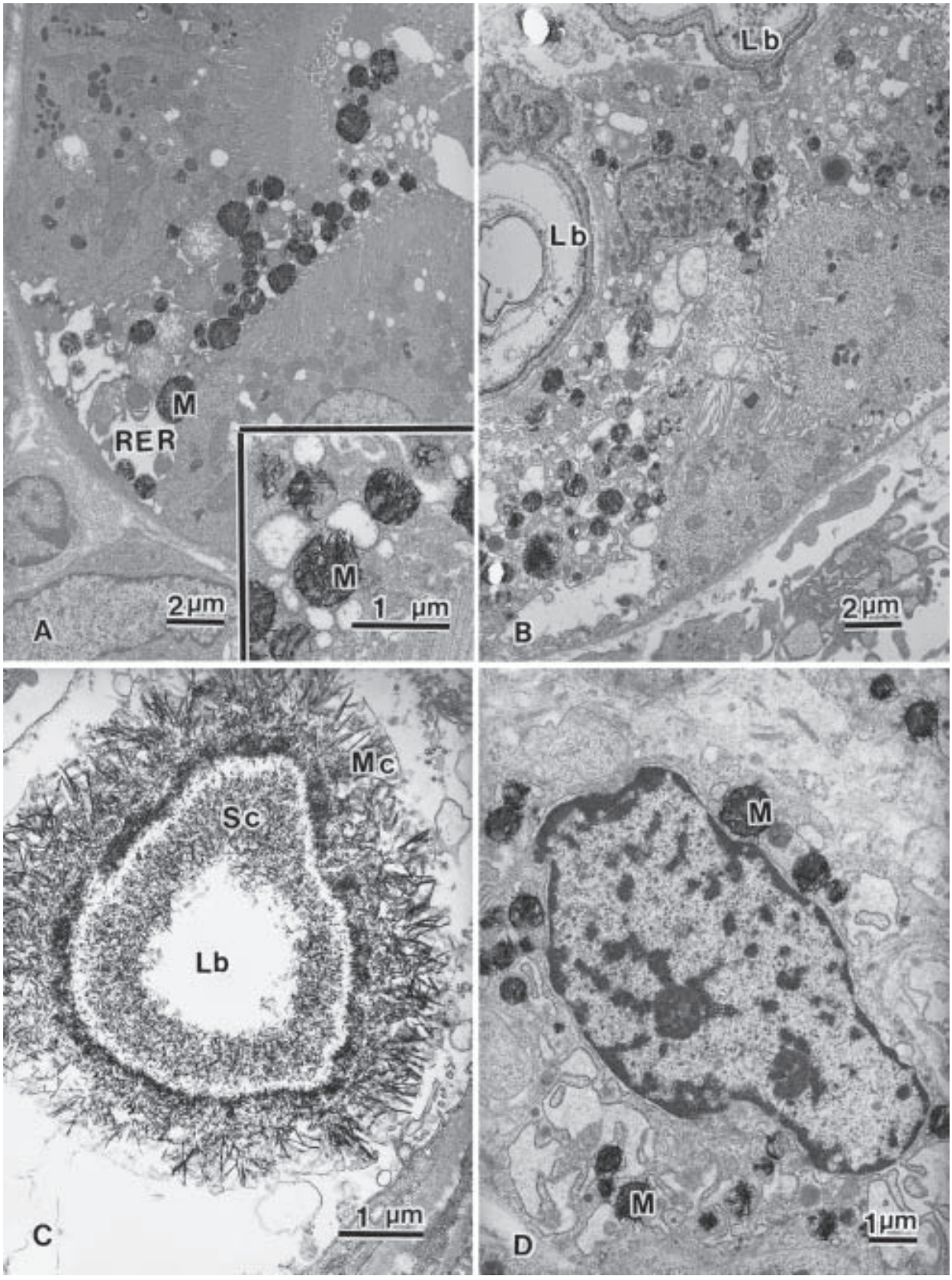


Fig. 4A–D. Electron micrographs showing calcinosis in a kidney from a rat fed the high phosphorus (1.5%) diet 2 weeks after the start of feeding. **A** Electron micrograph of a proximal tubule in a kidney from a rat fed the 1.5% phosphorus diet. Dilatation of the rough endoplasmic reticulum (*RER*) in the cells, and deposition of small needle-shaped hydroxyapatite crystals in the mitochondria (*M*) are evident (*insert*). **B** Calcified spherules, trilamellar bodies (*Lb*), are evident in the proximal tubule. **C** Trilamellar bodies with small needle-shaped crystals (*Sc*) and massive needle-shaped crystals (*Mc*) are evident in the collecting duct. **D** Deposition of small hydroxyapatite-like needle-shaped crystals in mitochondria (*M*) of interstitial cells in the cortex

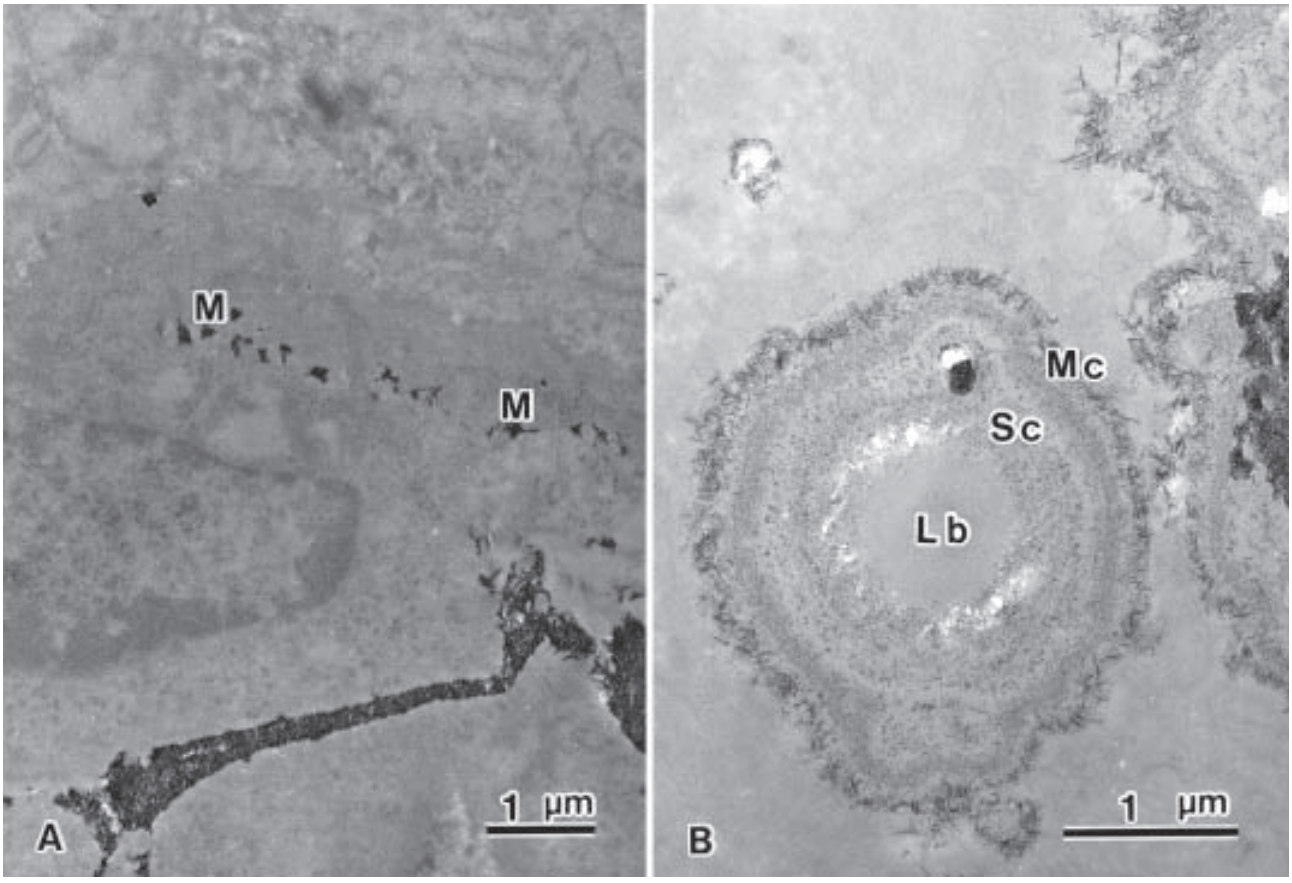


Fig. 5A,B. Micrographs of X-ray microanalysis. **A** Small crystals composed of hydroxyapatite, in the mitochondria (*M*) of a proximal tubule of a kidney from a rat fed a 1.5% phosphorus diet, 2 weeks after the start of feeding. **B** Trilamellar bodies (*Lb*) in a collecting duct of a kidney from a rat fed the 1.5% phosphorus diet, 2 weeks after the start of feeding. *Sc*, Small needle-shaped crystals; *Mc*, massive needle-shaped crystals

et al.[28] also reported that rats fed a high phosphorus diet had normal-appearing glomeruli in spite of an increase in urinary creatinine, which was attributable to impaired reabsorption in the proximal tubules. In their study, regeneration of microvilli and dilatation of the RER in epithelial cells of the proximal tubules were observed, but there was no damage in the distal tubules. Similarly, Matsuzaki et al.[29] observed no damage in the distal tubules.

Some reports have indicated a relationship between phosphorus and magnesium, in terms of the mechanisms by which calcinosis occurs upon intake of a high phosphorus diet. Magnesium plays a role in suppressing the development of calcinosis.[40] However, in rats fed a low magnesium diet, renal calcinosis and/or cell death has been observed.[41–43] Magnesium deficiency has been shown to cause a derangement in electrolyte transport[44] and mitochondrial dysfunction[43] in renal tubular epithelial cells. Furthermore, the intake of a high phosphorus diet has been found to cause a decrease in magnesium uptake in the intestine,[45–47] thereby resulting in a decrease in the concentration of magnesium in the urine.[48–51,43] The results of the present study suggest that rats given a high phosphorus diet might display the same status as patients with magnesium deficiency.

Phosphorus is reabsorbed with sodium via a cotransport system that is present in the brush-border membrane and in the apical vesicles in the proximal epithelial tubules.[52] In the present study, hydroxyapatite crystals were found in the mitochondria of proximal epithelial cells and interstitial cells. Mitochondria, which serve as storage organelles for heavy metals, may have accumulated excess calcium taken into the cells. However, hydroxyapatite crystals were rarely seen in the cells. Most of the hydroxyapatite was seen in the collecting ducts and in the proximal tubules as calcified spherules. This indicates that the glomerular filtrate contained a fairly substantial amount of phosphorus, and thus, the hydroxyapatite crystals may have been formed in the proximal tubules. In particular, as the high phosphorus diet contained a relatively low concentration of magnesium, it seems highly probable that phosphorus-calcium compounds could have been formed.[45–47] In the present study, impairment of the epithelial proximal tubules and destruction of the collecting ducts were observed. When they are attached to the membrane of the tubules, some crystals, such as those of calcium oxalate, denature lipids in the membrane and cause cell damage or cell death.[53–56] This mechanism may have been involved in the relationship between the hydroxyapatite deposits and the damage observed in cells in

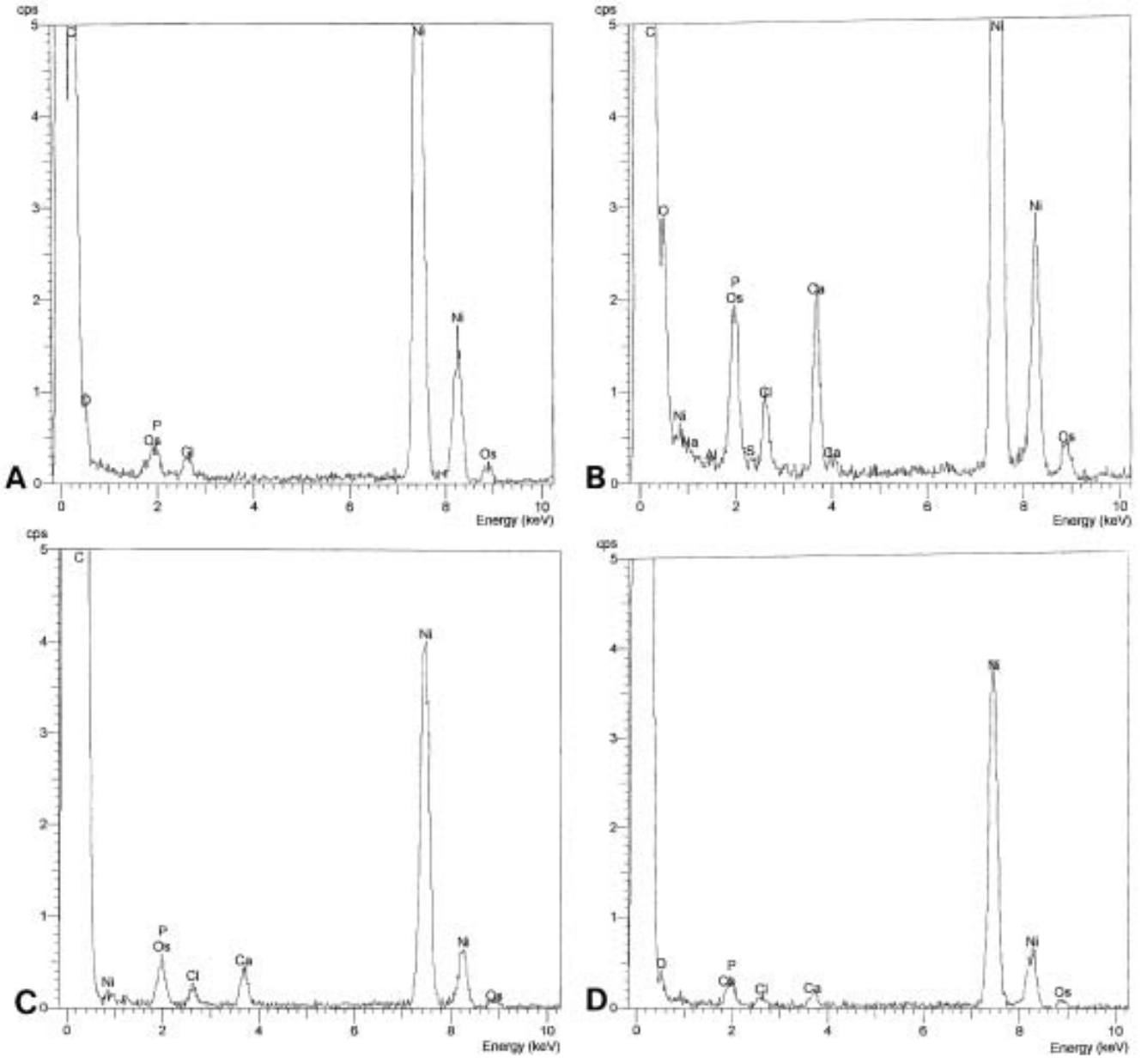
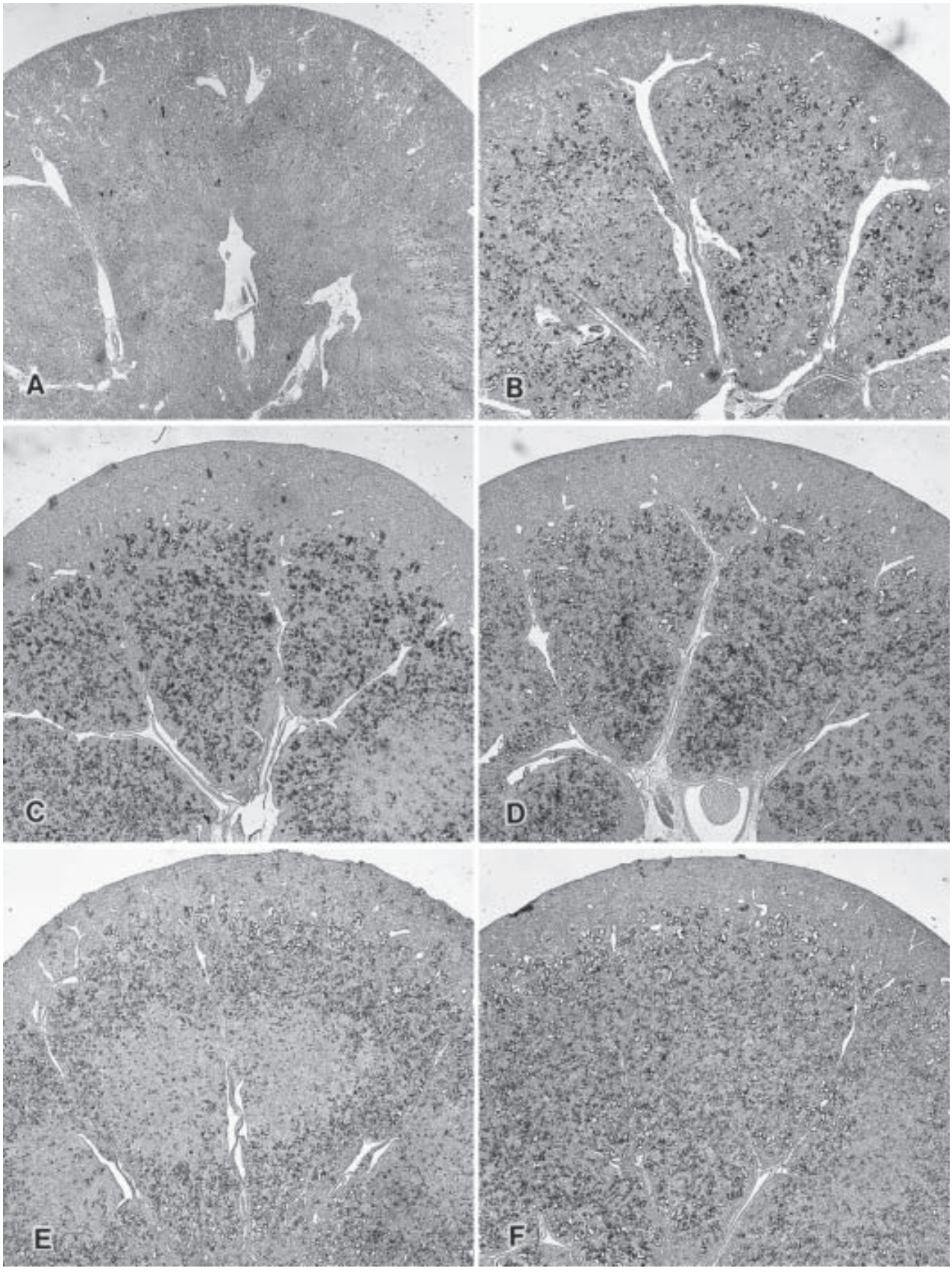


Fig. 6A–D. Results of X-ray microanalysis, showing peaks indicative of various minerals. **A** Results of X-ray microanalysis showing peaks indicative of various minerals in the nucleus of control distal tubular cells in the kidney, including peaks of P and Os. No Ca peak is evident. **B** Small needle-shaped crystals in the mitochondria of cells of a proximal tubule (see Fig. 5A). The Ca and P peaks are evident. **C** Massive needle-shaped crystals in the trilamellar bodies in a collecting duct (see Fig. 5B). The Ca and P peaks are evident. **D** Small needle-shaped crystals in the trilamellar bodies in a collecting duct (see Fig. 5B). The Ca and P peaks are evident

Fig. 7A–F. Light micrographs of kidneys from rats fed the 1.5% phosphorus diet supplemented with Wulingsan or one of its components, 2 weeks after the start of feeding. Section stained with von Kossa's stain, ×20. The grades correspond to those shown in Table 1. **A** Effect of Wulingsan on the development of calcinosis. Slight nephrocalcinosis was observed; grade 1. **B,C,D** Effect of Poria (**B**), Alismatis Rhizoma (**C**), and Atractylodis Rhizoma (**D**) on the development of calcinosis. In the cortex, no calcinosis was observed; in the corticomedullary junction there was grade 2 calcinosis in animals that received Poria (**B**) and Alismatis Rhizoma (**C**) and grade 4 calcinosis in animals that received Atractylodis Rhizoma (**D**) (see Table 1). **E,F** Effect of Polyporus (**E**) and Cinnamomi Ramus (**F**) on the development of calcinosis. Severe nephrocalcinosis was observed in all regions (grade 4; see Table 1). Conspicuous dilatation of the lumen of the tubules was observed in animals that received Polyporus (**E**)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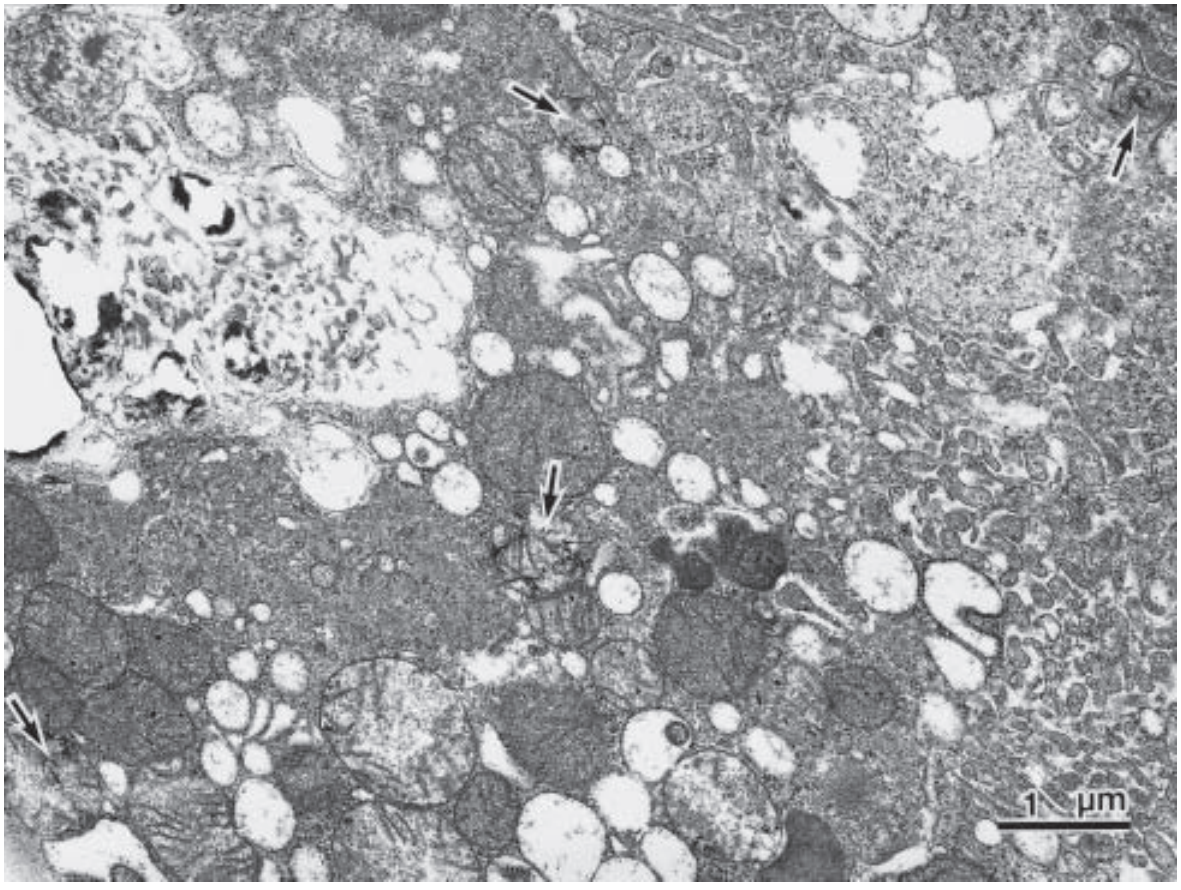

Fig. 8. Dilatation of the rough endoplasmic reticulum in the cells, and slight deposition of small needle-shaped crystals of hydroxyapatite in the mitochondria (*arrows*) are evident in the proximal tubule in rats fed the 1.5% P diet supplemented with Wulingsan, 2 weeks after the start of feeding

the proximal tubules and the collecting ducts in the present study. In particular, in the collecting ducts that were filled with hydroxyapatite, necrosis and infiltration of inflammatory cells around the necrotic region were often observed. The distal tubules were not damaged, and the reason for this might be that, in the distal tubules, in general, the level of reabsorption activity is not as high as that in the proximal tubules. Almost all of the PO_4 ions are reabsorbed in the tubules via a cotransport system, with Na^+ serving as a counter ion. Thus, in the distal tubules, the amount of hydroxyapatite formed and the amount of cell damage incurred might be substantially less than that in the proximal tubules. In experiments with established cell lines, two epithelial tubular cell lines, BSC-1, and MDCK, have been found to display similar adhesion to calcium monooxalate and hydroxyapatite.[57,58] In the body, however, each segment of tubules is affected differently, resulting in differences, in terms of the cellular responses to hydroxyapatite and the degree of cell damage incurred. Furthermore, in the present study, calcinosis in the collecting ducts seemed to have developed in a manner similar to that occurring clinically in general renal stone formation mechanisms.[59]

Effectiveness of Wulingsan and its components in suppressing the development of nephrocalcinosis

Wulingsan, when administered with the high phosphorus diet, was highly effective in suppressing the induction of the nephrocalcinosis that had been markedly evident in the rats fed the high phosphorus diet without Wulingsan. In the group fed the diet supplemented with Wulingsan, only slight calcinosis was observed in the corticomedullary junction, and there was no evidence of calcinosis in other regions. One component of Wulingsan, Poria, was slightly effective in suppressing the development of calcinosis (Table 1), but the other components, Alismatis Rhizoma, Atractylodis Rhizoma, Cinnamomi Ramus, and Polyporus, were not effective. As described above, the mechanism by which calcinosis occurs in the kidney upon the intake of a high phosphorus diet is related to the regulation of phosphorus and magnesium metabolism.[43,44] Neither Wulingsan nor any of its components, i.e., Poria, Alismatis Rhizoma, Atractylodis Rhizoma, Cinnamomi Ramus, and Polyporus, contains a sufficient amount of magnesium to cause a decrease in phosphorus levels. The seeds from which these

Chinese medicines are prepared are known to contain magnesium, but magnesium was not present in the powdered preparations used in this study. Although each of these Chinese medicines has a diuretic action, only Poria was effective in suppressing renal calcinosis, albeit only slightly. Alismatis Rhizoma slightly suppressed the development of calcinosis only in the medulla, compared with findings for the high phosphorus diet alone, but its effectiveness was far weaker than that of Wulingsan. Alismatis Rhizoma reportedly has the strongest diuretic action of these components, including Wulingsan itself. Thus, it is not likely that Wulingsan suppressed the development of calcinosis simply through its diuretic action in terms of the diuretic action of all its constituents described above. Alismatis Rhizoma is reportedly effective for dissolving kidney stones; however, in the present study, it was not effective in suppressing the development of calcinosis. Lieske et al.[60] proposed a mechanism by which hydroxyapatite accumulated in the BSC-1 epithelial tubular cell line. Hydroxyapatite-like calcium oxalate monohydrate, and anhydrous calcium oxalate, were found to adhere to the cell surface as tiny nascent crystals in the very early period before the large crystals were formed, and the tiny crystals were subsequently internalized.[53,57,61–63] Protein, heparin, glycosaminoglycan, and other polyanions present in the fluid in the tubules inhibited the adhesion of these tiny nascent crystals, which are positively charged, to the cell surface and let them flow away into the urine.[61,64,65] Hydroxyapatite was found to display the same behavior as calcium oxalate monohydrate.[60] In the present study, the feeding of excess phosphate necessarily led to supersaturation of the PO_4 concentration in the lumen, thus increasing hydroxyapatite crystal formation, and the internalization of hydroxyapatite into the tubular cells or the formation of bigger crystals in the lumen occurred. When rats were fed the high phosphorus diet supplemented with Wulingsan, the amount of hydroxyapatite crystals and the amount of calcified spherules in the lumen of the tubules decreased. Thus, it seems that Wulingsan may suppress crystal formation, and, thereby, prevent the development of calcinosis, causing a decrease in the concentration of PO_4 ions in the fluid in the tubules and inhibiting the internalization of hydroxyapatite into the cells.

There have been many reports of clinical, physiological, and biochemical studies indicating that Wulingsan, or a component of this medicine, has renal action, as described above; however, the precise mechanisms by which it suppresses nephrocalcinosis remain to be elucidated.

In conclusion, rats fed a high phosphorus diet serve as a model of nephrocalcinosis. Abundant needle-shaped crystals are formed in the lumen of the proximal tubules, in the mitochondria of proximal tubular epithelial cells, and in interstitial cells and/or collecting ducts in the corticomedullary junction and the medulla. These crystals consist of hydroxyapatite, as identified by X-ray microprobe analysis. The calcinosis induced by a high phosphorus diet was suppressed by supplementing the diet with Wulingsan, suggesting that Wulingsan may be a highly effective medicine in the treatment of phosphate-related diseases in humans, such as those described in the "Introduction."

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