



## Research Article

# Pyknotic Cell Features of Buccal Mucosal Smear in Various Oral Lesions of Betel Nut Chewers

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## Abstract

The habit of chewing betel nut is one of the predisposing factors that can trigger the formation of various oral lesions and develop into a malignant condition in the form of oral squamous cell carcinoma (OSCC). This malignancy has a low five-year survival rate as early detection is not widely carried out. Early detection can be conducted by examining pyknotic cells to describe the level of apoptosis. This study aims to examine the pyknotic cell features as a marker of apoptosis in various oral lesions found in betel nut chewers. This study is an analytic survey conducted once with a post-test control group design. The study was conducted on 17 betel nut chewers and 17 healthy people as controls. A buccal mucous smear was collected, and cytological slides were made with Papanicolaou stain. Pyknotic cells were counted using a light microscope and Optilab pro camera. This study found several oral lesions in betel nut chewers, namely exogenous pigmentation, lesions resembling leukoplakia, and lichenoid reaction. Furthermore, the result also showed that the frequency of pyknotic cells in betel nut chewers was lower than in the control group. Non-parametric Kruskal Wallis and post hoc Mann Whitney test showed significant differences in the number of pyknotic cells in various oral lesions with a p-value = 0,001. It can be concluded that the consumption of betel nut affected the level of apoptosis, characterized by a decrease in pyknotic cells and significantly different pyknotic cells depending on the type of oral lesions.

**Keywords:** apoptosis; betel nut; OSCC; pyknotic

## INTRODUCTION

Chewing betel nut is one of the top 4 habits people often carry out after consuming nicotine, alcohol, and caffeine, with an estimated 600 million people having this habit. The habit of chewing betel nut is widely practiced by people in several countries in South Asia and Southeast Asia, including Indonesia.<sup>1</sup>

The habit of chewing betel nut is mostly done by people in rural areas as a form of welcoming guests and appreciation for various activities such as festivals, weddings, and other traditional activities.

Betel nut consumption is considered to have a positive impact, such as a symbol of acceptance in society, strengthening teeth, curing various diseases, and increasing male sexual arousal.<sup>1,2</sup>

Betel nut, with or without tobacco, has been classified by the International Agency for Research on Cancer (IARC) as a group I carcinogen.<sup>3</sup> Long-term consumption of betel nut can increase the risk of developing several oral lesions, both benign lesions, premalignant lesions, and malignancy.<sup>4</sup> In several countries in South and Southeast Asia, 40% of cases of oral

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malignancy are associated with the habit of chewing betel nut with various compositions.<sup>5</sup>

The most common oral malignancy in betel nut chewers is oral squamous cell carcinoma (OSCC). This malignancy can be preceded by specific lesions such as leukoplakia, erythroplakia, or sub mucous fibrosis.<sup>4</sup> Oral Squamous Cell Carcinoma has a low five-year survival rate as early detection of malignancy is not widely performed.

Various methods of early detection of malignancy have been developed, such as cytological biomonitoring examination through buccal mucosal smears to check the various changes in the shape of the nucleus. One of the cytological examinations that can be done is the examination frequency of pyknotic cells. Pyknotic cells have a reduction in cellular volume.<sup>6</sup>

The pyknotic cell is one of the morphological characteristics of cells undergoing apoptosis. Evaluation of the level of apoptosis in cancer conditions is currently being developed as the level of apoptosis can evaluate cancer progression and resistance to cancer treatments.<sup>6</sup> This study aims to evaluate features of pyknotic cells as markers of apoptosis in various oral lesions found in betel nut chewers.

## MATERIALS AND METHODS

This research has received ethical approval for Health research from the Faculty of Medicine, Jenderal Soedirman University. This research is quantitative research with an analytical survey method conducted at once. The researcher did not give interventions to subjects and only took samples in the form of buccal mucosal smears.

First of all, the research subjects were given an explanation regarding the procedures, objectives, and benefits of the research and the signing of the informed consent was used as evidence of the willingness of the research subjects. Intraoral examination was then carried out to determine the health condition of the oral

cavity and to detect oral lesions that resembled malignancy as an effect of consuming betel nut.

Before collecting the buccal mucosal smears, the subjects were asked not to eat for at least 1 hour and the subjects were asked to rinse their mouth with clean water to remove debris in the oral cavity. A sampling of the buccal mucosal smear was carried out using a cytobrush with circular motion.

The cytobrush was then rotated on the end of the object glass, which was given drops of saline solution. The cytobrush was smeared all over the object glass with minimal pressure. The object glass was fixed by dripping 96% absolute alcohol solution, and then the object glass was sent to the laboratory to be stained for Papanicolaou stain. The stained cytology preparations were then observed under a light microscope and Optilab Pro Camera and pyknotic cells were counted for every 500 cells.

Observations were made with a magnification of 400 times and counted manually by multi observers. The calculated pyknotic cells were cells with intact cytoplasm, no overlapping with other cells, and minimal debris.<sup>7</sup> The research data were then processed and analyzed with the Statistical Package for Social Sciences (SPSS) software version 22. The normality test was analyzed using the Shapiro Wilk homogeneity Levene Test. The data were normally distributed but not homogeneous, although the data transformation had been carried out. Thus, the test was continued with the Kruskal Wallis and Mann Whitney post hoc.<sup>8</sup>

## RESULT

The subjects of this study consisted of two groups, namely the group with betel nut habits and the control group. Each group consisted of 17 females with an average age of 72 years. The subjects came from Banjarsari Wetan Village, Sumbang District, Banyumas Regency, Central Java, Indonesia.

On intraoral examination, various lesions on the oral mucosa were found in the betel nut chewers group. Oral lesions consisted of exogenous pigmentation, hairy tongue, leukoplakia-like lesions, and lesions resembling lichenoid reactions. No biopsy was performed to establish a definitive diagnosis of the oral lesions found, so the lesions were categorized according to clinical conditions. The lesions were scattered on the tongue's labial mucosa, buccal mucosa, palate, and dorsum. However, since the sample was taken from a buccal mucosal smear, the focus of the lesions studied were those on the buccal mucosa, which could be seen in Figure 1, Figure 2, and Figure 3.



**Figure 1.** Exogenous pigmentation lesions



**Figure 2.** Leukoplakia-like lesions

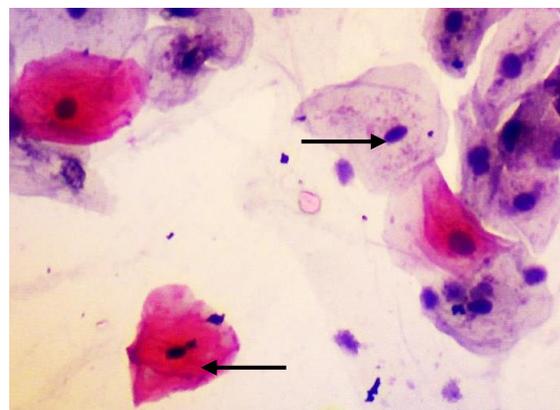


**Figure 3.** Lesions resembling lichenoid reactions

The examination of the buccal mucosal smear was then performed on the various oral lesions, and then the cytology preparation was stained by Papanicolaou (PAP) stain. Pyknotic cells were observed from the cytology slide and counted manually. The calculated pyknotic cells included cells with condensation of chromatin and nucleus. Pyknotic cells also decrease the size of the nucleus, as seen in Figures 4 and 5.



**Figure 4.** Pyknotic cells (black arrow) in the betel nut chewers group



**Figure 5.** Pyknotic cells (black arrow) in the control group

**Table 1.** Calculation of pyknotic cells

No.	Group	Number of the sample (n)	Mean $\pm$ SD
1.	Betel nut chewers group	17	31,86 $\pm$ 4,62
2.	Control group	17	51,13 $\pm$ 5,11

Pyknotic cells were found in this study in both the betel nut chewers and control groups. The mean of pyknotic cells was higher in the control group than in the betel nut group, as seen in Table 1.

A normality test using Shapiro Wilk was carried out on the number of pyknotic

cells in various types of lesions and obtained a p-value  $> 0.05$  to conclude that the data were normally distributed. Furthermore, the Homogeneity Levene test was carried out, and the p-value  $< 0.05$  was obtained. The data were transformed but still showed that it was not homogeneous, so the analysis test continued with the Kruskal Wallis test. The Kruskal Wallis test showed a p-value = 0.001 ( $p < 0.05$ ), indicating at least a difference in the number of pyknotic cells in the two types of lesions. The test was then continued with the Mann-Whitney Post Hoc Test, and the results were presented in Table 2 below.

**Table 2.** The result of the Mann-Whitney Post hoc Test of Pyknotic cells in various lesions

No	Oral Lesions	p-Value			
		Exogenous Pigmentation	Leukoplakia like lesions	Lesions resembling lichenoid reaction	No lesions
1.	Exogenous Pigmentation		0,45	1	1,46
2.	Leukoplakia like lesions	0,45		1	0,00*
3.	Lesions resembling lichenoid reaction	1	1		0,04*
4.	No lesions	1,46	0,00*	0,04*	

\*mean differs significantly

Based on Table 2, it can be seen that the number of pyknotic cells differs significantly in various oral lesions. The condition without lesions was significantly different compared to leukoplakia-like lesions and lesions resembling lichenoid reactions. It was evidenced by the value of  $p < 0.05$ . The number of pyknotic cells was not significantly different in the normal or no lesion condition compared to exogenous pigmented lesions.

## DISCUSSION

Betel nut is categorized as a carcinogen and can increase the risk of the oral cavity and pharyngeal cancer if consumed for a long time. Betel nut contains various compounds such as arecoline and arecaidine, which are capable of causing mutations in mammalian cells. Arecoline is also considered to be able to cause dysregulation in the cell cycle.<sup>3</sup>

Furthermore, the betel nut also contains quercetin, an antioxidant activity that can ward off free radicals and protect cells from necrosis and apoptosis.<sup>9</sup>

The carcinogenesis process triggered by betel nut exposure is complex, and no definite pathogenesis has yet been found. Several mechanisms are considered involved in the carcinogenesis of betel nut, namely epigenetic DNA modification, modulation of the formation of Reactive Oxygen Species (ROS) or free radicals, modification of tumor suppressor genes, and triggering of autophagy and hypoxia.<sup>10</sup> Betel nut consumption is considered capable of causing chronic irritation and inflammation that can damage epithelial cells in the oral mucosa so that the carcinogenic material from betel nut can diffuse into the oral mucosal tissue. Poor oral hygiene in betel nut chewers is considered a factor that can cause changes in the balance of the oral microflora that can

trigger inflammation and increase the risk of cancer development.<sup>11</sup>

Furthermore, the consumption of betel nuts can trigger the formation of various oral lesions. The most common benign oral lesions resulting from consuming betel nut is hyperkeratosis, while the most common premalignant lesions are leukoplakia, erythroplakia, and oral submucosal fibrosis (OSF).<sup>4</sup> These premalignant lesions can develop into malignancy in the form of OSCC. In this study, various oral lesions were found in the group with betel nut habits, including exogenous pigmented lesions, hairy tongue, lesions resembling leukoplakia and lesions resembling lichenoid reaction. A definitive diagnosis of these lesions can not be determined as the subjects did not perform biopsies, so the lesions were categorized based on clinical conditions and symptoms.

The progression of premalignant lesions to malignancy needs to be evaluated from the degree of dysplasia seen on biopsy examination. However, many recent studies have been carried out for the early detection of less invasive malignancies compared to biopsy examination, one of which is by examining buccal mucosal smears. The buccal mucosal epithelium is the main target of various genotoxic carcinogens that enter through the ingestion pathway, such as betel nut. The buccal mucosal epithelium is the primary barrier and is capable of metabolizing carcinogens into various reactive compounds.<sup>12</sup>

Examination of buccal mucosal smears is considered an early detection of genotoxic and carcinogenic exposure, such as examining various changes in the shape of the nucleus. Examination of the micronucleus frequency, cytoplasmic and nuclear ratio abnormalities, pyknotic, karyorrhexis, and karyolysis are frequently performed. Pyknotic cells are changes in cell morphology in which the nucleus undergoes chromatin condensation and volume reduction. Pyknotic is a marker of cell death. Programmed cell death or apoptosis plays an important role in tumor

pathogenesis. Reducing the rate of apoptosis can increase the formation and progression of tumors. It will cause tumor cells to have the opportunity to survive longer, protected from oxidative stress and hypoxia when the tumor mass expands.<sup>6</sup>

In this study, it was found that the mean of pyknotic cells was found to be lower in the betel nut chewers group compared to the control group. Besides, it was also known that the number of pyknotic cells differed in various oral lesions. Pyknotic cells differed significantly, especially in normal conditions or without lesions, compared to leukoplakia-like lesions and lesions resembling lichenoid reactions.

The number of pyknotic cells in leukoplakia-like lesions showed a significant difference, so it can be concluded that both leukoplakia-like lesions and lesions resembling lichenoid reactions have a risk of progression to premalignant or malignant lesions. Leukoplakia lesions have a progression to a malignant condition with a percentage of 17%.<sup>13</sup> In this study, the number of pyknotic cells was significantly different in lesions resembling lichenoid reactions. Lesions resembling Lichenoid reactions may accompany the initial condition of OSF, but the ability to progress to malignancy needs to be further proven through tissue biopsy to determine a definitive diagnosis and evaluate the degree of dysplasia of the case.<sup>14</sup> Meanwhile, the number of pyknotic cells in exogenous pigmentation oral lesions was not significantly different from normal conditions or the condition without lesions. It indicated that exogenous pigmented lesions were not suspected of being capable of developing into a malignant condition.<sup>15</sup>

## CONCLUSION

Based on the result of this study, it can be concluded that betel nut was one of the predisposing factors for the emergence of various oral lesions that could potentially develop into oral malignancy conditions.

Early detection of oral malignancy progression could be carried out by examining the morphological changes of buccal mucosal smear cells, one of which was pyknotic cells that described the rate of apoptosis. This study also showed a decrease in the number of pyknotic cells compared to the normal group, and the number of pyknotic cells in various types of oral lesions was significantly different. It indicated that apoptosis also decreased in lesions that could potentially become malignant. However, further investigation is needed to assess the degree of cell dysplasia and make a definitive diagnosis of the various lesions found.

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