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Research Article

Elevated Cadmium Exposure Associated with Oxidative Stress and Oxidative DNA Damage in Population of Cadmium-Contaminated Area

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ABSTRACT
We examined the association of elevated cadmium exposure with chronic kidney disease (CKD), increased oxidative stress and oxidative DNA damage in a total of 129 study residents 13 cadmium-contaminated villages. In bivariate correlation, elevated urinary cadmium excretion was significantly correlated with creatinine, N-acetyl- -D-glucosaminidase, glomerular filtration rate (GFR), malondialdehyde, 8-hydroxy-2'-deoxyguanosine (8-OHdG) and total antioxidant level. Overall findings showed that elevated urinary cadmium excretion appeared to increase risk of oxidative stress, oxidative DNA damage and CKD; ORs and 95% CIs were 2.64 (1.44, 4.82) and 2.18 (1.22, 3.89), respectively, after adjusting for CKD and other co-variables. Our study revealed that elevated cadmium exposure induces increased oxidative stress and oxidative DNA damage concomitant with CKD in these populations of environmentally contaminated area, might be increased in morbidity and mortality of all degenerative diseases in the future.

Keywords: Urinary cadmium, chronic kidney disease, N-acetyl- -D-glucosaminidase, oxidative stress, oxidative DNA damage, 8-hydroxy-2'-deoxyguanosine

INTRODUCTION
Cadmium (Cd) is a toxic metal associated with environmental and industrial pollution is an important public health concern due to its wide range of organ toxicity [1, 2]. Cadmium is a common by-product during the processing of zinc-bearing ores. Cadmium pollution has become more widespread, causing contamination in the food chain as well as water and air contaminations. In Mae Sot district, Tak province, northwestern Thailand, the Cd-contaminated areas were caused from the two creeks running through zinc mine and which affected 13 rural villages of the district as in our previous study [3]. Metallic Cd is absorbed by the body via enteral and pulmonary routes from dietary sources, drinking water, and polluted air [1, 4]. These resident-villages may be at high risk of the Cd toxicity from rice and other crops consumption. Acute intoxication is responsible for injuries to the testes, liver and lungs [2, 4]. Chronic exposure leads to obstructive airway diseases, emphysema, neurologic, end-stage renal failures, diabetic, and renal complications, cardiovascular, deregulated blood pressure, bone disorders, urinary stone and immune-suppression [1-4]. These negative effects on human's health are due to the low biological excretion rate of the element. Cadmium that accumulates in the body tissues has a biological half-life of 15-20 years [5], and its accumulation in the organism. Blood Cd is thought to be a marker of ongoing exposure but also appears to reflect body burden from long-term retention of Cd in the liver and kidney; urinary Cd reflects kidney cadmium concentrations with a half-life of 15–30 years. Urinary Cd is thought to more specifically be a marker of cumulative exposure [6, 7]. One of the major targets of Cd-induced damage are the kidneys, where it accumulates preferentially in the proximal tubule, which may ultimately result in renal failure [2, 4].
At the cellular level, Cd-induced nephrotoxicity is reflected by the formation of reactive oxygen species (ROS) [8] and cell death induction [9, 10]. The mechanisms responsible for the oxidative toxicity of Cd are uncertain. It does not appear to generate free radicals by itself, but lipid peroxidation is increased in various tissues soon after Cd exposure [11, 12]. Moreover, it has been shown that

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Table 1 Comparison of general characteristics of the Cd-exposure with non-Cd-exposure control population

Parameter	Non exposure (n=166)	Cd exposure (n=129)	*p*-value
Age (yr)	48.09 ± 8.26	53.45 ± 10.15	<0.001
Systolic BP (mmHg)	123.0(115.0-133.0)	126.0(116.0-139.0)	0.105
Diatolic BP (mmHg)	76.0(69.0-83.0)	78.0(70.0-86.0)	0.222
BMI (kg/ m^2)	23.48 ± 3.62	22.23 ± 4.27	0.007
Cd (µg/g CT)	2.48(1.68-3.29)	8.18(6.15-11.0)	<0.001
eGFR (ml/min/1.73 m^2)	80.4(69.7-92.9)	59.1(51.8-74.9)	<0.001
NAG (U/gCT)	3.00(2.67-3.12)	4.28(3.66-5.39)	<0.001
8-OHdG (ng/mg CT)	15.33(11.49-19.56)	34.93(23.22-41.73)	<0.001
Glucose (mg/dl)	84.0(80.0-90.0)	84.0(79.0-91.0)	0.929
BUN (mg/dl)	14.5(12.0-17.0)	14.3(11.9-17.6)	0.879
CT (mg/dl)	0.80(0.70-0.90)	0.80(0.70-1.00)	0.002
MDA (µmol/l)	4.02(3.18-5.30)	5.20(4.40-5.65)	<0.001
TAC (mmol/l Trolox Equiv/l)	520.0(406.0-580.0)	410.0(395.0-420.0)	0.001
Smoking	44 (26.51%)	59 (45.74%)	0.001
Alcohol drinking	43 (25.90%)	46 (35.66%)	0.046
Hypertension	34 (20.48%)	51 (39.53%)	<0.001
Type 2 diabetes	9 (5.42%)	28 (21.71%)	<0.001

Data are mean±SD or median (interquartile range) for variables with a skewed distribution and n (%)
P values are given for comparisons between non-exposure and Cd exposure groups tested with t test, non-parametric and Chi square tests

various antioxidants and cell defense systems are reduced from Cd-induced toxicity [13]. Cadmium is also classified as a class 1 carcinogen and suspected of inducing cancer in many organs, including the kidney [14]. Cadmium carcinogenesis involves multiple mechanisms, including DNA strand breaks as well as inhibition of DNA repair [15, 16] and also has been shown to induce formation of 8-hydroxy-2’-deoxyguanosine (8-OHdG) adducts in human–hamster hybrid A_L cells [17], and in human lymphoblastoid cells [18]. Some studies on Cd intoxication indicate an association with lung, prostate and kidney cancers [19] and possible occurrence of liver, pancreas and stomach cancers, but these observations are uncertain [20]. The *in vitro* effect of Cd on mammalian cells showed increased levels of DNA strand breaks [21]. Therefore the aims of the present study to validate a possible association between cadmium exposure with chronic kidney disease (CKD) and concomitant with increased oxidative stress, oxidative DNA damage in these populations environmentally exposed to cadmium.

MATERIALS AND METHODS

Study population: This cross-sectional study was based on health evaluation. A total 295 volunteer subjects who were 30 years or older participated in the present study. One hundred twenty nine subjects were randomly selected from 13 Cd-contaminated villages (during January 2010–January 2011) and 166 subjects from non-Cd-polluted village located in the same province were selected as the control group. A questionnaire survey was conducted by trained health workers about demographic characteristics, occupational history, residency time, medical history of diabetes, hypertension, renal diseases, cancers, smoking and alcohol consumption. We excluded the 82 subjects with known end stage renal failure, cancer, infection and any life threatening diseases from the study. The study protocol was approved by the Ethic committees of Naresuan University (51-02-04-0042) and the permission for research problem from the Provincial Governer of Tak. All subjects provided written informed consent and they all agreed to participate and to provide blood and urine samples for their follow-up health check. Blood pressure was taken after the participants were seated and rested for 5 minute in the morning before blood sample taken. All blood pressure measurements were twice at 5 minute intervals with a digital blood pressure monitor, ES-P 110 (Terumo cooperation, Japan). The average of the two measurements was used for data analysis.

Blood and urine samples collection: Fasting venous blood was collected from all participants. Plasma glucose (Glu), serum blood urea nitrogen (BUN) were measured by using enzymatic colorimetric method, serum and urine creatinine (CT) concentration was estimated based on the Jaffe reaction procedures with an auto-analyzer (Hitachi 912, Roche Diagnostic, Switzerland) at laboratory of Department of Medical Technology, Faculty of Allied Health Sciences. For determination of MDA and TAC, serum was immediately separated and stored at -70°C without the addition of exogenous antioxidants before MDA and TAC analysis. Urine samples were collected in polyethylene bottles after the subject’s physical examination, wherein anthropometric measurements and blood taken. The urine sample from each subject was divided into three aliquots (3–5 ml each); one for urine microscopic examination and other aliquots were frozen and stored at −70 C without the any addition for later analysis of Cd, NAG and creatinine.

Malondialdehyde (MDA) assay: After thawing the samples, MDA level was determined by using the thiobarbituric acid substances (TBARS) assay, a spectroscopic techniques as our previously report [23]. The method is based on the formation of red (pink) chromophore following the reaction of TBA with MDA and the other breakdown products of peroxidized lipids

called MDA. One molecule of MDA reacts with 2 molecules of TBA to yield a pink pigment with absorption maximum at 532 nm.

Total antioxidants capacity (TAC) assay: The assay is based on the reaction of metmyoglobin with hydrogen peroxide to form ferryl myoglobin, a free radical species. A chromogen 2, 2'-amino-di-[3-ethylbenzthiazole sulphonate] is incubated with ferryl myoglobin to produce a radical cation which has a relatively stable blue-green color that can be measured at 600 nm. Antioxidants in the added serum can suppress this color production to a degree proportional to their concentration. The assay was calibrated using 6-hydroxy-2, 5, 8-tetramethylchroman-2-carboxylic acid (Trolox), and results were expressed as mmol/l trolox equivalent [24]. The within–run coefficient of variation for the TAC assay in control material assay was 4.8% (n=10).

Urinary cadmium determination: Urinary cadmium concentration was determined by a graphite tube atomic-absorption spectrometer (Varian Model AA280Z, USA) at the Bangkok-Pathology Laboratory,_a private reference Clinical Laboratory. All specimens were analyzed in duplicate. The laboratory has been certified and participated as the member of the toxicological analyses in biological materials by the External Quality Assessment Scheme of Medical Sciences Center, Thailand. The within-run assay coefficients of variation (CV) ranged from 2.8% to 13.6%. Additionally, in an external quality assurance program from the External Quality Assessment Scheme of Medical Sciences center of Thailand, laboratory measures were within 10% of reference means for urinary cadmium ($r^2 = 0.97$).

N-acetyl- -D-glucosaminidase (NAG) assay: The method was as described by Horak et al. [22]. The NAG in urine is added to an enzyme reaction mixture that consists of the substrate (p-nitrophenyl-N-acetyl- -D-glucosaminide) dissolved in sodium citrate buffer (pH 4.4). During incubation at 37 ºC, NAG hydrolysis of the substrate liberates p-nitrophenylate ion. The reaction is stopped by adding 2-amino-2-methyl-1-propanol (AMP) buffer (pH 10.25), and the reaction product is measured by spectrophotometry at 405 nm. Urine NAG activity is proportional to the absorbance of the liberated p–nitrophenylate ion, after correction for absorbance of a urine "blank" sample. The within–run and between-run coefficient of variation for NAG assay in control material assay was 3.14% and 4.11% (n=10).

Urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) assay: The urine concentration of 8-OHdG was determined using the competitive enzyme-linked immunosorbent assay (ELISA). The detail procedures were described by Chiou et al. [25]. The determination range was 0.5–200 ng/ml for 8-OHdG. The intra-assay coefficient of variation (CV) of 8-OHdG was range from1.8% to 5.5%, while the inter-assay CV ranged from 2.8% to 7.9%. The urinary concentrations of 8-OHdG were corrected by using individual urinary creatinine concentrations.

Renal function: All participants had clinically normal renal function, defined as a serum creatinine concentration below 159.12 μmol/l (1.8 mg/dl) and serum BUN concentration below 7.14 mmol/l (20 mg/dl). We used the Cockroft-Gault formula to calculate the estimated glomerular filtration rate (eGFR) which incorporates age, body weight and sex [26]. The formula is as follows:

eGFR = [(140-age) * Weight (kg) * constant]/ serum creatinine (μmol/L)

Where constant is 1.23 for men and 1.04 for women; serum creatinine in μmol/L.

The classification of eGFR by stages was done according to the Kidney Disease Outcome Quality Initiative [27] criteria as follow: Stage I: normal eGFR (≥90ml/min/1.73 m^2); Stage II: mildly eGFR (60-89 mL/min/1.73 m^2); Stage III: moderately eGFR (30-59 ml/min/1.73 m^2); Stage IV: severely eGFR (<30 ml/min/1.73 m^2), and Stage V: end-stage renal disease: eGFR (<15 ml/min/1.73 m^2). eGFR lower than 60 ml/min/1.73 m^2 (moderately eGFR) was defined as chronic kidney disease (CKD).

STATISTICAL ANALYSIS

Categorical data are presented as continuous and percentages. Continuous data are presented as mean± standard deviation (SD) or median and interquartile range for non-normally distributed data, were tested by using Shapiro-Wilk test. A Student's *t*-test was used to analyze the differences for normally distributed data and Mann-Whitney U-test was used to analyze the differences for non-normally distributed data. Correlation between Cd exposure index with renal toxicity (NAG levels and eGFR), oxidative stress and oxidative DNA damage were analyzed with Spearman's rho correlation test. Odds ratios (OR) from logistic regression analyses were used to estimate the risk of oxidative stress, oxidative DNA damage and CKD that was associated with elevated urinary Cd excretion. The results of all analyses were evaluated for statistical significance using p-value <0.05 and the 95% confidence intervals (CI). The analysis was performed using the SPSS computer program version 17.0 (SPSS, Chicago, IL).

RESULTS

A total of 129 residents (aged 53.45 ± 10.15 yrs) who lived in the Cd-contaminated villages, had elevated urinary-Cd and 166 residents (aged 48.09 ± 8.26 yrs) in the non-Cd contaminated villages participated as control in this study. Of the participants, 81 (27.5%) were men [of which 27 (13.2%) were exposed] and 214 (72.5%) were women [of which 102 (47.7%) were exposed]. The characteristics of the study population are shown in Table 1. Residents of Cd-exposed area were significantly higher in age, urinary Cd, NAG, 8-OHdG, creatinine, MDA and lower in BMI, eGFR and TAC than residents of control area (p<0.05). Our finding were also found that clinical characteristics of Cd-exposed men and women were not significantly difference, only CT levels higher than women (p<0.001).

Bivariate correlation, urinary-Cd levels showed significant positive correlations with NAG/gCT (r =0.229, p=0.009), 8-OHdG/mgCT (r =0.396, p<0.001), MDA (r =0.278, p=0.001), CT (r =0.364, p<0.001) and significant negative correlation with eGFR (r = -0.302, p=0.001) and TAC (r =-0.243, p=0.005) and correlations of the other variables

Table 2 Comparison of general characteristics of the Cd-exposure-men with Cd-exposure-women

Parameter	Cd-exposed men (n=27)	Cd-exposed women (n=102)	*p*-value
Age (yr)	55.04 ± 7.90	53.03 ± 10.66	0.283
Systolic BP (mmHg)	130.0(120.0-140.0)	126.0(115.0-138.0)	0.385
Diatolic BP (mmHg)	79.0(70.0-88.0)	78.0(70.0-85.3)	0.610
BMI (kg/ m^2)	21.36 ± 3.76	22.46 ± 4.38	0.201
Cd (µg/g CT)	8.18(6.13-10.00)	8.17(6.17-11.5)	0.543
eGFR (ml/min/1.73 m^2)	58.6(47.4-75.0)	59.4(53.2-75.1)	0.675
NAG (U/gCT)	4.47(3.91-6.45)	4.24(3.58-5.21)	0.285
8-OHdG (ng/mg CT)	34.36(21.04-40.49)	35.02(25.06-41.85)	0.702
Glucose (mg/dl)	86.0(80.0-91.0)	84.0(78.8-91.0)	0.658
BUN (mg/dl)	14.5(11.4-20.0)	14.0(12.0-17.0)	0.427
CT (mg/dl)	1.00(0.80-1.10)	0.80(0.70-0.90)	<0.001
MDA (µmol/l)	5.30(5.00-6.10)	5.10(4.00-5.60)	0.153
TAC (mmol/l Trolox Equiv/l)	420.0(401.0-426.0)	406.0(395.0-420.0)	0.260
Smoking	17 (62.96%)	42 (41.18%)	0.036
Alcohol drinking	14 (51.85%)	32 (31.37%)	0.042
Hypertension	14 (51.85%)	37 (36.27%)	0.106
Type 2 diabetes	3 (11.11%)	25 (24.51%)	0.104

Data are mean±SD or median (interquartile range) for variables with a skewed distribution and n (%)
P values are given for comparisons between non-exposure and Cd exposure groups tested with t test, non-parametric and Chi square tests

Table 3 Bivariate correlation of all variables in population of elevated Cd-exposure

Correlation between parameters		Correlation coefficient r	*p*-value
Cd/g CT	CT	0.364	<0.001
	eGFR	-0.302	0.001
	MDA	0.278	0.001
	TAC	-0.243	0.005
	NAGgCT	0.230	0.009
	8-OHdG	0.396	<0.001
eGFR	Age	-0.530	<0.001
	Systolic BP	-0.231	0.008
	BUN	-0.417	<0.001
	CT	-0.416	<0.001
	MDA	-0.330	<0.001
	8-OHdG	-0.187	0.034
NAGgCT	Diastolic BP	0.232	0.008
	BUN	0.228	0.009
	CT	0.190	0.031
MDA	BUN	0.242	0.006
	CT	0.320	<0.001
	NAGgCT	0.205	0.020
8-OHdG	TAC	-0.196	0.026
	BMI	0.304	<0.001
CT	BUN	0.311	<0.001
	NAGgCT	0.190	0.031
	eGFR	-0.416	<0.001

were demonstrated in Table 2. Multiple logistic regression analyses were used to test an association between elevated urinary Cd with oxidative stress, oxidative DNA damage and CKD after adjusting for their covariates. As shown in Table 3, the risk for oxidative stress (elevated MDA) OR is 2.64 (95% CI: 1.44-4.82) after adjusting for CKD, BMI,

age and gender. The risk for oxidative DNA damage OR is 2.18 (95% CI: 1.22-3.89) after adjusting for CKD, BMI, age and gender. The risk for CKD OR is 4.80 (95% confidence interval (CI): 2.36-9.78) after adjusting for oxidative stress, BMI, age and gender (Table 3).

DISCUSSION

In our present study, the resident of the contaminated area, urinary Cd concentration was significantly higher than from the control area. An early sign of Cd-induced nephrotoxicity is the renal tubular dysfunction, demonstrated by an increased urinary excretion of NAG and decreased eGFR could be observed in individuals with elevated urinary Cd as shown in Table 1. We also found MDA, 8-OHdG, and lower TAC levels higher in population of Cd contaminated area, the mechanisms are uncertain. According to the animal studies, treatment of rats with a single dose of Cd chloride (30 nmol/kg) was reported to be carcinogenic. This single dose induces increased lipid peroxidation, iron content and elevated production of H_2O_2 in testicular Leydig cells, the target for Cd-induced carcinogenesis [28]. There was a several mechanisms for Cd-increased oxidative stress: (i) In exposed cells and tissues Cd affects cellular thiol redox balance that leads to decreased intracellular glutathione (GSH) content [29] and reduced activities of cellular antioxidant enzymes (i.e. superoxide dismutase, peroxidase and catalase), which in turn results in the accumulation of ROS and an increase in intracellular oxidative stress [30, 31]. (ii) Another possible mechanism is the substituting of Cd for zinc in critical metabolic processes, in the presence of metallothionein, and the displacement of endogenous redox metals, such as iron and copper [11, 32, 33] from different cytoplasmatic and membrane proteins leading to increased concentration of these ions that can participate in ROS production through Fenton reactions. Cd has been shown to promote a time dependent iron release from biological membranes [34]. These results also support the idea that ROS and in particular peroxides may play a crucial role in Cd-induced carcinogenesis in this target cell population. The present study noted that lipid peroxidation level was significantly increased and the levels of total antioxidant capacity were significantly decreased in Cd-exposed populations. The level of plasma lipid peroxidation was positively and the total antioxidant capacity was negatively and significantly correlated with the levels of Cd in urine.

From the experimental evidence indicates that Cd induced genotoxic effects are oxidative stress mediated. In addition, Cd was shown to block apoptosis in cultured cells, most likely by inhibiting caspase-3, a central mediator of apoptosis [35]. Cultured cells exposed to Cd respond in a biphasic manner regarding cellular GSH contents. An initial time and dose-dependent increase was followed by a decrease at higher doses, presumably due to the production of ROS at a rate that exceeds the ability of the cells to regenerate the oxidized GSH [36] as mentioned above. If generation of ROS is one of the major pathways for Cd mediated genotoxicity and mutagenicity, then it should be expected to induce specific DNA lesions consistent with oxidative damage. Cd-induced oxidative stress causes the production of typical oxidatively generated mutagenic lesions such as 8-oxo-7, 8dihydro-2 -deoxyguanosine (8-oxodG) adducts in the DNA of human lymphoblastoid cells after exposure with 5–35 μM of Cd [18]. Nevertheless, in occupationally exposed individuals, Palus et al. [37] found positive correlation between Cd blood levels and genotoxic effects in peripheral blood mononuclear cells. DNA lesions arising after Cd exposure are not due to direct effects. They appear to be mainly mediated by an indirect production of ROS, which at least in part, is due to the inhibition of cellular antioxidants and constitutes oxidative stress. Numerous oxidative DNA adducts have been identified and their frequency is estimated to be 10^4 lesions/cell/day in humans [38, 39]. Thus, oxidative DNA damage induced by •OH is a key factor influencing the mutagenic and carcinogenic load in living organisms.

Oxidative stress is known to be involved in many human pathological processes, such as hypertension, atherosclerosis, cardiovascular disease, diabetes, neurological disorders and cancer [40-43]. Populations of Cd-contaminated area are at higher rate of CKD, renal tubular dysfunction, hypertension, diabetes and the other degenerative diseases. The co-occurrence in our study, much evidence shows that CKD is also associated with increased oxidant production and decreased antioxidant defenses. There are many potential sources of increased oxidative stress in CKD patients: uremic toxins, iron overload, angiotensin II, proinflammatory cytokines and decreased level of antioxidants [41]. *In vivo* studies have found accumulated oxidative damage occurs from decreased levels of these endogenous anti-oxidants rather than increased ROS production [44]. The kidney function is to filter waste products that build up in the blood. These

Table 4 Association of elevated Cd-exposure with CKD, oxidative stress (MDA) and oxidative DNA damage after adjusted with their variables

Variables	Elevated urine Cd-excretion			Variables	Elevated urine Cd-excretion		
Model *	OR	95% CI	*P-value*	Model *	OR	95% CI	*P-value*
Elevated oxidative stress	2.64	1.44-4.82	0.002	Elevated oxidative DNA damage	2.18	1.22-3.89	0.009
CKD	4.80	2.36-9.78	<0.001	CKD	5.27	2.60-10.68	<0.001
BMI	0.92	0.86-0.99	0.039	BMI	0.92	0.85-0.99	0.036
Age	1.02	0.99-1.06	0.163	Age	1.02	0.99-1.06	0.169
Gender	3.03	1.52-6.05	0.002	Gender	2.72	1.37-5.40	0.004

*Model * after adjusted with CKD, BMI, gender, age*

waste products are not removed completely or sufficiently. People with CKD have a higher risk of death from stroke or heart attack, and CKD may also progress to total and permanent renal failure (end-stage renal disease). These conditions are also consistent with the increased Cd-exposed caused CKD or renal insufficient and co-increased ROS formation and oxidative stress in the population of contaminated area noted in the present study. Then, populations of Cd-contaminated area are at higher risk for all degenerative diseases and concomitant with CKD may cause future morbidity and mortality. Cd-contamination in these areas was still health problems nowadays. Then environmental protection and education on healthy lifestyle are warranted to protect residents in Cd contaminated areas from further exposure.

In conclusion, elevated Cd-exposure associated with increased oxidative stress and oxidative DNA damage concomitant with cadmium-induced CKD and may play a central role of the other degenerative diseases.

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List of abbreviation**:** *BP,* blood pressure; *BMI,* body mass index; *Cd,* cadmium; */g CT,* per gram creatinine; *eGFR,* estimated glomerular filtration rate; *NAG,* N-acetyl- -D-glucosaminidase; *CKD,* chronic kidney disease; *8-OHdG,* 8-hydroxy-2'-deoxyguanosine*; BUN,* blood urea nitrogen; *CT,* creatinine; *MDA,* malondialdehyde; *TAC,* total antioxidant capacity; *DM,* diabetes mellitus; *HT,* hypertension

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