Estimation of serum levels of copper, zinc, and iron and cytological study of the impact of smoking on oral keratinocytes in a sample of tobacco smoke among Iraqi men

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Abstract
Tobacco use is a major public health problem worldwide. Its products encompass diverse types of chemicals, heavy metals, and toxic mineral elements. Trace elements are very important for cell functions at biological, chemical and molecular levels; where, at normal levels, they are important for stabilization of the cellular structures, but in deficiency, states may stimulate alternate pathways and cause diseases. Tobacco smoking may influence the concentrations of several trace elements, which in turn may cause changes in oral keratinocytes. This study is designed to estimate levels of copper, zinc, and iron in serum and to demonstrate impacts of smoking on oral keratinocytes of tobacco smoke men by utilizing cytological study; furthermore, to link each level of serum element on the possible changes in oral keratinocytes. Fifty men, their age range 20-40 years were participated in this study and classified into two groups (25 men each): Group I- Healthy men who were non-smokers, non-exposed to tobacco smoke from the environment, and non-alcoholic, and with clinically normal oral mucosa were served as controls. Group II- Healthy men who practice tobacco smoking for more than 9 years of approximately more than 9 cigarettes/day, but who had no clinically visible alteration in the oral mucosa, served as the tobacco-using group. Venous blood samples were withdrawn from each man and utilized for the assessment of serum copper, zinc and iron levels; additionally, men were asked to rinse their mouth to remove debris. Oral smears were then collected with a Transport Swab. This study showed that there was a significant elevation in serum levels of copper and iron; but, a significant reduction in serum zinc level (P<0.05) in smoker men compared to the corresponding serum levels in non-smokers. Moreover, there was a non-significant difference in nuclear diameter, and nuclear area (P>0.05); but, there is a significant reduction in cellular diameter and in the cellular area (P<0.05) in buccal mucosa of smokers compared to those of non-smokers. Furthermore, non-significant correlations between each of the serum levels of copper, zinc, and iron and nuclear-to-cellular area ratio in smoker men were observed. In conclusion, it could be concluded that alterations in serum levels of copper, zinc, and iron; and in cellular diameter and cellular area but not on the nuclear-diameter and -area in the buccal mucosa of smokers' men were observed; this indicates that oral mucosa exposed to tobacco smoke is altered by tobacco smoking and there is no correlation between levels of elements (copper, zinc, and iron) with the oral keratinocytes.

Key words: Tobacco smoke, copper, zinc, iron, oral keratinocytes

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Introduction
Tobacco smoking consists of drawing into the mouth and usually, the lungs, smoke from burning tobacco; and the type of product smoked is most commonly cigarettes, and it can also include cigarillos, cigars, pipes or water pipes [1]. Cigarette smoking is the major universal public health problem and the adverse effects of cigarette smoke on human health were widely recognized by many researchers [2]; also, the rise in tobacco smoking's prevalence is the cause of premature death worldwide [3]. Tobacco smoke is a complex, dynamic and reactive mixture containing an estimated 5,000 chemicals. The toxic and carcinogenic mixture is probably the most significant source of toxic chemical exposure and chemically-mediated disease in humans [4]. Palakurthy P, et al., (2017) reported that the utilization of tobacco is the primary cause of numerous oral lesions; where, it
is a risk factor for oral cancer, oral cancer recurrence, adults periodontal diseases, in addition to congenital defects such as cleft lip and palate in children whose mothers smoke during pregnancy; moreover, the response of the immune system can be suppressed in persons with tobacco use thus, persons may prone to oral infections, and there may be a delay in the healing process (5).

Copper (Cu) is an essential trace element in both humans and animals, and it is only needed in trace amounts (6). It was reported that Cu has dual functions as essential- or toxic- element to living systems; as an essential function, Cu is required for -adequate growth, -cardiovascular (CV) integrity, -lung elasticity, -neovascularization, neuroendocrine function, and -iron metabolism; moreover, Cu is involved in the functions of many Cu-dependent proteins, enzymes, and it is also important for immune function (7, 8).

Zinc (Zn) is an essential element whose significance to health is increasingly appreciated and whose deficiency may play an important role in the appearance of diseases. Such element is existing as Zn+2, which is a strong electron acceptor in biological systems without the risk of causing oxidant damage to cells; furthermore, Zn+2 was reported to be essential for many biological functions in the body which can act as a catalytic component of more than 300 different enzymes and it is in the structure of proteins, encompassing almost every aspect of biology, including growth, immune defense, cognitive function, and bone health; and because such essential ion can play a fundamental role in the survival of humans, its concentration in the body must be adequate and well-controlled; and also such element has been reported to have antioxidant and anti-atherosclerotic effects (9,10).

Iron is an essential element in biology, and it is the most abundant essential trace element in the human body. The total content of iron in the body is about 3–5g with most of it in the blood and the rest in the liver, bone marrow (BM), and muscles in the form of the heme (11). Iron is particularly contained within the functional heme group (a component in the electron transport chain) as well as within the oxygen-carrying molecule Hb; where, most of the iron in the human body (approximately 65%) is contained within the Hb-carrying red blood cells and thereby facilitates the transport, transitional tissue storage, and cellular use of oxygen; moreover, it also has important roles in cytochromes within mitochondria, mediating the transfer of electrons in the electron transport chain; furthermore, Fe is also part of heme-containing enzymes such as catalase, xanthine oxidase, and glutathione peroxidase (GP) and acts as an enzymatic cofactor for several enzymes including -aconitase, -nicotinamide dinucleotide dehydrogenase (NADH), -succinate dehydrogenase, and –glycerol-3-phosphate dehydrogenase (GPDH); furthermore, even though Fe is plentiful on earth, most of it is in the largely insoluble and biologically unavailable Fe3+ state, hence organisms have evolved intricate mechanisms of acquiring iron from their environment (12, 13).

Aims of the study
This study is designed to estimate serum levels of Cu, Zn, and Fe; and to demonstrate impacts of smoking on oral keratinocytes of tobacco smoke men by utilizing cytological study; where, nuclear diameter and area in addition to cellular diameter and (N/C) area ratio in the mucosal buccal sample to be calculated, and to link each level of serum element mentioned above on nuclear-to-cellular (N/C) area ratio.

Subjects
This study was carried out participating fifty (50) men, their age range 20-40; a performance was framed and all the relevant information of each man was recorded. The procedure was described to men and written consent was obtained. The study was approved by the Scientific and the Ethical Committees of the Department of Pharmacology and Toxicology, College of Pharmacy, University of Baghdad. Participants (Men) were classified into two groups (25 men each) as follows: Group I- Healthy men who were non-smokers, non-exposed to tobacco smoke from the environment, and non-alcoholic, and with clinically normal oral mucosa were served as controls. Group II- Healthy men who practice tobacco smoking, but who had no clinically visible alteration in the oral mucosa, served as the tobacco-using group.

Exclusion Criteria: This study excludes the following: 1- Men older than 40 years of age and less than 20. 2- Men who quit smoking for 3 months and more. 3- Men using nicotine replacement therapy, or Nargile (Shisha). 4- Alcoholics. 5- Men who were working in an industry that generates dust or fumes. 6- Men with a history of any recent systemic disease and those on medications. Chemicals utilized in this study are of the highest available purity.

Blood sampling
Venous blood (5ml) was collected under aseptic precautions from the forearm of all men who participated in this study by plastic disposable syringes. Each blood sample was placed in EDTA-free plastic tube and left at

room temperature for complete clotting to obtain serum, which was aspirated after centrifugation of the blood at 3000 rpm for 10 minutes; all samples refrigerated and transferred by ice bag into ministry of Science and Technology, Food Contamination Research Center, Baghdad-Iraq. Frozen serum was allowed to thaw at room temperature; assessment of trace elements (Cu, Zn, and Fe) was performed by Flame Atomic Absorption Spectrophotometry (FAAS) instrument (Shimadzu, Japan) which is based upon the fact that metal atoms absorb light energy strongly at a discrete characteristic wavelength which coincides with the emission spectra line of that particular element, respectively (14-16).

**Cytological study**

Oral smears were obtained from the mucosa of all men who participated in the study. Men were asked to rinse their mouth to remove debris. Oral smears were then collected with a transport swab (AFCO, Jordan). Exfoliative cytology is a painless, non-invasive procedure, and is very well accepted by subjects due to little discomfort. Smears were prepared from the buccal mucosa of all participants using a transport swab applying gentle pressure. The collected cells were smeared over the microscopic slide, fixed in 99.9% absolute ethanol, and stained using Papanicolaou stain (Pap stain) (ABCAM, England). The stained smears were cytomorphometrically analyzed for the nuclear and cellular parameters using image analysis software (5) performed in the Food Contaminations Research Center at the Iraqi Ministry of Science and Technology. Following calculations for the area (µ²), the same cells were subjected to calculations of diameter (µ). Furthermore, the nucleus and the cellular diameter; in addition to nuclear-to-cellular area ratio was calculated (17).

\[
\frac{\text{Nuclear area}}{\text{Cellular area}} = \text{Nuclear-to-cellular (N/C) area ratio}
\]

**Statistical analysis**

The significance of differences between the mean values was calculated using Mann-Whitney analysis. The numeric data were expressed as mean±standard deviation (SD). P-values less than 0.05 were considered significant for all data presented in this study. Furthermore, Pearson correlation coefficient (r) was used to test the relation between the mean of nuclear and cellular area, nucleus and cellular diameter, and serum levels of each element (Cu, Zn, and Fe).

**Results**

**Levels of serum copper (Cu) in smoker men compared to non-smokers**

Table 1 showed that in smokers men their age range (20-40), there was significant elevation \((P<0.05)\) in serum levels of Cu compared to the corresponding serum levels in non-smokers at the same aged range; where, serum copper levels were respectively, 36.22±7.990 and 14.52±3.306.

<table>
<thead>
<tr>
<th>Group of men</th>
<th>Copper levels (Cu) µg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smokers (control)</td>
<td>14.52±3.306</td>
</tr>
<tr>
<td>Smokers</td>
<td>36.22±7.990*</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SD.  
*: \(P<0.05\): Significantly different compared to non-smoker men  
N= Number of men.

**Levels of serum zinc (Zn) in smoker men compared to non-smokers**

Table 2 showed that in smokers men their age range (20-40), there was a significant reduction in serum Zn level compared to the corresponding levels in non-smokers at the same aged range \((P<0.05)\); where, serum zinc levels were respectively, 42.52±23.386 and 85.60±19.476.
Table 2. Levels of serum zinc (Zn) in smoker men (age range 20-40) compared to non-smokers men (age range 20-40).

<table>
<thead>
<tr>
<th>Group of men</th>
<th>Zinc levels (Zn) μg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smokers (control)</td>
<td>85.60±19.476</td>
</tr>
<tr>
<td>Smokers</td>
<td>42.52±23.386*</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SD.

*: P<0.05: Significantly different compared to non-smoker men

N= Number of men.

Levels of serum iron (Fe) in smoker men compared to non-smokers

Table 3 showed that in smokers men their age range (20-40), there was a significant elevation in serum Fe level compared to the corresponding serum levels in non-smokers at the same aged range (P <0.05 ); where serum iron levels were respectively, 186.36+10.275 and 96.92+37.010.

Table 3. Levels of serum iron (Fe) in smoker men (age range 20-40) compared to non-smokers men (age range 20-40).

<table>
<thead>
<tr>
<th>Group of men</th>
<th>Iron levels (Fe) μg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smokers</td>
<td>96.92±37.010</td>
</tr>
<tr>
<td>Smokers</td>
<td>186.36±10.275*</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SD.

*: P<0.05: Significantly different compared to non-smoker men

N= Number of men.

Nuclear diameter in smoker men compared to non-smokers

Figure 1 showed the nuclear and cellular parameters (nuclear diameter, nuclear area, cellular diameter, and cellular area) in a tobacco smokers men their age range (20-40 year) compared to non-smokers of the same aged-range (figure 2).

Figure 1. The nuclear (N) and cellular (C) parameters (nuclear diameter, nuclear area, cellular diameter, cellular area) in a tobacco smoker.

Nuclear diameter (μ) = 9.83±0.312. Nuclear area (μm²) =68.49±2.92.
Cellular diameter (μ) =79.61±2.61. Cellular area (μm²) =3783.83±181.51
Figure 2. The nuclear (N) and cellular (C) parameters (nuclear diameter, nuclear area, cell diameter, cell area) in a nonsmoker.

Nuclear Diameter (μ) =8.09±0.149  
Nuclear Area (μm²) =67.33±1.29  
Cellular Diameter (μ) =75.05±1.21  
Cell Area (μm²) =3668.44±71.90

Table 4 showed that in smokers men their age range (20-40), there was a non-significant difference in nuclear diameter of buccal mucosa compared to the corresponding diameter in buccal mucosa of non-smokers at the same aged range (P >0.05 ); where nuclear diameters were respectively, 186.36+10.275 and 96.92+37.010.

Table 4. Nuclear diameter in smoker men (age range 20-40) compared to non-smokers men (age range 20-40).

<table>
<thead>
<tr>
<th>Group of men</th>
<th>Nuclear diameter (μ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smokers (control) N=25</td>
<td>8.3936±0.70759</td>
</tr>
<tr>
<td>Smokers N=25</td>
<td>8.3848±1.16812</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SD. 
N= Number of men, μ= Micron.

Nuclear area in smoker men compared to non-smokers
Table 5 showed that in smokers men their age range (20-40), there was a non-significant difference in nuclear area of the buccal mucosa compared to the corresponding area in the buccal mucosa of non-smokers at the same aged range (P>0.05 ); where the nuclear area was respectively, 66.9780+1.53761 and 66.7748+1.38678.

Table 5. Nuclear area in smoker men (age range 20-40) compared to non-smokers men (age range 20-40).

<table>
<thead>
<tr>
<th>Group of men</th>
<th>Nuclear area (μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smokers (control) N=25</td>
<td>66.7748±1.38678</td>
</tr>
<tr>
<td>Smokers N=25</td>
<td>66.9780±1.53761</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SD. N= Number of men, μm²= Square micron.

Cellular diameter in smoker men compared to non-smokers
Table 6 showed that in smokers men their age range (20–40), there was a significant reduction in cellular diameter in buccal mucosa compared to the corresponding diameter in the buccal mucosa of non-smokers at
the same aged range (P<0.05); where cellular diameters were respectively, 73.3756±3.52789 and 77.0532±1.85524.

**Table 6.** Cellular diameter in smoker men (age range 20-40) compared to non-smokers men (age range 20-40).

<table>
<thead>
<tr>
<th>Group of men</th>
<th>Cellular diameter(µ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smokers (control) N=25</td>
<td>77.0532±1.85524</td>
</tr>
<tr>
<td>Smokers N=25</td>
<td>73.3756±3.52789*</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SD.  
*: P<0.05: Significantly different compared to non-smoker men.  
N= Number of men.µ= Micron

**Cellular area in smoker men compared to non-smokers**

Table 7 showed that in smokers men their age range (20-40), there was significant reduction in cellular area of the buccal mucosa compared to the corresponding area in the buccal mucosa of non-smokers at the same aged range (P<0.05); where, cellular area were respectively, 3615.0724±73.298 and 3713.9352±104.637.

**Table 7.** Cellular area in smoker men (age range 20-40) compared to non-smokers men (age range 20-40).

<table>
<thead>
<tr>
<th>Group of men</th>
<th>Cellular area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smokers (control) N=25</td>
<td>3713.9352±104.637</td>
</tr>
<tr>
<td>Smokers N=25</td>
<td>3615.0724±73.298*</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SD.  
*: P<0.05: Significantly different compared to non-smoker men.  
N= Number of men.µm²= Square micron

**Correlation analysis**

**Correlation between serum copper (Cu) and nuclear-to-cellular (N/C) area ratio in smoker men**

Figure 3 showed that there was non-significant correlation between serum Cu levels and nuclear-to-cellular area (N/C) ratio in smoker men (r = 0.105, P =0.617).

![Scatter chart showing the correlation between serum copper (Cu) levels to nuclear-to-cellular area (N/C) ratio in smoker men. Their age range (20-40 years).](http://doi.org/10.36295/ASRO.2020.232131)

r: Correlation coefficient= 0.105  
P: Significant difference= 0.617
Correlation between serum zinc (Zn) and nuclear-to-cellular area ratio in smoker men
Figure 4 showed that although there is a negative correlation between serum Zn levels and nuclear-to-cellular area ratio in smoker men but it is non-statistically different (r= -0.142, P =0.498).

![Figure 4](image1)

**Figure 4.** Scatter chart showing the correlation between serum zinc (Zn) levels to nuclear-to-cellular area (N/C) ratio in smoker men. Their age range (20-40 years). r: Correlation coefficient= - 0.142. P: Significant difference= 0.498.

Correlation between serum iron (Fe) and nuclear-to-cellular (N/C) area ratio in smoker men
Figure 5 showed that although there is a negative correlation between serum Fe levels and nuclear-to-cellular area (N/C) ratio in smoker men but it is non-statistically different (r= -.050 , P =0. 812).

![Figure 5](image2)

**Figure 5.** Scatter chart showing the correlation between serum iron (Fe) levels to nuclear-to-cellular area (N/C) ratio in smoker men. Their age range (20-40 years). r: Correlation coefficient=- 0.050 P: Significant difference=0. 812

Discussion
Levels of copper (Cu), zinc (Zn), and iron (Fe) in serum of smoker men compared to non-smokers
In this study, levels of serum Cu (36.22+7.990), table 1, in smoker men (their age range 20-40years) were significantly elevated (P<0.05) compared to the corresponding serum levels in non-smokers (14.52+3.306). Such levels in all men participating in this study are above the international value (10-15 μg/L) (18). Moreover, the results of this study are coinciding with those of several investigators who reported that the level of Cu increased in adult smokers. But are inconsistent with those of Ali Aycicek, *et al.*, (2015) (19). Furthermore, in the current study, serum Zn levels (42.52+23.386), table 2, in smoker men were significantly reduced (P<0.05) compared to the corresponding serum levels in non-smokers (85.60+19.476). Results of the present study are in tune with those of other studies; where, Zn level was reduced in adult smokers; moreover, researchers also reported that the hypozincemia that is often observed in adult smokers have been attributed to the acute-phase response that can be triggered by tissue damage (20). Owing to the fundamental role that Zn plays in cellular metabolism, its effect is substantial in cells with a rapid turnover such as the immune system and is therefore said to modulate host resistance to various infections (21).
Besides, in this study, serum Fe levels (186.36±10.275) in smoker men were significantly elevated (P<0.05) compared to the corresponding serum levels in non-smokers (96.92±37.010) (table 3). Such results are inconsistent with those of others (19). The elevation in Cu and Fe levels with a reduction in Zn levels in adult smokers may be dependent on several factors. One factor, toxic heavy metals that are naturally found in tobacco, especially in the tar phase, acts antagonistically to Zn (22). Another factor may be dependent on the inflammatory response due to tobacco smoking; where, it has been reported that tobacco smoke increased the inflammatory processes and caused elevation in the expression of inflammatory mediators such as interleukin-1 (IL-1), IL-6, IL-8, and serum eosinophil cationic protein (23, 24).

Nuclear diameter, nuclear area, cellular diameter, and cellular area in smoker men compared to non-smokers

In this study, there was a non-significant difference in nuclear diameter in the buccal mucosa sample in smokers men (3.848±1.16812) compared to the corresponding diameter in buccal mucosa of non-smokers (3.936±0.70759) (table 4), at the same aged range (P>0.05). Similarly, there was a non-significant difference in the nuclear area of the buccal mucosa sample of smokers (66.9780±1.53761) compared to the corresponding area in the buccal mucosa of non-smokers (66.7748±1.38678), table 5, at the same aged range (P>0.05). The results of this study are inconsistent with those of others (25). In the current study, there was a significant reduction in cellular diameter in buccal mucosa in smokers (73.3756±3.52789) compared to the corresponding diameter in the buccal mucosa of non-smokers (77.0532±1.85524) (table 6), at the same aged-range (P<0.05). Similarly, the results of this study showed that there was a significant reduction (P<0.05) in the cellular area of the buccal mucosa of smokers (3615.0724±73.298) compared to the corresponding area in the buccal mucosa of non-smokers (3713.9352±104.637) (table 7). The results of this study are in agreement with those of others (26). Moreover, Cançado, et al., (2004) reported that the increase in cellular diameter and increase in the nuclear size are considered as two significant changes that may occur in actively proliferating cells and this, in turn, may consequently result in malignancy (26, 27).

Correlation between each of serum -copper (Cu), -zinc (Zn), and -iron (Fe) levels and nuclear-to-cellular (N/C) area ratio in smoker men

Results of the present study showed that there was a non-significant correlation between serum Cu levels and nuclear-to-cellular area ratio in smoker men (r = 0.105, P =0.617) (figure 3). Furthermore, there is a negative correlation between serum Zn levels and nuclear-to-cellular area ratio in smoker men but it is non-statistically different (r = -0.142, P =0.498) (figure 4). Also, there is a negative correlation between serum Fe levels and nuclear-to-cellular area ratio in smoker men but it is non-statistically different (r= -.050, P =0. 812) (figure 5). To the best of our knowledge, this is the first study that investigated the relationship between serum level of each element (Cu, Zn, and Fe) with the nuclear-to-cellular area ratio in a mucosal sample of smoker men.

In conclusion, according to the results obtained from this study, one can conclude that alterations in serum levels of copper, zinc, and iron; and in cellular diameter and cellular area were observed but not on the nuclear-diameter and -area in the oral keratinocytes of smokers’ men; this indicates that oral mucosa exposed to tobacco smoke is altered by tobacco smoking; thus, cytomorphometrically evaluation of keratinocytes can serve as a useful diagnostic adjunct for early detection of oral cancer; furthermore, there is no correlation between levels of elements (copper, zinc, and iron) with the oral keratinocytes.

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