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ORIGINAL PAPER

Urinary 8-hydroxydeoxyguanosine is elevated in patients with nephrolithiasis

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Abstract 8-hydroxydeoxyguanosine (8-OHdG) is an oxidatively modified guanosine, which has been widely used as an oxidative DNA damage marker in various diseases. The present study aimed to determine urinary 8-OHdG in nephrolithiasis patients and evaluate its clinical significance. Thirty-six nephrolithiasis patients and 30 healthy subjects were recruited. Urine volume, creatinine, malondialdehyde, *β-N*-acetylglucosaminidase (NAG) activity and proteins were measured in 24 h urine samples. Urinary 8-OHdG was determined by competitive enzyme-linked immunosorbent assay. Mineral composition of stones was analyzed using Fourier-transformed infrared spectroscopy. Nephrolithiasis patients excreted urinary 8-OHdG significantly higher than healthy controls. Urinary 8-OHdG levels compared among patients with calcium oxalate, struvite and uric acid stones were insignificantly different. The urinary NAG activity correlated positively with urinary 8-OHdG. Multiple linear regression showed that urinary NAG activity was an independent predictor of urinary 8-OHdG level. Receiver operating characteristic analysis revealed that the urinary 8-OHdG test was adequate for diagnosing nephrolithiasis. At 10 μg/g creatinine cutoff, the 8-OHdG test imparted high specificity (96.67%) and a positive predictive value (91.67%). In conclusion, this is the first report of elevated urinary 8-OHdG excretion in nephrolithiasis patients indicating increased oxidative DNA damage. Increased renal tubular damage was independently associated with elevated urinary 8-OHdG. Elevated urinary 8-OHdG levels adjunct with metabolic profile may be useful for identifying people at risk of stone development.

Keywords Nephrolithiasis · Kidney stone · Oxidative stress · 8-OHdG · ROC analysis · Diagnostic values

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Introduction

Although the actual mechanism of stone formation is not fully understood, oxidative damage is considered to play an important role in the pathogenesis of nephrolithiasis [1]. In chronic supersaturated urine, lithogenic crystals are formed. These crystals as well as lithogenic ions such as oxalate directly induce the production of reactive oxygen species (ROS) in renal tubular cells leading to oxidative damage [2]. For clearance purposes, crystals are internalized by renal tubular cells to be digested in the lysosome and the residues are exocytosed to the interstitial space leading to immunoactivation. In vitro evidence demonstrated that pro-inflammatory mediators such as monocyte chemoattractant protein-1 [3] and interleukin-6 [4] were released from renal tubular cells after lithogenic crystals or oxalate challenge. These events are believed to occur in vivo in order to recruit leukocytes into the renal interstitium to phagocytose the interstitial crystals and initiate inflammation [5]. The oxidative burst produced by leukocytes in turn extends the oxidative damage of renal tubular

cells. Renal tubular damage provides sites for crystals adhering. Nidi are formed, grow and become calculi. Reduction of oxidative stress using various antioxidants has been suggested to weaken the potentiality of calculi formation and recurrences.

Oxidative stress is an imbalance of oxidants and antioxidants causing various reactive species such as ROS to be overwhelmingly generated. ROS rapidly attack cellular macromolecules such as proteins, lipids, carbohydrates and nucleic acids leading to oxidative damage. Attack of ROS on DNA (both in the nucleus and mitochondria) causes a number of oxidative DNA lesions, notably an oxidized form of guanosine, 8-OHdG [6]. To fix the damaged DNA, an excision repair system is activated to cleave out 8-OHdG and replace it with an intact nucleotide. Since 8-OHdG is readily water-soluble, it is directly excreted via the urine. The 8-OHdG is the most frequently detected DNA lesion in urine. It is widely used as a biomarker of oxidative DNA damage in many pathological conditions [7].

Elevated levels of urinary 8-OHdG indicate the vast extent of oxidative DNA damage. This has been documented in various oxidative-mediated diseases such as diabetes [8] and cancers [9, 10]. Hitherto, the urinary excretion of 8-OHdG in nephrolithiasis has not been investigated. The present study measured urinary 8-OHdG in nephrolithiasis patients and evaluated the clinical usefulness of this urinary DNA lesion.

Materials and methods

Subjects

Thirty healthy controls and 36 nephrolithiasis patients were recruited for the study. The healthy group consisted of 11 males (37%) and 19 females (63%) while the nephrolithiasis group consisted of 15 males (42%) and 21 females (58%). All patients underwent surgical removal of stone by either open surgery, percutaneous nephrolithomy or shock-wave lithothripsy. Nephrolithiasis patients with anatomical abnormality of the kidney, urinary infection and obstruction and malignancy were excluded. Stone types of nephrolithiasis patients were classified according to the majority of mineral component into three types, calcium oxalate (CaOx), magnesium ammonium phosphate (MAP) and uric acid (UA). Informed consent was received from all participants prior to specimen collection and the research protocol was approved by the Ethical Committee, Faculty of Medicine, Chulalongkorn University, Bangkok.

24-hour urine and stone specimen collection

Twenty-four hour urine specimens were collected from all subjects using thymol as preservative. In nephrolithiasis patients, the urine specimens were collected pre-operatively. The urine samples were kept at −20°C until testing. Since 24 h creatinine level varies among different laboratories, the normal value can range from 0.5–2.0 g/day. In this study, any specimens with urinary creatinine less than 0.5 g/day were regarded as inadequate collection and excluded.

Stone specimens obtained from nephrolithiasis patients were washed, dried, grinded into powder and kept at −20°C. Fourier-transform infrared spectroscopy was employed for mineral composition analysis.

Urine biochemistry

A 24 h urine volume was measured. Malondialdehyde (MDA) as a lipid peroxidation marker was determined by thiobarbituric acid (TBA) method, in which MDA reacts with TBA in the presence of butylated hydroxytoluene and ferric chloride to form a colored complex with a maximum absorbance of 532 nm [11]. Indicators of renal tubular damage used in this study were urinary protein content and *β-N*-acetylglucosaminidase (NAG) activity. NAG activity was measured by the spectrophotometric method established by Horak et al. [12]. Urinary protein content was determined by dye-binding method (Bradford protein assay).

Urinary 8-OHdG was determined by competitive enzyme-linked immunosorbent assay (ELISA) (New 8-OHdG Check ELISA kit, JaICA, Japan). The procedure was performed according to the manufacturer's instruction.

Statistical analysis

Mean and standard deviation (SD) were used as representatives of the data. Comparisons of mean between two groups of subjects and three types of stones were performed using Student's *t*-test and analysis of variance (ANOVA) tests, respectively. Also, Mann-Whitney and Kruskal-Wallis tests, the equivalent non-parametric tests of Student's *t*-test and ANOVA, respectively, were performed and similar conclusive results were obtained. Scatter plots were created using Microsoft Excel and bivariate correlation was assessed by Pearson's correlation test. Box-Whisker plots were created by STATA version 8.0 software (Collage Station, TX, USA).

Multiple linear regression was modeled to evaluate the contribution magnitudes of urinary parameters (MDA, NAG activity, proteins and urine volume) on urinary excretion of 8-OHdG controlled for age, sex and body mass index (BMI). In order to directly compare the magnitude of correlation of each parameter, concentrations of urinary parameters were standardized (mean = 0; SD = 1) and the normalized β-coefficient of each urinary parameter was computed.

A receiver operating characteristic (ROC) curve analysis is used to evaluate the ability of a test to discriminate diseased cases from non-diseased cases. How well the test can distinguish between two diagnostic groups is indicated by an area under curve (AUC). An AUC of 0.5 indicates that the test cannot distinguish between the two groups. On the other side, an AUC of 1.0 indicates that the test can perfectly separate diseased cases from non-diseased cases. A perfect separation between the two populations is rarely observed, meaning that incorrectly classified cases are found in every selected cutoff point. In an attempt to assess the potential of urinary 8-OHdG in distinguishing nephrolithiasis from healthy subjects, ROC analysis was carried out. An appropriate cutoff was chosen from the ROC curve to calculate the diagnostic value of urinary 8-OHdG determination. Two-side analysis at a significance level of $\alpha = 0.05$ was set for all statistical tests.

Results

Table 1 displays the demographic data and urine biochemistry of subjects compared between healthy controls and renal stone patients. The healthy group contained 37% males and the nephrolithiasis group consisted of 42% males. Means of age and body mass index (BMI) were not significantly different between healthy and renal stone groups. Likewise, excretion of creatinine was insignificantly different between two groups. In the healthy group, males excreted creatinine significantly higher than females ($P = 0.009$).

Twenty-four hour urine volume compared between nephrolithiasis and healthy groups was not statistically different. Assessment of renal tubular damage using urinary NAG activity and proteins as indicators found that both urinary NAG activity and protein content were significantly higher in nephrolithiasis patients than in healthy controls. Urinary excretion of MDA in the renal stone group (9.45 ± 7.93 μM/g creatinine) trended to be higher than in the healthy group (6.22 ± 1.40 μM/g creatinine). However, the observation was not statistically significant.

The urinary excretion of 8-OHdG compared between healthy and nephrolithiasis groups is shown in Fig. 1. Nephrolithiasis patients (8.26 ± 4.94 μg/g creatinine) excreted 8-OHdG significantly higher than healthy controls (5.27 ± 2.77 μg/g creatinine) ($P = 0.004$). There was no gender difference in urinary 8-OHdG excretion in both healthy (male versus female; 5.18 ± 2.66 vs 5.23 ± 2.90 μg/g creatinine) and nephrolithiasis groups (male vs female; 8.48 ± 5.04 vs 8.11 ± 4.99 μg/g creatinine).

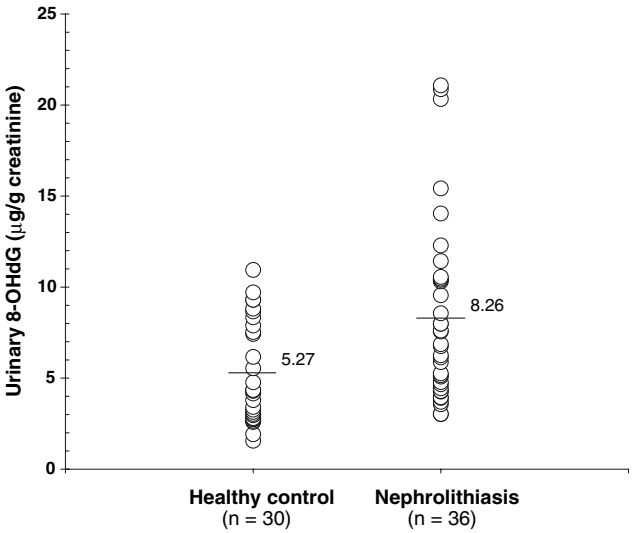

Fig. 1 Dot plot shows comparison of urinary excretion of 8-OHdG between healthy and nephrolithiasis groups. The level of urinary 8-OHdG in nephrolithiasis (8.26 ± 4.94 μg/g creatinine) was significantly higher than that of healthy controls (5.27 ± 2.77 μg/g creatinine) ($P = 0.004$). The horizontal lines with values indicate the mean of each group

Table 1 Basic clinical characteristics and urine biochemistry of subjects

Variables	Healthy control			Nephrolithiasis			*P**
	Total	Male	Female	Total	Male	Female	
n (%)	30 (100)	11 (37)	19 (63)	36 (100)	15 (42)	21 (58)	
Age (years)	41.43 ± 10.34	40.09 ± 12.83	42.21 ± 8.89	46.89 ± 11.75	45.33 ± 12.37	48.00 ± 11.46	0.052
BMI (kg/m^2)	22.56 ± 3.16	23.46 ± 2.47	22.04 ± 3.45	23.68 ± 4.30	23.93 ± 4.15	23.51 ± 4.49	0.238
24-H urine volume (ml)	1858 ± 762	1943 ± 561	1808 ± 868	2003 ± 648	1858 ± 595	2108 ± 678	0.404
Urinary creatinine (g/day)	1.08 ± 0.42	1.39 ± 0.45	0.90 ± 0.28	0.97 ± 0.42	1.05 ± 0.53	0.92 ± 0.32	0.296
Urinary NAG activity (U/g creatinine)	3.43 ± 2.30	3.64 ± 2.92	3.30 ± 1.92	14.73 ± 16.78	16.01 ± 21.10	13.82 ± 13.38	<0.001
Urinary protein content (g/g creatinine)	0.10 ± 0.06	0.06 ± 0.03	0.12 ± 0.07	0.40 ± 0.48	0.32 ± 0.43	0.45 ± 0.52	0.001
Urinary MDA (μM/g creatinine)	6.22 ± 1.40	5.93 ± 1.14	6.40 ± 1.54	9.45 ± 9.73	9.35 ± 1.00	9.53 ± 9.78	0.077

* *P* values of Student's *t*-test: total healthy control versus total nephrolithiasis

The types of stone were subdivided according to the principal mineral component into three stone types viz. CaOx, MAP, and UA stones. CaOx, MAP, and UA stones accounted for 70.59% (24/34), 14.71% (5/34), and 14.71% (5/34), respectively. Means of urinary 8-OHdG in patients with CaOX, MAP, and UA stones were 8.15 ± 4.90, 10.02 ± 7.24, and 6.75 ± 3.92 μg/g creatinine, respectively. Urinary excretions of MDA, proteins, NAG activity, and 8-OHdG (Fig. 2) did not vary significantly among the three types of stone. Therefore, we opted to treat nephrolithiasis patients, with various stone types, as a single group in the subsequently statistical analysis.

To see the correlation between excretion of 8-OHdG and urine biochemistry in nephrolithiasis patients, scatter plots were created and a bivariate correlation test was performed. A positive correlation between urinary NAG activity, and 8-OHdG level was observed ($r = 0.68$, $P < 0.001$) (Fig. 3). Urinary proteins were associated with urinary 8-OHdG excretion ($r = 0.47$, $P = 0.004$). Urine volume ($r = 0.14$, $P = 0.403$) and urinary MDA ($r = (0.01$, $P = 0.949$) were not significantly associated with urinary 8-OHdG.

In an attempt to quantify the contribution magnitude of urinary variables (urine volume, MDA, NAG activity, and proteins) on the predictive value of 8-OHdG levels in renal stone patients, multiple linear regression was modeled. The multivariate analysis adjusted for age, gender and BMI revealed that urinary NAG was the only significant predictor of urinary 8-OHdG (Table 2). Overall, the model explained 56% of variability in urinary 8-OHdG ($n = 36$, model $R^2 = 0.56$, $P < 0.001$). Urinary NAG activity (β-coefficient = 0.171, 95% CI: 0.055, 0.289, $P = 0.006$) was

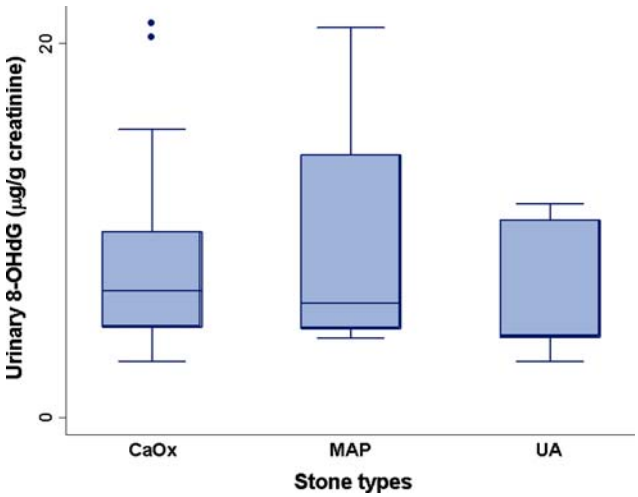

Fig. 2 Box-Whisker plot of urinary 8-OHdG compared among stone types. Medians (ranges) of urinary 8-OHdG in patients with CaOx ($n = 24$), MAP ($n = 5$) and UA ($n = 5$) stones were 6.81 (3.02, 21.08), 6.14 (4.26, 20.87) and 4.44 (3.03, 11.43) μg/g creatinine, respectively. The excretions of urinary 8-OHdG among three stone types tested by Kruskal–Wallis test were not statistically different ($P = 0.473$). *CaOx* calcium oxalate, *MAP* magnesium ammonium phosphate, *UA* uric acid

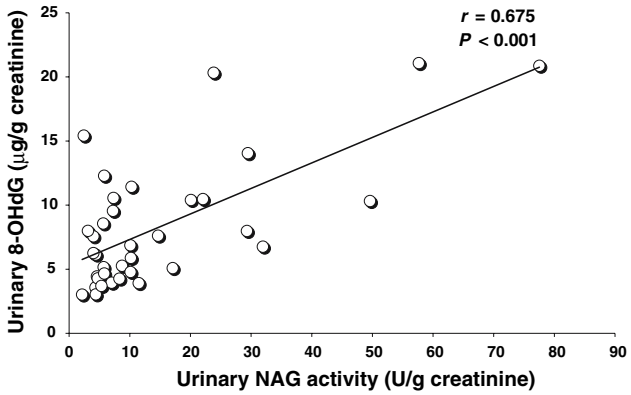

Fig. 3 Bivariate analysis using scatter plot and Pearson's correlation test to assess the correlation between urinary excretion of 8-OHdG and urinary NAG activity. Level of urinary 8-OHdG was significantly correlated to urinary NAG activity ($r = 0.675$, $P < 0.001$)

positively related to urinary 8-OHdG. To make the β-coefficients of the predictive model directly comparable, all independent variables were standardized (mean = 0; SD = 1) and the normalized β-coefficient of each variable was computed [13]. Normalized β-coefficient of urinary NAG activity was 0.583. In other words, an increase in urinary NAG activity of 1 SD (1 U/g creatinine) was associated with an increase in urinary 8-OHdG of 0.583 μg/g creatinine.

In order to evaluate the clinical value of urinary 8-OHdG in separating nephrolithisis from healthy subjects, an ROC curve was generated; a cutoff point was chosen to calculate the diagnostic value. The ROC curve of urinary 8-OHdG provided an AUC of 0.709 (95% CI; 0.583–0.835). The best accuracy of the 8-OHdG test (69.70%) was obtained when using a cutoff point of 3.50 μg/g creatinine (Table 3). This cutoff provided a sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of 94.44, 40.00, 65.38 and 85.71%, respectively. To obtain the high specificity (high true negative rate) and high PPV, the cutoff was set at 10.00 μg/g creatinine, which was approximately 2 SD above the healthy mean. This cutoff imparted a sensitivity, specificity, PPV and NPV of 30.56, 96.67, 91.67 and 53.70%, respectively. However, the accuracy of the test was reduced to 60.61%.

Nephrolithiasis patients with positive urinary 8-OHdG (>10.00 μg/g creatinine) accounted for 30.56% (11/36) while urinary NAG activity higher than the healthy range (8.02 U/g creatinine) was found in 50% (18/36) of cases.

Discussion

Our data showed an elevated excretion of urinary 8-OHdG in patients with renal calculi, indicating that oxidative

Table 2 Multiple linear regression model to assess the contribution of urinary volume, MDA, NAG activity and protein content to the prediction of urinary 8-OHdG in nephrolithiasis patients controlled for age, sex and BMI ($n = 36$, model $R^2 = 0.56$, $P < 0.001$)

Variables	β-coefficient	SE	P	95% CI	β-Coefficient[a]
Age (years)	0.103	0.057	0.085	0.015, 0.220	0.244
Gender	0.904	1.341	0.506	3.651, 1.844	−0.091
BMI (kg/m^2)	0.106	0.153	0.496	0.420, 0.208	−0.092
24-H urine volume (ml)	0.002	0.001	0.095	0.0003, 0.004	0.225
Urinary MDA (μM/g creatinine)	0.022	0.067	0.741	0.114, 0.159	0.044
Urinary NAG activity (U/g creatinine)	0.171	0.057	0.006	0.055, 0.289	0.583
Urinary protein content (g/g creatinine)	1.198	1.931	0.540	2.758, 5.154	0.117

SE Standard error

[a] β-coefficient for standardized variables (mean = 0, SD = 1)

Table 3 Diagnostic values of urinary 8-OHdG determination in distinguishing nephrolithiasis patients from healthy individuals

Diagnostic values	Cutoff points (μg/g creatinine)	
	3.50	10.00
Sensitivity (%)	94.44	30.56
Specificity (%)	40.00	96.67
Positive predictive value (%)	65.38	91.67
Negative predictive value (%)	85.71	53.70
Accuracy of test (%)	69.70	60.61

damage to DNA was increased in these patients. Increased renal tubular damage was independently associated with an elevated level of urinary 8-OHdG. Based upon ROC analysis, the urinary 8-OHdG at 10 μg/g creatinine provided a high specificity for distinguishing nephrolithiasis patients from the healthy population.

Oxidative stress mediates the development of many pathological conditions and the urinary excretion of oxidatively modified products of lipids (MDA and isoprostanes) and DNA (8-OHdG) is currently used to assess the overall oxidative stress in diseases. Evidence from both in vitro and in vivo data clearly demonstrates that oxidative stress plays a significant role in the pathogenesis of nephrolithiasis [2, 14–16]. Urinary MDA is frequently measured in nephrolithiasis patients to indicate the extent of lipid peroxidation and to be reflective of oxidative stress [15, 16] while the excretion of urinary 8-OHdG, which is an established biomarker of oxidative DNA damage, has not been explored in patients with nephrolithiasis.

The present study found that the level of urinary 8-OHdG in nephrolithiasis patients was significantly higher than that in healthy controls. This result suggested that urinary 8-OHdG was able to be an indicator of overall oxidative stress in renal stone patients and also implied that these patients had increased oxidative damage to DNA. It is surprising that urinary MDA did not differ significantly between healthy and nephrolithiasis subjects. Moreover, it bares no correlation with urinary NAG activity. Perhaps urinary 8-OHdG might represent an oxidative stress biomarker better than urinary MDA.

It has been proposed that the urinary 8-OHdG level may be affected by from either diet [17, 18] or cell death [19, 20]. However, a recent review provides evidence indicating that the 8-OHdG levels in urine principally reflects oxidative DNA repair in the body [21]. Our finding implied that oxidative DNA repair was increased in nephrolithiasis patients.

A comparison of urinary 8-OHdG levels among three types of stones demonstrated that excretion of urinary 8-OHdG was not associated with the type of stone. We concluded that the presence of stones rather than stone constituents per se had an impact on the extent of oxidative damage to DNA. This implied that mechanical irritation by stones partly contributed to the production of reactive radical species. However, the speculation needs to be experimentally elucidated.

Although different stone types have distinct etiologies, crystal-induced inflammation has been believed to be a common pathology [5, 22]. Thus, inflammatory cells may be another source of ROS that attacks DNA. A positive correlation between the number of infiltrating-CD68+ cells and the number of 8-OHdG adducts in liver tissue of patients with hepatitis has been demonstrated. This suggested that viral-induced inflammation causes oxidative DNA damage [23]. In ethylene glycol-induced nephrolithiasis rats, an increased infiltration of CD45+ (leukocyte common antigen) cells in the kidney and the deposition of calcium oxalate crystals surrounding with ED1+ cells (monocytes/macrophages) were found [24]. Although the direct evidence has not been provided, oxidative burst produced by infiltrating phagocytes in the stone-containing kidney may enhance the production of ROS and consequently damage to DNA.

Both bivariate analysis and multivariate linear regression showed that urinary NAG activity was an independent predictor of urinary 8-OHdG in nephrolithiasis patients. Urinary NAG activity was positively correlated to urinary 8-OHdG. This data underlined the relationship between oxidative stress and renal tubular damage. We concluded that increased oxidative stress, manifested by elevated urinary 8-OHdG, enhanced the damage of renal tubular cells.

At the cutoff of 10 μg/g creatinine, determination of urinary 8-OHdG provided a low false positive rate with PPV of 92%. This means that patients with a positive result of a urinary 8-OHdG test had a probability of 92% to have kidney calculi. At this cutoff point, almost all of the healthy subjects (97%, 29/30) were negative for the urinary 8-OHdG test and over a half of nephrolithiasis patients (69%, 25/36) were also negative. This suggested that the oxidative stress status of most nephrolithiasis patients was low or even in the physiological or normal range.

Chiou and coworkers [10] used an in-house ELISA kit and reported a significant difference of the normal ranges of urinary 8-OHdG for males (29.6 ± 24.5 μg/g creatinine, $n = 548$) and females (43.9 ± 42.1 μg/g creatinine, $n = 486$). They verified that their assay found urinary 8-OHdG values approximately three times higher than the Japanese kit that was used in this study. We did not find a significant difference of urinary 8-OHdG between males and females. The discrepancy may be explained by a small sample size. However, Pilger and colleagues [25] reported an insignificant difference of urinary 8-OHdG excretion between healthy men and women after adjustment for body weight and creatinine. A recent study also showed no significant difference of this DNA lesion in urine of Japanese males and females [26]. The present finding corresponded with these studies.

Cigarette smoking has been reported to be associated with an increase of urinary 8-OHdG levels [27]. The data on smoking habits was not available as it was omitted in our multivariate analysis. A study using a multi-biomarker approach concluded that oxidative stress imposed by cigarette smoke had a low impact on pathways involved in DNA damage [28]. No significant association between smoking status and oxidative damage indices was reported in female dry cleaners [29]. In addition, Kimura et al. [26] reported that mean urinary 8-OHdG was not significantly different in terms of smoking and alcohol consumption. These studies corroborated the minute impact of smoking on urinary 8-OHdG excretion.

In conclusion, our study demonstrated that the determination of urinary 8-OHdG has clinical utility in estimating oxidative stress and predicting renal tubular damage. Thus, urinary 8-OHdG may be useful for evaluating oxidative severity or monitoring the effectiveness of treatment and predicting the recurrence of stone.

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