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The preventive effect of sodium pentosan polysulfate against renal stone formation in hyperoxaluric rats

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Abstract Sodium pentosan polysulfate (SPP), a semi-synthetic glycosaminoglycan, was administered to rats with hyperoxaluria, induced by a vitamin B6 deficient diet, as a model of calcium oxalate stone formation. We studied the preventive effects of SPP on stone formation as well as its inhibitory effects on stone growth by autoradiography and radioluminography after intravenous injection of ^{14}C-oxalate. The rats were divided into non-treated and SPP-treated groups. The non-treated rats were divided into three groups: one group was fed a regular diet, while the other two groups were fed a vitamin B6 deficient diet for 2 and 4 weeks, respectively. The SPP-treated rats were divided into two groups: one group was intravenously injected with SPP from the start of the vitamin B6 deficient diet for a total of 4 weeks and the other group was injected with the same amount of SPP after 2 weeks of the diet for 2 weeks. ^{14}C-oxalate renal macroautoradiograms were prepared, and calcium oxalate deposits in the renal tissues were compared between the non-treated and SPP-treated groups. The preventive effects on calcium oxalate stone formation were clearly observed in the group injected with SPP for 4 weeks. Even in the other SPP-treated group, in which the administration of SPP was started at 2 weeks after the start of the diet when calcium oxalate stone formation was already observed, the size of the calcium oxalate deposits observed after 4 weeks was smaller than that in the non-treated group fed a vitamin B6 deficient diet for 4 weeks. In conclusion, our results show that SPP has not only preventive effects on calcium oxalate stone formation but also growth inhibitory effects on stones in hyperoxaluric rats.

Keywords Sodium pentosan polysulfate · Calcium oxalate · Hyperoxaluria · Rat · Radioluminography · Macroautoradiography

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Introduction

Although urine is often supersaturated with calcium oxalate even in healthy subjects, many researchers have suggested that crystals do not form because factors that inhibit stone formation are present also in the urine [2, 21]. One such inhibitory factor is thought to be glycosaminoglycan (GAG), an acidic mucopolysaccharide, much of which is present in renal interstitial tissues. Preliminary reports have shown that sodium pentosan polysulfate (SPP), a semi-synthetic GAG, has strong inhibitory effects on calcium oxalate crystal formation and growth, even at low concentrations. It acts by blocking the adherence of bacteria and other ions to the urothelium [13, 16]. Although SPP has proved to be clinically effective to some degree, there have been few reports elucidating its preventive effects on renal stone formation or its inhibitory effects on stone growth in actual renal tissues by administering SPP in a urolithiasis model [6, 15].

We used rats with hyperoxaluria, induced by feeding on a vitamin B6 deficient diet, as a model of calcium oxalate stone formation and prepared ^{14}C-oxalate macroautoradiograms from the removed kidneys. In this experimental system, urinary oxalate excretion increases from the second week after the start of the vitamin B6 deficient diet and oxalate deposits are observed from the fourth week. In this study, the preventive effects of SPP on renal stone formation were evaluated by administering SPP to hyperoxaluric rats

from the start of the vitamin B6 deficient diet and then comparing the results with those of macroautoradiography in hyperoxaluric rats not treated with SPP. Similarly, by administering SPP after inducing renal stone formation, its inhibitory effect on stone growth was evaluated.

Materials and methods

Preparation of ^{14}C-oxalate macroautoradiograms using hyperoxaluric rats

Establishment of non-treated and SPP-treated groups

A total of 30 male Sprague-Dawley rats (Charles River, Japan) were divided into the following three non-treated groups each containing ten rats: the first group was fed a regular diet for 4 weeks (0W-NT group), the second group was fed a vitamin B6 deficienct diet for 2 weeks (2W-NT group) and the third group was fed a vitamin B6 deficiency diet for 4 weeks (4W-NT group) (Fig. 1). After each feeding period, the rats were intravenously injected with 7.4 MBq of ^{14}C-oxalate/kg body wight via a tail vein (molecular weight: 93.5 kDa, specific activity: 4.03 GBq/ mmol; Amersham, England) for three consecutive days. At 18 h after the last injection of ^{14}C-oxalate, the rats were killed by laparotomy and exsanguinated under diethyl ether anesthesia. Bilateral nephrectomy was immediately performed.

For the SPP-treated groups, 20 male Sprague-Dawley rats were given a vitamin B6 deficient diet for 4 weeks and divided into the following two groups of 10 rats each (Fig. 1): the first group was given 10 mg of SPP (Sigma, St. Louis, USA) dissolved in 0.2 ml physiological saline via a tail vein every other day from the start of the vitamin B6 deficient diet for 4 weeks (4W-SPP group). The second group was given the same amount of SPP 2 weeks after the start of the vitamin B6 deficient diet for 2 weeks (2W-SPP group). After each treatment period, the rats were injected with ^{14}C-oxalate and nephrectomized in the same manner as the non-treated groups. In all groups, the rats were killed at the age of 10 weeks.

Preparation of macroautoradiograms

After removal, the kidneys were rapidly frozen using dry-ice acetone and stored at –80°C for 24 h. They were then embedded in carboxy methylcellulose paste and again rapidly frozen using dry-ice acetone and stored at –80°C. After 24 h, consecutive, sagittal kidney sections, 40 μm in thickness and including the cortex, medulla and papilla, were prepared using a microtome (Cryo-Microtome 450MP, PMV, Stockholm, Sweden). After the sample sections were lyophilized on adhesive tape (Sarotape RI-70, Hisamitsu Pharmaceutical, Tokyo) for 48 h, a Lumirror film (6 μm thick, Toray, Tokyo) was placed on the samples for protection. To obtain the macroautoradiograms from the unilateral kidney, the sample sections were exposed on X-ray film for 2 weeks. This was then developed and fixed. The contralateral kidney sections were exposed on imaging plates (20×40 cm, Fuji Film, Tokyo) as described below.

Evaluation of renal stone formation prevention and growth inhibitory effects of SPP in hyperoxaluric rats

The prepared macroautoradiograms were evaluated as follows: the total number of ^{14}C-oxalate deposits on ten consecutive macroautoradiograms obtained from the unilateral kidney of each rat in the non-treated and SPP-treated groups as well as the maximum area of the deposits in all sample sections were calculated. These parameters were first compared among the non-treated groups in order to confirm whether ^{14}C-oxalate was deposited in the hyperoxaluric rats. Next, by comparing the 4W-SPP group with the non-treated groups, the preventive effects of SPP on renal stone formation could be statistically analyzed. Comparing the 2W-SPP group with the non-treated groups, enabled us to determine the inhibitory effects of SSP on stone growth. In order to accurately measure the radioactivity of ^{14}C-oxalate in each sample section, radioluminography using imaging plates was conducted [1]. By exposing the contralateral kidney sections to imaging plates for 24 h, but not on X-ray film, the radioactivity of ^{14}C-oxalate in the sample section was accumulated on the plate particles. Measurement of the immunofluorescence released from these particles when stimulated with 633 nm scanning visible laser beam radiation enabled the energy or radioactivity retained by the particles to be detected using a Bioimaging Analyzer (BAS2000, Fuji Film,

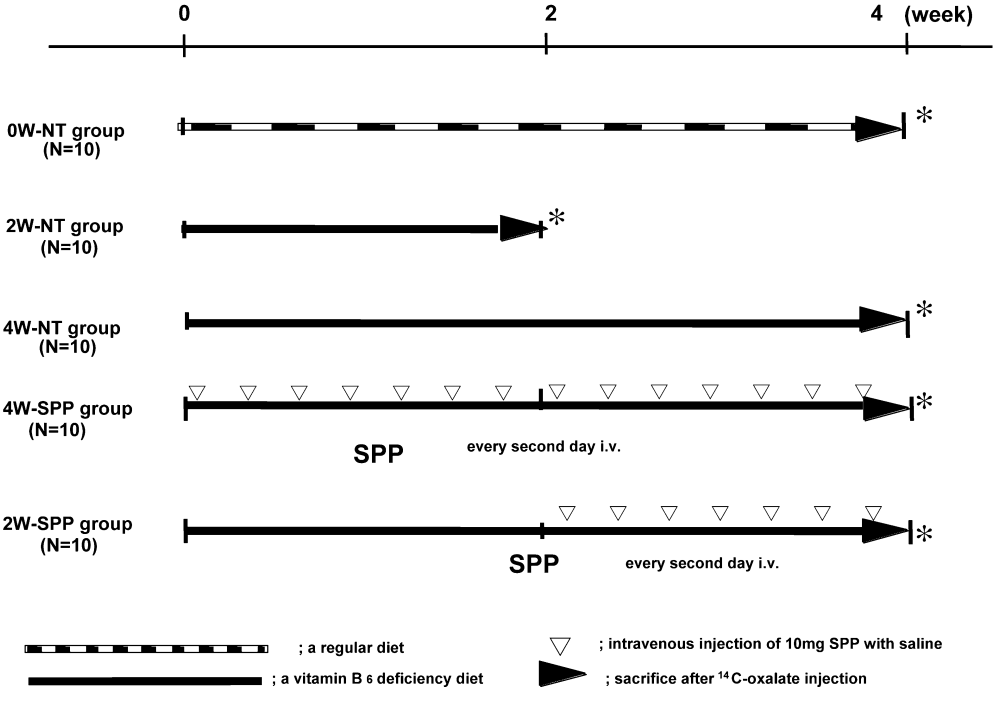

Fig. 1. Sodium pentosan polysulfate (SPP) regimen in the five groups. For the non-treated groups, the first group was fed a regular diet for 4 weeks (0W-NT group, $n = 10$), the second group was fed a vitamin B6 deficient diet for 2 weeks (2W-NT group, $n = 10$) and the third group was fed a vitamin B6 deficient diet for 4 weeks (4W-NT group, $n = 10$). For the SPP-treated groups, the first group was administered 10 mg of SPP intravenously every other day from the start of the vitamin B6 deficient diet for 4 weeks (4W-SPP group, $n = 10$) and the second group was administered the same amount of SPP 2 weeks after the start of the vitamin B6 deficient diet for 2 weeks (2W-SPP group, $n = 10$). The rats in each group were killed at the *asterisk*

Tokyo). For each rat, the total radioactivity of the ^{14}C-oxalate deposits in ten sample sections, as well as the maximum radioactivity in all sample sections, were calculated and quantitatively compared and analyzed among the groups.

Effects of SPP on urinary oxalate and calcium excretion and renal function

Daily urinary calcium and oxalate excretion were measured at the start of and 2 and 4 weeks after the start of the vitamin B6 deficient diet in all groups, and BUN and serum creatinine levels were measured 4 weeks after the start of the diet. Urinary oxalate excretion was measured using high performance liquid chromatography [18], and BUN and serum creatinine levels were measured by the enzyme method. Statistical analysis was done using the Mann-Whitney U-test.

Results

^{14}C-oxalate macroautoradiograms of hyperoxaluric rats

^{14}C-oxalate deposits were not observed in the 0W-NT group. In the 2W-NT group, some scattered black spots considered to be ^{14}C-oxalate deposits were confirmed. In the 4W-NT group, similar deposits were observed mainly in the papilla, but the number and diameter were clearly larger (Fig. 2).

In the 4W-SPP group, the deposits were scattered mainly in the renal medulla, but the number and diameter were smaller compared to those of the 4W-NT group (Fig. 3). In the 2W-SPP group, similar deposits were observed in the medulla, but the maximum diameter was smaller compared to that of the 4W-NT group. However, when the 2W-SPP and 4W-SPP groups were compared, there was no clear difference in the number, size and distribution of the deposits.

Prevention of renal stone formation and growth inhibitory effects of SPP in hyperoxaluric rats

As scattered black spots considered to be ^{14}C-oxalate deposits were not observed in the 0W-NT group, the 2W-NT and 4W-NT groups were compared. The total number of deposits in ten sample sections, maximum area of the deposits in all sample sections, total radioactivity of the deposits in ten sample sections and maximum radioactivity of all sample sections were significantly greater in the 4W-NT group compared to those in the 2W-NT group (Table 1).

In the 4W-SPP group, all parameters including the number and maximum area as well as total and maximum radioactivity were significantly less than those of the 4W-NT group as shown in Table 2. In the 2W-SPP group, the number of deposits was less than that of the 4W-NT group but there was no statistically significant difference (Table 3). However, the maximum area as well as total and maximum radioactivity in the 2W-SPP group were significantly less than those of 4W-NT. This shows that the administration of SPP 2 weeks after the start of the vitamin B6 deficient diet, allowed no increase in the amount of oxalate deposits in the renal tissues 2 weeks later.

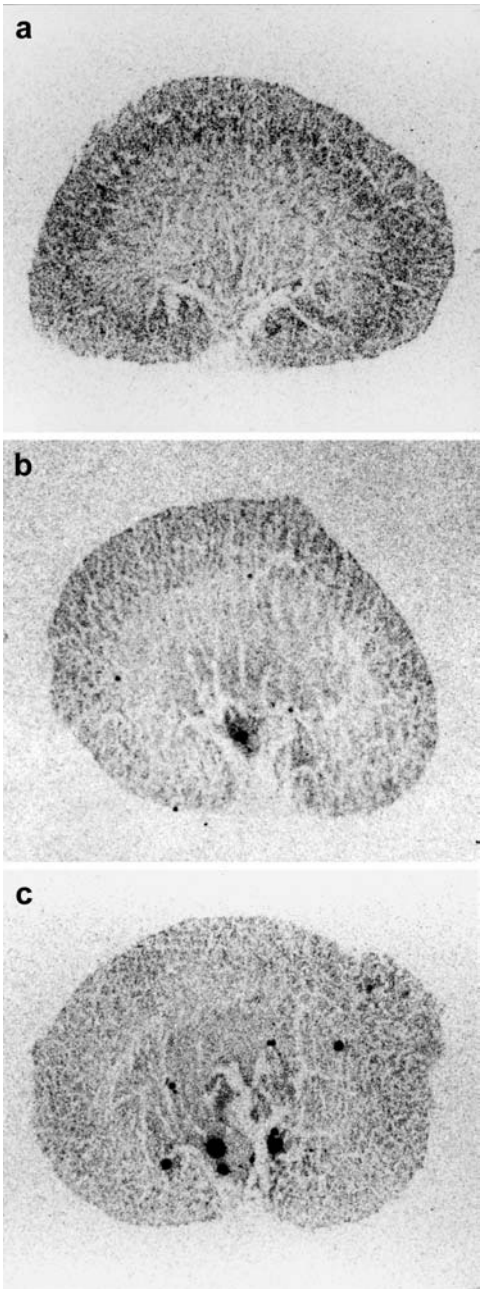

Fig. 2. Typical renal macroautoradiograms of ^{14}C-oxalate in: **a** the 0 W-NT, **b** the 2 W-NT and **c** the 4W-NT groups. ^{14}C-oxalate deposits were not observed in the 0W-NT group. In the 2W-NT group, some ^{14}C-oxalate deposits were confirmed. In the 4W-NT group, similar deposits were observed, mainly in the renal papilla, but the number and diameter were clearly larger than in the 2W-NT group

Effects of SPP on urinary oxalate and calcium excretion and renal function

There was no significant difference in daily urinary calcium excretion among the 4W-NT, 4W-SPP and

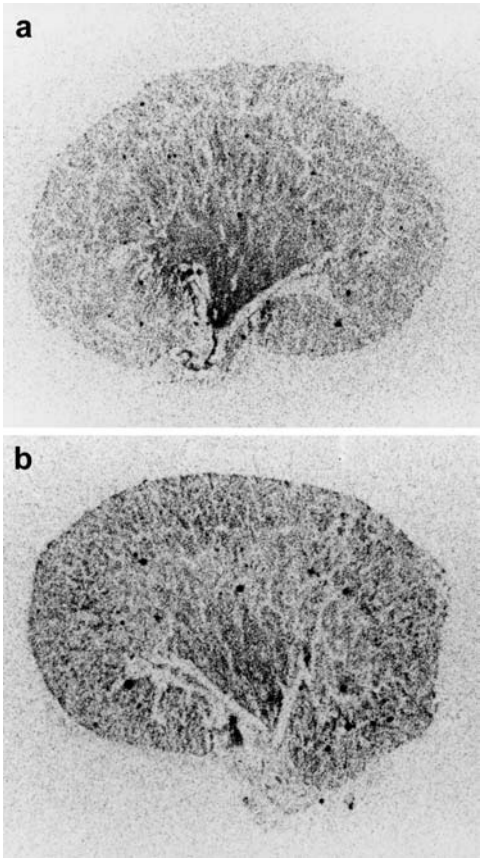

Fig. 3. Typical renal macroautoradiograms of ^{14}C-oxalate in: **a** the 4W-SPP group and **b** the 2W-SPP group. In the 4W-SPP group, ^{14}C-oxalate deposits were scattered mainly in the renal medulla, but the number and diameter were smaller compared to those of the 4W-NT group. In the 2W-SPP group, similar deposits were observed in the medulla, but the maximum diameter was smaller compared to that of the 4W-NT group

Table 1. Comparison of the total number, maximum area, total radioactivity and maximum radioactivity of ^{14}C-oxalate deposits in renal macroautoradiograms between the 2W-NT and 4W-NT groups. Values are expressed as mean ± SE. The statistical significance (P) was determined by the Mann-Whitney U-test. $n = 10$ for all groups. Total number and maximum area indicate the total number of deposits in ten renal macroautoradiograms, and the maximum area of deposit in these macroautoradiograms, respectively. Total radioactivity and maximum radioactivity indicate total radioactivity of deposits in ten kidney specimens, and the maximum radioactivity of deposit in those, respectively. Radioactivity was measured using imaging plates

	Total number	Maximum area (mm^2)	Total radioactivity (dpm)	Maximum radioactivity (dpm)
2W-NT	32.8 ± 3.0	0.30 ± 0.04	275.2 ± 69.2	7.24 ± 1.48
4W-NT	84.1 ± 12.0	0.89 ± 0.17	2,416.8 ± 641.2	149.76 ± 30.04
P	($P < 0.01$)	($P < 0.01$)	($P < 0.01$)	($P < 0.01$)

2W-SPP groups as shown in Fig. 4. Daily urinary oxalate excretion tended to increase similarly in all groups. Urinary oxalate excretion increased slightly 2 weeks after the start of the diet and increased twofold after 4 weeks. This increase was statistically significant. Renal function was normal 4 weeks after the start of the diet in all three groups (Table 4).

Table 2. Comparison of total number, maximum area, total radioactivity and maximum radioactivity of ^{14}C-oxalate deposits in renal macroautoradiograms between the 4W-NT and 4W-SPP groups. Values are expressed as mean ± SE. The statistical significance (P) was determined by Mann-Whitney U-test. $n = 10$ for all groups

	Total number	Maximum area (mm^2)	Total radioactivity (dpm)	Maximum radioactivity (dpm)
4W-NT	84.1 ± 12.0	0.89 ± 0.17	2,416.8 ± 541.2	149.76 ± 30.04
4W-SPP	20.9 ± 2.8	0.15 ± 0.02	93.6 ± 34.0	3.32 ± 1.52
P	($P < 0.01$)	($P < 0.01$)	($P < 0.01$)	($P < 0.01$)

Table 3. Comparison of total number, maximum area, total radioactivity and maximum radioactivity of ^{14}C-oxalate deposits in renal macroautoradiograms between the 4W-NT and 2W-SPP groups. Values are expressed as mean ± SE. The statistical significance (P) was determined by Mann-Whitney U-test: NS; not significant. $n = 10$ for all groups

	Total number	Maximum area (mm^2)	Total radioactivity (dpm)	Maximum radioactivity (dpm)
4W-NT	84.1 ± 12.0	0.89 ± 0.17	2,416.9 ± 541.2	149.76 ± 30.04
2W-SPP	61.4 ± 18.7	0.34 ± 0.07	702.0 ± 282.4	49.96 ± 24.72
P	(NS)	($P < 0.01$)	($P < 0.01$)	($P < 0.01$)

Discussion

Vitamin B6 acts as a co-enzyme in transamination between glyoxalate, which is a precursor of oxalate, and glycine. Its deficiency enhances glyoxalate accumulation and endogenous oxalate production, inducing hyperoxaluria. In the present study, rats were fed a vitamin B6 deficient diet, after which ^{14}C-oxalate was administered for three consecutive days. After the rats were killed 18 h later, macroautoradiography was performed. Pharmacokinetic studies have revealed that the half-life in blood of ^{14}C-oxalate is approximately 20 min and its urinary recovery rate is about 95% at 4 h [19]. As the kidneys were removed after three consecutive days of ^{14}C-oxalate administration in our study, the ^{14}C-oxalate deposits observed in the macroautoradiograms were thought to be oxalate accumulated or deposited in the renal tissues and not oxalate flowing in the nephron. In an experiment in which ^{45}Ca was administered to similar rats and macroautoradiography was performed, radioactive deposits were observed in similar areas [9]. In addition, as it was confirmed that calcium oxalate crystals form in the area of ^{14}C-oxalate deposits by microautoradiography using polarization microscope and X-ray analysis [8], it was assumed that the ^{14}C-oxalate deposits observed in our study consisted of calcium oxalate. Moreover, the injected ^{14}C-oxalate was thought to have the same action as endogenous oxalate, because

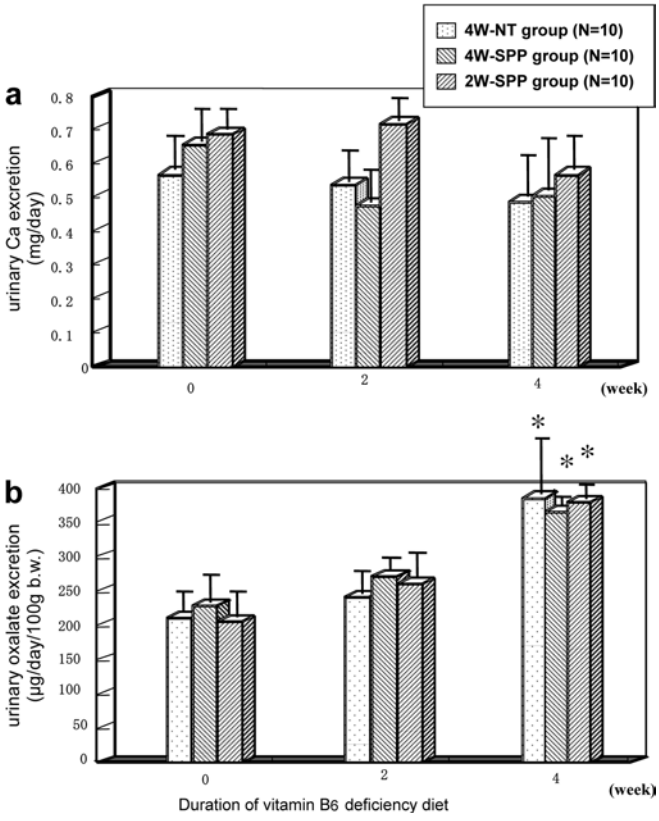

Fig. 4. Effects of SPP on urinary excretion of: **a** calcium and **b** oxalate in the 4W-NT, 4W-SPP and 2W-SPP groups. There was no significant difference in daily urinary calcium excretion among the three groups. Daily urinary oxalate excretion tended to increase similarly in all groups. Values are expressed as the mean ± SE. The significant difference from each group on week 0 was determined by the Mann-Whitney U-test: an *asterisk* indicates $P < 0.05$

Table 4. Evaluation of renal function 4 weeks after the start of the vitamin B6 deficient diet in the 4W-NT, 4W-SPP and 2W-SPP groups. Values are expressed as mean ± SE. $n = 10$ for all groups

	BUN (mg/dl)	Serum creatinine (mg/dl)
4W-NT	17.21 ± 0.78	0.44 ± 0.03
4W-SPP	15.77 ± 0.58	0.48 ± 0.03
2W-SPP	15.08 ± 1.05	0.48 ± 0.04

oxalate does not combine with protein in the blood of rats and is the final metabolite [7]. In our experiment, the amount of ^{14}C-oxalate administered was 7.4 MBq/kg body weight, which is equivalent to an acute oxalate load of approximately 40 μg per rat when calculated from specific activity. When Sugimoto et al. [19] measured inulin and oxalate clearance at the same oxalate load, neither showed much change. Therefore, the amount of ^{14}C-oxalate injected in our present experiment was considered not to affect the renal excretory dynamics of endogenous oxalate.

As calcium oxalate stone formation models, massive administration of exogenous oxalate or oxalate precursors such as ethylene glycol have been reported, but in these models, deposits of calcium oxalate were observed in the entire kidney and renal function was remarkably reduced [10]. However, in our model using a vitamin B6 deficient diet, urinary oxalate excretion was about twice that of normal rats, which is a slight increase compared to that of other models, and renal function can be maintained. Therefore, our hyperoxaluric rats, induced by a vitamin B6 deficient diet, can be regarded as a more clinically appropriate urolithiasis model.

In our study, macroautoradiography was performed on rats fed a regular diet (0W-NT group), rats fed a vitamin B6 deficient diet for 2 weeks (2W-NT group) and rats fed a vitamin B6 deficient diet for 4 weeks (4W-NT group), and the number and maximum area as well as total and maximum radioactivity of the ^{14}C-oxalate deposits were first compared and analyzed among these non-treated groups. ^{14}C-oxalate deposits were not observed in any of the macroautoradiograms of the 0W-NT group, but in the 2W-NT group, some deposits were confirmed. In the 4W-NT group, the number of ^{14}C-oxalate deposits in ten sample sections, maximum area of the deposits in all sample sections as well as total radioactivity of the deposits in ten sample sections and maximum radioactivity of all sample sections were significantly larger compared to those of the 2W-NT group. These results show that calcium oxalate deposits began to occur within 2 weeks of the start of the vitamin B6 deficient diet, and that the amount of the deposits in the renal tissues increased with time.

For the 4W-SPP group, SPP was intravenously injected via a tail vein every other day from the start of the vitamin B6 deficient diet for 4 weeks, and the number and maximum area as well as total and maximum radioactivity of the ^{14}C-oxalate deposits were compared to those of the non-treated groups. Thus the preventive effects of SPP on renal stone formation could be studied. Furthermore, by starting the administration of SPP after 2 weeks from the start of the vitamin B6 deficient diet in 2W-SPP, when calcium oxalate deposits began to occur or when the deposits began to form in the renal tissues, we also studied whether SPP could inhibit the growth of these deposits. When the ^{14}C-oxalate deposits of the treated group of 4W-SPP were compared to those of the non-treated group of 4W-NT, not only the number and maximum area but also total and maximum radioactivity were significantly suppressed. This suggests that the amount of calcium oxalate deposited in the renal tissues was less after SPP treatment,strongly indicating that SPP has a preventive effect on calcium oxalate stone formation. Although there was no significant difference in the number of deposits when the 2W-SPP and 4W-NT group were compared, the maximum area, total radioactivity and maximum radioactivity were significantly smaller in the 2W-SPP group compared to those of the 4W-NT group. This result suggests that the growth of calcium oxalate deposits in the kidney was inhibited by the administration of SPP.

SPP, the structure of which is a linear polymer of 1–4 linked ß-D xylopyranose residues (molecular weight: app. 5,000 Da), is a structural analog of heparin, although its anticoagulant properties are far less. It has a wide spectrum of biological effects and has recently been used to treat a number of diseases, including interstitial bladder cystitis and chronic nonbacterial prostatitis [3, 4, 5, 11]. Its dynamics have not been clarified, but it has been reported that urinary excretion is 10% during the first 12-h period after intravenous injection and 1–2% of the delivered dose is recovered unchanged in the urine after oral intake [6]. Although the mechanism has not been elucidated, it is known to have strong inhibitory effects on calcium oxalate crystal formation and growth in the urine, even at low concentrations [16, 20]. As for the method of administration, as urinary excretion is higher when administered intravenously compared to when administered orally, we chose to administer SPP by intravenous injection. Suzuki et al. [20] have reported that when the urinary concentration of SPP exceeds 5 μg/ml, it strongly inhibits calcium oxalate crystal formation and growth. We therefore selected the dose of 10 mg every other day, taking into consideration the urinary recovery rate after intravenous injection and the urinary volume of the rats. At this dose, there were no side effects such as bleeding.

Scurr et al. [14] have reported that macromolecular substances such as GAG adhere reversibly to the surface of calcium oxalate crystals and by decreasing the zeta-potential of the crystal surface and strengthening the electrical, repulsive force among the crystals, the aggregation and growth of the crystals are inhibited. It has also been reported that the strength of its inhibitory effects may depend on the negative charge density it retains or the number of sulfate ions it contains as well as the distance between sulfate ions in the molecule [2]. As SPP is a polysaccharide polymer which possesses negatively charged sulfates, Norman et al. have reported that SPP exerts its inhibitory effects on stone formation by a mechanism similar to that of GAG [12]. However, many of these studies are in vitro experimental results using artificial urine and other methods [2, 17, 20]. Although there have been reports of SPP administered to urolithiasis patients, and its effectiveness has been indicated to some extent [6], there are few reports showing that SPP actually inhibits the deposit and growth of calcium oxalate in renal tissues. In our experiment, in which SPP was administered to hyperoxaluric rats, calcium oxalate deposits were suppressed without affecting the amount of urinary oxalate or calcium excretion. In addition, the growth of calcium oxalate already deposited in the renal tissues was inhibited. These results suggested that despite its low urinary recovery rate by oral administration, SPP may play an important role in the prevention and treatment of calcium oxalate stone formation in the kidney.

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