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Short Report

Nucleolar Organizer Regions of Oral Epithelial Cells in Crack Cocaine Users

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ABSTRACT

Background: The health risks of crack cocaine smoking on the oral mucosa has not been widely researched and documented. **Objective:** The purpose of this study was to analyze the proliferative activity of oral epithelial cells exposed to crack cocaine smoke using silver nucleolar organizer region (AgNOR) staining. **Methods:** Oral smears were collected from clinically normal-appearing buccal mucosa by liquid-based exfoliative cytology of 60 individuals (30 crack cocaine users and 30 healthy controls matched for age and gender) and analyzed for cytomorphologic and cytomorphometric techniques. **Results:** Crack cocaine users consumed about 13.3 heat-stable rocks per day and the time consumption of the drug was of 5.2 (± 3.3) years. Mean values of AgNOR counting for case and control groups were 5.18 ± 1.83 and 3.38 ± 1.02 ($P<0.05$), respectively. AgNOR area and percentage of AgNOR-occupied nuclear area were increased in comparison with the control ($P<0.05$). There was no statistically significant difference in the mean values of the nuclear area between the groups ($P>0.05$). **Conclusion:** This study revealed that crack cocaine smoke increases the rate of cellular proliferation in cells of normal buccal mucosa. *Iran. Biomed. J. 17 (2): 107-111, 2013*

Keywords: Crack-Cocaine, Mouth mucosa, Cell proliferation

INTRODUCTION

Cocaine is a powerfully addictive drug of abuse. Historically, it has been used by some ancient civilizations and many indigenous tribes as a stimulating agent. The drug was introduced to medicine and dentistry in the late 19th century due to its anesthesiological properties [1-5]. Pharmacologically, cocaine is classified as a central nervous system stimulant. Its effects include euphoria, supreme confidence, loss of appetite, insomnia, alertness and increased energy, followed by anxiety, depression and fatigue. When utilized in high doses, cocaine is able to trigger hallucinations and psychotic behavior [3, 6]. The epidemic of cocaine abuse has been a major public health issue worldwide.

Crack cocaine or 'crack' is a solid, smokable form of cocaine. This drug can be inhaled, smoked or used intravenously [3]. Before reaching the lungs, crack cocaine smoke comes into contact with the oral mucosa. Currently, there are few studies about the effects of crack on the oral tissues [5]. There are some cases of oral, oropharynx and laryngopharynx lesions associated with the use of the different forms of cocaine, such as dental enamel erosion caused by the cocaine hydrochloride applied intraorally, necrotic lesions on the tongue and epiglottis related to smoking free-base cocaine and larynx mucosa burns caused by crack use [7-10].

Some histopathological changes have already been identified in the epithelium of the tracheobronchial mucosa of crack cocaine users, such as basal cell hyperplasia, squamous metaplasia, mitosis figures, variation in nuclear morphology, increased nucleus/cytoplasm ratio, thickening of the basal membrane and submucous inflammation [1]. Lima *et al.* [11] have demonstrated that there are inflammatory changes in the oral mucosa of crack cocaine users. Besides, the oral epithelial cells suffer a decreased in the nuclear area and in the nucleus/cytoplasm ratio,

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associated to an increase in the cytoplasmic area [12]. These findings raise the hypothesis that crack cocaine may be able to increase cellular proliferation of the oral mucosa.

Nucleolar organizer regions (NOR) are chromosomal segments, which contain ribosomal genes. NOR also contains a set of acidic, non-histone proteins that bind to silver ions and are selectively visualized by silver methods in routinely processed cyto-histological samples [13]. The NOR stained by silver and the argyrophilic NOR-associated proteins are called “AgNOR” and “AgNOR proteins”, respectively. The study of AgNOR represents a valuable parameter of cell kinetics, which is significantly associated with the rapidity of cell duplication [14, 15]. Therefore, the aim of this study was to compare the argyrophilic NOR of oral epithelial cells collected from crack cocaine users and non-users.

MATERIALS AND METHODS

The experimental protocol of this study was approved by the Committee of Ethics in Research at the Pontifícia Universidade Católica do Paraná–PUCPR, Curitiba/Brazil (Process number 1422/08).

Subjects. A number of 30 thirty adult crack cocaine users (experimental group) and 30 non-users (control group) participated in this study. Crack is the free-base form of cocaine that is smoked by users through pipes. The crack cocaine users were treated for crack cocaine intoxication at the Instituto de Pesquisa e Tratamento do Alcoolismo (IPTA, Campo Largo/PR, Brazil). Crack cocaine consumption was defined as at least five heat-stable rocks per day. Name, age, occupation and relevant medical history were recorded for each participant. The selected control group subjects were free of oral complaints and matched with the experimental group by sex, race, marital and religious status, month salary income, and educational level.

Cell collection. Exfoliated cells of clinically normal oral mucosa were collected by oral liquid-based exfoliative cytology from both groups. Initially, the mouth was rinsed with water to remove excess of debris and bacteria within the oral cavity. The squamous epithelial cells were collected using a cytobrush and kit Universal Collection Medium™ (Digene, São Paulo, Brazil).

Cytological preparations. The DNA-Citoliq System™ allows thin-layer slide. An aliquot of 200 µL of Universal Collection Medium was filtered through Filtrogene polycarbonate membrane filters™ (Digene, São Paulo, Brazil), 5 µm pore size, 25 mm diameter placed in a prepgene press™ (Digene, São Paulo, Brazil) and attached to glass slides. For staining AgNOR, the smears were fixed in ethanol 95% for 12 hours. Then, the impregnation by silver histochemical technique was carried out in a dark room at 45ºC for 30 min, in accordance with the technique introduced by Trerè *et al.* [14].

AgNOR analysis. The quantitative analysis of the AgNOR was carried out through light microscopy using an Olympus BX50 binocular microscope (Olympus, Japan), adapted with a WH 10×-H/22 ocular and PLAN 100×/0.25. Prior to the analysis, the slide identification were covered to avoid bias. The parameter utilized for the AgNOR counting was defined as well-defined blackened points in the interior of the nucleoli in accordance with the criteria established by Crocker *et al.* [15]. The same observer carried out the process of counting the AgNOR. The number of dark points colored by silver was evaluated in 100 cell nuclei for each smear. Nuclei with the absence of AgNOR were not counted. The nucleoli colored by silver similar to a ring were counted as if they were one. A value of one AgNOR was assigned during analysis when the assemblage of blackened points could not be viewed as AgNOR [14, 15]. For morphometric analysis of AgNOR, 50 randomly selected cells were measured in a stepwise fashion. Cell images were captured for digitalizing by a Sony CCD-IRIS color video camera (Sony Model DXC-107A, Japan) at 400× magnification. The nuclear and AgNOR areas were obtained by drawing around the nuclear areas and AgNOR using a digitizer cursor and by measuring the mode of Image-Pro plus image analysis system™ (Media Cybernetics, Silver Spring MD, USA), version 4.5.029 for Windows 98/NR/2000. Moreover, the percentage of AgNOR-occupied nuclear area was calculated.

Statistical analysis. All data were tabulated and statistical tests were performed with SPSS for Windows 13.0 (SPSS Inc., Chicago, IL, USA). Significant statistical differences between groups were examined using Student’s *t*-test and Mann-Whitney test. Differences were considered statistically significant when $P<0.05$.

RESULTS

All screened patients were males. The mean age for the case and control groups was 28.36 years (21-45). The average amount of crack cocaine consumed was 13.3 (± 12.3) heat-stable rocks per day. The average

Table 1. Mean values of AgNOR area, percentage of AgNOR-occupied nuclear area, number of AgNOR dots per nucleus, and nuclear area in controls and crack cocaine users

Variables	Case group mean ± SD	Control group mean ± SD	*P* value
AgNOR Area (μm²)	24.38 ± 10.23	15.3 ± 4.54	0.000*
AgNOR-occupied nuclear area (%)	10.43 ± 4.65	6.98 ± 3.12	0.007**
Number of AgNOR	5.18 ± 1.83	3.38 ± 1.02	0.000*
Nuclear area (μm²)	246.5 ± 35.25	247.27 ± 37.15	0.941

*Statistical difference ($P<0.05$), *Student's *t*-test and ** Mann-Whitney test

time of consumption was 5.2 (± 3.3) years. In the case group, 16 participants (53.3%) were only crack cocaine users, whereas 8 participants (23.5%) were tobacco users, 3 participants (10%) had the habit of inhaling cocaine and 6 participants (20%) were alcohol consumers and tobacco users. Table 1 shows the main sociodemographic characteristics of the participants of the experimental and control groups.

The AgNOR were located in the nucleus and visualized as small structures with a predominantly rounded morphology. The color varies from light brown to black in various forms and numbers (Fig. 1).

The AgNOR number was significantly higher in the case group when compared to the control group (5.18 ± 1.83 vs. 3.38 ± 1.02) ($P<0.001$). Besides, there was a significant augment in the AgNOR area for crack cocaine users ($P<0.001$). The percentage of AgNOR-occupied nuclear area in case group was increased in comparison with the control group ($P = 0.007$). No statistical difference was observed in the nuclear area for crack cocaine users and non-users. Mean values of nuclear area, AgNOR area, percentage of AgNOR-occupied nuclear area, and number of AgNOR dots per nucleus in crack cocaine users and controls have been presented in Table 2.

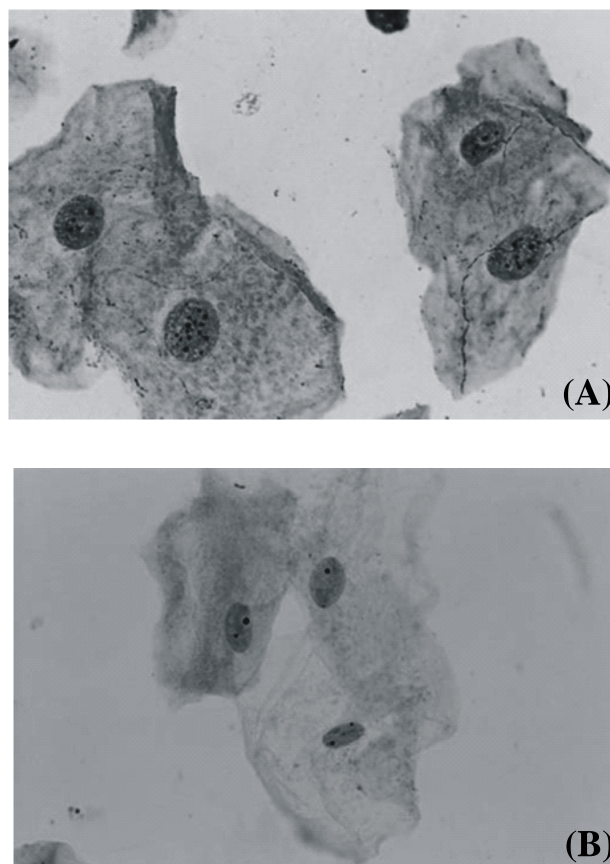

Fig. 1. Nuclei containing **(A)** several blackened points (AgNORs) in the epithelial cells of crack cocaine user and **(B)** reduced number of AgNOR in oral smear of crack cocaine non-user (AgNOR, 1,000×).

DISCUSSION

This study investigates the AgNOR in the nucleus of oral mucosa epithelial cells of crack cocaine users and non-users. The NOR represent DNA segments responsible for the transcription of the RNAr during the first steps of the ribosome synthesis. The analysis of NOR is one of the methods used for the quantitative evaluation of cell proliferation. The AgNOR number per nucleus has demonstrated a correlation with cell proliferation and differentiation degree of oral tissues [16-21].

Despite numerous publications in this field, there is not enough studies on the effects of crack cocaine in the proliferation degree of oral mucosa. The results of this study reveal that the mean AgNOR number and area in the group of crack cocaine users are significantly higher when compared with non-users. Therefore, our results reinforce the findings of the study developed by Woyceichoski *et al.* [12], which utilized cytomorphometric techniques to evaluate the oral epithelial cells of crack cocaine users. They demonstrated that the chronic use of crack cocaine causes decreasing the nuclear area and consequently, altering the nucleus/cytoplasm ratio.

Table 1. Sociodemographic characteristics of users and non-users of crack cocaine

Characteristics	Experimental and control groups*	
	n	%
Sex		
Male	30	100.0
Female	-	-
Age (years)		
21 – 31	24	80.0
32 – 41	5	16.7
42 – 51	1	3.3
52 – 61	-	-
Education		
Illiterate	2	6.7
Literate	28	93.3
Marital status		
Single	2	6.7
Married	24	80.0
Widowed / separated / divorced	4	13.3
Occupational status		
Employed	25	83.3
Unemployed	5	16.6

*The data of experimental and control groups are completely similar

The number, size and arrangement of AgNOR have shown a correlation with the indicators of cellular proliferative indicators. Therefore, quantitative analysis of AgNOR per cell nucleus in squamous cell carcinoma, salivary gland cancer, dysplastic, hyperplastic and normal epithelium have demonstrated a strong correlation with AgNOR, cellular proliferation and malignancy increase [22, 23]. AgNOR in normal cells appeared to be of regular size and round shape, but in malignant tissue they appear less uniform in size and shape and often take on bizarre forms. In this study, the morphology of AgNOR was predominantly rounded and arranged in big and small black dots.

All of these modifications in the oral epithelial cells may be the reflex of an adaptive response of the mucosa to an aggression caused by the chemical injury or by heating during the use of crack cocaine. A similar study was also developed by Kadivar and Attar [24] on cigarette smokers, opium addicts and non-smokers. They have observed that cigarette smoking and particularly opium abuse increase the number of AgNOR and consequently, the rate of cellular proliferation in cells of oral mucosa.

Several studies that utilize the AgNOR technique have demonstrated that the mucosa increases its proliferative activities when irritated by several agents, such as alcohol, tobacco, gasp 'golden glue' and cocaine [20, 24, 25]. All of these substances have been capable of increasing the AgNOR number significantly when compared with oral mucosa cells of individuals who do not make use of them. Therefore, one limitation of this study was the inability to obtain buccal swabs of patients who consumed just the crack. As mentioned in the results, only 16 (53.3%) participants exclusively used crack. In this way, it is not possible to discard the possibility of the influence of alcohol, tobacco cigarettes and cocaine on the findings of this study.

It is believed that the effects of cocaine in the mouth depend on the administration route of the drug [8]. According to Mitchell-Lewis *et al.* [5], the lesions that took place in the oral mucosa are caused by smoking cocaine and probably by heat generated on the mucosa during its use than the chemical action of the drug. Studies performed on oral and tracheobronchial mucosa smears have demonstrated that the crack smoke may be capable of producing significant cellular alterations [1, 11, 12]. In mouth, the chronic use of crack cocaine is also capable of causing inflammatory changes in the oral mucosa [11].

Some molecular and histopathological alterations have been observed in bronchial epithelium of marijuana, cocaine and cigarette smokers. In one study, Barsky *et al.* [1] have demonstrated that there is an abnormal expression of Ki-67 in samples of drug users when compared to non-users. Ki-67 is a cellular proliferation marker, which reflects the percentage of cells involved in cell division. The results of this study raised the hypothesis that using crack, in the same way as tobacco, exerts field cancerization effects on the bronchial epithelium.

The quantitative analysis of AgNOR allows to evaluate the level of cellular proliferation and to monitor the lesions with a propensity to malignancy [26, 18, 21]. In Brazil, the use of crack cocaine has become a serious public health problem that affects patients of both sexes and all ages. Therefore, the clinician must be alert to and notice the patients previously experienced the use of crack cocaine.

Although our study is preliminary, these results suggest a change in the proliferation pattern of the oral epithelium in crack cocaine users. Meanwhile, additional studies should be performed to elucidate the real mechanisms involved in the oral mucosa changes induced by this drug.

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