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A RESEARCH ARTICLE

Interplay Between Serum Zinc and Urinary Cadmium in Smokers: Potential Modulatory Effects on Serum CC16

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Abstract

Smoking-related metal exposure, especially cadmium, is associated with lung epithelial injury. CC16, also known as club cell protein, is a microprotein secreted by Clara cells that play multiple roles, including host defense and immune response, and emerged as a biomarker for lung epithelial injury. Zinc is an essential trace element that might play a vital role in protection against cadmium toxicity. Objectives: This study aimed to test the hypothesis that Cd from smoking is associated with club cell damage and intravascular leakage of CC16. Furthermore, examine the role of zinc in protection against it. Methodology: This study is a case-control design, where serum CC16, urinary cadmium (Cd), and serum zinc levels were measured in two groups: smokers and non-smokers (the control group). Result: The serum levels of CC16 are significantly lower in smokers compared to non-smokers ($p<0.0001$). Also, there is a significant increase in the levels of urinary Cd in smokers compared to non-smokers ($p = 0.0013$). The levels of serum zinc are significantly lower in smokers compared to non-smokers ($p = 0.0186$). There is a significant positive correlation between serum zinc and urinary cadmium (Cad) ($r = 0.3037$ and $p = 0.032$). Conclusion: Clara cell protein (CC16) is a promising biomarker for Cd inhaled toxin-induced cell damage; zinc may exert a protective effect against the damage caused by Cd or potentially modulate the damage caused by Cd through increasing the level of Cd in the urine.

1. Introduction

Club cell secretory protein (CC16), formerly known as (CC10), secretoglobin or uteroglobin, is a micro dimeric protein about 16 kDa [1]. It is secreted by a specific epithelial cell called Clara cells which are located in respiratory tract and it is the major component of airway secretions and eliminated by the urinary tract [2,3]. It can be detected in the bronchial fluid, sputum, nasal fluid, and also in urine. The biological activities of CC16 and its pathways have not been completely understood, but many studies suggest that CC16 has anti-inflammatory and anti-oxidative effects [1]. There are evidences suggesting that Clara cell protein CC16 have additional roles in host defense, immune response, and airways[4]. CC16 can diffuse passively into plasma and serum CC16 (sCC16)is one of the peripheral markers of respiratory epithelial injury detecting Clara cell impairments that has recently been proposed[5, 6]. It supposes that CC16 expression decreases with lung injury and smoking [7]. It is encoded by the SCGB1A1 gene, that is located on chromosome 11, p12-q13, beside the location of genes which regulate allergy and inflammation [1].

WHO estimates that 1.1 billion people globally smoke; in addition, it is one of the main preventable causes of premature death and disease worldwide [8]. Smoke inhalation is well-recognized as a cause of airway injuries [9]. Cigarettes contain more than 7000 compounds, and their intoxicants, especially cadmium (Cd) that have deleterious health effects [10]. Smoking is a well-known causative agent for many diseases like lung disease, cancer, cardiovascular disease, stroke, metabolic diseases like diabetes mellitus, and other noninfectious diseases [8]. Moreover, cigarette smoking is well known as the most important risk factor for many lung diseases, especially obstructive

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lung diseases. Besides the direct toxins of smoking, it is also associated with inflammation and oxidative lung damage [11].

Cadmium (Cd) is one of the important environmental pollutants and is classified in group 1 of human carcinogens [12]. Long-term Cd exposure is closely related to many diseases, such as autoimmune diseases, cancer, cardiovascular diseases (CVD), lung diseases, and hepatic dysfunction [13]. A lot of studies indicate that exposure to Cd, a constituent of cigarette smoke, is associated with oxidative stress and chronic inflammation [11]. Increasing evidence indicates that Cd may play a role in smoking-induced disorders, including impaired lung function [10,11]. The body rapidly chelated cadmium from blood once entering the body; Cd is bound to a plasma protein, mainly metallothionein [14]. Metallothionein is a protein rich in cysteine and a scavenger of OH radicals [11,15]. The essential adaptation to Cd poisoning due to its binding to metallothionein prevents free Cd ions from exerting their toxicity [10,11]. The use of this protein in treating cadmium poisoning comes at the cost of increasing hydroxyl free radicals, as this protein is also used to eliminate hydroxyl free radicals; thus, exposing tissues to hydroxy radical damage from other sources [11].

Zinc (Zn) is an essential metal that plays key roles in protein structure, catalysis, and regulation of their function [11]. Numerous studies have shown that Zn can reduce Cd toxicity; In general, Zn and Cd are similarly in chemical structures and charges so Zn reduce Cd toxicity by direct competition between the two metals, Zn-mediated metallothionein induction, and Zn-mediated redox homeostasis however, the underlying mechanisms have not been extensively explored [11,13].

Although cadmium (Cd) is a well-known smoking derived pulmonary toxin, its impact on CC16 levels in smokers and the potentially protective role of Zinc (Zn) remain not fully understood.

This study evaluates the hypothesis that smoking-derived Cd causes epithelial club cell damage and CC16 leakage into the blood stream. Furthermore, it investigates the protective role of Zn against Cd-smoking-induced damage on epithelial club cells by comparing levels of serum CC16, zinc, and urinary Cd between smokers and non-smokers and comparing the levels of serum CC16 and urinary Cd in high-zinc and low-zinc subgroups of smokers. Additionally, the study performs a correlation between these three parameters.

2. Materials and Methods

The type of this study is a case-control study; 90 healthy volunteers enrolled in this study after written consent; they were divided into two groups. 50 smokers and 40 nonsmokers (control group). The smoker group was further subdivided into two groups: Low zinc and high zinc, based on their serum zinc concentrations. The classification was determined using the median value or a cutoff of 70 µg/dL as the threshold.

2.1. Inclusion criteria

This study includes all adults aged from 29 to 46 years old, healthy volunteers, with no acute respiratory illness and currently smoking (at least one cigarette per day for at last 6 months).

2.2. Exclusion criteria

Chronic respiratory diseases (COPD, interstitial lung disease and asthma patients), acute respiratory illness, renal diseases, liver diseases, ex-smokers and occupational exposure to heavy metals (especially Cd from industrial work) are excluded from participating in this study. All participants should avoid taking multivitamins or zinc for 2 weeks before the time of sample extraction, as the concentrations of zinc may fluctuate during the first 1–2 weeks post discontinuation of supplementation [16,17].

2.3. Research Ethics

The department of medical biochemistry, Assiut Faculty of Medicine and the ethical committee approved the study. And written consent was taken from all participants. Each participant completed a questionnaire regarding, age, sex, Smoking habit include, duration of smoking in years

and number of cigarettes per day. Body weight, height, body mass index (BMI), systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded.

2.4. Blood sample collection

From each participant a blood sample (3 ml) in a metal-free polypropylene tube. Samples were left to clot for 20 min, then centrifuged at 3000×g for 10 min. The serum was used to measure CC16 and zinc concentration.

2.5. Urine sample collection

a spot midstream urine sample (at least 10 ml) in a metal-free polypropylene container, which is used for to measure Cd concentration.

2.6. Chemicals, reagents and labware

Cd and zinc stock solutions (1000 mg/L or 1000 ppm), concentrated nitric acid (HNO3) (trace metal grade), high purity deionized water, beakers and volumetric flasks of various sizes. All labware, especially new ones, is completely submerged in the acid bath of 10% nitric acid. Ensure all surfaces are in contact with the acid. Soak the labware for a minimum of 24 hours (≥ 48 hours is recommended for all plastic labware), then rinse with deionized water and leave it to dry [18]. Standard curve preparation: dilute the stock solution by using 1% of nitric acid to prepare the standards used in the standard calibration curve.

2.7. Sample Digestion and Metal Quantification

Take 5 ml of urine sample or 1 ml of serum into the digestion flask and add double the sample volume (10 ml for urine or 2 ml for serum sample) of the concentrated acid mixture (4parts HNO3, 1part HClO4) in the digestion flask. Gently heat the mixture, using a water bath or heating mantle [12, 19-21], in a fume cupboard designed for perchloric acid use. Continue heating until the organic matter is completely digested, indicated by a clear or light-colored solution and the appearance of dense white fumes of perchloric acid. Sample dilution: Cool the digested sample at room temperature and transfer the sample to a 25 ml volumetric flask for urine or 5 ml for serum, and dilute the sample with 1% nitric acid to the final flask mark. The use of nitric acid for dilution is better than deionized water because it prevents precipitation of metals in the sample [22]. The final concentration after measurement is multiplied by the dilution factor as seen in Equation 1.

$$\text{Dilution factor} = \frac{\text{The final volume of the sample after digestion}}{\text{The initial volume of the sample}} \quad \text{.....(1)}$$

Measurement of Cd and zinc was performed using a Buck Model 210 VGP Atomic Absorption spectrophotometer with hollow cathode lamps. The wave length and current of the lamp for Cd and zinc are 228.8 and 4 mA, 213.9 nm and 3 mA respectively. All samples and standards were done in replicates, quality control standard and blank samples used to monitor quality control. Also, urine and serum matrix were spiked with known concentrations of standards and then measured; the recovery of spiked samples was from 88 to 103, and this ensured the quality of the data.

Creatinine concentration in urine is measured by the alkaline picrate method [23]; the Cd levels were adjusted for the urine creatinine concentration to compensate for dilutional variation; the corrected Cd concentration in urine is calculated by dividing actual urinary Cd by urinary creatinine [24] and expressed as µg/g as seen in Equation 2.

$$\text{Corrected urinary Cd concentration (µg/g)} = \frac{\text{Cd concentration in urine µg/L}}{\text{Creatinine concentration in urine g/L}} \quad \text{.....(2)}$$

2.8. CC16 Assay

Club cell protein (the human Clara cell) (CC16) was measured in serum in smokers and in controls using Human Clara Cell Protein ELISA kits from Bioassay Technology Lab, China, according to the manufacturer's instructions.

Statistical analysis was done by using GraphPad Prism software version 10.6.1; the results were expressed as mean ± SD. The $p < 0.05$ was considered statistically significant. EndNote reference management program (Clarivate Analytics, version 2025.1). The software was used to organize, store, and cite the references throughout this study. which reduced human error in citation formatting and guaranteed accuracy and consistency.

3. Results and Discussion

Table 1. Shows the demographic data, all subjects are males, and there is no significant difference in the mean of age, BMI, SBP and DBP among smokers and control group.

Table 2 shows the serum levels of CC16, zinc and the urinary level of Cd in smokers and nonsmokers or control groups. The serum level of CC16 is 10.32 ± 4.387µg/L in smokers and 14.69 ± 9.32 µg/L in non-smokers. The urine Cd was 2.038 ± 0.678 µg/gram of creatinine in smokers versus 0.427 ± 0.168 µg/gram of creatinine in nonsmokers. The serum zinc is 69.34 ± 7.99 µg/dl in smokers versus 92.53 ± 10.61 µg/dl in nonsmokers.

Table 3 shows the correlation between different parameters: serum levels of CC16 and zinc and urinary Cd. There is a weak significant positive correlation between serum zinc and urinary Cd ($r = 0.3037$ and $p = 0.032$). There was a nonsignificant positive correlation between CC16 and serum zinc ($r = 0.08694$ and $p = 0.5483$). And also, there was a nonsignificant positive correlation between CC16 and urinary Cd with ($r = 0.02574$ and $p = 0.859$).

Table 4 shows the serum levels of CC16, and the urinary level of Cd in low and high zinc subgroups of smokers. The serum level of CC16 was 9.755 ± 3.880 µg/L in low-zinc smokers versus 11.11 ± 4.983 µg/L in high -zinc smokers. The urine Cd was 0.4448 ± 0.1418 µg/gram of creatinine in low-zinc smokers versus 0.5254 ± 0.1674 µg/gram of creatinine in high -zinc smokers.

Table 1. The demographic data in smokers and nonsmokers (control group). BMI (Body mass index). SBP (systolic blood pressure). DBP (Diastolic blood pressure).

Demographic data	Smokers Mean ± SD n (50)	Non-smokers (Control group)Mean ± SD n(40)
Age (years)	37.5 ± 8.5	36.5 ± 7.5
BMI (kg/m^2)	24.9± 3.9	25.6 ± 2.7
SBP (systolic blood pressure)	120.32 ± 4.1	121 ± 2.6
DBP (Diastolic blood pressure)	80.42 ± 2.5	81 ± 1.8
Duration of Smoking (years).	12.5 ± 7.3	-
Number of Cigarettes per Day.	14.6 ± 5.4	-

Data expressed as Mean ± SD

Table 2. The differences in CC16, Cd and Zn between smokers and non-smokers (control group).

	Smokers Mean ± SD(n=50)	Non-smokers (Control group) Mean ± SD(n=40)
Serum CC16 µg/L	10.32 ± 4.387	14.69 ± 9.34
Urine Cd µg/g creatinine	0.5025 ± 0.1706	0.2205 ± 0.0917
Serum Zinc µg/dL	69.34 ± 7.099	92.53 ± 10.61

Data expressed as Mean ± SD

Table 3. The Pearson correlation between serum CC16, serum zinc and urinary Cd in smokers.

	Serum CC16		Serum Zinc		Urinary Cd	
	r	p	r	p	r	p
SerumCC16			0.08694 ns	0.5483	0.02574 ns	0.8592
Serum zinc	0.08694 ns	0.5483			**0.3037** *	**0.032**
Urinary Cd	0.02574 ns	0.8592	**0.3037** *	**0.032**		

$p < 0.05$ was considered statistically significant

Table 4. The differences in CC16, urinary Cd between low and high Zinc groups of smokers.

	Smokers with low zinc Mean ± SD(n=28)	Smokers with high zinc Mean ± SD(n=22)
Serum CC16 µg/L	9.755 ± 3.880	11.11 ± 4.983
Urine Cd µg/g creatinine	0.4448 ± 0.1418	0.5254 ± 0.1674

Data expressed as Mean ± SD

CC16 protein is the main secretory product of club, or Clara cells, which are the epithelial cells lining the lung airways. Cub cell damage led to the release of CC16, which crosses through the bronchoalveolar/blood barrier and diffuses passively into plasma. Thus, Clara cell impairments or damage has recently been proposed to be detected using serum CC16 as a biomarker [25]. In this study we measured the serum level of CC16 and also investigated the association of this protein with serum zinc and Cd levels in urine in smokers' subjects.

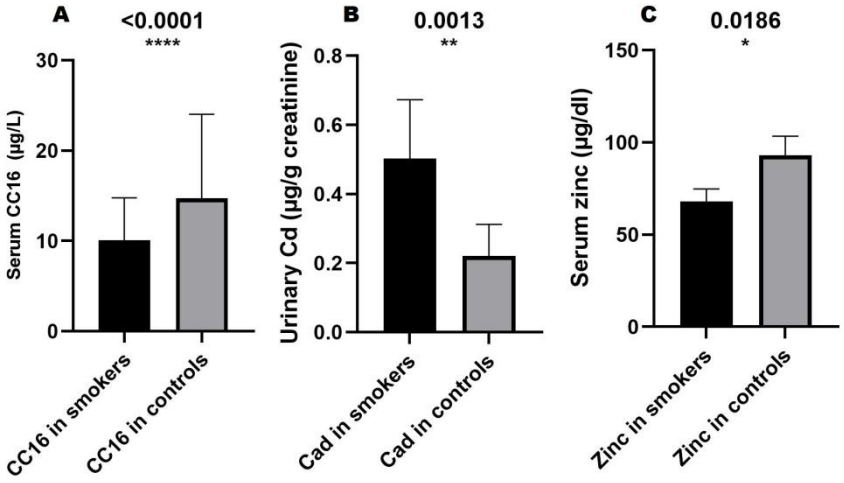

Figure 1 (A) The differences in serum levels of CC16 in smokers and non-smokers (controls). (B) The differences in urinary levels of Cd in smokers and non-smokers (controls). (C) The differences in serum levels of zinc in smokers and non-smokers (controls).

As shown in Figure 1 (A), there was a significant decrease of serum CC16 in smokers compared to non-smokers ($p<0.0001$), which is probably caused by chronic damage to club secretory cells. In early exposure to smoke, the injury to these cells lead to subsequent increase of CC16 protein in blood, but with chronic exposure to smoke, club cell damage become more porfound resulting in the decrease of this protein [26]. In this regard, most studies have shown lower serum level of CC16 in smokers compared to non-smokers, supporting the idea that severe or massive club cell damage is caused by chronic smoking, which leads to a decrease of its level. For instance, Dell'Omo M et al.[6] reported that CC16 is lower in smokers than in non-smokers (11.3 µg/L vs 14.6 µg/L) ($p = 0.005$). similar finding were observed by Robin et al.[27] who reported that CC16 was lower in tobacco smokers, with levels of 7.6 (range 6.0 -11.2) µg/L in smokers vs 10.6 (range 8.7-14.6) µg/L in non-smokers. Also, Bernard et al [5] reported lower levels of CC16 in smokers versus non-smokers, with levels of 14.7 vs 21.9 µg/L. Conversly, some studies show a transient increase in serum CC16 in smokers versus non-smokers; for instance, Ściskalska & Milnerowicz [25] reported an increase in CC16 concentration in the group of smoking women compared to non-smoking women. similarly, Bernard et al. [26] reported that the concentration of CC16 in the serum of firefighters after the fire was significantly higher as compared to controls ($P = 0.04$), which had returned to the concentration level of controls 10 days later. The finding in those two studies is attributed to the initial epithelial barrier disruption in acute exposure to smoking [25,26].

As shown in Figure 1(B), there is a significant increase in the urinary Cd in smokers compared to non-smokers ($p = 0.0013$). These results are inconsistent with Mannino et al.[28], who reported that smokers had higher mean urinary Cd/creatinine levels (0.46 µg/g) than never smokers

(0.23 µg/g). Ferraro et al. [29] and Rokadia & Agarwal [30] also reported higher levels of urinary Cd in smokers as compared with non-smokers. The levels of serum zinc.

As shown in Figure 1(C), were significantly lower in smokers compared to non-smokers (p -value = 0.0186). These results are in line with finding reported by Skalny et al. [31] and Theint Hlaing [32].

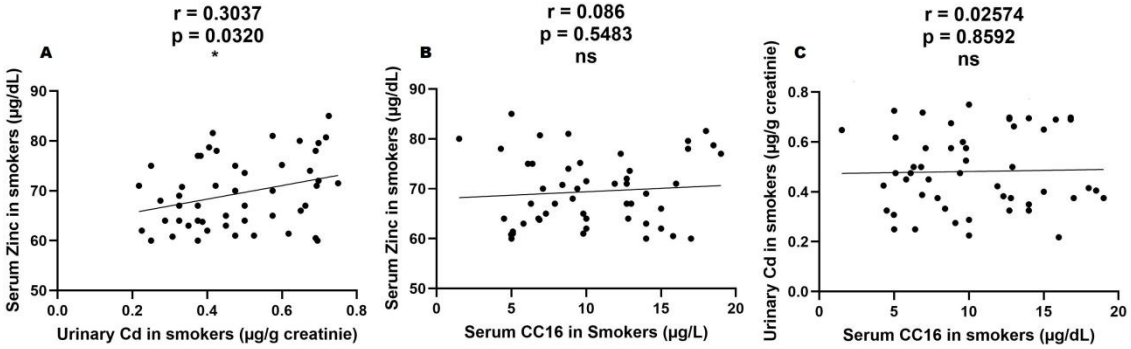

Figure 2. (A) The Pearson correlation between in urinary Cd and Zn in smokers. (B) The correlation between in serum CC16 and Zn in smokers.

The levels of serum zinc correlate positively with levels of urinary Cd (r = 0.3037, p = 0.032), as shown in Figure 2(A). This finding is consistent with Vance & Chun [33], who reported that a 10% increase in serum zinc is associated with a 4.09% increase in urinary Cd and there is a strong positive correlation between serum zinc and urinary Cd (p-value = 0.0001) [33]. As Cd and zinc are chemically more closely related than other transition metals, Cd absorption and deposition are partially done by zinc transporters such as ZIP8 and ZIP14 [34, 35]. It is worth noting that zinc can increase urinary Cd in many ways, primarily by competitively inhibiting Cd reabsorption in renal proximal tubules, which is the main site of Cd reabsorption [36]. In addition, zinc interferes with the damage caused by Cd in the kidney and increases its excretion [37]. These mechanisms might explain the positive correlation between the level of serum zinc and the level of urinary Cd. Therefore, zinc may ameliorate or protect against the Cd-induced damage.

The levels of serum CC16 correlate positively but not significantly with serum zinc, as shown in in Figure 2(B), this positive correlation albeit nonsignificant, it could be attributed to many factors: zinc is an essential trace element that has antioxidant and anti-inflammatory roles and promotes healing of the smoking damage of the epithelial cells [38]; moreover, zinc increases CC16 production by these epithelial cells with subsequent release into the bloodstream upon disruption by smoking.

The levels of serum CC16 correlates positively with urinary Cd but did not reach the statistical significance, as shown in Figure 2(C), this trend aligns with the findings of Ściskalska and Milnerowicz who reported upregulation of CC16 protein in response to Cd exposure from smoking [25]. Such increase is an adaptive process as, CC16 is an antioxidant and anti-inflammatory lung protein secreted by epithelial club cells of the lung airway, which increases in response to inhaled toxins in smoking, such as Cd [39,40]., Accordingly, CC16 is a potential sensitive early marker for cadmium-induced-damage.

Additionally, in an attempt to investigate the potential protective role zinc against smoking induced epithelial damage, smoking group was subdivided into high and low serum zinc groups, the data showed a non-significant trend toward higher CC16 levels (p = 0.3008) and higher urinary Cd (p = 0.0879) in the high serum zinc group, as show in Figure 3(A) and 3(B) and in Table 4, but this was not statistically significant. So, the protective effect of zinc on Clara cell function might be marginal compared to damaging effect of smoking; Also, the high degree of variability or the wide spread and broad distribution of data within each group and the overlapping of both groups, as shown in the violin plots (Figure 3(A) and (B)), masks any potential effect of zinc. Thus, this study might be underpowered to detect a true biological difference due to a limited sample size, furthermore other factors such as smoking intensity (pack-years) or age may have a more dominant influence than zinc status.

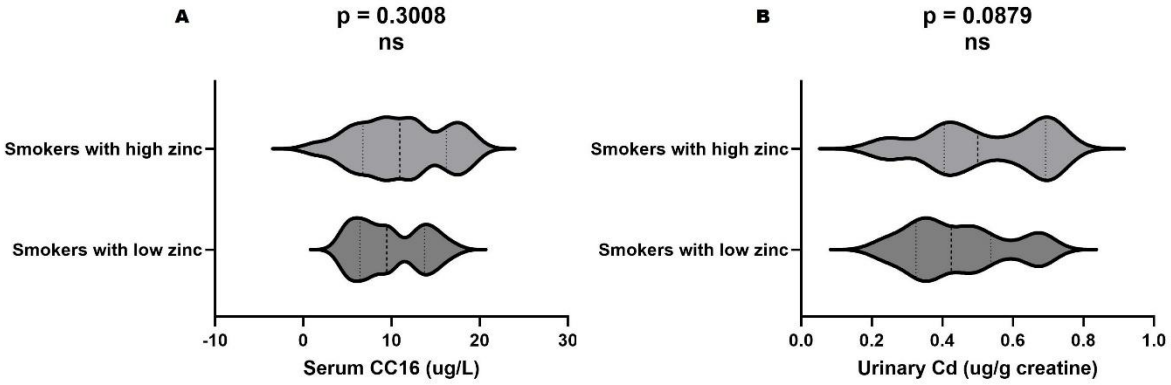

Figure 3. (A) The levels of urinary Cd in smokers with low and high serum zinc. (B) The levels of serum CC16 in smokers with low and high serum zinc.

4. Conclusions

CC16 is a promising biomarker of Cd-inhalation-induced cell damage, as it is a protective protein that has anti-inflammatory and antioxidant activities. Zinc supplementation may exert a protective effect against or potentially alleviate the Cd-smoking-induced club cell damage. It is recommended to conduct further research on a larger longitudinal cohort study with a larger sample size to achieve greater statistical significance, taking into consideration the other confounding factors such as age of the participant, and smoking intensity.

Acknowledgments

None Applicable

Conflicts of Interest

The author declares no conflict of interest.

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التفاعل بين الزنك في المصل والكادميوم في البول لدى المدخنين: التأثيرات التنظيمية المحتملة على بروتين كلارا-16 في المصل

الملخص

يرتبط التعرض للمعادن الناتجة عن التدخين، وخاصة الكادميوم، بتلف ظهارة الرئة. يُعدّ بروتين CC16، أو بروتين الخلايا الناديّة، بروتينًا دقيقًا تفرزه خلايا كلارا، وله أدوار متعددة، بما في ذلك الدفاع المناعي والاستجابة المناعية، وقد برز كعلامة حيوية لتلف ظهارة الرئة. يُعدّ الزنك عنصرًا أساسيًا من العناصر النزرة، وقد يلعب دورًا حيويًا في الحماية من سمية الكادميوم. الأهداف: هدفت هذه الدراسة إلى اختبار فرضية أن الكادميوم الناتج عن التدخين يرتبط بتلف خلايا كلارا وتسرب بروتين CC16 إلى الأوعية الدموية. بالإضافة إلى ذلك، دراسة دور الزنك في الحماية من ذلك. المنهجية: هذه الدراسة هي دراسة حالة-مراقبة: تم قياس مستوى بروتين CC16 في مصل الدم، ومستوى الكادميوم في البول، ومستوى الزنك في مصل الدم لدى مجموعتين: مجموعة المدخنين ومجموعة غير المدخنين (مجموعة المراقبة). النتائج: كانت مستويات بروتين CC16 في مصل الدم أقل بشكل ملحوظ لدى المدخنين مقارنةً بغير المدخنين ($p<0.0001$). كما أن هناك زيادة كبيرة في مستويات الكادميوم البولي لدى المدخنين مقارنة بغير المدخنين ($p = 0.0013$). مستويات الزنك في مصل الدم أقل بكثير لدى المدخنين مقارنةً بغير المدخنين ($p = 0.0186$). توجد علاقة ارتباط إيجابية قوية بين الزنك في مصل الدم والكادميوم في البول ($r = 0.3037$ و $p = 0.032$). الخلاصة: يمكن اعتبار بروتين خلايا كلارا (CC16) مؤشرًا محتملاً لتلف الخلايا الناتج عن استنشاق سموم الكادميوم؛ إذ قد يحمي الزنك من التلف الناجم عن الكادميوم أو يُقلله عن طريق زيادة مستوى افرازه في البول.

الكلمات المفتاحية: بروتين خلايا النادي، بروتين خلايا كلارا، CC16، الكادميوم البولي لدى المدخنين، الزنك لدى المدخنين.