ORIGINAL

Utilization of titanium oxide-like compound as an inorganic phosphate adsorbent for the control of serum phosphate level in chronic renal failure

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Abstract : Hyperphosphatemia adversely affects the prognosis of patients with chronic renal failure (CRF). We synthesized a titanium oxide-like compound (TAP) as a phosphate adsorbent for treatment of hyperphosphatemia in CFR patients. We evaluated the ability of TAP to adsorb inorganic phosphate *in vitro* and *in vivo*. TAP was shown to contain sulfate and hydroxyl groups by thermal analysis, which probably involved in phosphate adsorption through an ionic exchange mechanism. TAP constantly adsorbed phosphate (66.20-72.84 mg/g TAP) over a wide pH range (1.22-7.27) *in vitro*. To evaluate the phosphate binding potential of TAP *in vivo*, adenine-induced CRF rats were fed AIN-76 diet containing 3% TAP, 10% TAP, 3% sevelamer hydrochloride (clinical phosphate adsorbent), or 3% calcium carbonate, and serum levels of phosphate and calcium and urinary phosphate were compared with those in untreated CRF rats. Orally administered TAP showed the inhibitory effect on serum phosphate level in adenine-induced CRF rats, which was equivalent to that of sevelamer hydrochloride. These results indicate that TAP is a useful alternative phosphate-binder with fewer side effects than sevelamer hydrochloride and calcium carbonate. J. Med. Invest. 57 : 275-283, August, 2010

INTRODUCTION

Patients with chronic renal failure (CRF) show increased serum phosphate levels due to impaired phosphate excretion (1). Hyperphosphatemia induces secondary hyperparathyroidism (2-4) and renal osteodystrophy (5), which adversely affect the patient's prognosis and quality of life (6, 7).

Aluminum gel and calcium carbonate have been used clinically as phosphate-binding inorganic compounds for treatment of hyperphosphatemia. However, administration of aluminum gel is now prohibited due to the toxicity of aluminum, which may induce encephalopathy or osteopathy (8-12). On the other hand, the use of calcium carbonate induces hypercalcemia, which accelerates the calcification of blood vessels (13-15). To overcome these problems, sevelamer hydrochloride (16), which is a phosphate-binding organic polymer, has been developed. However, this compound also frequently induces adverse reactions, such as constipation, abdominal pain, and abdominal fullness (17, 18). Therefore, the development of alternative phosphate binders with fewer side effects is required.

In the present study, we synthesized a titanium oxide-like compound, TAP [titanium (IV) oxide adsorbing phosphate], as a candidate phosphate adsorbent and evaluated its applicability for the treatment of hyperphosphatemia in CRF using adenine-induced renal failure model rats. We propose that TAP is an alternative phosphate-binding inorganic compound useful for prevention of hyperphosphatemia in CRF patients.

MATERIALS AND METHODS

Preparation of TAP

We chemically synthesized the novel titanium oxide-like compound TAP as follows. Briefly, 171 g of ammonium sulfate was dissolved in 400 ml of water by agitation, and 200 g of titanium tetrachloride solution (65.4% $TiCl_4$) was added. The solution was heated at $100°C$ for 3 h under strongly acidic conditions (pH< 1.0). The obtained sediment was recovered by filtration, washed with distilled water, and dried at $60°C$.

Reagents

Sevelamer hydrochloride (Renagel®) was purchased from Chugai Pharmaceutical, Co., Ltd, Japan. Calcium carbonate used in this study was Japanese pharmacopoeia grade.

Phosphate adsorption test in vitro

Phosphate solutions containing various concentrations (5, 10, 20, 40, or 60 mM) of NaH_2PO_4 were used, and their pH values were adjusted using HCl or NaOH. TAP (0.5 g) was added to 50 ml of each phosphate solution, and the mixtures were agitated in a water bath at $37°C$. The mixtures were then filtered with 0.2-μm nylon membranes. The phosphate concentrations in the flow-through fractions were measured, and the rates of phosphate adsorption were calculated.

Analytical procedure

The concentrations of phosphate and sulfate were measured by the ion chromatography method using an AS4A column (DIONEX) and AMMS® (anion micro membrane suppressor) under the following conditions. The AS4A column was injected with 25 μl of the sample and operated at a flow rate of 1.5 ml/min. Elution was performed with elution buffer (10 mM sodium bicarbonate, 15 mM sodium carbonate, and AMMS®) at a flow rate of 4.0 ml/min.

In chemical regeneration mode, 12.5 mM sulfuric acid was used as a regenerant.

Phosphate adsorption test of TAP in CRF model rats

Eighteen 8-week-old male Crj : CD (SD) IGS rats were purchased from Charles River Japan (Tokyo, Japan). We designed minimum number of experimental animal (three rats per group) due to the capacity of our metabolic cage. Rats were housed individually in plastic cages and acclimated for five days before starting the experiment. Distilled water and AIN-76 (19)-based diet were provided *ad libitum*. The room was maintained at a temperature of $23±2°C$, humidity of $55±10\%$, and a 13-h light/11-h dark cycle. All of the animal experiments were performed according to the guidelines for animal experiments approved by the University of Tokushima. Adenine-induced CRF model rats were generated by referring to the literature (20-22). Briefly, after acclimation, the rats were divided into six groups without deviation on the mean body weights. Four groups were fed AIN-76 plus 0.675% adenine supplemented with TAP (3% or 10%), sevelamer hydrochloride (3%), or calcium carbonate (3%), respectively, and one group was fed AIN-76 plus 0.675% adenine alone (control group) for 31 days. The remaining three rats were fed only AIN-76 diet throughout the experiment (normal group). Dietary intakes of the rats were recorded every day.

Blood analysis

Orbital blood was collected from all of the rats on days 1, 14, 24, and 31 after administration of the test reagents, and the serum levels of phosphate, calcium, blood urea nitrogen (BUN), and creatinine were measured with Hitachi 7600 automatic analyzer by employing Fiske-Subbarow method, Arsenazo-III method, Urease-GLDH method, and enzymatic method, respectively.

Urinalysis

At 24 and 31 days after administration of the test reagents, the rats were settled into metabolic cages, 24-h urine was collected, and the urinary phosphate levels were measured (Fiske-Subbarow method : Hitachi7600 automatic analyzer).

Statistical analysis

Data are expressed as the means± standard deviation. Differences between the control group and treated groups were assessed by unpaired *t*-test. $P< 0.05$ was considered to indicate significance.

RESULTS

Properties of TAP

The chemical characteristics of TAP and a comparison of the X-ray diffraction patterns of TAP and TiO_2 are shown in Table 1 and Figure 1, respectively. TAP is a titanium oxide-like compound containing TiO_2 (63.2%), SO_4 (13.7%), and H_2O (13.3%). The X-ray diffraction pattern indicated that TAP is a TiO_2 with an anatase structure and extremely low crystal formation (Figure 1).

Table 1. Chemical characteristics of TAP

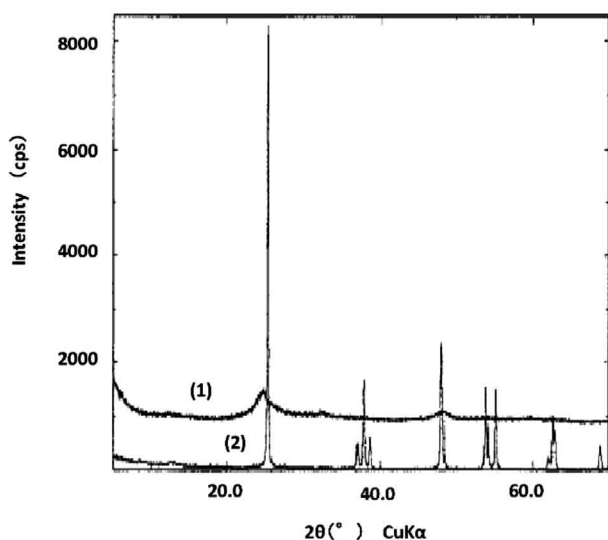| Subjects	Analysis data
TiO_2 content (%)	63.2
SO_4 content (%)	13.7
Dry loss ($105°C$, 2 h) (%)	13.3
Surface area (m^2/g)	165.9
Mean pore size (μm)	11.8
Apparent density (g/ml)	0.74

Fig. 1.   X-ray diffraction patterns of TAP (1) and TiO_2 of Japanese pharmacopoeia grade (2).

Phosphate adsorption of TAP in vitro

The phosphate-binding capacity of TAP was assessed using phosphate solutions of various concentrations (5, 10, 20, 40, and 60 mM NaH_2PO_4) at pH1.2 and 6.8 (Table 2). As the phosphate concentration increased, the amount of phosphate adsorbed by TAP increased. On the other hand, the proportion of the phosphate adsorbed by TAP decreased to 34.8% when 60 mM phosphate solution was used, indicating that most of the TAP in the 60 mM phosphate solution was saturated. There were no differences in the phosphate-binding properties of TAP at pH1.2 and 6.8. The final pH of the mixtures decreased when phosphate solutions at pH6.8 were used. We measured the sulfate concentrations in the mixtures after addition of TAP (Figure 2). TAP
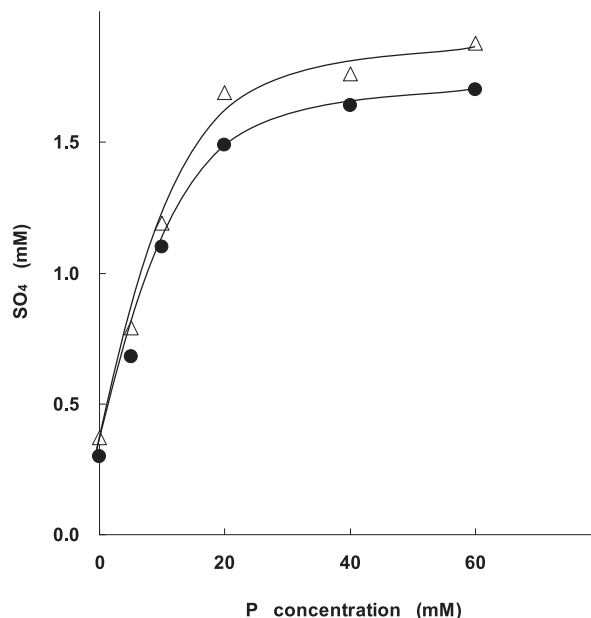

Fig. 2.   Ion exchange between sulfate groups in TAP and exogenous phosphate ions. The concentration of sulfate released from TAP in solutions of various phosphate concentrations were measured at pH1.2 (closed circles) and 6.8 (open triangles).

Table 2.   Phosphate adsorption of TAP

	Condition	Phosphate concentration of solution (mM)				
		5	10	20	40	60
pH1.2	Adsorbed P (mg/g)	16.11	32.56	52.15	60.87	66.20
	Adsorption rate [a] (%)	100.0	100.0	83.1	47.1	34.8
	Final pH [b]	1.22	1.16	1.15	1.13	1.14
pH6.8	Adsorbed P (mg/g)	16.26	32.08	55.26	61.45	68.38
	Adsorption rate [a] (%)	100.0	100.0	87.4	48.8	36.4
	Final pH [b]	2.23	2.20	2.37	3.58	5.34

[a] Adsorption rate indicates the percentages of TAP-adsorbed phosphate (P) to total P content in the test solution.
[b] The decrease in final pH value was derived from the release of sulfate ions into the test solution due to ionic exchange between sulfate groups of TAP and phosphate ions.

released 0.30 to 0.37 mM sulfate in the absence of phosphate, indicating that the sulfate groups in TAP are easily released. The released sulfate increased in proportion to the amount of TAP-adsorbed phosphate and almost reached a plateau at phosphate concentrations above 40 mM.

Phosphate adsorption rate of TAP in vitro

The phosphate adsorption rate of TAP was measured at $37°C$ in 60 mM phosphate solution adjusted to pH1.2 or 6.8. As shown in Figure 3, at both pH values, TAP showed rapid phosphate adsorption, which reached a plateau after 2 h of incubation.
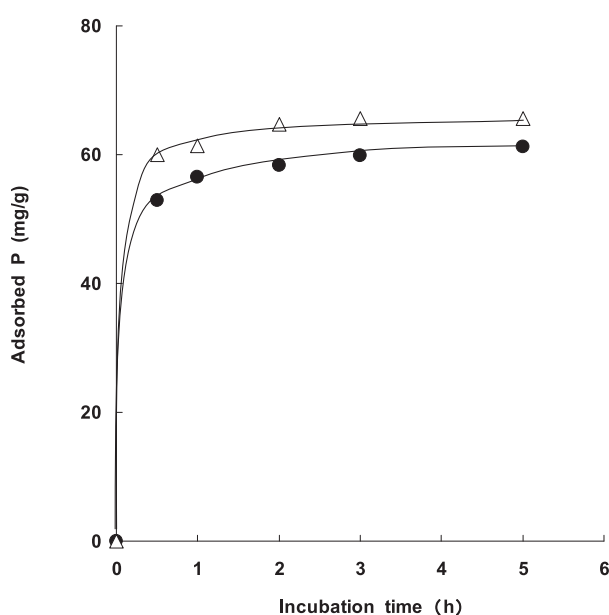

Fig. 3. Phosphate adsorption rate of TAP. The amounts of phosphate adsorbed by 1 g of TAP after incubation for 0.5, 1, 2, 3, and 5 h at $37°C$ in 60 mM phosphate solution (NaH_2PO_4) pH adjusted to 1.2 (closed circles) or 6.8 (open triangles).

Effects of pH on in vitro phosphate adsorption of TAP

The effects of pH on phosphate adsorption capability of TAP were determined using 60 mM phosphate solutions adjusted to various pH values (1.2, 3.0, 5.0, 6.8, 8.0, 9.0, 10.0, 11.0, or 12.0). As shown in Figure 4, the capacity of TAP to adsorb phosphate ranged from 66.20 to 72.77 mg/g at final pH values ranging from 1.22 to 7.27. TAP showed stable phosphate adsorption under acidic to neutral conditions. However, the phosphate adsorption of TAP was markedly reduced at pH10.21, indicating that the phosphate-binding of TAP is reduced under strongly basic conditions.
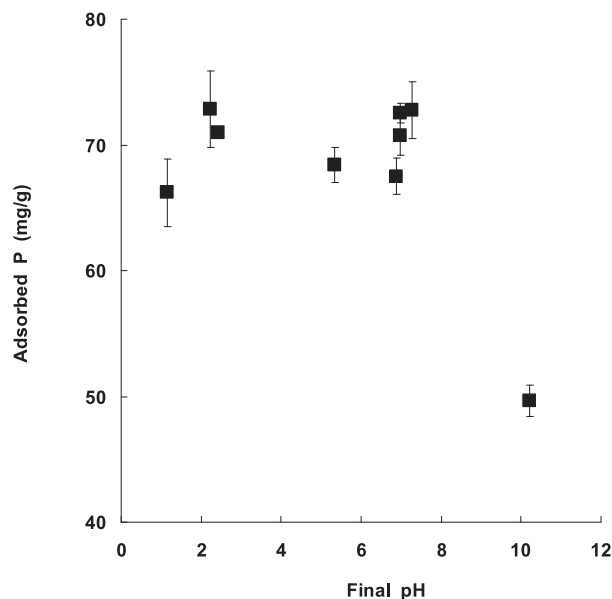

Fig. 4. Optimal pH range for the phosphate adsorption by TAP. The amounts of phosphate adsorbed by 1 g of TAP at pH ranging from 1.22 to 10.21 were calculated. The results are given as means$\pm$ standard deviation.

Effects of TAP on serum phosphate levels in CFR model rats

We evaluated the potential of TAP as a therapeutic agent to prevent hyperphosphatemia in CRF patients using experimental CRF model rats. Adenine-induced CRF model rats were fed a diet supplemented with TAP (3% or 10%), sevelamer hydrochloride (3%), or calcium carbonate (3%) for 31 days, and the serum phosphate levels were compared.

Table 3 shows the daily dietary intake of the rats in each experimental group during the experimental period of 31 days. The dietary intake of CRF model rats was significantly lower than that of normal controls. Among the CRF groups, the mean dietary intake of the 10% TAP-treated group was significantly lower than that of the control group.

Table 3. Average dietary intakes during experimental period

| Group	Average dietary intakes (g/31days)
Normal	22.6$\pm$ 0.35
Control	14.5$\pm$ 2.14
3% TAP	11.2$\pm$ 2.03
10% TAP	7.4$\pm$ 1.05*
3% Sevelamer	12.1$\pm$ 1.99
3% CaCO$_3$	11.7$\pm$ 0.84

*Significantly different from control group ($P < 0.05$).

The changes in serum levels of BUN and creatinine are shown in Figure 5. The serum levels of BUN and creatinine in CRF model rats increased in a time-dependent manner, while those in the normal rats were constant. Among the CRF model rats, BUN levels in those treated with 3% calcium carbonate were significantly elevated at 14, 24, and 31 days, while those in TAP-treated rats at 24 days were significantly decreased in comparison with the control CRF group (Figure 5a). Serum creatinine levels showed a similar tendency to those of BUN (Figure 5b). Serum creatinine levels at 31 days in rats treated with calcium carbonate were significantly higher than those in the control group, while

those in 10% TAP and 3% sevelamer groups were significantly lower than those in the control group.

The changes in serum phosphate and calcium levels in each group are shown in Figure 6. The serum phosphate levels of the rats in the control group increased in a time-dependent manner and reached 17.0 ± 2.55 mg/dl at 31 days (Figure 6a). With the exception of 3% sevelamer, all of the test reagents significantly inhibited the elevation of serum phosphate level in CRF model rats. Calcium carbonate markedly reduced the serum phosphate level to less than that in the normal group. On the other
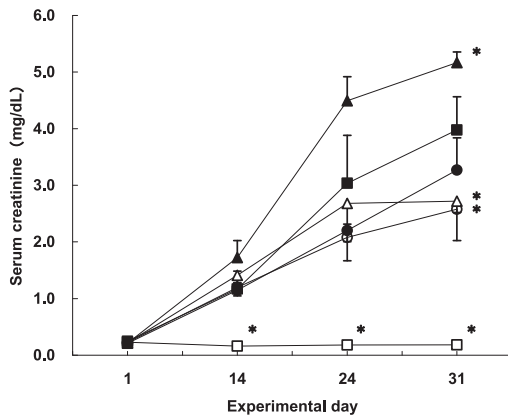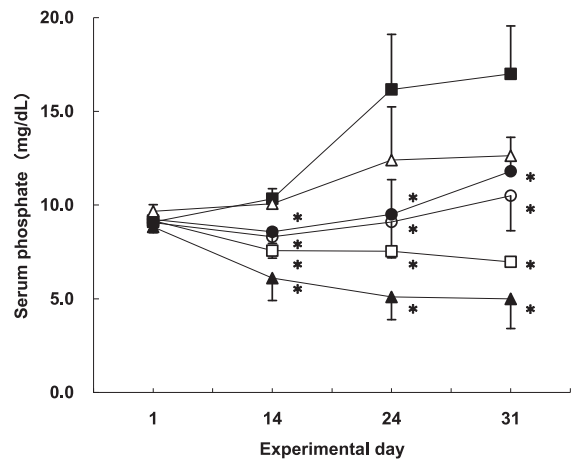
(a)

(a)

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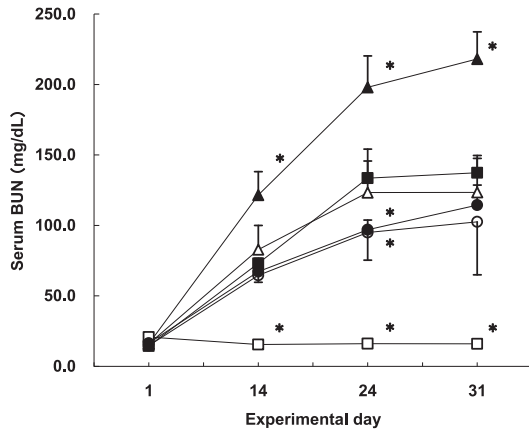
(b)

Fig. 5.   Effects of TAP on serum levels of blood urea nitrogen (a) and creatinine (b) in rats with adenine-induced renal failure. The results are shown as means$\pm$ standard deviation. *Significantly different from control group ($P < 0.05$). Normal rats and control CRF model rats are indicated by open and closed squares, respectively. Other symbols indicate CRF model rats treated with $CaCO_3$ (closed triangles), 3% sevelamer (open triangles), 3% TAP (closed circles), and 10% TAP (open circles), respectively.
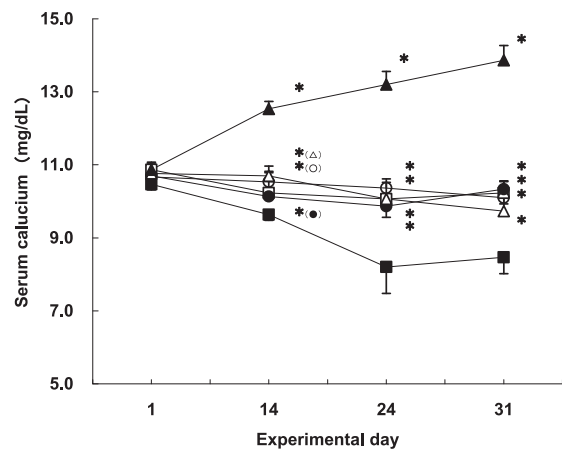
Fig. 6.   Effects of TAP on serum levels of phosphate (a) and calcium (b) in rats with adenine-induced renal failure. The results are shown as means$\pm$ standard deviation. *Significantly different from control group ($P < 0.05$). Symbols are the same as in Figure 5.

hand, serum calcium levels in rats treated with calcium carbonate were significantly higher than those in the control group (Figure 6b). The serum calcium levels in the control group decreased in a time-dependent manner, which was indicative of the progression of renal dysfunction. On the other hand, the serum calcium levels of TAP- and sevelamer-treated groups were stable throughout the experiment. The values of calcium by phosphate (Ca×P) are plotted in Figure 7. The Ca×P values in TAP-treated groups were significantly lower than those in the control group only at 14 days, while those in calcium carbonate-treated rats were low similarly to that of normal rats. On the other hand, the Ca×P values in sevelamer-treated group were similar to those in controls.
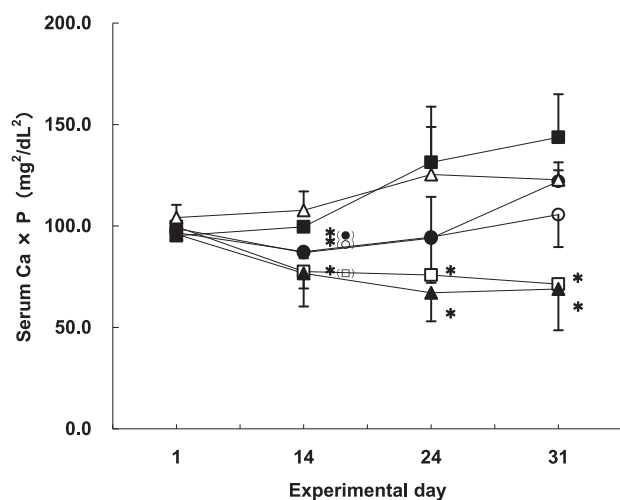

Fig. 8. Effects of TAP on urinary phosphate levels in rats with adenine-induced renal failure. The results are shown as means± standard deviation. *Significantly different from control group (24day) ($P<0.05$). **Significantly different from control group (31day) ($P<0.05$).

Fig. 7. Effects of TAP on serum Ca×P (mg^2/dL^2) in rats with adenine-induced renal failure. The results are shown as means± standard deviation. *Significantly different from control group ($P<0.05$).

Figure 8 shows the phosphate concentration in 24-h urine. Control CRF model rats showed a similar urinary phosphate level to the normal group although the dietary intake of CRF model rats was significantly reduced in comparison with normal rats (Table 3), reflecting hyperphosphatemia in the control CRF group. Treatment with TAP (both 3% and 10%) and 3% calcium carbonate significantly reduced urinary phosphate level. Treatment with 3% sevelamer also decreased urinary phosphate level but the difference was not significant in comparison with the control group. The results regarding urinary phosphate level correlated well with those of serum phosphate level (Figure 6a).
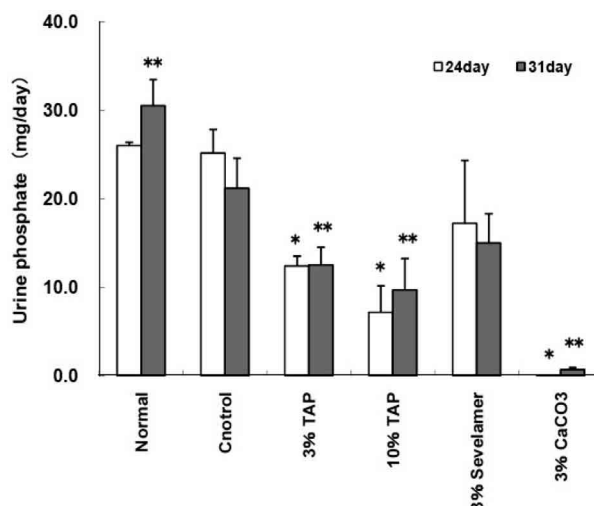
DISCUSSION

In this study, we developed TAP as an inorganic phosphate adsorbent that has desirable characteristics for treatment of hyperphosphatemia in CRF patients. TAP showed rapid phosphate adsorption *in vitro* and adsorbed over 65 mg of phosphate per gram of TAP over a wide pH range under pH7.0, indicating that TAP is a suitable phosphate-binder in the human gastrointestinal tract. As ion exchange through hydroxyl groups was markedly affected by pH, the wide pH optima of TAP with regard to phosphate adsorption is probably due to ion exchange through stable sulfate groups rather than through hydroxyl groups, as reported for titanium oxide monohydrate (23, 24).

We used adenine-induced CRF model rats to evaluate the capability of TAP to prohibit hyperphosphatemia in CRF by comparison with the effects of other phosphate adsorbents (sevelamer hydrochloride and calcium carbonate). More than 3-week administration of adenine is required to induce the significantly higher serum phosphate level than that in untreated control, and the treatment with adenine must be continued to maintain CRF condition (22). However in this model, it has been reported that 19% and 67% of the rats died at 4 and 6 weeks after administration of adenine, respectively (20). Therefore, we designed the experiment of 31 days and started the administration of adenine and test reagents simultaneously to avoid the loss of the rats

due to the prolonged treatment with adenine.

The dietary intakes of adenine-treated groups were significantly lower than that of untreated normal group. In adenine-treated groups, the dietary intake of 10% TAP- treated group was significantly lower than that in control group (Table 3). This was probably due to the irritation by strong acidity of TAP (pH of 10% TAP suspension is 2.2), and the addition of TAP at 10% concentration into AIN-76 powder diet resulted in the decrease of dietary intake in this group.

The administration of adenine induced renal failure in rats, in which serum BUN and creatinine levels were elevated in a time-dependent manner (Figure 5). In calcium carbonate-treated group, serum BUN and creatinine levels were extensively higher than those in other groups. This reason was unclear but high calcium intake possibly affects the renal function synergistically with adenine because the dietary intake of this group was similar to other adenine-treated groups.

Serum phosphate levels increased in CRF model rats in comparison with untreated normal controls. TAP treatment inhibited the elevation of serum phosphate level, especially after 24 days of adenine administration. Serum phosphate levels of all of the phosphate adsorbent-treated rats were consistently lower than those in control CRF rats throughout the experiment. In comparison with calcium carbonate, TAP- and sevelamer-treated groups showed higher phosphate levels, but both phosphate adsorbents showed sufficient control of serum phosphate level. This effect was also reflected in 24-h urinary phosphate level. Both serum and urinary phosphate levels indicated that TAP adsorbed phosphate *in vivo* more effectively than sevelamer hydrochloride. However, serum levels of BUN and creatinine in TAP- and severamer-treated groups were lower than those in control group, indicating that the administration of these test reagents inhibit the progression of renal failure by treatment with adenine. Therefore, it is possible that the other factors than phosphate adsorption by TAP and sevelamer involved in the inhibitory effects of these reagents on serum phosphate levels observed in this study. In addition, it was difficult to evaluate the effect of 10% TAP on serum phosphate level due to the significantly lower dietary intake than those in other groups. Improvement of administration method (e.g. coating of TAP particles with cellulose) to reduce the irritation by TAP is necessary to evaluate the effect of high-dose TAP on serum phosphate level

in CRF model rats.

Serum calcium level was markedly elevated in rats treated with calcium carbonate. As hypercalcemia induces calcification of blood vessels (25, 26), the elevated serum BUN and creatinine levels in calcium carbonate-treated rats may indicate that hypercalcemia induced kidney malfunction in these animals. The elevated $Ca \times P$ value is a predictive risk factor for ectopic calcification (27-29). The $Ca \times P$ values in TAP-treated rats were stably lower than those in control CRF model rats throughout the experiment, indicating that TAP has a low risk of inducing ectopic calcification.

In summary, TAP has a high capability for adsorption of inorganic phosphate over a wide pH range. TAP also showed this ability in adenine-induced CRF model rats, where it inhibited the elevation of serum phosphate level without influencing serum calcium level. These results indicate that TAP is an effective phosphate adsorbent working throughout the intestinal tract. In addition, TAP has none of the risks associated with aluminum toxicity, hypercalcemia, or ectopic calcification. Sevelamer hydrochloride is a widely used phosphate adsorbent in CRF patients but constipation is a frequent adverse reaction due to the volume expansion of this polymer. On the other hand, TAP does not expand in volume in the intestinal tract, and is expected to be a phosphate adsorbent with less side effects, especially constipation. TAP is expected to be a good lead compound from which to develop a novel phosphate adsorbent for use in CRF patients with lower adverse effects.

ACKNOWLEDGEMENT

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