International Journal of Health Sciences and Research
www.ijhsr.org ISSN: 2249-9571

Original Research Article

Quantitative Analysis of Apoptotic Cells in Normal Mucosa, Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma Using Methyl Green-Pyronin Stain

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ABSTRACT

Background: Apoptosis is a form of physiologic cell death which involves coordinated events and responsible for mediating cell death in a variety of physiologic and pathologic process. Apoptotic cells can be identified by routine haematoxylin and eosin staining, but can be identified with more precision by methyl green-pyronin staining. The added advantage of this stain is that it is simple and cost effective method as compared to other advanced diagnostic methods available for identifying apoptotic cells.
Aim: To identify the expression of apoptotic cells in normal epithelium, oral epithelial dysplasia and in oral squamous cell carcinoma using methyl green-pyronin stain.
Materials and methods: The study included a total of thirty samples. Ten cases each of oral epithelial dysplasia and oral squamous cell carcinoma made up the study group whereas, ten cases of normal mucosa constituted the control group. Apoptotic cells were identified and apoptotic index was calculated using methyl green-pyronin stain. Results were statistically analysed and compared between the groups.
Results: The mean apoptotic index was found to be increased from normal mucosa to oral squamous cell carcinoma. Oral squamous cell carcinoma showed higher mean apoptotic index (3.89±0.63) followed by oral epithelial dysplasia (2.06±0.13) and normal mucosa (1.50±0.28).
Conclusion: Methyl green-pyronin staining method can be used as a routine stain in laboratory in identifying the apoptotic cells.

Key words: Apoptosis, Oral epithelial dysplasia, Oral squamous cell carcinoma, Methyl green-pyronin stain

INTRODUCTION

The majority of the oral cancers are epithelial in origin, known as oral squamous cell carcinoma. Early diagnosis of oral cancer improves the prognosis as well as reduces the mortality and morbidity rate. [1] Apoptosis is a well-defined, planned active physiologic process required for the regulation of tissue homeostasis. [2] Apoptosis is involved in removing or eliminating the tumour cells. Hence, regulation of apoptosis plays a key role in carcinogenesis, tumour progression and metastasis. Accumulation of genetic or epigenetic alterations, eventually lead to deregulation of apoptosis resulting in uncontrolled cell proliferation leading to cancer, autoimmune diseases and

neurodegenerative diseases. Apoptotic cell identification is one such early diagnostic marker to identify cancer. [1]

Histochemical identification and quantitative assessment of expression of apoptotic cells have gained importance as they can help in identifying high risk individuals and also aids in the prognosis. The most commonly used techniques to identify the apoptotic cells include terminal deoxynucleotidyl transferase-mediated d-UTP biotin nick end labelling (TUNEL), cytofluorometric assay and DNA diffusion method. But these methods also have limitations being expensive, time-consuming, technique sensitive and production of artefacts. The use of histochemical stains like methyl green-pyronin (MGP) can provide a simple effective method in identifying the apoptotic cells. [3] Therefore, we intended to use methyl green-pyronin stain to identify the apoptotic cells and to assess their expression in normal mucosa, oral epithelial dysplasia and Oral squamous cell carcinoma.

MATERIALS AND METHODS

The present study was carried out in the department of oral pathology and microbiology. Archival tissues from paraffin embedded blocks from the department and also recent cases in the dental OPD were considered. The study group consisted ten tissue blocks each of histopathologically confirmed cases of oral epithelial dysplasia (OED) and oral squamous cell carcinomas (OSCC). Ten cases of normal mucosa constituted the control group. From each tissue block two sections each of 3-4 micron thickness were obtained. One set of sections were stained with haematoxylin & eosin stain to histopathologically confirm the diagnosis and the other set of sections were stained with methyl green-pyronin staining technique for apoptotic cells.

Staining technique

The improved method of methyl green-pyronin technique for apoptotic cells was done according to the standard protocol recommended by Potvin C (1979). [4] The sections were dewaxed in xylene for about 3-5 minutes and then rehydrated through decreasing grades of ethanol and hydrated to distilled water for 8-10 minutes. The slides were then stained with methyl green-pyronin solution for 10 minutes followed by dehydration using acetone, acetone: xylene (1:1) and finally cleared in xylene for 5 minutes and mounted in DPX (Fig 1). MGP stains DNA (nuclei) bluish green and RNA intensely red.

Fig 1: Photograph showing the armamentarium for methyl green-pyronin staining

Counting of apoptotic cells

The apoptotic cells were identified and counted based on features described by Kerr et al (1972). [5] The methyl green-pyronin stained sections were examined in by research light microscope under x400 magnification and the number of apoptotic cells in 1000 normal/neoplastic cells from three random fields were counted and the apoptotic index (AI) was calculated by using formula,

$$AI = \frac{\text{Number of apoptotic cells}}{\text{Total number of cells counted}} \times 100$$

Photomicrographs of histopathological sections showing apoptotic cells were obtained using a research microscope.

Evaluation of apoptotic cells

Apoptotic Index was expressed in terms of mean ± Sd. Data obtained was analysed using, statistical package for social sciences (SPSS) version 16 for windows operating system. One way ANOVA (Post hoc) followed by Dunnet-t was applied for

statistical significance between the groups at 95% confidence intervals and $p<0.05$ was considered to be statistically significant.

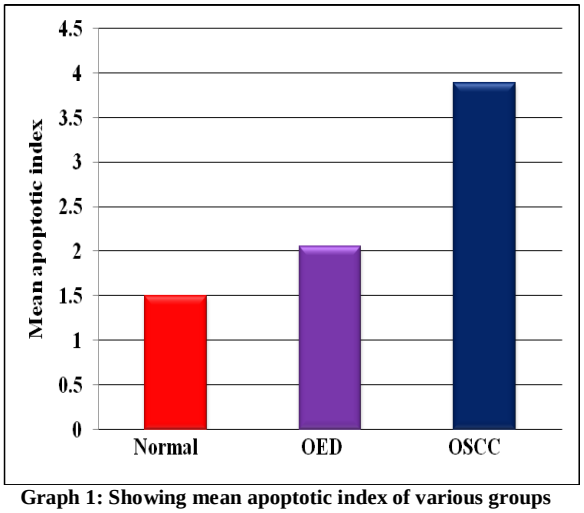
RESULTS

In the present study the OED samples were in the age range of 20–50 years with a mean age of 35 years and there was a male predilection in the ratio of 2:1. Similarly the samples with OSCC were in the age range of 45–75 years with the mean age of 60 years and there was male predominance with a male to female ratio of 3:1. Documentation of the associated habits and common site of the lesion showed tobacco and betel nut chewing as the most prevalent cause and buccal mucosa as the most favourable site for both OED and OSCC.

Table 1: Multiple comparison of mean apoptotic index between groups

Groups	Apoptotic index (%) (MEAN±SD)	p value
Normal mucosa	1.50±0.28	0.001
OED	2.06±0.13	
OSCC	3.89±0.63	

The apoptotic cells were counted by two observers' in-order to eliminate the intra-observer bias. Apoptotic cells were found to be increasing from normal mucosa to OED to OSCC (Table 1). The increase of mean of apoptotic index was statistically significant between the groups. Apoptotic cell count was found to be higher in oral squamous cell carcinoma than in oral epithelial dysplasia (Graph 1).



Graph 1: Showing mean apoptotic index of various groups

DISCUSSION

Apoptosis plays an important role in the process of morphogenesis and homeostasis. Flow cytometry, electrophoresis, in situ end labeling of fragmented DNA and the TUNEL technique are excellent in identifying the apoptotic cells. But these methods have limitations like expensive, technique sensitivity. [3] Jain et al (2009) demonstrated the use of H&E stain and found that the apoptotic index increased as the lesion progressed from premalignant lesions to oral squamous cell carcinoma. [6]

Mohtasham et al (2010) measured nuclear parameters (nuclear area, numbers & diameter) in normal mucosa, dysplastic mucosa and in varying grades of OSCC using MGP stain. [7] Viswanathan et al (2015) conducted a study to evaluate the apoptotic indices and proliferative indices in premalignant and malignant squamous cell lesions of the oral cavity. They showed that apoptotic index was gradually increased from normal to dysplasia to carcinoma with the highest being well differentiated squamous cell carcinoma. This observation gives us an indication that as the apoptosis became generalized, the severity also increased and apoptotic index could be a better marker in assessing the disease progression. [8]

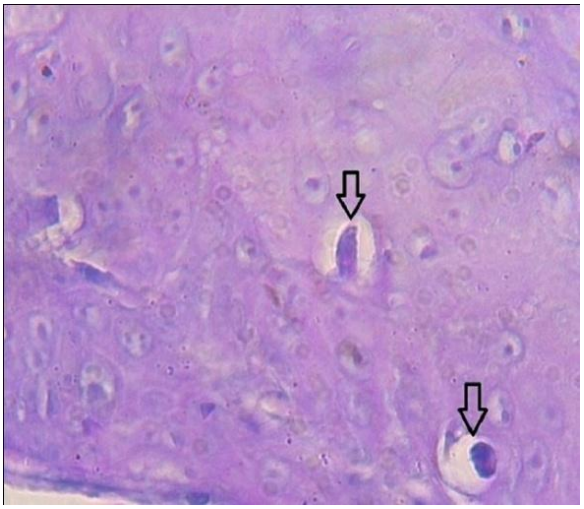


Fig 2: Photomicrograph showing apoptotic cells in epithelial dysplasia (MGP staining, x400)

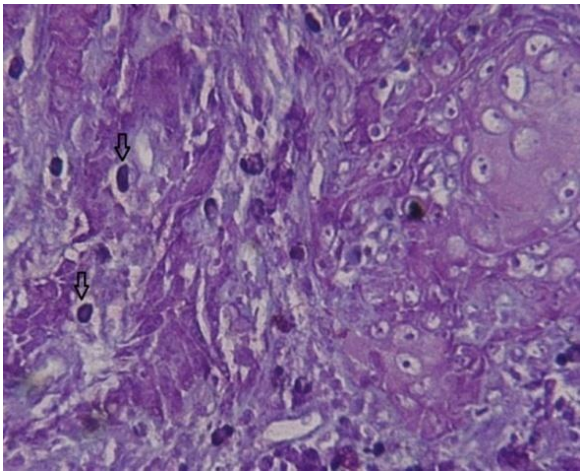


Fig 3: Photomicrograph showing apoptotic cells in squamous cell carcinoma (MGP staining, x400)

Nambiar et al (2016) measured apoptotic index in premalignant and malignant oral lesions and concluded that apoptosis was found to be increasing from dysplasia to oral squamous cell carcinoma. In this study, they concluded that due to the large tumor size and aggressive biological behavior, the tumor undergoes hypoxia leading to increased apoptosis. [9] Nayak A et al (2016) identified the apoptotic cells in gingival epithelium using methyl green-pyronin and haematoxylin & eosin stain. They concluded that apoptotic cells are easily identified by using MGP stain when compared to H&E stain. [10]

The present study findings also suggest that MGP staining is an easier method of identifying the apoptotic cells in routine laboratory procedures as this stain stains the DNA bluish green and RNA reddish pink (Fig 2, 3). The study also revealed statistically significant difference between the groups. These observed findings suggest that apoptosis is increased from OED to OSCC indicating that apoptosis plays an important role in eliminating the excess tumor cells that have undergone chromosomal aberrations or genetic mutation. Analysis of the mean numbers of apoptotic cells demonstrated a progressive increase as the aggressiveness of the lesion increases. This indicates that apoptosis process is actively involved in eliminating the potentially malignant cells and the mutated cells.

CONCLUSION

Apoptotic index is an important diagnostic and prognostic tool in premalignant lesions and malignant oral lesions. The apoptotic cells can be easily detected by using MGP stain because of its peculiar feature of selective staining of nucleic acids. Therefore, MGP stain can be used in laboratory as it is simple, cost effective method. Recently, researches are being carried out to modify the apoptotic programme as potential target in the treatment of the diseases.

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How to cite this article: Simila. CSA, Joseph. TI, Prasanth. T et.al. Quantitative analysis of apoptotic cells in normal mucosa, oral epithelial dysplasia and oral squamous cell carcinoma using methyl green-pyronin stain. Int J Health Sci Res. 2018; 8(9):52-56.

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