Inhibitory effects of female sex hormones on urinary stone formation in rats

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Background. The effects of female sex hormones on urinary stone formation are not known. This study was conducted to investigate the effects of these hormones on stone formation by using an ethylene glycol (EG) and vitamin D-induced rat urolithiasis model.

Methods. Adult female Wistar rats were fed the same diet for four weeks and were then divided into four groups ($N = 10$ each). One group was administered 0.5 ml of olive oil three times per week for four weeks as a control. The other three groups were administered 0.5 µg of vitamin D_3 and 0.5 ml of 5% EG three times per week for four weeks. The rats in two of these three groups were oophorectomized, and the rats of the remaining group underwent a sham operation on the day before the start of the four-week treatment period. One of the two oophorectomized groups was then administered a supplementation of female sex hormones (0.1 mg of estrogen and 2.5 mg of progesterone 3 times per week for 4 weeks). On the first day of the fifth week of the experimental period, the degree of crystal deposition was determined histologically, and the calcium content in renal tissue was measured. We also investigated the level of osteopontin (OPN) mRNA in renal tissues by Northern blot analysis. OPN is a matrix protein thought to be a promoter of stone formation.

Results. The urinary oxalate excretion, crystal deposition and calcium content in renal tissue and the expression of OPN-mRNA were greater in the oophorectomized rats compared with the controls, and the same parameters were inhibited by the female sex hormone supplementation.

Conclusions. These results suggest that female sex hormones can inhibit renal crystal deposition in EG-treated rats by suppressing the urinary oxalate excretion and the expression of OPN.

Many causes have been considered for urolithiasis in the upper urinary tract, however, the mechanism of urinary stone formation has not yet been adequately elucidated. Male gender is generally thought to be one of the risk factors of urolithiasis. The male-to-female ratio of patients with urolithiasis in several countries has been approximately 2:1 to 3:1 in recent years [1–3]. However, by age group, there is no gender difference in childhood or climacterium [4], whereas the male-to-female ratio in the reproductive stage is approximately 3:1 [3]. This suggests that female sex hormones are involved in inhibiting urinary stone formation. Moreover, the crystalline composition of upper urinary stones differs according to the reproductive condition versus postmenopausal condition [5]. Some studies have indicated that testosterone promotes renal crystal deposition because glycolic acid oxidase is involved in the metabolism of ethylene glycol (EG) to oxalate, and the activity may be enhanced by testosterone [6–8]. In light of these observations, both male and female sex hormones may have important roles in the pathogenesis of urolithiasis. In this study, we investigated the effects of female sex hormones on urinary stone formation in rats with EG-induced stones.

METHODS

Ten-week-old female Wistar rats (CLEA, Tokyo, Japan) were fed a standard commercial diet (CE-2, CLEA) and were maintained under constant conditions (temperature 23 to 24°C, humidity 50 to 60%, 12-hr illumination). They were given distilled water *ad libitum.*

When the rats reached 12 weeks of age (mean body weight, 210.5 g), they were divided into four groups of 10 rats each: the (*1*) Ooph+EG group and (*2*) Ooph+EG+FH group were treated by bilateral oophorectomy, and the (*3*) sham+EG group was treated by sham operation. In the oophorectomy, the bilateral ovaries, oviduct, and ovarian arteries and veins were ligated and cut out under anesthesia by an intraperitoneal injection of pentobarbital. To induce urinary stone formation, 0.5 µg of 1,25-dihydroxy vitamin D_3 (vitamin D_3; Chugai,

Tokyo, Japan) and 0.5 ml of 5% EG (Wako, Osaka, Japan) were administered three times per week via a stomach tube to each of the three groups. The administration period was four weeks. The Ooph+EG+FH group was additionally given estrogen in the form of estradiol dipropionate (Teikoku Hormone Mfg., Tokyo, Japan) and progesterone in the form of hydroxyprogesterone caproate (Teikoku Hormone), which were intramuscularly injected to the femoral region at 0.1 and 2.5 mg, respectively, three times per week for four weeks, as the supplementation of female sex hormones. In the (4) control group, 0.5 ml of olive oil was administered via a stomach tube three times a week for four weeks. These animals did not undergo a bilateral oophorectomy or sham operation.

On the first day of the fifth week of the experimental period (two days after from the last administration of EG and vitamin D_3), 24-hour urine samples were collected from all rats. The urinary creatinine level was determined by the alkaline picrate method, the urinary calcium level by the o-cresolphthalein complexone (OCPC) method, the urinary oxalic acid and citric acid levels by high-performance liquid chromatography [9], and the urinary magnesium level by the xylidyl blue method. After the 24-hour urine sampling, blood samples were collected by laparotomy, following the induction of anesthesia by an intraperitoneal injection of pentobarbital. The serum creatinine level was determined by the alkaline picrate method, serum calcium level by OCPC, and serum estradiol level by radioimmunoassay.

Immediately after the blood and urine sampling, the animals' right kidneys were removed and cut longitudinally. Half of the right kidney tissue was then fixed with 10% formaldehyde for hematoxylin and eosin (HE) and von Kossa staining. After thin sections were prepared from tissues, including the renal papilla, the existence and the frequency of crystal deposition in the renal tissue were observed in each group by light microscopy. The frequency of stone deposition was graded semiquantitatively in four categories. If there were 0, 1 to 5, 6 to 10, and more than 11 stone depositions on 10 fields (×100), the stone depositions were graded as (−), (±), (+), and (++), respectively.

After the left kidneys were weighed, they were minced in a beaker to which 2 ml of 0.5 N nitric acid was added. The beaker was then heated until the liquid became transparent. Up to 5 ml of distilled water was added to the solvent extract with stirring. After calibration using the standard calcium solution, the calcium content was determined by atomic absorption spectroscopy [10]. The calcium content of the kidney was expressed as μg/g wet tissue of the kidney.

The other halves of the right kidneys were immediately frozen in liquid nitrogen for a Northern blot analysis to determine the degree of osteopontin mRNA (OPN-mRNA). OPN-mRNA was measured in each kidney in each group.

Northern blot analysis

Total RNA was prepared by the lithium chloride (LiCl)-urea method [11], and was then electrophoresed in a 1.2% agarose-7.0% formaldehyde gel in 1 × MOPS buffer, pH 7.0 [20 mM MOPS, 5 mM sodium acetate, 1 mM ethylenediaminetetraacetic acid (EDTA)]. After the electrophoresis, the gel was soaked twice with 20 × standard saline citrate (SSC) for 15 minutes and was stained with size markers (28 S, 4.7 kb; 18 S, 2.2 kb). RNA was transferred to a nylon membrane (Gene Screen®; DuPont-New England Nuclear Research Products, Boston, MA, USA) by capillary action in 20 × SSC. Baked filters were prehybridized for four hours at 65°C in a hybridization solution containing 50% formamide, 5 × SSC, 10 × Denhardt's solution, 10 mM sodium phosphate buffer, pH 6.5, 0.5% sodium dodecyl sulfate (SDS), and 0.5 mg/ml sonicated salmon sperm DNA. Hybridization proceeded at 65°C for 16 hours in the same buffer containing 100 ng/ml digoxigenin (DIG)-11-UTP-labeled RNA probe. The filters were then washed in 2 × SSC/0.1% SDS, 0.2 × SSC/0.1% SDS twice at 65°C for 20 minutes each time. The washed filters were then soaked in DIG buffer 1 (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) for five minutes. Then the filters were washed briefly with 10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA (TNE) and treated by RNase A (10 μg/ml) in the same buffer at 25°C for 60 minutes. After RNase A treatment, the filters were washed with TNE to remove RNase.

The immunodetection of hybridized DIG-labeled RNA probe was performed by using a DIG Luminescent Detection Kit (Boehringer Mannheim, Mannheim, Germany) with some modifications [12]. The filters were incubated with 1.0% blocking reagent in DIG buffer 1 at room temperature for 60 minutes. The filters were then incubated with 0.1 unit/ml of polyclonal sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase in DIG buffer 1 at room temperature for 30 minutes. Excess antibody was removed by washing with DIG buffer 1 twice for 15 minutes each time. The washed filters were equilibrated for five minutes with DIG buffer 3 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl₂) and assay buffer (100 mM diethanolamine, 2 mM MgCl₂) twice for three minutes each time. An alkaline phosphatase reaction was obtained by incubating the filters in 0.1 mg/ml 3-(2′-spiroadamanatane)-4-methoxy-4- (3′-phosphoryloxy)-phenyl-1,2-dioxethane (AMPPD) in assay buffer at 37°C for 10 minutes. After the excess substrate was removed, the filters were exposed to x-ray film for 30 minutes at room temperature and were developed. The blots were also stripped and rehybridized with a human β-actin oligonucleotide to normalize for mRNA.

Data are presented as mean values ± sᴅ. Statistical

Table 1. Serum concentrations of estradiol and calcium at sacrifice, and the urinary excretions of calcium, oxalate, magnesium and citrate and creatinine clearance in each group of rats

	Group			
	Ooph + EG	Sham + EG	Ooph + EG + FH	Control
Serum estradiol *p/ml*[b]	1.29 ± 0.24[e]	15.0 ± 14.7[c]	160.9 ± 42.8[d,f]	15.8 ± 19.5[c]
Serum calcium *mg/dl*[b]	10.7 ± 0.5[f]	9.7 ± 0.5[d,e]	11.1 ± 0.5[c,f]	9.2 ± 0.4[d]
Urine volume *ml/day*[b]	31.3 ± 7.5[f]	23.6 ± 6.5[d,f]	30.9 ± 4.4[f]	16.5 ± 3.2[d]
Urinary calcium *mg/day*[b]	14.7 ± 5.1[f]	12.3 ± 4.9[f]	13.0 ± 3.3[f]	0.9 ± 0.4[d]
Urinary oxalate *mg/day*[b]	12.2 ± 8.1[f]	4.7 ± 0.6[d]	6.0 ± 1.9[d]	1.5 ± 0.9[d]
Urinary citrate *mg/day*	15.1 ± 4.4	13.8 ± 6.2[e]	13.1 ± 5.6[e]	19.3 ± 3.0
Urinary magnesium *mg/day*[a]	6.1 ± 3.5[e]	5.4 ± 4.2	8.6 ± 4.0[f]	1.8 ± 1.2[c]
C_{Cr} *ml/min*	1.36 ± 0.20	1.27 ± 0.20	1.31 ± 0.15	1.37 ± 0.22

Abbreviations are: Ooph, bilateral oophorectomy; EG, ethylene glycol; FH, female hormones; C_{Cr}, creatinine clearance.
[a] $P < 0.05$, [b] $P < 0.01$ (one-way ANOVA)
[c] $P < 0.05$, [d] $P < 0.01$, compared to the Ooph + EG group
[e] $P < 0.05$, [f] $P < 0.01$, compared to the Control group

analyses were performed with one-factor analysis of variance with a post hoc test (Fisher's protected least-significant difference test), and the level of crystal deposition was evaluated by the Mann–Whitney *U*-test.

RESULTS

The serum estradiol level in the Ooph+EG group was significantly lower than those in the sham+EG group and the control group, and thus the suppression of female sex hormones in the Ooph+EG group by oophorectomy was sufficient. The serum estradiol level in the Ooph+EG+FH group was significantly higher than those in the other groups. The supplementary dose of female sex hormones was approximately five times the normal level in human females, adjusted for weight, and this dose was thought to be sufficient to provide female sex hormones in the oophorectomized rat. The serum calcium levels in the Ooph+EG group, the sham+EG group, and the Ooph+EG+FH group were significantly higher than those in the control group. There were no significant differences among the Ooph+EG group, the sham+EG group, and the Ooph+EG+FH group in urinary calcium and magnesium excretions, but those in the control group were smaller than those of the other groups. There were no significant differences among the groups in creatinine clearance or in urinary citric acid excretion. The urinary excretion of oxalate in the Ooph+EG group was significantly higher than that in the other groups (Table 1).

The HE staining of the excised kidneys revealed substances stained purple in the distal tubule of the medulla (Fig. 1A). Because the same substances were stained dark brown by von Kossa stain, they were concluded to be stones containing calcium (Fig. 1B). The Ooph+EG group showed increased crystal deposition in the inner zone of the distal tubule of the medulla. Although crystal depositions were observed in the sham+EG group and the Ooph+EG+FH group, the volumes were small and their distribution was limited. No crystal depositions were observed in the control group. Crystals were observed in the inner zone in 6 of the 10 kidneys (60%) in the Ooph+EG group, and they reached the medullary outer zone in two of those six kidneys. In contrast, crystals were present in the inner zone in only 20%, 20%, and 0% of the 10 kidneys in the sham+EG group, the Ooph+EG+FH group, and the control group, respectively. No crystals were observed in the medullary outer zone in the sham+EG group, the Ooph+EG+FH group, and the control group (Table 2).

As shown in Table 3, the calcium contents in renal tissue in the Ooph+EG group were significantly higher than those in the other three groups, whereas there were no significant differences among the sham+EG group, the Ooph+EG+FH group, and the control group.

The expression of OPN m-RNA in renal tissue in the Ooph+EG group was clearly higher than that in the sham+EG group. The expression was markedly suppressed in the Ooph+EG+FH group, at a level that was not significantly different from that of the control group (Fig. 2). The expression of OPN mRNA in renal tissue was enhanced by the presence of calcium deposition.

DISCUSSION

In this study, we clarified the role of female sex hormones in the pathogenesis of urolithiasis, and gave progesterone with estrogen to the oophorectomized rat (Ooph+EG+FH group) as the female sex hormone. The reason is that progesterone affects the calcium metabolism in a similar fashion to estrogen [4, 13]. The mechanism underlying the inhibitory effect of female sex hormones on urinary stone formation is not yet known, but is generally thought to be due to the enhancement of the urinary excretion of citric acid. Urinary citric acid is regarded to be an inhibitor of Ca-containing stone formation because it shows a chelating activity against Ca ions. The urinary excretion of citric acid in stone formers is
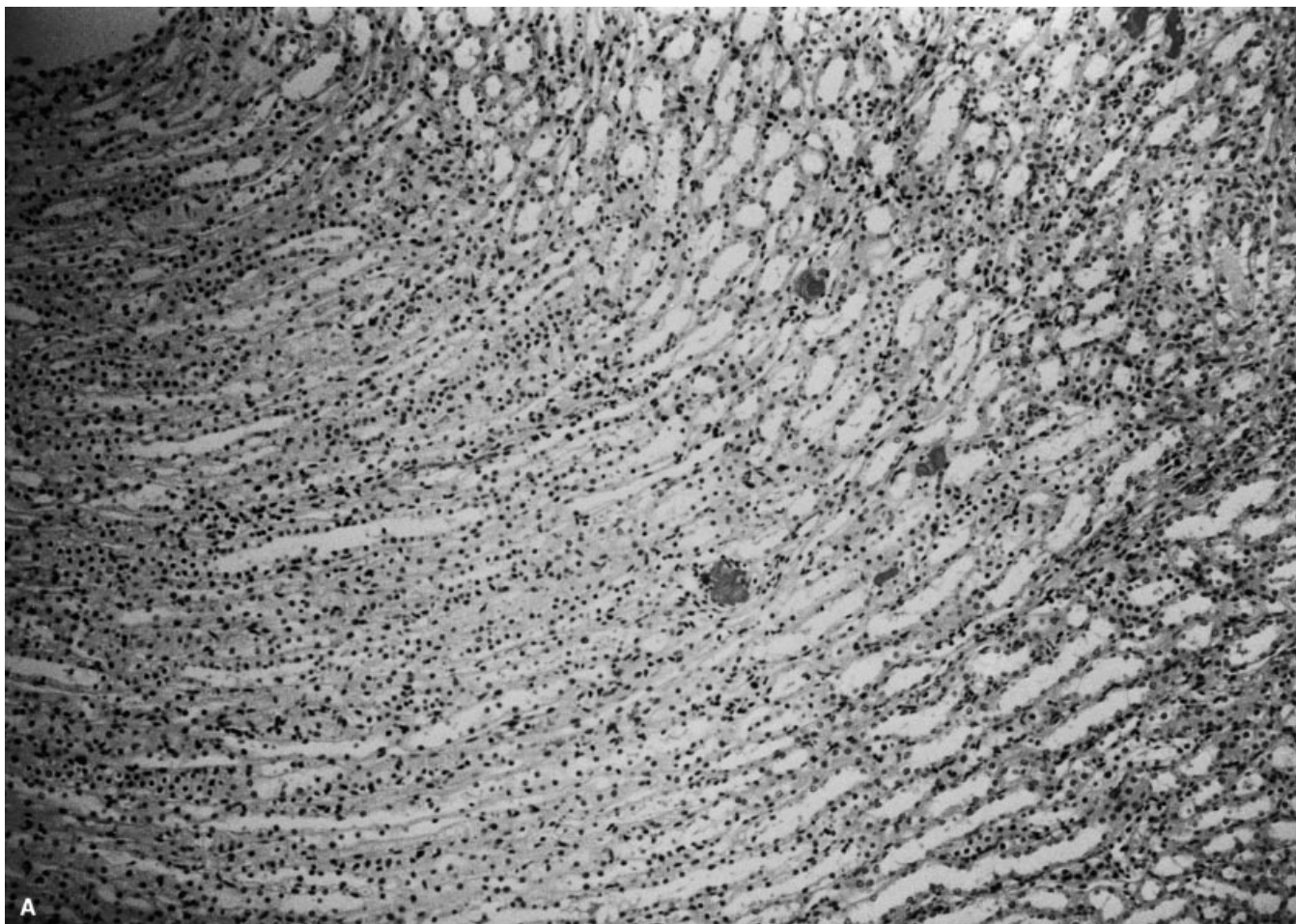
Fig. 1. (*A*) **Substances stained purple were observed in the distal tubule of the medulla (HE stain, ×100, the sham+EG group).** (*B*) These substances were stained dark-brown (von Kossa stain, ×120, the Ooph+EG group).

distinctly less than that in healthy adults [14, 15]. The urinary excretion of citric acid has been confirmed to vary with the estrus cycle and is markedly increased in postovulatory or later periods when the level of estrogen secreted increases. The excretion of citric acid is also higher in women of childbearing age than in men and is markedly decreased in postmenopausal and later periods of growth [14]. The urinary excretion of citric acid was most influenced by the variation of the acid-base equilibrium [16], and the influence of citric acid on crystal formation was much less than those of oxalic acid and calcium. In the experimental urolithiasis model used in this study, no significant differences of urinary citric acid excretion between the sham+EG group and oophorectomized rats (the Ooph+EG group and the Ooph+EG+FH group) were observed.

In this experiment, crystal depositions were induced in rats by modifying the method described by Okada et al [17]. They produced hyperoxaluria as well as hypercalciuria in rats by administering EG (0.5%, in drinking water administered *ad libitum*) and 1-α(OH)D₃ (0.5 µg/

rat given every other day), respectively, for three to four weeks. Although the frequency of crystal deposition reached 77% in their method, the rats also developed nephropathy because of the nephrotoxicity of EG, and the inulin clearance fell to 25% in that experiment. In our study, therefore, the dose of EG was reduced and fixed to 1.5 ml of 5% EG per week to avoid this disadvantage. This administration volume of EG was approximately 20% of that used in the Okada study. As a result, although only 20% of the sham+EG group rats demonstrated crystal deposition, reductions of creatinine clearance in the Ooph+EG group, the sham+EG group, and the Ooph+EG+FH group were not observed.

Lee et al recently showed that testosterone plays an important role in the promotion of stone formation, and found that the role of female sex hormones is smaller than that of testosterone [7, 8]. Lee et al used an EG (0.5%, in drinking water administered *ad libitum*)-treated Sprague-Dawley rat model and observed the crystal deposition in kidney tissue in order to evaluate the influence of testosterone. They found that 70% of the intact
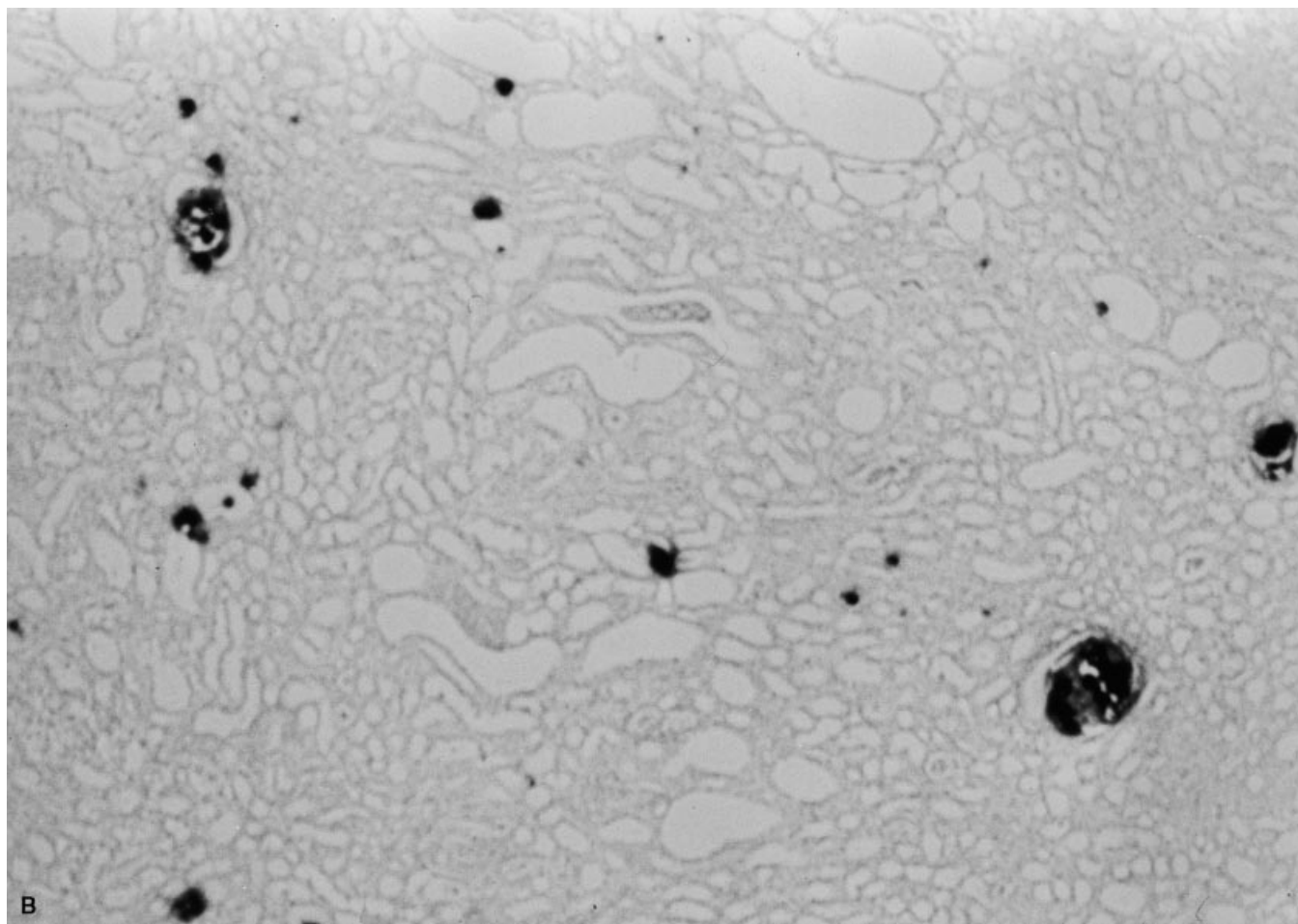
Fig. 1. Continued.

Table 2. Crystal formation in the renal medulla in each group

| | Group | | | |
	Ooph + EG	Sham + EG	Ooph + EG + FH	Control
Stone incidence %	60	20	20	0
Frequency of stone deposition %				
(−)	40	80	80	0
(±)	10	10	20	0
(+)	30	10	0	0
(++)	20	0	0	0
P value[a]		< 0.05	< 0.05	< 0.05

[a] Compared to the Ooph + EG group

Table 3. Kidney weight and renal calcium contents

	Left kidney wet weight *g*	Calcium contents[a] *µg/g wet tissue*
Ooph + EG group	0.89 ± 0.10	490 ± 69[d]
Sham + EG group	0.88 ± 0.06	376 ± 138[b]
Ooph + EG + FH group	0.86 ± 0.05	384 ± 160[b]
Control group	0.84 ± 0.07	315 ± 12[c]

[a] $P < 0.05$ (one-way ANOVA)
[b] $P < 0.05$, [c] $P < 0.01$, compared to the Ooph + EG group
[d] $P < 0.01$, compared to the control group

male rats fed 0.5% EG had renal stones, and the incidence of renal crystal deposition of orchiectomized male rats decreased to 10%. They also found that oophorectomized female rats fed 0.5% EG had no renal stones or crystal deposition. Although we did not evaluate the effects of testosterone on stone formation in this study and could not compare the effects of testosterone and female hormones, our study shows that stone deposition in the distal tubule was distinctly greater in oophorectomized-only rats than in sham-operated rats, and crystal deposition was inhibited by the addition of female sex
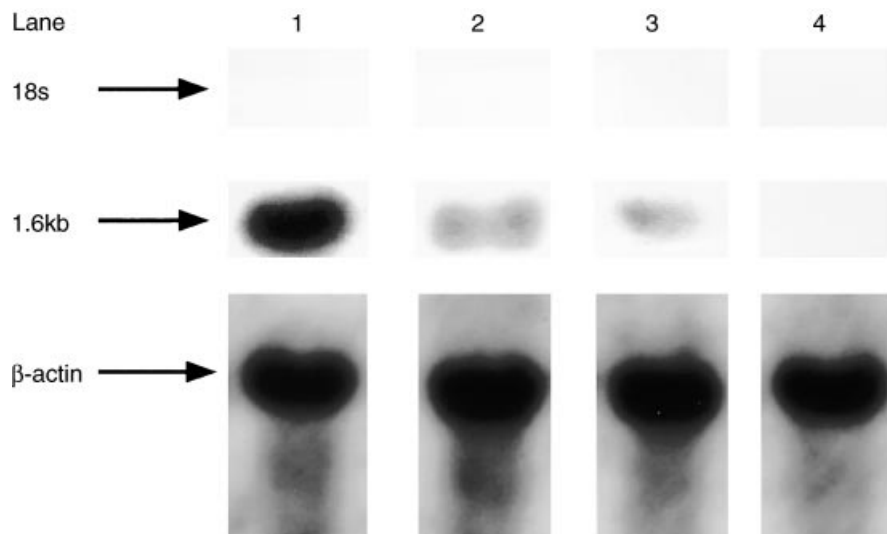
Fig. 2. Northern blot analysis of the level of osteopontin (OPN) mRNA. The level of OPN mRNA was higher in the Ooph+EG group than in the sham+EG group. OPN mRNA expression was markedly reduced in the Ooph+EG+FH group. Total RNA was isolated from the kidney and electrophoresed on a 1.2% agarose gel (total RNA 20 μg/lane). The arrows indicate 1.6 kb, 18 S and β-actin.

hormones to oophorectomized rats. The cause of the difference in the results of this study and those of Lee et al is unclear, but it is possible that the differences in the strain of rats and the administered volumes of EG and vitamin D_3 contributed to the different results. Although estrogen is generally thought to inhibit the excretion of urinary calcium, the excretion in the Ooph+EG+FH group was not decreased by the treatment with female sex hormones in this study. We suspect that the reason for this lack of influence is that the effect of the vitamin D_3 supplementation was greater than that of female sex hormone supplementation because the urinary calcium excretion in the control group was significantly lower than that of the groups fed EG and vitamin D_3. However, the female sex hormone supplementation inhibited the excretion of urinary oxalate in this study. Although the reason for this inhibition is unclear, this result indicates that a suppression of female sex hormones will promote the stone formation by increasing the urinary excretion of oxalate. This result also agrees with the finding that calcium oxalate stones are predominant in postmenopausal women [18].

Although many investigators have reported the importance of stone matrix to the pathogenesis of urolithiasis, its exact role has remained unclear. Our previous study revealed that the cDNA sequence of OPN encoded the urinary oxalate stone protein, suggesting that OPN is involved in stone formation as the stone matrix [19], and the expressions of OPN mRNA and protein were observed in stone-forming kidneys [19]. In this study, we did not determine the cell types of OPN mRNA-positive cells by hybridization. We previously observed in a rat model of nephrolithiasis that OPN mRNA was sporadically present in the distal tubules of the normal rat kidney, and it was increased with stone formation [20].

Moreover, we reported the role of OPN as a promoting factor of stone formation in the model of Madin-Darby canine kidney cells using OPN antisense oligonucleotide [21]. These findings suggest that OPN is strongly related to stone formation in calcium stone formation. In this study, the expression of OPN m-RNA in renal tissue was enhanced in the oophorectomized rats and suppressed by female sex hormone supplementation. OPN is a matrix component of the calcification of arteriosclerosis [12], and estradiol inhibits fibrous plaque formation, as a result of promoting the degeneration of elastin and collagen by estradiol in the rat aorta [22]. In light of these previous and current findings, the presence of female sex hormones is thought to play a very important role in the pathogenesis of calcification, particularly in the inhibition of urolithiasis.

Conclusion

In the rat groups administered EG and vitamin D_3, urinary excretion of oxalate in the group without female sex hormones caused by oophorectomy was increased compared with those in groups undergoing sham operation and receiving supplementation of female hormones. Moreover, the stone matrix in renal tissue, that is, OPN, was increased in the group without female sex hormones because of oophorectomy. As a result, the frequency of crystal deposition was increased in that group.

These results indicate that female sex hormones play an important role in the pathogenesis of urolithiasis.

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