

**CYTOMORPHOMETRIC STUDY OF NORMAL BUCCAL MUCOSA  
OF ALCOHOLIC INDIVIDUALS**

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**Summary**

Alcohol consumption was a limited risk factor for several oral conditions and, including oral cancer. This study assessed the effect of alcoholism in nuclear (NA), and cytoplasmic area (CA) and nucleus-to-cytoplasm area ratio (NA/CA) of oral squamous epithelial cells of adult male individuals. Oral smears were collected from clinically normal buccal mucosa by liquid-based exfoliative cytology of 60 individuals (30 alcohol users and 30 non-users). Glass slides were processed, stained by Papanicolaou technique and analyzed for quantitative techniques using an image analysis system (Image-Pro Plus). Mean values of NA for experimental and control groups were, respectively,  $67,05 \pm 10,79 \mu\text{m}^2$  and  $57,91 \pm 8,12 \mu\text{m}^2$  ( $P=0.000623$ ). The variable CA showed the following mean values: experimental group =  $2313,99 \pm 231,28 \mu\text{m}^2$  and control group =  $2122,95 \pm 215,71 \mu\text{m}^2$  ( $P=0.001973$ ). The mean value for NA/CA in both experimental and control groups was 0.2 ( $P=0.2182$ ). This study revealed that alcoholism was able to induce significantly changes on the oral epithelial cells. Since this drug is normally used in association with others risk factors for oral cancer (tobacco), alcohol abusers should be submitted frequently to preventive oral exams.

Key words: Oral mucosa, epithelium, cytology, alcoholism, oral disease.

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### **Introduction**

Studies have indicated a strong association between alcohol use and various oral lesions, especially oral cancer (1-2-3). Excessive alcohol ingestion is a problematic issue. Alcoholism is a chronic and progressive psychiatric illness. It is characterized by a loss of control over the use of alcohol, resulting in impaired social functioning, and the consequent development of medical illnesses (4).

Ethanol or alcohol is the most widely used and abused agent throughout the world. In Brazil, studies report prevalence rates for alcoholism between 3.0% and 6.0% among the general population. Alcoholism is the third most frequent reason for work absenteeism and is responsible for high rates of early retirement and work and traffic accidents and for a considerable proportion of the occupation of hospital beds (5-6). Alcohol ingestion may change the health of tissue and organs in human. The toxic effects of alcohol consumption were related, principally, on the nervous system (7) and digestive system (8).

Use of alcoholic beverages is associated with an increased incidence of cancer of the oral cavity, pharynx, esophagus, liver, and possibly the breast. Although alcohol is not a direct-acting carcinogen, one of its metabolites, acetaldehyde, may act as a tumor promoter (9).

Some studies have suggested that a reduced nuclear size and increased cytoplasmic size are useful early indicators of malignant transformation (10-11). Thus, exfoliative cytology is a relevant technique to analyse the external and systemic factors that might change oral mucosa and predispose oral cancer.

The aim of this study was to assess the effect of alcoholism on the nuclear (NA), and cytoplasmic area (CA) and nucleus-to-cytoplasm area ratio (NA/CA) of oral squamous epithelial cells by computerized cytomorphometric analysis.

### **Methods**

The experimental protocol of the present study was approved by Ethics Committee on Human Research at Pontifical Catholic University of Paraná – Curitiba/PR, Brazil (Protocol n°. 1373).

#### *Subjects*

Thirty adult alcohol users (experimental group) and thirty non-users (control group) participated in this study. These alcohol users were been treated for intoxication at the Instituto de Pesquisa e Tratamento do Alcoolismo (IPTA, Campo Largo/PR, Brazil). Alcohol using was defined as >60mL per day. Name, age, occupation, and relevant medical history were recorded.

#### *Cells collection*

Exfoliated cells of the clinically normal buccal mucous membrane were obtained by liquid-based exfoliative cytology. 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 cytobrush and kit UCM (Universal Collection Medium of DNA-Citoliq System<sup>®</sup>, Digene Brazil).

#### *Cytological preparations*

The DNA-Citoliq System<sup>®</sup> allows thin-layer preparations to be provided thanks to a filtration process. An aliquot of 200 $\mu$ L of UCM was filtered through filtrogene<sup>®</sup> - polycarbonate membrane filters, pore size 5 $\mu$ m, diameter 25 mm- (Digene, Brazil), placed in prepgene press<sup>®</sup> (Digene, Brazil) attached to glass slides. Glass slides were immediately fixed in absolute alcohol for 20 minutes. Smears were then stained with routine Papanicolaou stain.

#### *Morphometric analysis*

Each slide was assessed using the light microscopy by binocular Olympus BX50 microscopy<sup>®</sup> (Olympus, Japan). Fifty randomly selected cells were measured in a stepwise fashion. Cell images were captured for digitalizing by *Sony CCD Iris Color Video Camera*<sup>®</sup> (Sony Model DXC-107A, Japan) at x400 magnification. The nuclear (NA) and cytoplasmic (CA) areas were obtained by drawing around areas the nuclear and cell boundaries using the digitizer cursor and measuring mode of *Image-Pro Plus* image analysis system (Media Cybernetics, Silver Spring MD, USA), version 4.5.029 for Windows 98/NR/2000.

#### *Statistical analysis*

All data were tabulated and statistical tests were performed with SPSS for Windows 13.0 (SPSS Inc., Chicago, Illinois, USA). Significant statistical differences between groups were examined using *t*-test for equality of means. Differences were considered statistically significant when  $P < 0.05$ .

## **Results**

All screened patients were male and the mean age for both alcohol users and non-users individuals was 44years-old. The mean time of alcohol consumption was 18 years for the experimental group.

AN, AC and AN/AC values were calculated of 3000 cells. All smears showed normal epithelial cells (Figure 1). The mean values for the AN, AC and AN/AC ratio are illustrated in table 1. The normality test of Kolmogorov-Smirnov and homogeneity of variance Levene's test revealed that data showed a normal distribution and homogeneous variances between groups ( $P > 0.05$ ). Using *t*-test for equality of means, a highly significant increase in both nuclear area ( $P = 0.000623$ ) and cytoplasm area ( $P = 0.001973$ ) resulted from alcohol using. No statistical difference was observed in AN/AC ( $P > 0.05$ ).

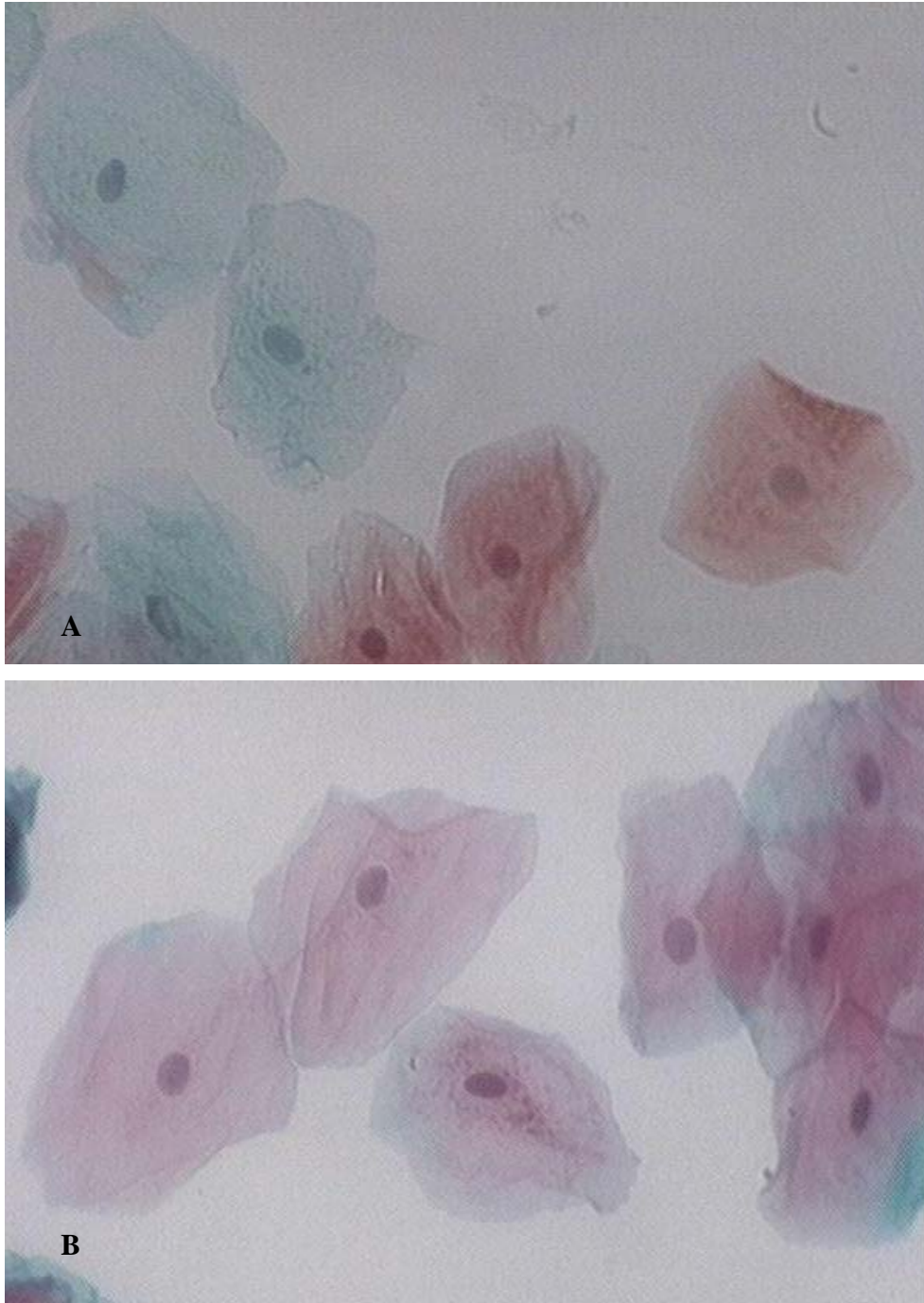


Figure 1 – Cytological smears of the healthy oral mucosa. A – Normal epithelial cells (control group). B – Increased nuclear and cytoplasmic area of epithelial cells (experimental group). Papanicolaou staining (X400).

Table 1. Average of AN, AC and AN/AC to users or non-users of alcohol.

Groups	Non-users	Users	P value
Variable	Mean $\pm$ SD	Mean $\pm$ SD	
NA	57,91 $\pm$ 8,12 $\mu\text{m}^2$	67,05 $\pm$ 10,79 $\mu\text{m}^2$	0.000623*
CA	2122,95 $\pm$ 215,71 $\mu\text{m}^2$	2313,99 $\pm$ 231,28 $\mu\text{m}^2$	0.001973*
NA/CA	0,0275	0,0292	0.2182

\*Statistics difference (P&lt;0,05)

### Discussion

Ethanol, also known as ethyl alcohol or grain alcohol, is a flammable, colorless, slightly toxic chemical compound with a distinctive perfume-like odor, and is the alcohol found in alcoholic beverages. In common usage, it is often referred to simply as alcohol. It consists of a hydroxyl group attached to a two-carbon hydrocarbon chain. It is moderately polar, it forms hydrogen bonds easily, and it is infinitely water-soluble. These physical properties help to clarify the *in vivo* phenomena of absorption, distribution, excretion, and metabolism of alcohol (12).

The main effects of alcohol are observed in central nervous system, where its depressor actions look like to the volatil anesthetics ones (13). Meanwhile, it actually affects almost every organ in the body (12).

The oral mucosa is covered by epithelial cells arranged in different layers and lamina propria. The integrity of this mucosa membrane is fundamental for the maintenance of oral health (14). However, epithelial cells changes according to oral diseases, infections, traumatic agents or metabolic conditions resulting in several clinic alterations and neoplasms (15).

According to Müller et al. (16), there are two types of alcohol-toxic tissue and organ damage in chronic alcoholic ingestion: the direct effect of ethanol by the contact with the mucous membrane and the indirect action by the absorption of the ethanol in the blood and subsequently by all tissues. The damage, in the both cases, is proportional to the degree of ethanol concentration.

In this study, the results revealed a significant elevation in NA and CA for alcohol users. Probably, these findings can be attributed to the cytoplasmatic changes that are produced by chronic alcohol consumption. Contrarily, Ogden et al. (17) observed a statistically significant reduction in mean CA and NA for the oral epithelial cells of alcoholics. These differences in the results possibly can be attributed to the different methodologies employed.

Cytological preparations are of established value in the diagnosis of a variety of disorders – local and systemic, neoplastic, infectious, endocrine, genetic, etc. In this study, all the smears were obtained by liquid-based cytology, a new method of preparing oral and cervical samples for cytological examination (18). This technique results in slides with high a cellularity dispersed in a homogeneous thin layer. Blood, inflammatory cells and mucus are reduced and distributed randomly throughout the slide. The clear background obtained enhances sensitivity and quality of the results (19).

The chronic use of alcohol in rats have demonstrated that size of the basal cell nuclei of the oral mucosa from the floor of the mouth, the edge of the tongue, and the base of the tongue was significantly increased. Thus, chronic alcohol consumption causes oral mucosa atrophy associated with hyperregeneration, which may result in an enhanced susceptibility of the mucosal epithelium to chemical carcinogens (20). Ultrastructural changes were observed in all epithelial layers of the palatine mucosa in rats after alcohol consumption (21). Histologically, the intercellular spaces between basal cells were wider and epithelial cells exhibited small cytoplasmatic lipid droplets, large vacuoles, picnotic nucleus and autolysis cells (22).

In human, several cytological changes were observed, such as pyknosis, karyorrhexis and karyolysis in increased numbers of tongue and buccal mucosa cells. These features do not indicate carcinogenic action and may be understood as induction of the keratinization process, with the purpose of protecting non-keratinized oral mucosa from injury caused by ethanol (23). Besides, an increased incidence of abnormal nucleus/cytoplasm ratio was also noticed in the exfoliated cells of the alcohol users (23).

Hillman and Kissin (24) demonstrated that, in alcoholic patients, NA and CA can be altered according to the nutritional status. Patients with poor diet patterns showed larger nuclei than those with better eating habits. All the patients included in this study were in treatment for alcohol intoxication and, practically, showed similar diet pattern.

The most important risk factors for oral cancer development are smoking and alcohol intake (25-26-27). It was observed in a retrospective study that alcoholic beverage drinking was the major risk factor for oral and pharyngeal cancer in persons that have never smoked. Alcoholic patients that use daily more than six doses of alcoholic beverages (with high levels of alcohol) showed a higher probability to develop oral cancer than no-users (28). Nevertheless, the literature relates that exist a synergism between alcohol and tobacco that increase about 100 times the probability of oral cancer developing (29).

There are several mechanisms by which ethanol may have an influence on the oral mucosa. Among these are the oxidizing activity leading to changes by direct injury to DNA, and increased carcinogen penetration across the oral mucosa, by raising

carcinogen solubility or perhaps by increasing mucosa permeability (30-31). Alcohol activity associated with oral carcinogenesis generally occurs when the daily ethanol intake exceeds 45 ml (32).

There is increasing evidence that acetaldehyde, the first metabolite of ethanol, is responsible for the major part of the carcinogenic potency of ethanol (33-34). Acetaldehyde has been shown to be highly toxic, mutagenic and carcinogenic in different cell cultures and animal models (35-33). This could allow significant acetaldehyde accumulation in oral tissues during chronic ethanol consumption and may explain some of the cytologic anomalies in the oral mucosa of non-smoker alcoholics. Several researchers quote these anomalies, and include the following among them: increase in the nuclear area (36), epithelial atrophy due to a decrease in basal cellular size (37), dysplastic changes with keratosis and increased number of mitotic figures (38).

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#### References

- 1- Saraswathi TR, Ranganathan K, Shanmugam S, Sowmya R, Narasimhan PD, Gunaseelan R. Prevalence of oral lesions in relation to habits: Cross-sectional study in South India. *Ind J Dent Res* 2006; 17(3):121-5.
- 2- Harris CK, Warnakulasuriya KA, Johnson NW, Gelbier S, Peters TJ. Oral health in alcohol misusers. *Community Dent Health* 1996; 13(4):199-203.
- 3- Harris CK, Warnakulasuriya KA, Cooper DJ, Peters TJ, Gelbier S. Prevalence of oral mucosal lesions in alcohol misusers in south London. *J Oral Pathol Med* 2004; 33(5):253-9.
- 4- Friedlander et al. Alcohol abuse and dependence: Psychopathology medical management and dental implications. *J. Am Dental Assoc.* 2003; 134(6)731-740.
- 5- Almeida LM, Coutinho ESF. Prevalência de consumo de bebidas alcoólicas e de alcoolismo em uma região metropolitana do Brasil. *Rev Saúde Pública* 1993; 27:23-9.
- 6- Baus J, Kupek E, Pires M. Prevalência e fatores de risco relacionados ao uso de drogas entre escolares. *Rev Saúde Pública* 2002; 36:40-6.
- 7- Andersen BB. Reduction of purkinje cell volume in cerebellum of alcoholics. *Brain Res.* 2004; 1007, 10-18.
- 8- Blagaic AB, Blagaic V, Romic Z, Sikiric P. The influence of gastric pentadecapeptide BPC 157 on acute and chronic ethanol administration in mice. *Eur. J. Pharmacol.*, 499, 285-290.
- 9- Albert D, Diaz-Rohena R. Historical review of sympathetic ophthalmia and its epidemiology. *Surv Ophthalmol* 1989; 34(1):1-14.

- 10- Cowpe JG, Longmore RB, Green MW. Quantitative exfoliative cytology of abnormal oral smears. *J R Soc Med.* 1988;81(9):509-13.
- 11- Ramaesh T, Mendis BR, Ratnatunga N, Thattil RO. Cytomorphometric analysis of squames obtained from normal oral mucosa and lesions of oral leukoplakia and squamous cell carcinoma. *J Oral Pathol Med.* 1998;27(2):83-6.
- 12- Becker CE. Review of pharmacologic and toxicologic effects of alcohol. *J Am Dent Assoc* 1979; 99(3):494-500.
- 13- Robbins, et al., *Pathologic Basis of Disease*, 6<sup>a</sup> ed., P. 410-412, 1999.
- 14- Wolff A, Ship JA, Tylanda CA, Fox PC, Baum BJ. Oral mucosal appearance is unchanged in healthy, different-aged persons. *Oral Surg Oral Med Oral Pathol* 1991; 71(5):569-72.
- 15- Sognaes RF, Albright JT. Electron microscopic study of the epithelial lining of the human oral mucosa. *Oral Surg Oral Med Oral Pathol* 1958; 11:62-73.
- 16- Müller P, Hepke B, Medau U, Raabe G. Tissue damage in the rabbit oral mucosa by acute and chronic direct toxic action of different alcohol concentrations. *Exp. Pathol.* 1983; 24:171-181.
- 17- Ogden GR, Wight AJ, Rice P. Effect of alcohol on the oral mucosa assessed by quantitative cytomorphometry. *J Oral Pathol Med* 1999; 28(5):216-20.
- 18- Payne N, Chilcott J, McGoogan E. Liquid-based cytology for cervical screening. *Cytopathology* 2000; 11(6):469-70.
- 19- Mehrotra R, Gupta A, Singh M, Ibrahim R. Application of cytology and molecular biology in diagnosing of premalignant or malignant oral lesions. *Mol Cancer* 2006; 23:5-11.
- 20- Born IA, Zoller J, Weidauer H, Maier H. Effects of chronic alcohol drinking on mouth mucosa. A morphometric study. *Laryngorhinootologie* 1996; 75(12):754-8.
- 21- Martinez M, et al., Ultrastructural changes on the hard palatine mucosa of calomys callosus after 120 days of experimental chronic alcoholism. *J. Submicrosc. Cytol. Pathol.* 2005; 37(1), 59-65.
- 22- Martinez M, et al., Morphological changes on the hard palatine mucosa of rats (*Rattus norvegicus albinus*) after chronic alcohol consumption. *J. Submicrosc. Cytol. Pathol.* 1998; 30(3), 379-384.
- 23- Reis SRA, do Espírito Santo AR, Andrade MGS, Sadigursky M. Cytologic alterations in the oral mucosa after chronic exposure to ethanol. *Pesqui Odontol Bras* 2006; 20(2):97-102.
- 24- Hillman RW, Kissin B. Oral cytologic patterns and nutritional status: some relationships in alcoholic subjects. *Oral Surg* 1980; 49(1):34-6.
- 25- Cohen SM, Ellwein LB. Cell proliferation in carcinogenesis. *Science* 1990; 249(4): 1007-11.
- 26- Schottenfeld D. Alcohol as a co-factor in the etiology of cancer. *Cancer* 1979; 43(5):1962-6.
- 27- Fioretti F, Bosetti C, Tavani A, Franceschi S, La Vecchia C. Risk factors for oral and pharyngeal cancer in never smokers. *Oral Oncol* 1999; 35(4):375-8.



- 28- Graham, S, Dayal, H, Rohrer, T, Swanson M, Sultz H, Shedd D, Fischman S. Dentition, diet, tobacco and alcohol in the epidemiology of oral cancer. *J Natl Cancer Inst* 1977; 59(6):1611-8.
- 29- Boyle, P, Macfarlane, GJ, Zheng, T, McGinn R, Maisonneuve P, LaVecchia C, Scully C. Recent advances in the etiology and epidemiology of head and neck cancer. *Curr Opin Oncol* 1990; 2(3):539-45.
- 30- Lieber CS. Herman award lecture, 1993: a personal perspective on alcohol, nutrition, and the liver. *Am J Clin Nutr* 1993;58(3):430-42.
- 31- Wight AJ, Ogden GR. Possible mechanisms by which alcohol may influence the development of oral cancer: a review. *Oral Oncol* 1998;34(6):442-7.
- 32- Ziegler RG. Alcohol-nutrient interactions in cancer etiology. *Cancer* 1986;58(8 Suppl):1942-8.
- 33- Homann N, Jousimies-Somer H, Jokelainen K, Heine R, Salaspuro M. High acetaldehyde levels in saliva after ethanol consumption: methodological aspects and pathogenetic implications. *Carcinogenesis* 1997;18(9):1739-43.
- 34- Obe G, Ristow H. Acetaldehyde, but not ethanol, induces sister chromatid exchanges in Chinese hamster cells *in vitro*. *Mutat Res* 1977;56(2):211-3.
- 35- Dellarco VL. A mutagenicity assessment of acetaldehyde. *Mutat Res* 1988;195(1):1-20.
- 36- Maier H, Weidauer H, Zoller J, Seitz HK, Flentje M, Mall G, *et al.* Effect of chronic alcohol consumption on the morphology of the oral mucosa. *Alcohol Clin Exp Res* 1994;18(2):387-91.
- 37- Mascres C, Ming-Wen F, Joly JG. Morphologic changes of the esophageal mucosa in the rat after chronic alcohol ingestion. *Exp Pathol* 1984;25(3):147-53.
- 38- Muller P, Hepke B, Meldau U, Raabe G. Tissue damage in the rabbit oral mucosa by acute and chronic direct action of different alcohol concentrations. *Exp Pathol* 1983;24(2/3):171-81.