COMPARATIVE EVALUATION OF SALIVARY PROTEIN PROFILE AND OXIDATIVE STRESS STATUS AMONG YOUNG HEALTHY ADULT SMOKERS AND NON-SMOKERS

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ABSTRACT
Aim: To evaluate salivary protein-profile and oxidative stress status in young healthy adult smokers and non-smokers.
Method: Saliva samples were collected from a total of 46 individuals, including non-smokers (n=26) and smokers (n=20) through drooling method. All participants were males, healthy and aged 18-35 years. Salivary flow rate and pH was noted. Total salivary protein concentration, oxidative stress status and amylase activity was measured by spectrophotometric assays. Salivary protein-profile was evaluated by SDS-PAGE.
Results: Our results showed that salivary pH was significantly lowered in smokers. Significant increase in levels of reactive oxygen species (ROS) were found in smokers as compared to non-smokers (p<0.05). Significant difference was not found in total salivary protein concentration and amylase activity. However, differences were observed in frequency and intensity of protein bands. Seven predominant protein bands of molecular weight ranging from <11-100kDa were observed and frequency of <11kDa protein bands were found to be higher in smokers.
Conclusion: Increased ROS levels in smokers indicate an oxidative imbalance in these subjects. Differences observed in salivary protein-profile needs further investigations centered on identifying whole salivary proteome which may help in determining early biomarkers for tobacco related oral diseases.
Keywords: Saliva, Oxidative stress, Protein profile, Cigarette smoke, Reactive Oxygen Species

INTRODUCTION
Human saliva is a heterogeneous fluid which is collectively generated by the secretions from different salivary glands1,2. Saliva has a pivotal role in maintaining the oral homeostasis. This aqueous fluid not only lubricates the mouth but also helps in eating, swallowing, speaking and provides protection to the epithelial and mucosal tissue against toxic effects of micro-organisms or any chemical that comes in contact with the oral cavity3.
Saliva is comprised of several organic and inorganic biomolecules including a large number of proteins and peptides; which play a significant role in protecting the oral cavity against infections and maintaining the oral health4,5. The protective and defensive system of saliva is governed by the presence of immunoglobulins, enzymes, glycoproteins and antimicrobial peptides6. Antioxidant function of saliva is due to the presence of number of antioxidant compounds and enzymes, these molecules protects the oral cavity against the detrimental effects of free radicals such as reactive oxygen species (ROS) which are produced in saliva either naturally or by exposure to environmental chemicals7. Saliva provides defense against ROS and any imbalance in the composition of saliva that suppresses the salivary antioxidant system, may favor the production of oxidative stress8. Oxidative stress plays a critical role in the pathogenesis of many oral diseases including oral squamous cell carcinoma9. Recent studies have shown a higher incidence of oral cancer in regular smokers as compared to non-smokers8.
Cigarette smoke contains around 4000 toxic chemicals including carcinogens and oxidants. Free radicals in cigarette smoke can alter the salivary protein expression and structure thus affecting their protective function and may lead to several oral diseases9,10. Smoking is combustion and inhalation of tobacco or some other substance for recreational purpose. Smoking increases the risk of cancer, cardiovascular and respiratory diseases and therefore is a major cause of morbidity and mortality globally11. Survey studies in different countries have found that young adult age group has the highest current smoker numbers in comparison to other groups12,13.
There has been an increased research interest in the last decade in identifying disease related biomarkers in saliva. As saliva is the first body fluid that comes in contact with smoke, it is expected to show differential compositional patterns that can be used for early diagnosis4. However, limited studies are available regarding the effect of cigarette smoking on the expression of salivary proteins. This study aimed to explore the salivary protein profile and oxidative stress status of young healthy adult smokers and non-smokers, to detect the probability of any smoking associated changes in oral cavity.

MATERIALS AND METHODS
Study Subjects and Sample: Saliva samples were collected from 46 healthy young adult males (26 non-smokers and 20 smokers), aged between 18-35 years during April to September 2019. Samples were collected from healthy male subjects who do not have any medical history of systemic diseases and neurological problems. Subjects were recruited after signing an informed consent and were asked to fill a brief questionnaire regarding their demographic details, oral hygiene, smoking habits and tobacco intake. Body mass index (BMI) was recorded for each participant. Study was conducted after taking ethical approval (IRB-1327/DUHS/Approval/2019) from university’s institutional review board.
Saliva Collection by Swabbing and Drooling Method: Saliva samples were collected by swabbing and drooling method. Participants were asked to keep the cotton swab in the mouth and saliva until they feel that they have collected enough saliva in their mouth. The accumulated saliva was collected in two ways, first the participant drools out the collected saliva into the 50ml sterile falcon tube and secondly, the saliva soaked cotton which was removed from the participants mouth was extorted in the same falcon by placing it in sterile needleless syringes. The total volume of the collected saliva, time of collection and pH of the saliva was noted. Saliva samples after collection was centrifuged at 3000 rpm for 20 min to settle any debris present in the saliva and cleared supernatant was stored at -20°C until further use.

Estimation of Total Salivary Protein levels and Salivary Amylase Activity: Total salivary protein concentration was determined by Bradford assay. Salivary amylase activity was determined by the method reported by Sahni et al.

Estimation of total reactive oxygen species (ROS): ROS was measured by Ferrous Oxidation Xylenol-Orange (FOX) assay to evaluate the oxidative stress status, using hydrogen peroxide as a standard (0-40µM). FOX reagent, a mixture of FOX A (25mM ammonium ferrous sulphate and 2.5M sulphuric acid) and freshly made FOX B (100mM sorbitol and 125µM Xylenol orange) were mixed in a ratio of 1:10. Saliva sample was added to FOX reagent in 1:10 dilution and incubated in dark for 1 hr. After incubation color change was observed and absorbance was measured at 560nm.

Salivary proteins separation by sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE): Salivary protein profile was determined by SDS-PAGE. Individual saliva samples were added to reducing sample buffer and boiled. Samples containing 5-10µg protein were then loaded on the gel and was resolved on a 12% acrylamide gel. Gels were stained with Coomassie Brilliant Blue G250 for 1 hour and detained with 10% acetic acid until the bands were completely visible.

Gels were imaged and analyzed for the presence of different protein bands in the samples. Number of protein bands present in each subject was counted and scored as “1” and “0” indicating the presence and absence of bands respectively. Frequency of bands was calculated for each individual. Molecular weight of the separated proteins was determined by comparing their distance of migration in the gel with that of the corresponding molecular weight ladder.

Statistical Analysis: Data was analyzed using SPSS version 16 and Microsoft Excel. Descriptive statistics was used for analysis. Frequencies were generated for the categorical variables. Independent student’s t-test was used to compare the difference in variable means between the two study groups. Differences in the categorical data were compared by Chi-square test. Sialochemical analysis among different sub-groups of smokers was analyzed by ANOVA followed by post-hoc comparison. P-value of <0.05 was considered statistical significant. Standard calibration curves and all the charts were prepared using Excel.

RESULTS
In total we have evaluated 46 young healthy adult male participants (26 non-smokers and 20 smokers). Briefly, the mean age of the participants was 22.6±4.8 years, most of them were undergraduate students (~74%) and maintained a good oral hygiene which was evaluated by their brushing frequency per day (61% brush their teeth twice a day). Significant difference was not observed in demographic characteristics of non-smokers and smokers (Table 1).

Physiological & Biochemical Health Parameters of Smokers and Non-Smokers: Table 2 shows the comparison of the physiological and biological health parameters between smokers and non-smokers. Sialometrical analysis showed a decreased salivary flow rate in smokers (0.69±0.25 ml/min) as compared to non-smokers (0.80±0.40 ml/min); however the difference was not found statistically significant. When salivary pH was compared between the two study groups, smokers were found to have significantly lower salivary pH (p=0.005) (Table 2).

Comparison of total salivary protein levels and amylase: There was no significant difference (p=0.78) observed in total protein concentration; average protein concentrations were 1.04±0.55 mg/ml and 0.92±0.5 mg/ml between non-smokers and smokers respectively (Table 2). Similarly, no significant difference was found among the study groups for amylase activity (Table 2).

Oxidative stress measurement: Total Reactive oxygen species (ROS) were measured to evaluate the oxidative stress among study groups. There was significant difference (p=0.03) in the mean total ROS levels among non-smokers (8.5±6.5 µM) and smokers (16.0±15.8 µM). Smokers have significantly higher total ROS levels as compared to non-smokers.

Sialochemical analysis among different sub-groups of smokers: Smokers were further divided into three groups based on the frequency of cigarettes they smoke per day. The three sub groups of smokers were light smokers (smokes <5 cigarette/day, n=7), moderate smokers (smokes 5-15 cigarette/day, n=7) and heavy smokers (smokes >5 cigarette/day, n=6). Total protein concentration was found to be 1.28±0.4, 0.94±0.51 and 0.89±0.57 mg/ml in light, moderate and heavy smokers respectively. Total ROS levels were 12.5±6.9, 21.9±20 and 13.3±12.9 µM, whereas amylase activity was found to be 104.0±18.3, 75.4±54.2 and 86.3±47.9 U/ml in light, moderate and heavy smokers respectively.

Comparison of protein concentration among non-smokers and subgroups of smokers showed insignificant difference (p=0.53), the amylase activity and total ROS levels were also found insignificant (p=0.74 and p=0.06 respectively). Similarly, no significant differences were observed among different subgroups of smokers.

Sialovary protein profiling: Electrophoretic pattern indicating the differences in salivary protein profile of non-smokers and smokers is shown in Figure 1. Seven predominant protein bands of molecular weight ranging from <11-100 kDa were observed (Figure 1). Differences were observed in the frequency and intensity of protein bands between smokers and non-smokers (Table 3). Protein bands of molecular weight 48-63 kDa were found to be present in all individuals. Frequency of occurrence of <11 kDa protein bands were found to be higher in smokers (Table 3).
Table 1: Demographic details of non-smokers and smokers

<table>
<thead>
<tr>
<th>Variables</th>
<th>All study Participants (n=46)</th>
<th>Non-Smokers (n=26)</th>
<th>Smokers (n=20)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>22.6 ± 4.8</td>
<td>21.8 ± 3.3</td>
<td>23.6 ± 6.3</td>
<td>0.22</td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undergraduates</td>
<td>34 (73.9)</td>
<td>20 (76.9)</td>
<td>14 (70.0)</td>
<td>0.54**</td>
</tr>
<tr>
<td>Graduates/Postgraduates</td>
<td>11 (23.9)</td>
<td>6 (23.1)</td>
<td>5 (25.0)</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Underweight (&lt;18.5kg/m²)</td>
<td>5 (10.9)</td>
<td>3 (11.5)</td>
<td>2 (10.0)</td>
<td>0.92**</td>
</tr>
<tr>
<td>Normal weight (18.5-24.9kg/m²)</td>
<td>33 (71.7)</td>
<td>19 (73.1)</td>
<td>14 (70.0)</td>
<td></td>
</tr>
<tr>
<td>Overweight/Obese (&gt;25kg/m²)</td>
<td>8 (17.4)</td>
<td>4 (15.4)</td>
<td>4 (20.0)</td>
<td></td>
</tr>
<tr>
<td>Daily Brushing Frequency</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Once a day</td>
<td>15 (32.6)</td>
<td>7 (26.9)</td>
<td>8 (40.0)</td>
<td>0.23**</td>
</tr>
<tr>
<td>Twice/Thrice a day</td>
<td>31 (67.4)</td>
<td>19 (73.1)</td>
<td>12 (60.0)</td>
<td></td>
</tr>
<tr>
<td>Dental visit for oral examination</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Visit</td>
<td>38 (82.6)</td>
<td>20 (76.9)</td>
<td>18 (90.0)</td>
<td>0.18**</td>
</tr>
<tr>
<td>Yearly (Once/Twice)</td>
<td>4 (8.7)</td>
<td>4 (15.4)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Only in case of problem</td>
<td>4 (8.7)</td>
<td>2 (7.7)</td>
<td>2 (10.0)</td>
<td></td>
</tr>
</tbody>
</table>

Results are presented as Mean ± Standard Deviation & n (%)
* Independent Student's t-test
** Chi-square test.
p-value < 0.05 is termed as significant.

Table 2: Comparison of Physiological & Biochemical Parameters of Smokers and Non-Smokers

<table>
<thead>
<tr>
<th>Variables</th>
<th>All Participants (n=46)</th>
<th>Non-Smokers (n=26)</th>
<th>Smokers (n=20)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salivary pH</td>
<td>6.93 ± 0.63</td>
<td>7.15 ± 0.50</td>
<td>6.64 ± 0.68</td>
<td>0.005*</td>
</tr>
<tr>
<td>Salivary Flow Rate (ml/min)</td>
<td>0.74 ± 0.35</td>
<td>0.80 ± 0.40</td>
<td>0.69 ± 0.25</td>
<td>0.29</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.7 ± 0.06</td>
<td>1.74 ± 0.06</td>
<td>1.72 ± 0.05</td>
<td>0.25</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>65.6 ± 10.2</td>
<td>65.7 ± 9.4</td>
<td>65.4 ± 11.4</td>
<td>0.91</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.98 ± 3.4</td>
<td>21.79 ± 2.9</td>
<td>22.3 ± 3.9</td>
<td>0.67</td>
</tr>
<tr>
<td>Salivary total protein (mg/ml)</td>
<td>1.02 ± 0.53</td>
<td>1.04 ± 0.55</td>
<td>0.99 ± 0.51</td>
<td>0.78</td>
</tr>
<tr>
<td>Salivary Amylase Activity (U/ml) (n=36)</td>
<td>0.31 - 2.19</td>
<td>0.33 - 2.19</td>
<td>0.31 - 1.84</td>
<td></td>
</tr>
</tbody>
</table>

*p-value <0.05=Significant

Table 3: Comparative summary of the expression of protein bands between smokers and non-smokers.

<table>
<thead>
<tr>
<th>Protein Band MW (kDa) (Corresponding Protein)</th>
<th>All (n=46)%</th>
<th>Non-Smokers (n=26)</th>
<th>Smokers (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>75-100 (Immunological Components, e.g. IgA)</td>
<td>26 (56.5)</td>
<td>12 (46.2)</td>
<td>14 (70.0)</td>
</tr>
<tr>
<td>63-75 (Lactoferrin &amp; Albumin)</td>
<td>41 (89.13)</td>
<td>22 (84.6)</td>
<td>19 (95.0)</td>
</tr>
<tr>
<td>48-63 (Amylase family)</td>
<td>46 (100.0)</td>
<td>26 (100.0)</td>
<td>20 (100.0)</td>
</tr>
<tr>
<td>35-48 (Carbonic anhydrase)</td>
<td>17 (36.9)</td>
<td>4 (15.38)</td>
<td>13 (65.0)</td>
</tr>
<tr>
<td>25-35 (Proline Rich Proteins)</td>
<td>28 (60.8)</td>
<td>13 (50.0)</td>
<td>15 (75.0)</td>
</tr>
<tr>
<td>17-11 (Cystatins)</td>
<td>45 (97.8)</td>
<td>26 (100.0)</td>
<td>19 (95.0)</td>
</tr>
<tr>
<td>&lt;11 (Histatins)</td>
<td>11 (23.9)</td>
<td>2 (7.69)</td>
<td>9 (45)</td>
</tr>
</tbody>
</table>

*MW=Molecular weight

Figure 1: Representative Electrophoretic (SDS-PAGE) pattern of salivary proteins from non-smokers and smokers.
Lane 2 contains protein ladder of known molecular weight. Lane 3 contain sample from non-smoker (NS) and lane 4-9 was loaded with the sample of smokers (S). Seven protein bands of molecular weights of 75-100kDa, 63-75kDa, 48-63kDa, 35-48kDa, 25-35kDa, 17-11kDa & <11kDa were observed.
**DISCUSSION**

Cigarette smoke contains thousands of chemical toxins including polycyclic aromatic hydrocarbons, ammonia, acetalddehyde, carbon monoxide, hydrogen cyanide and nitrogen oxides\(^1\). These toxic chemicals can cause structural and functional changes in the salivary molecules and can lead to altered oral physiology. Present study was designed to compare the behavior and alteration of saliva and its components in young healthy smokers and non-smokers.

Smoking habit can affect multiple components of the saliva, including its flow rate and pH. Inhalation of cigarette smoke can result in imbalance of saliva’s environment. In this study, comparatively lower salivary flow rate was observed in smokers although the difference was found non-significant statistically. These results were consistent with previous findings that shown decreased salivary flow rate in smokers compared to non-smokers\(^{20,21}\). Reduced salivary flow rate can affect the protective function of saliva. The decrease in salivary secretions in smokers indicates towards the dry mouth condition called xerostomia, which is a common symptom in smokers\(^{21,22}\).

Current study showed significant difference in salivary pH of smokers and non-smokers (p<0.05), which was in accordance with previous studies\(^{21,23}\). The decrease in the salivary flow rate may have an influence on the salivary pH. In addition, the presence of toxins such as nicotine and tobacco present in cigarette smoke alters the buffering capability of saliva and thereby promoting the acidic environment of saliva\(^{21,23}\). Further studies are required to determine the correlation between the salivary flow rate and salivary pH with the oral health status in smokers.

The imbalance of ROS species and antioxidants results in oxidative stress, which may originate from multiple reasons including exposure to environmental toxins and chemicals or genetic alterations etc\(^1\). Our results, showed significantly increased levels of ROS in smokers that indicate the oxidative imbalance in these subjects compared to non-smokers. Our results are in agreement of previous studies that have shown an altered oxidant status in smokers\(^{19,24}\). When comparisons were made within the different subgroups of smokers, ROS levels were found to increase with the intensity of smoking. Moderate smokers have highest ROS levels, compared to light and heavy smokers. This indicates that the duration and intensity of smoking significantly contributes to the accumulation of free radicals in oral cavity\(^7\). Due to the elevated ROS levels, smokers become more prone to develop multiple oral and systemic diseases including cancer, therefore strategies should be designed for early screening of any changes in salivary composition to prevent the oral malignancies\(^{25}\).

Human saliva along with other components is a mixture of large number of proteins and peptides that have multiple roles in oral cavity such as pH regulation, buffering, and part of the salivary antioxidant and immune system. Mean salivary protein concentration has been reported to be ranged from 0.72 to 2.45 mg/ml, salivary protein levels observed in this study lies within the reported values\(^{26,27}\). A trend of decrease in protein concentration with increasing levels of smoking was observed; saliva from heavy smokers. This indicates that extent and duration of smoking may reduce the expression of salivary proteins\(^{26,27}\).

Comparatively lower salivary amylase activity was observed in smokers, although the difference was not significant. These findings were in accordance to results from previous studies\(^{28,29}\). Lower salivary activity in smokers may indicate any psychological stress, as salivary amylase has also been used as a marker of physical and psychosocial stress\(^{30}\).

Limited literature is available that explored the profile of salivary proteins in different oral conditions and particularly in cigarette smoking\(^{27}\). We have observed differences in the intensity of salivary protein bands between smokers and non-smokers. The apparent molecular weight of the separated proteins ranged from 11kDa to 100kDa, and seven predominate bands were observed in both the study subjects (Figure 1). The protein bands between 75 and 100 kDa corresponds to immunological components of saliva such as IgA, furthermore 63-75kDa corresponds to lactoferrin and albumin. The predominant protein band around 48-63kDa corresponds to amylase. The bands observed around 35-48kDa, 25-35kDa, 6-11kDa & <11kDa corresponds to Carbonic anhydrase, Proline rich proteins (PRPs) fractions, Cystatins and Histatins respectively\(^{27}\). The protein band pattern of <11kDa among smokers and non-smokers were found different. Total 11 samples demonstrated <11kDa band (corresponds to histatins); out of which nine were smokers (Table 3). Our study involved young adult healthy smokers; one reason of increased expression of low molecular proteins in these subjects could be that these proteins are involved in preventing inflammation, and smoking increases the risk of gums inflammation which may explain their increased expression in smokers\(^3\). Future larger scale studies that correlate the salivary protein profile with different oral conditions are needed.

**CONCLUSION**

In conclusion, results of present study showed an increased ROS levels in smokers which is an indicative of an oxidative imbalance in these subjects. Differences observed in salivary protein profile in smokers and non-smokers needs further investigations centered on identifying the whole salivary proteome, which may help in determining the early biomarkers for tobacco related oral cavity diseases.

**Competing Interests:** No conflicts of interest.

**Acknowledgments:** N/A

**REFERENCES**


