

# Serum antioxidant enzyme levels are decreased in patients with urinary calcium oxalate stones

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**Purpose:** To compare the serum antioxidant enzyme levels between patients with urinary stone disease and healthy volunteers to determine the effect of cellular oxidative stress on urinary calcium oxalate stones formation.

**Materials & Methods:** A total of 51 patients with proven urinary calcium oxalate stones (female 35.3%, mean age: 49.3 years) and 37 healthy subjects (female 45.9%, mean age: 44.1 years) were included. The serum levels of antioxidant catalase, glutathione peroxidase, superoxide dismutase and lipid peroxidation were measured in serum samples taken from the peripheral venous circulation.

**Results:** Mean serum catalase level of patient group was insignificantly higher than healthy subjects (7.54 mmol-H<sub>2</sub>O<sub>2</sub>/mg/sec versus 6.16 mmolH<sub>2</sub>O<sub>2</sub>/mg/sec, respectively;  $P = .06$ ) whereas mean superoxide dismutase level (1.56 U/ml versus 3.86 U/ml,  $P = .047$ ), glutathione peroxidase level (6.70 U/ml versus 8.19 U/ml,  $P = .022$ ) and lipid peroxidation level (2.35 nmol/ml versus 3.31 nmol/ml,  $P = .034$ ) of patient group were significantly lower than healthy subjects. Patients with family history of urinary stone disease had significantly lower mean serum levels of catalase ( $P = .037$ ), superoxide dismutase ( $P = .047$ ) and glutathione peroxidase ( $P = .01$ ), compared with patients without family history.

**Conclusion:** The findings of this study provide evidence regarding the role of oxidative stress in the development of urinary calcium oxalate stones. Future clinical trials are necessary to elucidate the actual mechanisms of the calcium oxalate stone formation in the environment with increased oxidative stress.

**Keywords:** antioxidants; calcium oxalate; oxidative stress; reactive oxygen species; urinary stone disease.

## INTRODUCTION

With its increasing prevalence and economic burden, urinary stone disease continues to be a major health problem<sup>(1)</sup>. It is expected that a climate-related increase of 1.6-2.2 million lifetime cases of nephrolithiasis will happen by the year 2050, which will result in a cost increase of \$0.9-1.3 billion annually (year-2000 dollars)<sup>(1)</sup>. However, our understanding related to the stone formation pathophysiology remains limited in spite of the recent studies, which demonstrated the crystallization and plaque formation mechanisms<sup>(2)</sup>.

The urinary stone formation is considered as a complicated physicochemical disorder. The epithelial cells inside the renal tubules respond to alterations in the urinary environment<sup>(3)</sup>.

These most crucial changes in urinary complex in the case of Calcium Oxalate (CaOx) stone formation are

dysregulated mineral metabolism, abnormal levels of calcium, oxalate, phosphate and citrate. Moreover, the increased production of crystallization modulating macromolecules plays an important role in the formation CaOx stone<sup>(4)</sup>.

It is well known that, the reactive oxygen species (ROS) are involved in the process of CaOx stone formation as signalling molecules as well as agents of inflammation and injury<sup>(5)</sup>.

The plaque formation in kidney is triggered by ROS and the formation of oxidative stress (OS). Exposure of the renal epithelial cells to high levels of CaOx/calcium phosphate (CaP) crystals and oxalate generates excess ROS, causing injury and inflammation<sup>(6)</sup>.

Several authors demonstrated that reactive oxygen species (ROS) may be involved in urinary stone formation<sup>(5,7)</sup>. Some of these studies reported increased renal enzymes in the urine of patients with calcium oxalate

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**Table 1.** Demographic characteristic of the patients and healthy subjects.

	Patients (n=51)	Healthy Subjects (n=37)	P
Age (Mean $\pm$ SD)	49.3 $\pm$ 16.20	44.13 $\pm$ 13.050	.008*
Male	50.3 $\pm$ 17.28	44.24 $\pm$ 13.17	< 0.001*
Female	47.44 $\pm$ 14.3	44 $\pm$ 13.3	0.02*
Urinary stone family history; n (%)	31 (60.8)	8 (21.6)	< 0.001**
Smoking history; n (%)	26 (51)	20 (54.1)	0.68**
Alcohol history;n (%)	10 (19.6)	10 (27.2)	0.43**
Body mass index, Mean $\pm$ SD (kg/m <sup>2</sup> )	26.09 $\pm$ 3.2	22.97 $\pm$ 2.66	< 0.001*

\*Mann-Whitney U test

\*\*Chi-Square test

(CaOx) stone, indicating ROS associated renal damage<sup>(8)</sup>, whereas some others identified antioxidant enzymes in the inner core of CaOx stones, suggesting their role in the nucleation process leading to inner matrix formation<sup>(9)</sup>. Results of the recently conducted National Health and Nutrition Examination Survey III (NHANES III) which included 17,695 subjects confirmed that patients with a kidney stone history have significantly lower serum antioxidants levels<sup>(10)</sup>. However, the type of the stones was not specified in NHANES III.

This case-control study aims to compare the serum antioxidant levels between patients with urinary CaOx stones and healthy volunteers to determine the effect of cellular OS on the development of urinary stone disease. Considering the findings of this study, urologists may initiate antioxidant treatment in patients with CaOx stone disease.

## PATIENTS AND METHODS

### Patient selection

A total of 85 patients with the diagnosis of CaOx urinary stone disease who were treated at our institution were screened. Patients (n = 34) with any comorbidities (e.g. malignancy, hypertension, congestive cardiac failure, diabetes mellitus), history of ESWL treatment within the last 3 months, urinary tract infection and/or who were using antioxidant supplements were excluded.

After obtaining written informed consent of the patients, their medical history was taken and physical examination was performed. The patients provided their urine sample for urine analysis and urine culture. Midstream urine samples were collected and evaluated immediately for urinary tract infections. Patients with positive urine culture were excluded from the study. The presence of the urinary stones was confirmed with imaging studies such as kidney-ureter-bladder (KUB) radiography, urinary ultrasonography, intravenous pyelography (IVP) or non-contrast-enhanced abdominopelvic computed tomography.

The patients underwent ureterorenoscopy, percutaneous nephrolithotomy and open kidney stone surgery according to the size and location of the stone. The removed stone fragments were analysed with quantitative X-ray diffraction phase analysis and the stone type was assigned. Patients whose urinary stone was not pure-

ly comprised of CaOx were excluded from the patient group and 51 eligible patients with confirmed CaOx urinary stone were included.

A total of 37 healthy volunteers without any urinary stones according to the urine test, KUB radiography and medical history constructed the control group. Participants with any comorbidities and smoking habit were excluded.

The study protocol was approved by the institutional ethics committee of Istanbul Sisli Etfal Research and Training Hospital. Patients provided their informed consent prior to their enrolment into the study.

### Laboratory methods

Patients and healthy subjects provided their venous blood sample for the measurement of serum antioxidant levels, which indirectly reflect the OS of the patients and controls. Blood samples were taken from patients who underwent stone removal surgery in the morning of the surgery day before induction of anesthesia. Morning blood samples were taken from controls as well. The 2.5 ml blood samples were drawn from brachial vein into EDTA containing tubes. The samples were centrifuged at 1500 rpm for 5 minutes and extracted serum was preserved at -20° C until the levels of antioxidant catalase, superoxide dismutase, glutathione peroxidase and the level of malondialdehyde (MDA) as the product of lipid peroxidation were determined, respectively.

The serum samples were defrosted for the assessment of antioxidant enzyme activities with spectrophotometry (Shimadzu, Japan). Superoxide dismutase and glutathione peroxidase enzyme activities are analysed with specific kits in accordance with the instructions of the manufacturer (Randox Laboratories Limited, UK). The performance characteristics of superoxide dismutase and glutathione peroxidase kits and the other lab assays were shown in **Table 2**.

Catalase activity was evaluated in serum at 25° C. The reaction related to H<sub>2</sub>O<sub>2</sub> substrate was spectrophotometrically measured at 240 nm for 30 seconds and the activity was measured in MU/L. One unit of catalase activity was equal to the 1  $\mu$ mol H<sub>2</sub>O<sub>2</sub> synthesized per minute. Lipid peroxidation was assessed by the measurement of thiobarbituric acid reactive substances (TBARS) level inside the serum. MDA was measured spectrophotometrically in 532 nm wavelength after the reaction with thiobarbituric acid and the results were

**Table 2.** Antioxidant enzyme activities of the patients and healthy subjects.

	Patients (n=51)*	Healthy subjects (n=37)*	P
Plasma catalase(mmolH <sub>2</sub> O <sub>2</sub> /mg/sec)	7.54 ± 1.34	6.16 ± 0.72	0.062
Glutathione peroxidase (U/ml)	6.7 ± 1.61	8.19 ± 0.13	0.022
Superoxide dismutase (U/ml)	1.56 ± 0.46	3.86 ± 0.58	0.047
Malondialdehyde (nmol/ml)	2.35 ± 0.45	3.31 ± 0.4	0.034

\*Result are given as mean ±SD (Standard Deviation)

recorded as nmol/ml.

### Statistics methods

Kolmogorov-smirnov test was used to determine the distribution of the data. Student's T test was used for the comparison of the antioxidant enzyme levels in patients with urinary CaOx stones and healthy controls. Chi-square test was used for comparison of alcohol and smoking status. The statistical analyses were performed on IBM SPSS Statistics (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp).

## RESULTS

A total of 51 patients with CaOx stones (female 35.3%, mean age: 49.3 years) and 37 healthy subjects (female 45.9%, mean age: 44.1 years) were included in the study. The stone patient population was older, had a higher BMI and had a greater proportion of males than the control population (Table 1).

Mean serum catalase level of patient group was insignificantly higher than healthy subjects whereas mean superoxide dismutase level, glutathione peroxidase level and MDA level of patient group were significantly lower than healthy subjects (Table 2).

In addition, patients with family history of urinary stone disease compared to those without family history had significantly lower mean serum levels of catalase (6.77 mmol H<sub>2</sub>O<sub>2</sub>/mg/sec versus 8.23 mmol H<sub>2</sub>O<sub>2</sub>/mg/sec, respectively;  $P = .037$ ), mean superoxide dismutase level (1.44 U/ml versus 1.67 U/ml, respectively;  $P = .047$ ), mean glutathione peroxidase level (6.11 U/ml versus 7.11 U/ml, respectively;  $P = .01$ ). Although mean MDA enzyme activities were also lower among patients with family history of urinary stone disease, this difference did not reach statistical significance (2.45 nmol/ml versus 2.78 nmol/ml,  $P = .064$ ) (Table 3).

## DISCUSSION

The etiological factors of the urinary stone disease are one of the most popular research topics in the field of urology. Recent reports confirmed the possible role of the ROS in stone formation<sup>(5,7-9)</sup>. Although the exact mechanism, by which ROS contributes to the stone formation, is not clear, it is well known that, the ROS are involved in the process of CaOx stone formation as signalling molecules as well as agents of inflammation and injury<sup>(5)</sup>. The inflammation and OS markers have been detected in urine samples of stone patients and in the urine of rats with experimentally induced CaOx nephrolithiasis<sup>(6)</sup>. Moreover, studies using animal models and tissue cultures reported that; antioxidant treatments may reduce crystal and oxalate induced injury<sup>(4)</sup>. Several authors demonstrated that antioxidant containing fruit juices and diets are associated with reduced risk for kidney stones<sup>(11-15)</sup>.

Exposure of the renal epithelial cells to high levels of CaOx/calcium phosphate (CaP) crystals and oxalate generates excess ROS, causing injury and inflammation<sup>(6)</sup>.

The plaque formation in kidney is triggered by ROS and the formation of OS<sup>(6)</sup>. The major mechanism of action can be explained as follows: ROS regulate crystal formation, growth and aggregation by affecting the modulators responsible for the crystallization process. It is known that, there is an overproduction of ROS and a decrease in the antioxidant capacity resulting in OS, renal injury and inflammation, which may stimulate the CaOx stone formation<sup>(16)</sup>.

The availability of ROS is controlled by several scavenging systems such as superoxide dismutase, glutathione peroxidase and catalase. Superoxide dismutase eliminates superoxide anion (O<sub>2</sub><sup>-</sup>). However, glutathione peroxidase and catalase detoxify hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)<sup>(5,6)</sup>.

The level of OS was assessed by the lipid peroxidation

**Table 3.** Antioxidant enzyme activities of the patients with or without urinary stone family history.

	Patients w/ history (n=31)*	Patients w/o history (n=20)*	P
Catalase (mmolH <sub>2</sub> O <sub>2</sub> /mg/sec)	6.77	8.23	0.037
Glutathione peroxidase (U/ml)	6.11	7.11	0.010
Superoxide dismutase (U/ml)	1.44	1.67	0.047
Malondialdehyde (nmol/ml)	2.45	2.78	0.064

**Abbreviations:** w/ history, with urinary stone family history; w/o history, without urinary stone family history

\*Result are given as mean (min-max)

assay, by measuring the amount of malondialdehyde (MDA) due to oxidative stress as an end product of the lipid peroxidation process<sup>(17)</sup>.

There are only few reports, which assessed the serum antioxidant levels in kidney stone patients. The NHANES III previously reported that elevated levels of serum antioxidants were inversely related to the prevalence of kidney stones<sup>(10)</sup>. The authors evaluated the data of the 17,695 adult subjects and revealed the overall prevalence of kidney stones as 5.25%. Furthermore, the authors detected a significant association between lower levels of alpha-carotene, beta-carotene and beta-cryptoxanthin, and the prevalence of kidney stones. In a prospective study, Tracy et al.<sup>(18)</sup> demonstrated that recurrent stone formers have increased oxidative stress as measured by serum lipid peroxidation and thiobarbituric acid reactive substances levels. The authors also recorded that the antioxidant characteristics of the pomegranate extract supplementation may confer some modest benefit in preventing crystal formation among patients with CaOx stones.

To our knowledge, serum antioxidant levels have not been specifically studied in patients with urinary CaOx stone disease before. Our results demonstrated lower serum catalase, superoxide dismutase, glutathione peroxidase and MDA levels among CaOx stone disease patients compared with healthy controls. These differences were statistically significant in all antioxidants except catalase. Moreover, these antioxidants (except MDA) were significantly lower among patients who had a family history of urinary stone disease, compared to those without familial urolithiasis history. To our knowledge, this finding has not been reported before and it provides further evidence regarding the involvement of oxidative stress in the stone formation suggesting that hereditary disorders in the production of antioxidants may play role in the occurrence of CaOx stones. As MDA is an end product of lipid peroxidation process, the severity of OS can be indicated with higher MDA levels<sup>(6)</sup>. However in our study the MDA levels were reported lower in patients with CaOx stones. The small patient group should be the possible reason of that result.

Our study is not without limitation. First of all, having a larger study group may also detect lower catalase levels in patients with CaOx stones. Unfortunately, we did not calculate the sample size prior to the commencement of the study because of not having any estimation related to the antioxidant enzyme levels. Moreover, the patients were not matched with the control subjects in terms of demographic data. Therefore, future studies with matched-control group must be designed. Secondly, we could not assess the impact of hypertension and diabetes, both of which are linked to increased oxidative stress<sup>(19-22)</sup>, because of excluding all the stone patients with comorbidities. Performing a logistic regression analysis that includes these comorbidities along with age and BMI as confounding factors would be more appropriate to clarify the actual role of OS on the pathophysiology of CaOx stones. Lack of data on the markers of OS is another limitation of the study. Lower MDA levels detected in the patient group may also be considered as a limitation. Future studies with larger patient groups using additional oxidative stress parameters are needed to confirm rational values. Moreover, having the levels of urinary ROS and/or antioxidant levels would increase

the validity of our findings. Finally, assessment of the correlation between the stone volume and antioxidant levels would confirm the validity of our findings.

## CONCLUSIONS

The outcomes of this study provide evidence regarding the role of OS in the urinary CaOx stone disease. Future clinical trials are necessary to elucidate the actual mechanisms of the CaOx stone formation in the environment with increased OS.

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