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# PREVENTIVE EFFECTS OF GREEN TEA ON RENAL STONE FORMATION AND THE ROLE OF OXIDATIVE STRESS IN NEPHROLITHIASIS

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## ABSTRACT

Purpose: Urinary stones are similar to arteriosclerosis in epidemiology, mechanism, calcification composition and age at frequent occurrence. The calcification that occurs in arteriosclerosis is inhibited by antioxidants. Green tea leaves contain approximately 13% catechins, which have been shown to have antioxidant effects. We investigated the inhibitory, antioxidative effects of green tea on calcium urinary stone formation.

Materials and Methods: A total of 120 Wistar rats were divided into 4 groups, namely group 1—control rats receiving saline, group 2—stone group rats administered ethylene glycol (EG) and vitamin D3, group 3—drink group rats administered EG, vitamin D3 and green tea given as drinking water, and group 4—powder group rats administered EG, vitamin D3 and 2.5% powdered green tea leaves mixed in a powder diet. Pooled 24-hour urine samples and blood samples were collected and the 2 kidneys were excised 7, 14 and 21 days after administration, respectively. One kidney was used for immunohistological examination of osteopontin, superoxide dismutase (SOD), p65, p53 and bcl-2 expression, in situ hybridization of osteopontin and detection of apoptosis, while the other was used for quantitative analysis of SOD activity.

Results: Green tea treatment decreased urinary oxalate excretion and calcium oxalate deposit formation. Green tea treatment increased SOD activity compared with the stone group. The degree of apoptosis in the stone group was significantly increased compared with the drink and powder groups.

Conclusions: The inhibitory effect of green tea on calcium oxalate urolithiasis is most likely due to antioxidative effects.

KEY WORDS: kidney; kidney calculi; rats, Wistar; catechin; antioxidants

Calcium containing urinary stones are a common clinical problem. The recurrence rate is quite high, about 50% at 10 years and 75% at 15 years if untreated.[1] Matrix proteins form 1% to 5% of urinary stones and several studies suggest the importance of matrix proteins in stone formation.[2] Previously we have cloned and sequenced the cDNA encoding osteopontin (OPN), an important soluble protein component of calcium oxalate stone proteins.[3] OPN expression in the kidneys was significantly increased after hyperoxaluria.[4] However, OPN deficient mice demonstrated significant intratubular deposits of calcium oxalate crystals, whereas WT mice were completely unaffected.[5]

The incidence of urolithiasis has increased in industrialized nations. However, the pathogenesis of and methods for preventing urolithiasis remain to be clarified. Urolithiasis resembles arteriosclerosis in the mechanism, calcification composition and epidemiology. The components of urolithiasis and arteriosclerosis calcification include calcium, phosphate, OPN and matrix gla protein, and macrophage chemotaxis is involved in the pathogenesis.[6]

Green tea has been reported to inhibit oxidation/degeneration of low-density lipoprotein and, thus, show antiatherosclerotic effects.[7] Therefore, we administered green tea to rats with stone formation and investigated its inhibitory effects on urolithiasis and its mechanism of action.

## MATERIALS AND METHODS

*Animals.* All experimental procedures were performed with the approval of the Animal Care Committee of the Faculty of Medicine, Nagoya City University Graduate School of Medical Sciences. Male Wistar rats (Charles River Japan, Yokohama, Japan) at age 7 weeks weighing approximately 240 gm were used. Animals were provided with a standard mEq diet, including 1.01 gm Ca, 0.78 gm P, 0.21 gm Mg and 0.23 gm Na/100 gm (Oriental Yeast, Tokyo, Japan), and free access to water. To induce calcium oxalate deposition in the kidneys rats were given 2 doses of 0.12 ml 5% ethylene glycol (EG) (Wako, Tokyo, Japan) in 1.0 ml saline daily through a stomach tube. Saline was administered to the control group in 2 doses and 0.5 $\mu$g vitamin D3 (1$\alpha$[OH]D3, alfacalcidol) (Chugai Pharmaceutical, Tokyo, Japan) was given with oil every other day.

*Experimental protocol.* After 1 week of acclimatization 120 rats were divided into 4 groups, that is group 1—control group rats receiving 1.0 ml saline daily in 2 doses with free access to water, group 2—stone group rats administered EG and vitamin D3 with free access to water, group 3—drink group rats administered EG, vitamin D3 and green tea containing 40 mg catechin in 100 ml (Ito-en, Tokyo, Japan) given

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*Urinary and serum variables in control and experimental rats*

| Group (days) | Mean Vol ± SD (ml) | Mean pH ± SD | Mean Urine ± SD (mg/day) | | | Mean Serum ± SD (mg/dl) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Calcium | Oxalate | Citrate | Creatinine | BUN | Calcium | Phosphate | Uric Acid |
| Control: | | | | | | | | | | |
| 7 | 31.3 ± 8.8 | 7.4 ± 0.5 | 4.05 ± 2.32 | 1.6 ± 0.3* | 46.2 ± 10.8 | 0.21 ± 0.06 | 15.6 ± 2.3 | 9.96 ± 1.06 | 6.79 ± 0.54 | 1.65 ± 0.58 |
| 14 | 33.5 ± 7.5 | 7.3 ± 0.3 | 3.89 ± 1.98 | 1.7 ± 0.4† | 54.5 ± 14.2 | 0.21 ± 0.08 | 16.9 ± 2.5 | 10.85 ± 0.78 | 6.89 ± 0.48 | 1.67 ± 0.29 |
| 21 | 33.1 ± 8.0 | 7.3 ± 0.2 | 4.02 ± 2.13* | 1.8 ± 0.3† | 41.8 ± 9.5* | 0.21 ± 0.09 | 17.5 ± 1.9 | 10.67 ± 1.08 | 7.05 ± 0.87 | 1.76 ± 0.75 |
| Stone: | | | | | | | | | | |
| 7 | 30.4 ± 8.3 | 7.5 ± 0.5 | 4.75 ± 2.58 | 2.8 ± 1.0* | 66.0 ± 22.3 | 0.23 ± 0.09 | 19.8 ± 1.9 | 10.89 ± 1.09 | 7.15 ± 0.45 | 1.68 ± 0.58 |
| 14 | 32.4 ± 7.5 | 7.7 ± 0.5 | 5.05 ± 3.16 | 3.6 ± 1.3†‡ | 75.3 ± 24.0 | 0.25 ± 0.05 | 18.9 ± 2.3 | 11.56 ± 1.18 | 7.08 ± 0.67 | 1.73 ± 0.35 |
| 21 | 39.5 ± 7.5 | 8.1 ± 0.3 | 5.25 ± 3.47 | 3.8 ± 1.4†‡ | 99.3 ± 20.4*‡ | 0.21 ± 0.04 | 16.5 ± 2.5 | 12.63 ± 1.21‡ | 7.06 ± 0.41 | 1.89 ± 0.65 |
| Drink: | | | | | | | | | | |
| 7 | 32.0 ± 11.6 | 7.5 ± 0.5 | 4.18 ± 2.36 | 1.6 ± 0.5 | 46.2 ± 18.0 | 0.21 ± 0.07 | 19.5 ± 2.7 | 10.08 ± 0.89 | 6.72 ± 0.43 | 1.87 ± 0.39 |
| 14 | 38.3 ± 8.9 | 7.5 ± 0.3 | 3.98 ± 2.15 | 2.2 ± 0.5‡ | 51.1 ± 16.2 | 0.23 ± 0.08 | 20.1 ± 2.5 | 10.94 ± 0.96 | 7.06 ± 0.63 | 2.28 ± 0.74 |
| 21 | 37.6 ± 9.5 | 7.7 ± 0.3 | 3.34 ± 1.85 | 2.4 ± 0.7‡ | 48.6 ± 17.5‡ | 0.26 ± 0.07 | 20.8 ± 2.7 | 10.64 ± 0.61 | 7.25 ± 0.64 | 2.76 ± 0.60 |
| Powder: | | | | | | | | | | |
| 7 | 39.3 ± 12.8 | 7.5 ± 0.2 | 3.86 ± 1.81 | 1.7 ± 0.4 | 32.8 ± 12.8 | 0.20 ± 0.05 | 16.2 ± 2.1 | 10.84 ± 0.98 | 6.69 ± 0.42 | 1.85 ± 0.59 |
| 14 | 35.3 ± 8.5 | 7.5 ± 0.3 | 4.34 ± 2.37 | 2.0 ± 0.4‡ | 40.3 ± 14.2 | 0.23 ± 0.08 | 16.8 ± 2.4 | 9.98 ± 0.58 | 6.98 ± 0.55 | 2.03 ± 0.68 |
| 21 | 38.2 ± 9.5 | 7.7 ± 0.5 | 5.35 ± 2.89* | 2.2 ± 0.7‡ | 39.2 ± 8.5‡ | 0.20 ± 0.08 | 16.0 ± 1.3 | 9.56 ± 0.19‡ | 6.71 ± 0.52 | 2.29 ± 0.70 |

* Control vs stone and powder groups $p < 0.05$.
† Control vs stone and powder groups $p < 0.01$.
‡ Stone vs drink and powder groups $p < 0.05$.

as drinking water and group 4—powder group rats administered EG, vitamin D3 and 2.5% powdered green tea leaves containing 169 mg catechin in 100 gm mixed with the mEq powder diet and provided with free access to water. Rats were weighed weekly. Pooled 24-hour urine samples were collected using metabolic cages. Urine samples for the measurement of oxalate were collected in cups containing 5% HCl. After collecting blood samples from the inferior vena cava with the animal under ether anesthesia the 2 kidneys were immediately excised at 7, 14 or 21 days after administration. One kidney was used for immunohistological examination of OPN, superoxide dismutase (SOD), p65, p53 and bcl-2 expression, in situ hybridization of OPN and apoptosis detection, while the other kidney was used for quantitative examination of SOD activity.

*Measurement of urinary and serum variables.* Urinary volume and urinary pH were measured manually. Urinary calcium and serum creatinine, blood urea nitrogen (BUN), calcium, phosphate and uric acid levels were determined using an automated analyzer (Model 705, Hitachi, Tokyo, Japan). Urinary oxalate was determined using oxalate decarboxylase and citrate was determined by citrate lyase conversions to oxaloacetate.

*Number of calcium oxalate deposits.* Excised kidney tissue samples were fixed with 4% paraformaldehyde in 0.1 M phosphate buffered saline (pH 7.0), dehydrated in ethanol, embedded in paraffin under ribonuclease-free conditions and cut into 4 $\mu$m serial sections. Sections were stained using the method described by Pizzolato[8] to visualize calcium oxalate. Data presented are the number of detected calcium oxalate deposits per $cm^2$ (cut area).

*Reverse transcriptase-polymerase chain reaction (RT-PCR).* Tissue samples for RT-PCR were frozen with liquid nitrogen. Total RNAs were extracted from kidney tissues using a previously described method. Primers used to amplify OPN were sense primer 5′-CCATGAGACTGGCAGGGTT-3′ and antisense primer 5′-GGAACTGTGGTTTTGCCTCT-3′. Cycling parameters for PCR were denaturation at 94C for 30 seconds, annealing at 55C for 1 minute and extension at 72C for 1 minute. A total of 30 cycles were performed. Primers used to amplify SOD were sense primer 5′–ATGGTGGCCTTCTTGTTCT GC-3′ and antisense primer 5′–GTGCTGTGGGTGCGGCACACC-3′. Cycling parameters for PCR were denaturation at 94C for 1 minute, annealing at 61C for 1 minute

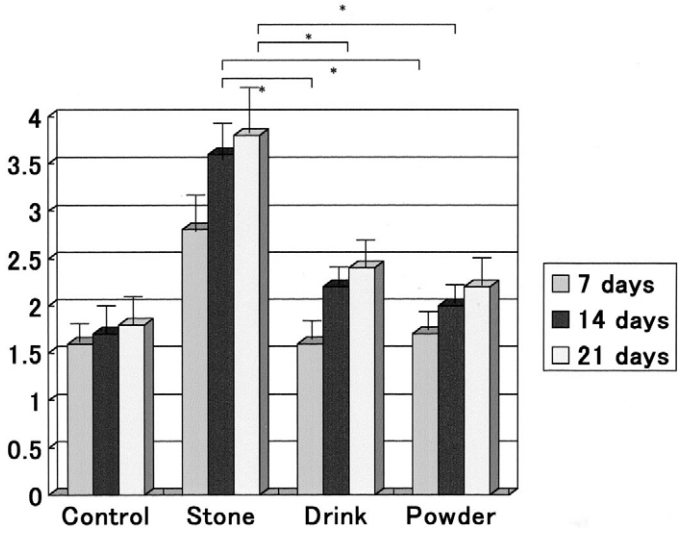


FIG. 1. Urinary oxalate excretion in stone group was significantly higher than in control group at 7, 14 and 21 days ($p < 0.05$, $< 0.01$ and $< 0.01$, respectively). Urinary oxalate excretion was significantly lower in drink and powder groups at 14 and 21 days vs stone group ($p < 0.05$). Asterisk indicates $p < 0.05$.

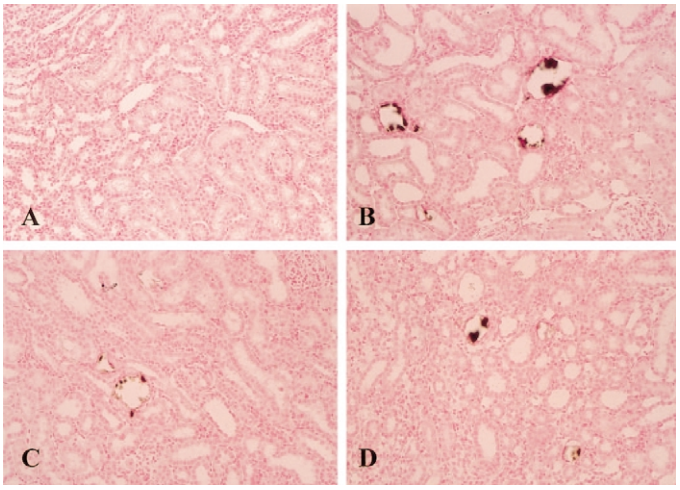


FIG. 2. Light microscopy of rat renal cortex and medulla showed calcium oxalate deposits in lumen of renal tubules at 21 days. There were no deposits in control group (*A*). In drink (*C*) and powder (*D*) groups deposition levels were decreased vs stone group (*B*). Pizzolato staining, reduced from ×200.

and extension at 72C for 1 minute. A total of 23 cycles were performed. Co-amplification with $\beta$-actin was included to ensure that equal amounts of RNA were reverse transcribed and amplified in each reaction tube. PCR products were visualized by ethidium bromide staining.

*In situ hybridization.* Digoxigenin-uridine triphosphate labeled, single strand RNA probes were prepared for hybridization using a DIG RNA labeling kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer protocol.

*Immunohistochemical staining.* Immunohistochemical staining was performed by the streptavidin-biotin method using a Histofine SAB-PO kit (Nichirei, Tokyo, Japan). AntiOPN, antiSOD, antinuclear factor (NF)-$\kappa$B (p65), antip53 and antibcl-2 antibodies were used as the primary antibodies at 1 $\mu$g/ ml.

*Detection of apoptosis.* We examined apoptosis by the TUNEL method.[9] In situ end-labeling was performed using an Apoptosis In situ Detection Kit (Wako, Osaka, Japan). To evaluate apoptosis microscopic fields were selected at random and the number of TUNEL positive staining cells per 100 distal and proximal convoluted tubules were counted as the apoptosis index.

*SOD activity.* SOD activities were determined using the method of Sohal et al.[10] SOD activity was calculated by dividing the change in optical density of the sample by the change in each unit of SOD from the standard curve.

*Statistical analysis.* All data are expressed as the mean $\pm$ SD. Differences between groups were examined for statistical significance using Student's t test with $p < 0.05$ considered statistically significant different.

## RESULTS

*Urinary and serum variables.* There were no changes in urine volume or urinary pH 7, 14 or 21 days after administration (see table). In the powder group urinary calcium excretion increased gradually after administration and it was significantly higher than in the control group 21 days after administration ($p < 0.05$).

In the stone group 24-hour urinary oxalate excretion was significantly higher than in the control group 7 days after administration ($p < 0.05$) and it was more than 2-fold higher than in the control group 14 and 21 days after administration ($p < 0.01$). In the drink and powder groups 24-hour urinary oxalate excretion increased gradually after administration and it was significantly lower than in the stone group at 14 and 21 days ($p < 0.05$, fig. 1). In the stone group 24-hour urinary citrate excretion was significantly higher than in the control group at 21 days. In the drink and powder groups 24-hour urinary citrate excretion levels were significantly lower than in the stone group 21 days after administration ($p < 0.05$). In the powder group the serum calcium level was significantly lower than in the stone group 21 days after administration ($p < 0.05$). There were no marked changes in serum creatinine, BUN, phosphate or uric acid levels 7, 14, or 21 days after administration.

*Histological examination.*The Pizzolato staining method[8] clearly showed calcium oxalate deposits in the kidneys of the stone, drink and powder groups 7, 14 and 21 days after administration (fig. 2). No deposits were detected in the control group. Quantitative analysis of the number of calcium oxalate deposits per $cm^2$ showed a significantly lower number of deposits in the drink and powder groups at 14 and 21 days compared with the stone group (fig. 3).

*In situ hybridization.* OPN mRNA expression was observed in the kidneys of all 4 groups 7, 14 and 21 days after administration. OPN mRNA was found in the distal and proximal convoluted tubules, the loops of Henle and the collecting ducts, whereas no staining for mRNA was detected in renal cortex glomeruli. In the control group only weak OPN staining was observed. However, expression was enhanced in the stone group. Green tea treated rats showed lower expression than those in the stone group (fig. 4).

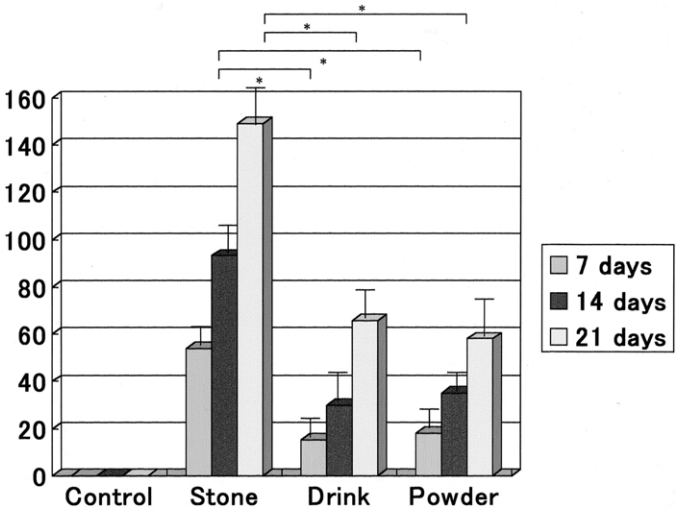


FIG. 3. No deposits were detected in control group. Quantitative analysis of number of calcium oxalate deposits per $cm^2$ showed significantly fewer deposits in drink and powder groups at 14 and 21 days vs stone group. Asterisk indicates $p < 0.05$.

*Immunohistochemical studies.* OPN protein expression was observed in the kidneys of all 4 groups 7, 14 and 21 days after administration. OPN protein was found in the distal convoluted tubules. In the control group weak OPN staining was observed. However, expression was enhanced in the stone group. Green tea treated rats showed lower expression than those in the stone group (data not shown).

In the control group SOD protein expression was observed in the proximal and distal tubules, and collecting ducts. In the stone group weak SOD staining was observed (fig. 5, *A*). Green tea treated rats showed higher expression than those in the stone group (fig. 5, *B*).

In the distal and proximal convoluted tubules p65 and p53 proteins were found. The expression of p65 and p53 proteins was enhanced in the stone group compared with the control group, and in the drink and powder groups expression was weak (fig. 5, *C* and *D*). The expression of bcl-2 protein was higher in the glomeruli than in the proximal and distal

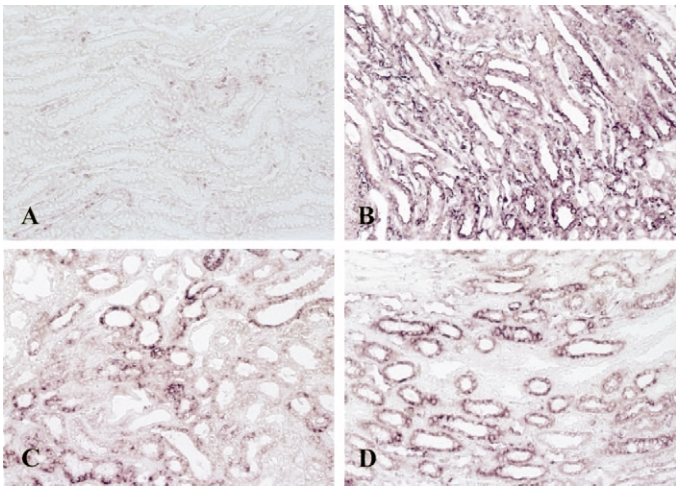


FIG. 4. In situ hybridization analysis of OPN mRNA at 21 days. In control group weak expression of OPN mRNA was observed in renal distal tubular cells (A). However, OPN mRNA expression was enhanced in stone group (*B*). Drink (*C*) and powder (*D*) groups showed weaker expression than in stone group. Reduced from $\times$200.

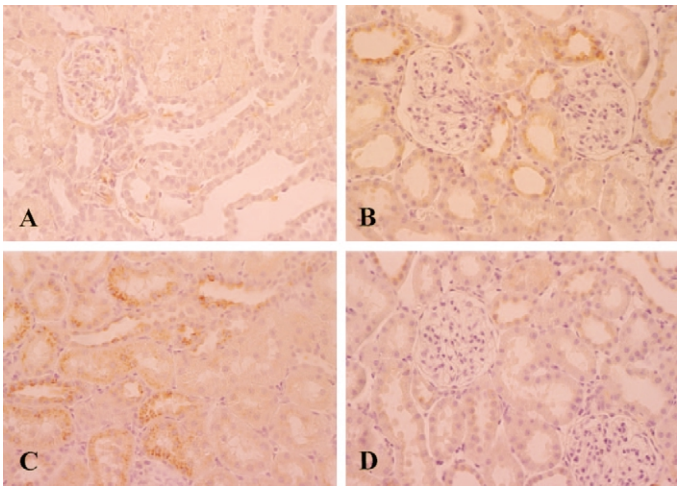


FIG. 5. Immunohistochemical staining revealed enhanced SOD protein expression in drink (*B*) vs stone (*A*) group. Expression of p53 protein was decreased in drink (*D*) vs stone (*C*) group. SOD polyclonal (*A* and *B*) and p53 polyclonal (*C* and *D*) antibody, reduced from ×400.

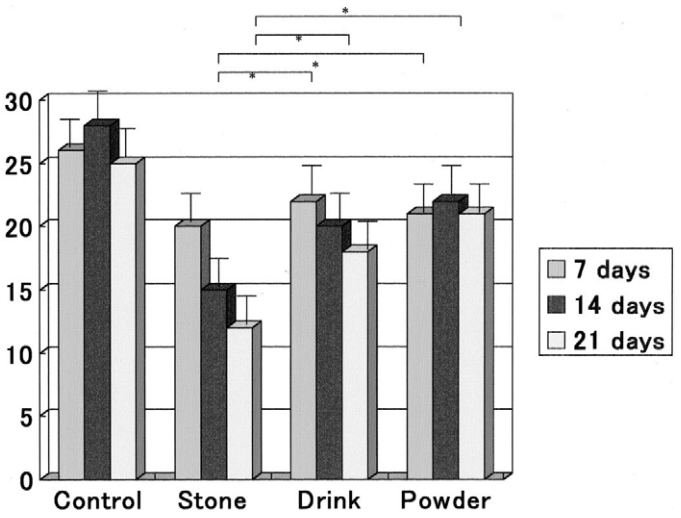


FIG. 7. Quantitative SOD activity in stone group was significantly decreased vs control groups at 14 and 21 days ($p < 0.05$ and $< 0.01$, respectively). Green tea treatment increased SOD activity vs stone group at 14 and 21 days ($p < 0.05$). Asterisk indicates $p < 0.05$.

tubules. In the stone group weak bcl-2 staining was observed. Green tea treated rats showed higher expression than those in the stone group (data not shown).

*RT-PCR.* RT-PCR was performed to detect sequences from OPN (979 bp) and SOD (447 bp). Analysis of SOD gene expression in rat kidneys using total RNA from kidney tissues showed clear bands in all groups 21 days after administration (fig. 6). Analysis of OPN gene expression in rat kidneys showed clear bands in all groups (data not shown).

*SOD activity.* Quantitative analyses showed that SOD activity decreased gradually in the stone group after administration and it was half that in the control group at 21 days. During the administration period there were no changes in SOD activity in the drink or powder groups. SOD activities in the drink and powder groups were higher than in the stone group and approached those in the control group (fig. 7).

*Apoptotic index.* In all groups TUNEL positive cells were observed in the convoluted tubules (fig. 8). The apoptotic index in the stone group was more than 10-fold higher than in the control group 14 and 21 days after administration. Green tea treatment decreased the apoptotic index compared with the stone group (fig. 9).

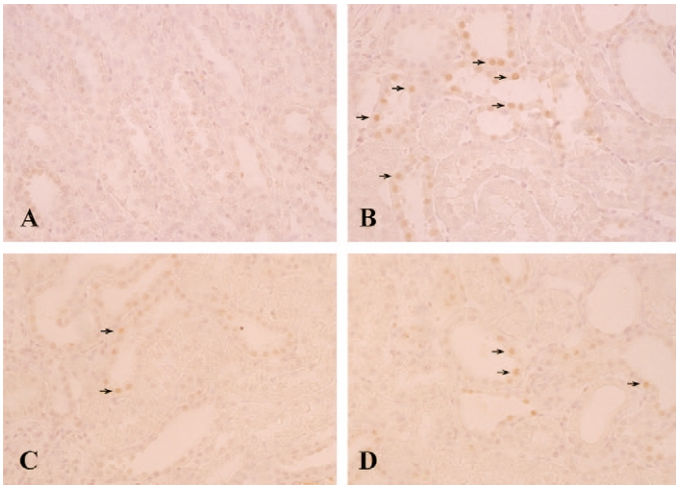


FIG. 8. In all groups TUNEL positive cells (arrows) were observed in convoluted tubules. In control group (*A*) rare TUNEL positive cells were visualized. Apoptosis was enhanced in stone group (*B*) vs drink (*C*) and powder (*D*) groups. Reduced from ×400.

## DISCUSSION

Urolithiasis resembles arteriosclerosis in the mechanism, calcification composition, epidemiology and gene relationship. It has been reported that the vascular endothelial growth factor gene polymorphism is a suitable genetic marker of urolithiasis.[11] Calcification in arteriosclerosis has been inhibited by antioxidants. In this study green tea treatment decreased the formation of calcium oxalate deposits in kidney tissue, and the expression of OPN and apoptosis, and increased SOD activity. Consumption of teas is generally known to increase urinary oxalate excretion. However, the oxalate concentration in the green tea administered to rats was 50 mg/l, which was lower than the concentration in the urine of control rats.

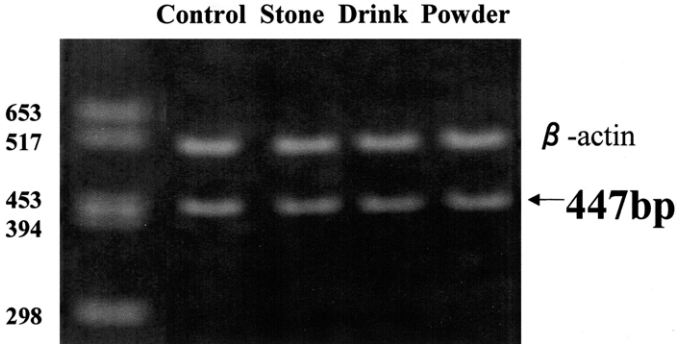


FIG. 6. RT-PCR amplification of SOD gene expression using total RNA from kidney tissues 21 days after administration. Ethidium bromide stained gel showed clear bands in all groups at 447 bp, representing SOD gene expression.

Antioxidant therapy with vitamin E has prevented calcium oxalate precipitation in the rat kidney and decreased urinary oxalate excretion in patients with kidney stones.[12] The renal antioxidants vitamin E, ascorbic acid and glutathione were significantly decreased on oxalate challenge.[13] Vitamin E administration in patients who underwent surgical stone removal rapidly restored antioxidant levels in the blood and decreased the urinary excretion of oxalate and calcium.[14] A previous study of antioxidant enzyme levels in rats with stone formation showed that almost all antioxidant enzyme activities were attenuated except that of catalase.[15]

Sarica et al reported that calcium oxalate crystals and

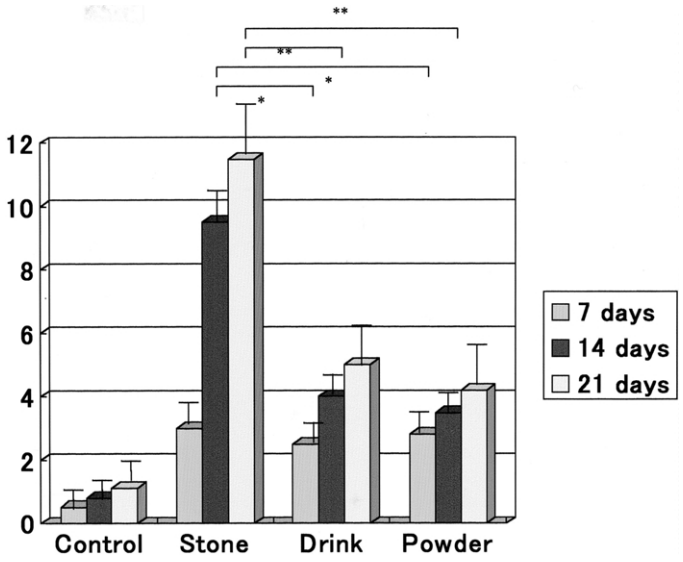


FIG. 9. Apoptotic index was significantly increased in stone group at 7, 14 and 21 days vs control group ($p < 0.05$, $< 0.01$ and $< 0.01$, respectively). Green tea treatment decreased apoptotic index significantly at 14 and 21 days vs stone group ($p < 0.05$ and $< 0.01$, respectively). There were no significant differences between drink and powder groups. Asterisk indicates $p < 0.05$. Double asterisks indicate $p < 0.01$.

hyperoxaluria may be injurious to renal tubular cells, as indicated by apoptotic changes in a urolithiasis rabbit model.[16] Sustained hyperoxaluria in association with calcium oxalate crystals induced apoptosis as well as necrosis.[17] Although cell death by hypoxia is a well-known type of oxidative stress, which has been generally believed to manifest as necrosis, recent biochemical observations suggest the possibility of hypoxia induced apoptosis.[18] Wu et al reported that NF-$\kappa$B activation by oxidative stress induced human aortic endothelial cell death and apoptosis through the suppression of bcl-2, bax translocation and p53 induction.[19] Apoptosis observed in this experiment was also thought to depend on the same mechanism. The blockade of NF-$\kappa$B activation by antioxidants has been suggested to be an effective strategy for the treatment of urolithiasis and arteriosclerosis.[20]

## CONCLUSIONS

The results of the current study demonstrate that the antioxidative effect of green tea decreased calcium oxalate stone formation, OPN expression and apoptosis, and increased SOD activity in rat kidney tissues. The inhibitory effect of green tea on calcium oxalate urolithiasis is most likely due to antioxidative effects.

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