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Effect of smoking cessation in saliva compounds by FTIR spectroscopy

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ABSTRACT

Introduction: Smoking is currently considered one of the biggest risk factors for the development of various diseases and early death. Fourier transform infrared (FTIR) spectroscopy is a valuable tool for analysis of biofluids such as saliva and is considered useful for diagnostic purposes. The aim of this study was to evaluate the effect of smoking cessation on saliva composition by FTIR spectroscopy.
Methods: We analyzed the saliva of participants in two groups: a smoker group made up of 10 chronic smokers and a former smoker group made up of 10 individuals who had stopped smoking. Members of both groups had similar smoking history.
Results: The results showed few differences in spectral intensity between the groups; however, spectral peaks were slightly increased in the group of smokers in the bands for DNA, indicating modification of its content or cell necrosis. They were also increased for the mannose-6-phosphatase molecule, which is expressed in prostate and breast carcinomas. In the former smoker group, the peak of thyociante was decreased and the band referring to collagen increased in intensity, which indicates a better tissue regeneration capacity.
Conclusion: Considering these results and the fact that tobacco intake was similar between the groups, it can be concluded that there was recovery of tissue regeneration capacity with smoking cessation during the study period, although the effects found in smokers persisted in the bodies of those who had given up smoking.

1. Introduction

Saliva is a complex body fluid [1] that contains a large number of different types of proteins, hormones and ions [2]. It is secreted primarily by three pairs of salivary glands (parotid, submandibular and sublingual), as well as numerous minor salivary glands [2], With the continuous advancement of proteomics technology, salivary diagnosis has become a focus of several studies [2] and, mainly because besides being considered a fluid with excellent indicators of plasma levels of various substances [1] its collection is simple and noninvasive. Thus, saliva has been used for the early diagnosis, prevention, and monitoring process of various diseases [2,3,4].

In recent years, various spectroscopic techniques have been studied in the biodiagnosis area and have greatly advanced in medical and diagnostic ability [5]. Fourier transform infrared (FTIR) spectroscopy is a vibrational spectroscopy method that can be used for qualitative and structural analysis and to determine structural changes in organic molecules [6]. It has been considered a powerful tool to analysis of biological samples such as plasma, serum, tissue, saliva, and urine and is already offered as a complementary technique for the clinical diagnosis and characterization of various types of diseases [6], including lesions which early diagnosis is a significant prognostic factor [7].

Habitual smoking is the most significant threat to the world's population, accounting for 30% of early death [8]. It is the biggest risk factor for cardiovascular disease and chronic obstructive pulmonary diseases [9,10] and malignancies [11], mainly because the substances contained in tobacco affect and damage several organs and tissues [8].

Since it is known the damage caused by tobacco systemically, the aim of this study was to compare the saliva of patients who smoke and patients who discontinued tobacco use for a period of at least 6 months and up to 3 years through FTIR. We proposed demonstrate

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significant differences in salivary content, detecting any changes at the molecular level as results of tobacco cessation, and then prove that the period during which the individual did not use cigarettes was sufficient to change the saliva composition and consequently general healthy.

2. Material and Methods

There were two groups of participants from the Smoking Cessation Program, Heart Institute (InCor), University of Sao Paulo Medical School, São Paulo, Brazil. The study was approved by the Ethics Committee of the Institute of Science and Technology, UNESP – Univ Estadual Paulista, São Paulo, Brazil, Protocol CAEE 45703114.6.0000.0077. It was conducted in accordance to Helsinki Declaration. Informed consent was obtained from all subjects prior to their participation in the study. All participants were subjected to extra- and intra-oral clinical examination. Patients were considered eligible for enrolment into the research if they fulfilled the following inclusion criteria: no history of malignant neoplasms and maximum weekly intake of 3 alcoholic drinks. The exclusion criterion was the presence of any visible sign of intra-oral alteration. Each group was composed by the selection of 20 consecutive patients as follows:

- Smoker group: 10 chronic smokers with consumption equal to or more than 20 cigarettes/day for more than 10 years;
- Former smoker group: 10 subjects who had stopped smoking for a period of at least 6 months and a maximum of 3 years;

Participants also completed a questionnaire regarding tobacco smoking. Data was used to calculate the smoking history according to Faria et al. [12]: number of cigarettes smoked per day divided by 20 (1 pack has 20 cigarettes in Brazil) multiplied by the total number of years of smoking (results in packs/year). Exhaled carbon monoxide (CO) concentrations were measured using a mini Smokerlyzer [13] to verify the accuracy of information on the smoking cessation rate.

2.1. Collection and Storage of Samples

Patients were instructed to refrain from eating, drinking and brushing their teeth for at least 1 h prior to saliva collection.

The gathering was held in the afternoon between 1 p.m. and 3 p.m. hours. Unstimulated saliva was collected from participants by spitting [14] into a sterile collection container. After collection, the samples were immediately stored in a cryogenic Nalgene® tube in the freezer (−80 °C). The previously prepared samples were transported into dry ice container (−20 °C), to the spectroscopy laboratory of the Federal University of ABC to be analyzed in an FTIR Spectrometer 660 - Varian Inc.

For analysis, samples were thawed at room temperature, and 30 μL were placed in a saliva sample portand placed in an oven for drying at 40 °C forming a thin film. This was necessary in order to reduce interference of water in the spectra acquisition.

2.2. Obtaining and Analysis of FTIR Spectra

The spectra were measured under the parameters of acquisition of 800 background scans, 4 min, background time, 200 scans, 4 cm^{-1}resolution, and 1 min total scan time (average). The measurement was performed by diffuse reflectance. It was obtained two to four spectra by sample (by participant), depending on the thickness and concentration of the film. From one of the samples of the former smoker group, it was not possible to obtain spectra. After correction and normalization and since spectra were very similar intra group (verified by PCA analysis, Supplementary Fig. 1) they were averaged by volunteer thus making possible a better understanding of the differences between the groups. Statistics was performed with 19 spectra (10 were from smoker group and 9 from the former smoker group).

Spectra were baseline corrected and vector normalized in Labspec 6 software. The regions of 400–750, 2270–2400 and 3700–4000 cm^{-1} were excluded from the principal component analysis (PCA). It was performed with the Minitab 17 software to originate a scatter plot and the loading plots from which it is inferred the main bands that are responsible for the separation between the samples. A binary logistic regression calculation was made from the Concordant Pairs PC1, PC2 and PC3, and it was obtained the concordant pairs percentage and the Pearson *P*-value. Thus, a receiver operating characteristic (ROC) curve graph was created, which gives the sensitivity and specificity of FTIR spectroscopy. For this calculation, it was used the PC1 and PC2 for reference, the state value used was the former smoker group, the threshold method was interpolation of data points, and the level of confidence was 95%. The Origin Lab 8.5 software was used to calculate the area under the curve (a. u. c.) between the 2000–2115 cm^{-1} of the19 spectra as well as the standard error for calculating the ROC curve as well as making the graphs. *t*-test of the areas was calculated two-tailed with unequal variance in Excel 2003.

3. Results

3.1. Sample Characterization

The smoker group consisted of 6 men and 5 women with a mean age of 54.55 ± 10.85 years (minimum = 33 and maximum = 72). The participants of this group smoked 25.64 ± 7.94 cigarettes/day for 35 ± 13.95 years, and tobacco intake was 43.68 ± 17.87 packs/year. The average CO ratio was 9.72 ± 2.8.

The former smoker group consisted of 2 men and 8 women with a mean age of 55.50 ± 11.1 years (minimum = 31 and maximum = 67). Participants of this group smoked 26 ± 13.10 cigarettes/day for 31.20 ± 13.86 years, but reported cessation was of 12.5 ± 9.31 months. Tobacco intake for this group was 43.20 ± 34 packs/year. The average CO ratio was 2 ± 0.66.

3.2. Analysis of the FTIR Spectra

Fig. 1A and B shows the raw spectra from each group and Fig. 1C shows the average of each group. It was possible to verify that there is no great difference in intensity of the mean spectra between groups analyzed. However, we observed that in the bands between the range of 1028–1160 cm^{-1} (shoulder at 1037 cm^{-1} and peaks at 1075, 1121 and 1170 cm^{-1}) and 2000–2115 cm^{-1} (peak at 2058 cm^{-1}), also in 1735 and 1750 cm^{-1} the intensity of spectra in the smoker group is slightly higher than the other group. However, from 1240 to 1670 cm^{-1} (peaks at 1314, 1341, 1401, 1455, 1542 and 1653 cm^{-1}) the intensity of spectra in the former smoker group is slightly higher. With this information it was possible to build Table 1 with information regarding the vibrational assignments (Table 1). It can be inferred by the intensity of the bands or peaks, the greater amount of polysaccharides, nucleic acids, and thiocyanate in the smoker group saliva than in the former smoker group. In the former smoker group, proteins in general are in greater amount than in the smoker group.

Since the differences in the average spectra are slight, we recurred to PCA and binary logistic regression to identify with more precision the changes between the groups. The principal components analysis (PCA) and loadings allowed us to evaluate which wavenumbers and structural components were more relevant for the discrimination between samples analyzed. It was found that the principal components (PCs) PC1 contains 98.2% of the data, PC2 1% and PC3 0.2%. In this study, PC1 and PC2 had 71% of concordant pairs and 0.25 Pearson *P*-value as verified by binary logistic regression (Fig. 1A). The PC2 loading