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. Clinically, nephrocalcinosis is reported to develop in children with X-linked familial hypophosphatemic rickets when they have been treated with phosphate or vitamin D administered orally. In other patients, nephrocalcinosis occurs as a secondary effect of hyperparathyroidism, endstage kidney disease, chronic glomerulonephritis, hypertension, or milk-alkali syndrome. Sarcoïdosis, idiopathic hypercalcaemia in infancy, sulfonamide toxicity, spongy kidney, toxicity affecting the thyroid (tertroxin), bone tumors, or primary hyperoxaluria.

In experiments using rats, nephrocalcinosis has been found to be induced by sodium phosphate, calcium gluconate, vitamin D, acetalazolamide, parathyroid hormone, oxamide, magnesium, and vitamin B₆. In these studies of nephrocalcinosis, the biochemical and histochemical changes have been well characterized, whereas the ultrastructural changes and the mechanism of progression of this disease have not been much studied. 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. In uremic patients, the degree of calcification was less in those undergoing dialysis, than in those not undergoing dialysis.

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., 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, without sources of calcium, magnesium and phosphorus, while the AIN-76A vitamin mixture 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 paraaffin 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.

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; 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.