1 Short communication

2 Cigarette smoking increases plasma levels of IL-6 and TNF-α

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5 Abstract

6 Background and objective: Cigarette smoking is a leading cause of a wide range of critical health problems such as cancers, especially those who are related to the respiratory system. Although studies are continuing on the smoking-related inflammatory responses, limited reports are there to explore how such responses can be affected by the smoking intensity. Therefore, the current communication aimed to shed light on how smoking and smoking intensity can affect some inflammatory and anti-inflammatory biomarkers.

7 Methods: A total of 159 subjects (108 smokers and 51 non-smokers) were enrolled in this cross-sectional study. Their sociodemographic, smoking intensity and blood samples were obtained and processed using approved methodologies. The blood plasma samples were used to quantify interleukin 6 (IL-6), IL-10, tumor necrosis factor-alpha (TNF-α), C-reactive protein, D-dimer, and ferritin by using ELISA. The gained data was then analyzed using GraphPad Prism software to assess the variations.

8 Results: The results showed that IL-6 and TNF-α are elevated markedly (p<0.001) in smoker subjects when compared with non-smoker ones (IL-6: 2.58±0.98 vs. 1.858±0.6256 pg/ml, TNF-α: 28.38±7.162 vs. 22.64±7.257). However, no significant differences were observed in other biomarkers comparing the groups, as well as no significant association was evidenced based on smoking intensity among smokers.

9 Conclusions: The findings might point to a relationship between smoking and the elevation of IL-6 and TNF-α levels in a cigarette dose-dependent manner.

10 Keywords: biomarkers; cytokines; inflammation; smoking

11 Introduction
Smoking is a leading cause of lung function decline and chronic obstructive pulmonary disease (COPD) development, and its relationship with inflammation is well established. In spite of controversial reports, inflammatory cells, oxidative stress, and inflammatory mediators are the major contributors in smoking-related inflammation in humans. The reported change in inflammatory cells included an increase in neutrophils in chronic and acute exposure to cigarette smoking in both peripheral blood and bronchoalveolar lavage fluid. Accumulated evidence has also shown that the acute exposure to cigarette smoking can result in immediate increase in oxidative stress and reactive species. Moreover, mediators of inflammation in humans as well as animal and in vitro models such as plasma neutrophil elastase and leukotrienes B4, D4, E4, and inflammatory cytokines (such as TNF-α) in acute smoking have also shown to be changed. However, limited reports are available on the relationship between the intensity of smoking and inflammatory responses.

This brief communication aimed to investigate the plasma levels of IL-6, IL-10, TNF-α, high sensitivity C-reactive protein (hsCRP), D-dimer, and ferritin in apparently healthy smoker subjects and how they can be affected by the smoking intensity.

**Materials and methods**

**Study design and subjects**

This communication is a part of cross-sectional study conducted at the Technical Institute of Baquba (Baqubah, Iraq) between February and April 2021. The study included 108 smoker subjects (active smoking) and 51 non-smoker healthy individuals. All the included subjects were males (students or staff at the institute).

Smokers with no history of cardiovascular diseases, any other chronic or acute diseases, as well as those who received no vitamin supplements or chemical treatment during the previous 6 months were included in this study. But, those who suffer from any chronic or acute disorders, or those who received medical treatment or vitamin supplements were excluded. The variables recorded were age, height, weight, smoking intensity (average of cigarettes smoked/day), occupation, educational level as well as medical history. The participants were subdivided according to smoking status (smokers and non-smokers), educational level (School, University) and occupation (employee, nonemployee). The Fagerstrom Test for Nicotine Dependence (FND) was modified and used to assess the smoking intensity. The smokers were divided accordingly into three groups: 1-20 cigarettes/day group (n=18), 21-40 cigarettes/day group (n=69), and >40 cigarettes/day group (n=21).

The Deanship of Technical Institute of Baqubah approved this study (No.: #1181, Date: 01-03-2021) as well as the Department of Chemistry, College of Science, University of Diyala (No.: #447, Date: 04-02-2021). Written informed consent from each participant was obtained.

**Blood sampling and analysis**

For each participant who eligible to be enrolled, 5-milliliters (mL) of whole venous blood were drawn and collected on K2.EDTA tube and serum separator gel tube. The centrifugation was used, to separate serum and plasma samples, at 500 rpm for 15 minutes. The gained serum
and plasma were then moved to another test tube to be preserved at freezer until chemical analysis day.

The biomarkers assessed were interleukin 6 (IL-6), interleukin 10 (IL-10), tumor necrosis factor alpha (TNF-α), high sensitivity C-reactive protein (CRP), D-dimer and ferritin. All the biomarkers were evaluated in plasma samples using enzyme-linked immunosorbent assay (ELISA) kits from Abcam (USA) and the manufacturer’s instructions were followed. About 5% of the samples were tested in twice to ensure the quality and performance of the kits.

**Statistical analysis**

The data was statistically evaluated using GraphPad Prism 8.0 (GraphPad Software, USA). Categorical data was presented in frequencies and percentages, and numerical data was presented using mean±SD (or median and interquartile range, IQR, as appropriate). Independent sample t-test (or Mann-Whitney’s U test) for two-group comparisons or One-way ANOVA (or Kruskal-Wallis test) for three and more group comparisons were used to assess the differences in means or median. In addition, Chi-square test was used to explore the difference between nominal variables. The p-value of ≤0.05 was considered statistically significant.

**Results and discussion**

**General characteristics of study participants**

The sociodemographic characteristics of participants were listed in Table 1. No significant differences were noticed between smokers and non-smokers in regards to age, BMI, educational level, and occupation status.

**Table 1: Sociodemographic characteristics of participants based on smoking status**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Smokers (n=108)</th>
<th>Non-smokers (n=51)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)†</td>
<td>22.5(20,26)</td>
<td>22.0(20,26)</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>25.39±3.24</td>
<td>25.62±3.24</td>
<td>NS</td>
</tr>
<tr>
<td>Educational level – n(%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary school</td>
<td>78(72.2)</td>
<td>37(72.55)</td>
<td>NS</td>
</tr>
<tr>
<td>Undergraduate school</td>
<td>30(27.8)</td>
<td>14(27.45)</td>
<td></td>
</tr>
<tr>
<td>Occupation – n(%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Employee</td>
<td>30(27.8)</td>
<td>14(27.28)</td>
<td>NS</td>
</tr>
<tr>
<td>Nonemployee</td>
<td>78(72.2)</td>
<td>37(72.55)</td>
<td></td>
</tr>
</tbody>
</table>

NS, non-significant (p value >0.05); † (median, IQR)

**Biomarkers based on smoking status**

Inflammatory responses and cellular releases are essential contributors to lung injury and pathogenesis. Inflammatory biomarkers, both anti- and proinflammatory, were reported to be disturbed in cigarette smoking. However, open literature search shows controversial
results. To explore the effect of smoking on the studied biomarkers, they were investigated in the plasma of all smoker and non-smoker subjects and analyzed using unpaired t-test.

The results showed that both IL-6 and TNF-α are dramatically elevated in smoker persons when compared to non-smokers (IL-6 in pg/mL: 2.582±0.9869 vs. 1.858±0.6256, p<0.001 and TNF-α in pg/mL: 28.38±7.162 vs. 22.64±7.257, p<0.001). Non-significant differences were noted in other biomarkers between smokers and non-smokers (IL-10 in pg/mL: 6.80±0.809, p=0.884; hsCRP in mg/dL: 0.41±0.108 vs. 0.38±0.113, p=0.255; D-dimer in mg/L: 0.20±0.0514 vs. 0.20±0.0591, p=0.934; and ferritin in μg/L: 69.51±20.62 vs. 63.42±21.13, p=0.09) (Figure 3).

The current results regarding IL-6 and TNF-α levels are in conformity with wide-range studies conducted previously such as that of Helmersson et al. (2005)10 concerning IL-6, which was increased in current smokers compared with non-smokers, and that of Petrescu et al (2010)17 in regards TNF-α. Petrescu et al found that TNF-α elevation is associated with smoking cigarettes in a dose-dependent manner. These two biomarkers are secreted by inflammatory cells in response to stimuli for the induction of inflammation.20 It is also well established that cigarette smoking activates oxidative stress and immune response, and this may interpret why these biomarkers are elevated. For other biomarkers that are not significantly varied, as in previous authors’ findings,19–21 our results may be limited with the sample size, race, gender, age, etc. of the study population. Accumulated evidence shows that race, gender and age have a significant influence on the inflammation, IL-10, hsCRP levels, D-dimer and ferritin levels.22–25

**Figure 3:** Plasma levels of inflammatory and hemostatic biomarkers in smokers and non-smoker participants. A) IL-6, B) IL-10, C) TNF-α, D) hsCRP, E) D-dimer, F) Ferritin. *** denote statistically significant change (p<0.001)

**Biomarkers based on smoking intensity**
To investigate the effects of smoking intensity on biomarkers, the smokers were classified into three subgroups as detailed in methods section and their biomarkers’ results together with those of non-smoker subjects were analyzed by using one-way ANOVA test (or Kruskal Wallis test). The results showed that IL-6 level, in pg/mL, in non-smokers is the lowest (1.87±0.62) and increased in smoking as the number of cigarettes smoked increased (1-20/day: 2.26±1.28, 21-40/day: 2.62±0.93, and >40/day: 2.72±0.84). When compared statistically, the significant increase is only seen in smokers with higher number of smoked cigarettes per day (21-40, p<0.001 and >40, p=0.001). The TNF-α was also revealed drop in its level in non-smoker subjects (22.64±7.25 pg/mL) when compared to smokers with low smoking intensity (30.02±7.22 pg/mL), moderate intensity (28.24±6.68 pg/mL), and intensive smoking (26.99±8.78 pg/mL). No significant differences were found between the smokers’ biomarkers and those of non-smokers (Figure 4).

These results are in line with those of Aldaham et al (2015) and Petrescu et al (2010) who found that IL-6 and TNF-α are increased in a dose-dependent fashion. The elevation in IL-6 and TNF-α level together with smoking intensity, in the current study, may indicate their significant role as a mediator of inflammation in cigarette smoking.

In contrast with previous studies by Dwivedi et al. (2014) for IL-10, by Ohsawa et al. (2005) and Helmersson et al. (2005) for hsCRP, and by Wannamethee D-dimer; our findings did not show significant differences in anti-inflammatory cytokine (IL-10) and inflammatory and hemostatic biomarkers. This may be affected by different small sample sizes of various studies. Besides, other racial and anthropometric variations may contribute to such inconsistencies.

**Figure 4:** Plasma levels of inflammatory and hemostatic biomarkers: A) IL-6, B) IL-10, C) TNF-α, D) hsCRP, E) D-dimer, and F) Ferritin in smokers based on smoking intensity. Letters denote statistically significant difference between the bar under letter and non-smokers or 0 bar (a when p<0.001 and b when p= 0.001 – 0.009)
Nonetheless, the inherent limitations of cross-sectional studies are well known and thus no conclusions about causality can be drawn from the current results. In particular, the small number of participants and especially in subgroups of smoking intensity and voluntary participation in the study may be the major limitations, as well as other sociodemographic variations in regards to environment, lifestyle and genetics.

Conclusions

Cigarette smoking is a leading cause of serious illness and cancers and its relation to inflammation is well established. This study investigated the influence of smoking and smoking intensity on the immune response in apparently healthy smoker males by measuring plasma levels of IL-6, IL-10, TNF-α, hsCRP, D-dimer, and ferritin. The results indicated significant rise in IL-6 and TNF-α of smoker persons’ plasma when compared with non-smokers. No significant changes in the studied biomarkers, based on smoking intensity, were found among smokers. As a proposed mediators of inflammation, it can be speculated that both IL-6 and TNF-α might be useful biomarkers in the evaluation of smoking intensity-related lung inflammation.

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Authors’ contributions

Conceptualization, data curation, resources: SAA. Formal analysis, investigation, methodology, project administration, software, validation: SAA and HAA. Funding acquisition: N/A. Supervision: NJH. Writing-original draft: SAA and HAA. Writing-review & editing: HAA, NJH and KBG.

Ethical approval and consent to participate

The Deanship of Technical Institute of Baqubah approved this study (No.: #1181, Date: 01-03-2021) as well as the Department of Chemistry, College of Science, University of Diyala (No.: #447, Date: 04-02-2021). Written informed consent was obtained from each participant before participation.

Conflict of interest

The authors declare no conflict of interest.

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References


