Assessment of Cytological Atypia and Agyrophilic Nucleolar Organizer Regions in Epithelial Cells of Oral Mucosa in Penitents Exposed to Snuff, Dental Teaching Hospital, Wad Medani, Gezira State, Sudan (2015-2017)

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(B.Sc in Histopathology and Cytology 2012)

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Submitted to the University of Gezira in Fulfillment for the Requirement for the Award of the Degree of Master of Science in Histopathology and Cytology

Department of histopathology and cytology
Faculty of medical laboratory sciences
University of Gezira

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Supervision Committee:

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<thead>
<tr>
<th>Name</th>
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<tbody>
<tr>
<td>Dr. Rania Mahgoub Sied Ahmed</td>
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</table>

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<table>
<thead>
<tr>
<th>Name</th>
<th>Position</th>
<th>Signature</th>
</tr>
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<tbody>
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<td>Dr. Rania Mahgoub Sied Ahmed</td>
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Authorization

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"submitted by me, under the supervision of Dr. Rania Mahgoub Siad Ahmed and prof. Mugahid Abdulruhman for the partial fulfillment for the award of Master degree in Medical Laboratory Sciences in Histopathology and Cytology. University of Gezira Faculty of Medical Laboratory Sciences Department of Histopathology and Cytology; Wad-Medani, Sudan.

Name and Signature of Candidate:

Hana Ibrahim Abdelhafiz ........................
Dedication

To my parents.
For encouraging me to believe that anything is possible

To my husband.
For making everything possible.

To my brother & my sisters.
I am really grateful to each one of you, you have been my inspiration and my soul mates.

To my beloved Asia & Aya.
Acknowledgement

Foremost, Praise be to Allah for his graces. I would like to express my sincere gratitude to my advisors Dr/ Rania Mahgoub SidAhmedand and prof/ Mugahid Abdulruhman for their continuous support, patience, motivation, enthusiasm, and immense knowledge. Their guidance helped me in all the time of research and writing of this dissertation. I could not have imagined having a better advisors and mentor for my research study.

Besides my advisors, I would like to thank all the members of the Department of Histopathology and Cytology, Faculty of Medical Laboratory Sciences, Gezira University for their technical help and assistance, encouragement, insightful comments, and hard questions.
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Abstract

Toombak plays a significant role in the etiology of squamous cell carcinoma. Specifically, nitrosamines present in Toombak possibly act as carcinogen. This case control study was conducted in Wad Medani Dental Teaching Hospital from 2015 to 2017, aimed to assess cellular proliferation among apparently healthy Toomak users using AgNOR counts and cytological atypia using H&E and Pap stains. Smears were collected from 100 of study subjects and 50 non-tobacco users as control. AgNOR were counted microscopically. The laboratory analysis showed that cytological atypia found in 6 cases (6%), 45 smears show inflammatory condition. The results of AgNOR count like the cytological diagnosis of H&E and Pap stain high count found in 6 cases (6%). Atypical cell in period (11-15yrs) frequent (6-10 times per day), inflammation high incidence in period (11-15yrs) frequent (6-10 times per day), 4 cases of atypia (67%) at age group (41-50) years. There is correlation between age, duration, frequency and cytological atypia with toombak dippers (p value 0.01). There is correlation between age, duration, frequency and high AgNOR count with toombak dippers (p value 0.01). The prolong exposure to toombak is a significant factor in increasing cellular atypia and the AgNOR counts. The study recommend the AgNOR count should be use with the routine cytology.
تقييم الاندماجية في الخلايا والنقاط النظامية النووية المحبة للفضة لظهور الغشاء المخاطي للفم المتعرض للتهاب، مستشفى الأسنان التعليمي، ومدني، ولاية الجزيرة – السودان في الفترة من (2015).

هناء إبراهيم عبد الحفيظ محمد خير

ملخص الدراسة

يعتبر التهاب الفم من أكثر المخاطر السرطان الفم لاحتراءه على أمينات النيتروز كمادة سامة. تم تطوير هذه الدراسة إلى تقييم الانتشار التكاثري الخلوي للغشاء المخاطي الفموي لمستخدمي التهاب الساخن باستخدام حساب النقاط النظامية النووية والخلايا الحمراء متماثلة باستخدام صبغة الهيماتوكزلين والألوس وصبغة البابانكولا. تم جمع العينات من 100 شرائح (عينة دراسة) و50 شرائح غير مستخدمي التتبغ (عينة ضابطة) مع حساب النقاط النظامية النووية باستخدام المجهر. التحاليل العقلية عرضت حالات الاندماجية الخلوية في الحالات 6 حالات تمثل 6% في فترة (11-15 سنة) وعدد رواج المستعمل في اليوم (11-15 رواج).

حالة احترام التهابات. نتائج الخلايا المحبة للفضة مماثلة لصبغة الهيماتوكزلين والألوس والبابانكولا. ظهرت في 3 حالات تمثل 3% من الاندماجية في الخلايا ظهرت في فترة 11-15 سنة وعدد رواج 10-6 مرات في اليوم، وعلى نسبة التهابات في فترة 11-15 سنة بمعدل 10-6 مرات في اليوم. 4 حالات للاندماجية خلوية (77%) في الفئة العمرية 41-50 سنة. هناك علاقة بين العمر - فترة الاستعمال - عدد مرات الاستعمال في اليوم والتسمم النمطي الخلوية مع مستخدمي التهاب وكمان هناك علاقة بين العمر – فترة الاستعمال - عدد مرات الاستعمال في اليوم وتسمم الخلايا المحبة للفضة مع مستخدمي التهاب.

الاختلاف إحصائياً بداء (0.001). التعرض للتهمباك لمدة طويلة عامل في زيادة الاندماجية الخلوية. هذه الدراسة توصى بحجة استخدام عدد الخلايا المحبة للفضة مع صبغات الروتين.
# Table of Contents

<table>
<thead>
<tr>
<th>No</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Authorization</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Dedication</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Acknowledgment</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Abstract in English</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>Abstract in Arabic</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>Table of Contents</td>
<td>VI</td>
</tr>
<tr>
<td></td>
<td>List of Tables</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>List of Figures</td>
<td>XI</td>
</tr>
<tr>
<td></td>
<td>List of Abbreviations</td>
<td>XI</td>
</tr>
<tr>
<td></td>
<td>Chapter One</td>
<td></td>
</tr>
<tr>
<td>1.1</td>
<td>Background</td>
<td>1</td>
</tr>
<tr>
<td>1.2.1</td>
<td>Justifications</td>
<td></td>
</tr>
<tr>
<td>1.2.2</td>
<td>Objectives</td>
<td>2</td>
</tr>
<tr>
<td>1.2.2.1</td>
<td>General Objectives</td>
<td></td>
</tr>
<tr>
<td>1.2.2.2</td>
<td>Specific Objectives</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Chapter Two Literature Review</td>
<td></td>
</tr>
</tbody>
</table>

VI
| 2.1 | Oral Mucosa | 4 |
| 2.2 | Pathology of Oral Mucosa | 6 |
| 2.3 | Epidemiology of Oral Mucosal changes caused by Tobacco use | 11 |
| 2.3.1 | Risk factors | 17 |
| 2.3.2 | Toombak, Snuff and Oral Cancer | 12 |
| 2.4 | Oral Cytology | 13 |
| 2.5 | Lab Diagnosis | 14 |
| 2.5.1 | H & E stain | 14 |
| 2.5.2 | Pap stain | 14 |
| 2.5.4 | Nuclear Organizer Regions (NORs) | 14 |

Chapter Three Materials and Methods

<p>| 3.1 | Materials | 15 |
| 3.1.1 | Study Design | 15 |
| 3.1.2 | Study Area | 15 |
| 3.1.3 | Study Duration | 15 |
| 3.1.4 | Study Setting | 15 |
| 3.1.5 | Study Population | 16 |
| 3.1.6 | Inclusion Criteria | 16 |</p>
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1.7</td>
<td>Exclusion Criteria</td>
<td>16</td>
</tr>
<tr>
<td>3.1.8</td>
<td>Sample Size</td>
<td>16</td>
</tr>
<tr>
<td>3.1.9</td>
<td>Types of Samples</td>
<td>16</td>
</tr>
<tr>
<td>3.2</td>
<td>Methods</td>
<td>16</td>
</tr>
<tr>
<td>3.2.1</td>
<td>Pre Procedural Evaluation</td>
<td>16</td>
</tr>
<tr>
<td>3.2.2</td>
<td>Sample Collection and Processing</td>
<td>17</td>
</tr>
<tr>
<td>3.2.3</td>
<td>Staining</td>
<td>17</td>
</tr>
<tr>
<td>3.2.3.1</td>
<td>H &amp; E staining Method</td>
<td>17</td>
</tr>
<tr>
<td>3.2.3.2</td>
<td>Papanicolaou Staining Method</td>
<td>19</td>
</tr>
<tr>
<td>3.2.3.3</td>
<td>AgNOR Staining Method</td>
<td>20</td>
</tr>
<tr>
<td>3.4</td>
<td>Assessment of Slides</td>
<td>21</td>
</tr>
<tr>
<td>3.5</td>
<td>Statistical Analysis</td>
<td>22</td>
</tr>
<tr>
<td>3.6</td>
<td>Ethical Approval</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Chapter Four Results and Discussion</td>
<td></td>
</tr>
<tr>
<td>4.1</td>
<td>Demographic Data of the Study Group</td>
<td>23</td>
</tr>
<tr>
<td>4.1.1</td>
<td>The Age of the Study Population</td>
<td>23</td>
</tr>
<tr>
<td>4.1.2</td>
<td>The Duration of the Snuffing of the Study Population</td>
<td>24</td>
</tr>
<tr>
<td>4.1.3</td>
<td>Frequency of Dipping Per day</td>
<td>25</td>
</tr>
<tr>
<td>4.2</td>
<td>The Cytological Diagnosis of Samples</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>4.2.1</td>
<td>Comparison between H&amp;E and Pap Stain in the Diagnosis of Samples</td>
<td>20</td>
</tr>
<tr>
<td>4.2.2</td>
<td>The Cytological Diagnosis of Samples Using AgNOR Count</td>
<td>26</td>
</tr>
<tr>
<td>4.1.5</td>
<td>Comparison of the Snuffing Duration with the Cellular Appearance Using H&amp;E and Pap stain and AgNOR Count</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Chapter Five Conclusion and Recommendations</td>
<td></td>
</tr>
<tr>
<td>5.1</td>
<td>Conclusion</td>
<td>30</td>
</tr>
<tr>
<td>5.2</td>
<td>Recommendations</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Chapter Six References</td>
<td></td>
</tr>
</tbody>
</table>
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Duration of snuffing among study group</td>
<td>74</td>
</tr>
<tr>
<td>4.2</td>
<td>Frequency of dipping per day</td>
<td>74</td>
</tr>
<tr>
<td>4.3</td>
<td>The cytological diagnosis of H&amp;E and Pap stain</td>
<td>75</td>
</tr>
<tr>
<td>4.4</td>
<td>Mean AgNOR count among study group</td>
<td>76</td>
</tr>
<tr>
<td>4.5</td>
<td>Correlate of snuffing duration with the cellular appearance using H&amp;E, Pap stain and AgNOR count</td>
<td>27</td>
</tr>
<tr>
<td>4.6</td>
<td>Correlate of snuffing frequency per day with the cellular appearance using H&amp;E, Pap stain and AgNOR count</td>
<td>28</td>
</tr>
</tbody>
</table>
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Changes in oral mucosa where toombak is dipped</td>
<td>13</td>
</tr>
<tr>
<td>3.1</td>
<td>H &amp; E stained slide normal appearance</td>
<td>18</td>
</tr>
<tr>
<td>3.2</td>
<td>Atypical cell Using H&amp;E stain</td>
<td>18</td>
</tr>
<tr>
<td>3.3</td>
<td>Pap stained slide normal appearance</td>
<td>19</td>
</tr>
<tr>
<td>3.4</td>
<td>Atypical cell Using Pap stain</td>
<td>20</td>
</tr>
<tr>
<td>3.5</td>
<td>Abnormal AgNOR count</td>
<td>21</td>
</tr>
<tr>
<td>3.6</td>
<td>Normal AgNOR count</td>
<td>21</td>
</tr>
<tr>
<td>4.1</td>
<td>Age distribution among study group</td>
<td>23</td>
</tr>
</tbody>
</table>

List of abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgNOR</td>
<td>Argyrophilic Nucleolar organizer regions</td>
</tr>
<tr>
<td>Pap Stain</td>
<td>Papanikolaou Stain</td>
</tr>
<tr>
<td>H&amp;E stain</td>
<td>Hematoxylin and Eosin Stain</td>
</tr>
</tbody>
</table>
Chapter one

Introduction

1.1 Background:

Oral mucosa is a specialized type of tissues that lines the oral cavity. This tissue is designed to provide protection for the body from infection and debris, and it is capable of producing secretions such as mucus, in addition to absorbing materials introduced into the mouth. The trait of absorption is used to apply certain types of medications, such as oral vaccines (American Heritage, 2002).

The color of the oral mucosa can vary, depending on the skin color of the body. In some people, it is a pale pink, while others have darker pink to brown tissue. Extremely pale mucosa can be a sign that someone is anemic, while patchy or dark ones can be signs of a medical problem (M.Collins 2013).

A classic problem that develops with the oral mucosa is leukoplakia, in which white plaques of material appear in the mouth. Also so many changes and lesions can appear in the oral mucosa that may or may not change the mucosal color (American Heritage, 2002).

Social habits of tobacco use and alcohol consumption have been shown to produce a variety of oral mucosal changes (Field A, Longman L, 2003).

In the Sudan, the high incidence of oral mucosal changes (dysplasia, hyperkeratosis and other potentially malignant oral mucosal lesions) and equally the high prevalence of these changes have been strongly attributed to the habit of snuff use which is dipped in the mouth and locally known as toombak (Field A, Longman L, 2003).

Toombak has been used in the Sudan for centuries and its use is widespread. A close relationship between use of toombak and development of oral mucosal changes has been reported. In addition, snuff contains other carcinogens including aliphatic and aromatic hydrocarbons, formaldehyde, ketones, alcohols, phenols, amines, amides, alkaloids, metals, radioelement. (e.g. polonium-210, uranium-235 and -238) and polyaromatic hydrocarbons (PAHs) (Dye BA, Dowden J, 2007).
Early diagnosis of symptomatic ulcers and asymptomatic (during routine dental check) is of great importance for oral mucosal changes and lesions

During the oral exfoliative cytology the presence of two or more of the following features were consistent with atypia in cells which is a sign of malignancy. (M.Collins 2013)

Nucleolar organizer regions (NORs) are located in the cell nucleoli during interphase. They are loops of DNA in which ribosomal RNA is encoded. Also changes can occur in the NORs counts that can be detected by the AgNOR test which is another method of oral exfoliative cytology (CrokerJ, 1989)

1-2 Justifications and Objectives

1-2-1 Justification:

The increased incidence of oral cancer among the toombak users in the last years, in which 30,000 new cases each year with 9,000 deaths per year (A.Babkier 2007).

Oral exfoliative cytology is a simple, painless and inexpensive method has become a preferred method for both early diagnosis of the lesion and for establishing quantitative techniques (D.Mongelluzzo,2000) (Prout ,M.N,sidari, 1997).

1-2-2 Objectives :

1-2-2-1 General Objectives

Assessment of cytological atypia using AgNOR in epithelial cells of oral mucosa of snuff users.

1-2-2-2 Specific Objectives:

Diagnosis of oral mucosal epithelial cells of clinically healthy toombak dippers using H&E stains.

Diagnosis of oral mucosal epithelial cells of clinically healthy toombak dippers using Pap stains.

To assess cellular proliferative activity of clinically healthy oral mucosal epithelial cells of toombak dippers by means of AgNOR counts.
To correlate the findings between H/E stain, PAP stain and silver nitrate to stain AgNOR.

To specify the best staining method that gives the accurate of diagnosis of clinically healthy oral mucosal epithelial cells of toombak dippers.

To find out the frequency of dipping toombak per day, and the effect of snuffing duration in cellular proliferative activity.
Chapter two

Literature Review

2-1 Oral Mucosa

The oral cavity (mouth) includes the lips, cheeks, palate (roof of the mouth), floor of the mouth and the part of the tongue in the mouth (oral tongue). Oral mucosa is a mucous membrane that lines and protects the inside of the mouth. The structures in the oral cavity play an important role in speech, taste and the first steps of digestion (American Heritage, 2002).

The buccal mucosa area of this membrane extends around the inside of the cheek and lower mouth area, the bottom of the tongue, out to the lips and to the back of the throat (American Heritage, 2002). This region is well known to dentists and oral surgeons, since it surrounds the tooth structures in the lower jaw and contains muscles used during chewing. It also contains a fat pad between the muscles, called the buccal fat pad, as well as nerves, blood vessels, and lymph nodes and salivary glands (Field A, Longman L, 2003).

The oral mucosa has several functions. Its main purpose is to act as a barrier; It protects the deeper tissues such as fat, muscle, nerve and blood supplies from mechanical insults, such as trauma during chewing, and also prevents the entry of bacteria and some toxic substances into the body (Dye B A, Dowden J, 2007).

The oral mucosa has an extensive innervation of nerves; which allows the mouth to be very receptive of hot and cold, as well as touch. Taste buds are also located in oral mucosa and are important for recognition of taste (Ramirez, Amador, 2000).

The buccal mucosal membrane secretes moisturizing and lubricating fluids for the mouth and upper throat. These fluids are necessary to prevent drying effects, since this mucosa is part of the membrane system that lines the entire gastrointestinal tract, and this is open to exterior surfaces at both ends (Ramirez, Amador 2000) (Guggenhemerj, 2000). A similar type of membrane also lines the exterior entrances to the respiratory system at the nose and throat areas. The major secretion associated with the oral mucosa is saliva, produced by the salivary glands.
salivary glands secrete most of the saliva via ducts that pass through the oral mucosa (D. Mongelluzzo, 2000).

There is a degree of permeability that allows for rapid absorption into the body in certain circumstances e.g. the permeability of the oral mucosa is utilised in the rubbing of orange juice, or another sugary drink when diabetics suffer from a low-blood sugar (Prout, M.N, sidari, 1997).

The oral mucosa consists of two layers: the corium (or lamina propria) and the epithelium. The epithelium is the surface layer, and where there is a disease of the oral mucosa, often it is the epithelium that is most affected (Diaz, G.L., 1998). The epithelial tissue (the tissue covering body surfaces) of the buccal mucosa is “squamous.” This means that this tissue consists of cells that are flattened, similar to the mesh in a fishnet but since squamous tissue has several layers, a more accurate description would be of several overlaid fishnets. Because the cells are flattened, however, they can more easily transfer substances such as saliva through out the cells due to their reduced vertical dimensions, and this aids the digestive process. (Castellanos, jL, 1991)

For each dividing cell, one cell is lost from the surface, thus, the integrity of that area is maintained. The rate in the oral cavity is much faster than on skin (approximately twice as fast), and areas such as the inside of the cheek will turnover in about 20 days (Kramer, IR, 1980). As cells mature in general, maturation can undergo two different patterns either keratinisation or non-keratinisation. The most common cells that are required to undergo cell turnover are called keratinocytes (Castellanos, jL, Kramer, IR, 1980). As the keratinocyte matures, it undergoes modification in its structure that causes it to progress towards the surface of the epithelium, and eventually die. Keratinocytes mature to different degrees (Diaz, GL, 2004). In some areas of the mouth, the keratinocytes will fully mature (orthokeratinisation), whereas in other areas the keratinocytes will only partially undergo keratinisation (parakeratinisation) (Diaz, GL, 2004).

The epithelial tissue of the buccal mucosa is non-keratinised, which means that these cells have a nucleus, or central generating core, as well as cytoplasm, which consists of all living structures in a cell apart from the nucleus. This is in contrast to squamous epithelial cells that cover drier areas of the body, such as the skin, which are keratinized and have lost cell regeneration capabilities. (Griffin so, Jones, 2012) Both types of epithelial tissue, however, are highly subject to cancers, since epithelial tissue is known as having a high cancer rate (Thornton, Evans, 2012).
2-2 Pathology of oral mucosa:
Include four types;

A- Local causes :

1-Infections related diseases:

A- VIRAL:

Couse fever blisters and cold sorese.g Herbes Zoster, Acute Herpetic Gingivostomatitis. acute herpetic gingivostomatitis with oral mucosal ulcers, Chicken pox which is caused by the varicella virus. (Griffin so ,Jones, 2012)

B- FUNGAL

like Angular chelitis- This condition is most often bilateral. The ulcerated, crusted, erythematos area of the right commissure is most often caused by Candidae. (Griffin S ,Barker, 2009)

C-BACTERIAL

Like Chancre- The lesion of primary syphilis of the tongue and secondary syphilis of the hands and palate caused by Treponema pallidum

Fistula (parulis or sinus tract):-Gingivitis- Periodontitis. A solitary painless ulcer on top of tongue (Griffin so ,Jones, 2012)

2- HORMONAL

Like Necrotizing Periodontal Diseases, the clinical picture showing punched out papillae, which are characteristic of this gingival condition.

Pregnancy tumor-A tumor that is histologically the same as a pyogenic granuloma and is a reactive inflammatory lesion. The patient exhibits a pregnancy tumor on the tongue (Gray SK ,Malvitz, 2009)
3- DERMATOLOGICAL

Allergy- This condition consists of a hypersensitive reaction acquired through exposure to a particular allergen. This series shows edema of the gingival (due to toothpastes), eyelids.
Chemical burns; This condition occurs when an exogenous chemical (aspirin, Clorox, nitroglycerin) (Griffin S, Barker, 2009) (Gray SK, Malvitz, 2009).

4-PHARMACEUTICAL

All pharmaceuticals have oral side effects in some manner ranging from xerostomia to lichenoid reactions to hyperplasias. (Gray SK, Malvitz, 2009)

B. Systemic Diseases

1-DIABETES: the number two source of oral disease conditions. Micro vasculature damage of the periodontal tissues, dental pulp and nerves.

2-HEPATITIS: the Clinical picture of the ventral surface of the tongue showing pallor and a yellow tone associated with alcoholic hepatitis with jaundice.

3-Iron deficiency anemia: Oral manifestations of iron deficiency anemia include angular chelitis and a smooth, red tongue with atrophy of the papillae. (Gray SK, Malvitz, 2009)

4-Vitamin deficiency: This smooth, dark red tongue resulted from a deficiency of the B-complex vitamins (Griffin S, Barker 2009, Jaramillo F, 2009)

C. GROWTHS

Exostoses- These are nodular projections of dense compact bone on the buccal and labial aspects of the maxilla or mandible, or both fibroma; A benign lesion with a smooth surface that is thought to be traumatically induced is called a fibroma. It is most often found near the occlusal plane (Gray SK, Malvitz, 2009).

D. PSYCHIATRIC DISORDERS

Anorexia nervosa (bulimia)- This disorder is associated with self-induce vomiting in which the gastric acids cause erosion of the dentition (Griffin S, Barker 2009).
E. CANCER

Is defined uncontrollable growth of cells that invade and cause damages to surrounding tissues. Oral cancer appears as a growth or sore in the mouth that does not go away (Chepaha DB, 2011). Oral cancer, which includes cancers of the lips, tongue, cheeks, floor of the mouth, hard and soft palate, sinuses, and pharynx (throat), can be life threatening if not diagnosed and treated early. Risk factors for the development of oral cancer include: Smoking; Cigarette, cigar, or pipe smokers are six times more likely than non-smokers to develop oral cancers (abdlalgaffer SA, 2011).

Smokeless tobacco users; Users of dip, snuff, or chewing tobacco products are 50 times more than non-users are likely to develop cancers of the cheek, gums, and lining of the lips (Chepaha DB, 2011) (Mendenhall WM, 2011).

Carcinoma; A malignant tumor of epithelium is a carcinoma with a 90% of all malignancies of the oral cavity are squamous cell carcinoma. 30,000 new cases each year with 9,000 deaths per year. Five year survival rate remains below 50%. Most all are associated in some way with tobacco (Mendenhall WM, 2011).

In Sudan, nasal use of snuff is found only in the far southern parts of the country while oral use widespread as described. Toombak is a native tobacco plant of the species Nicotiana rustica used for manufacture of snuff. Toombak is grown in silky or sandy soils which receive heavier rain falls in the north-west of the Sudan. After the end of the rainy season (September/October), toombak is planted during the months November/December and never irrigated. Harvesting starts in the months February/March when the leaves turn yellow and brownish spots start appearing (called the smallpox stage). Harvested leaves are left in the field for uniform drying, tied into bundles, moistened with sprinkling of water and stored for fermentation for a couple of weeks at temperature ranging from 30 to 45°C, during which bundles are separated for uniform drying during the months April/May. Tobacco Leaves are ground and stored for a year for ageing introduction of this tobacco plant to the Sudan was attributed by a quarnic (Islamic) teacher, who came to the Sudan either from Egypt, Timbuktu of Mali or Morocco. It has also been suggested that toombak was introduced to the Sudan from Turkey or Arabia. Processing of toombak for sale is usually carried out manually in toombak shops by toombak vendors. It is performed by preparing four parts of a coarse powder of dried toombak leaves in a bowl and in another the concentrate of sodium bicarbonate is added.
gradually in small amounts to the tobacco. While adding the solution, the product is mixed vigorously by both hands and concurrently tested by sensation of the tips of the fingers until it becomes moist and hardened. The output is then transferred to special air-tight containers which are then covered firmly for about 2 hours, thereafter the product becomes ready for sale or use. Before buying, users generally ask for a bit to smell or taste, since the aroma and taste decide the quality rank of the product. Currently, toombak is sold in small plastic bags each taking about 100g (Idris AM, 1989). Oral snuff, known locally as toombak, is home-made from finely ground leaves of Nicotiana rustica, a tobacco species with a particularly high content of nicotine and minor alkaloids. This tobacco is mixed with Natron or atron (sodium bicarbonate) (Hoffmonn D, 1992) (about 4:1), then water is added to the mixture, and after a period of about 2 hours or longer the mixture, called “saffa”.

**Natron:** Natron or atron (sodium bicarbonate \(Na_2H(CO_3)2.2H_2O\). Atron, opposed to lime in other parts of the world, is probably added to toombak for its alkaline effects. It has been shown that at high pH (11.0 - 11.8) nicotine is completely protonated and its rate of absorption is increased (Hoffmonn D, 1992) (Djordjevic MV, 1993). Atron probably quickens absorption of nicotine from toombak to the central nervous system (Chepeha DB, 2011). Natron or atron (sodium bicarbonate, also called sodium hydrogen carbonate), is a mineral rock with the chemical formula \(Na_2H(CO_3)2.2H_2O\). Its color is grey to yellowish white, and is of alkaline pH. There is no information on either the history or reasons behind use of atron as an additive to toombak. It may be used to homogenise the leaves to a fine sticky form as atron is used in the Sudan to homogenise vegetables during cooking. (Idris AM, 1989) Atron, opposed to lime in other parts of the world, is probably added to toombak for its alkaline effects. It has been shown that at high pH \((11.0\pm11.8)\) nicotine is completely protonated and its rate of absorption is increased. Studies of nass, a type of snuff used in the former USSR which contains lime and has high pH \((11\pm11.8)\), have shown that when the product is placed in the mouth, nicotine reaches the central nervous system very quickly. Thus, pH value in tobacco products continuance the absorption and thereby the extent of pharmacological activity of nicotine. Atron probably quickens absorption of nicotine from toombak to the central nervous system. (Idris AM, 1989).

**N-nitrosamines:** there were studies analyzed the Tobacco Specific Nittrose Amine (TSNA) levels in toombak and found unusually high levels of these TSNAs compared to the reported levels in any snuff. These high levels of TSNAs found in toombak were partially attributed to the use of tobacco species, Nicotiana rustica, and fermentation of toombak at elevated temperature,
prolonged storage, and contamination during processing (Babekir AR, 1989) (Idris AM, Ibrahim, 1989). Therefore, assuming chronic toombak use, the minimum daily dose of NNK to which these users were exposed was 0.12 - 0.44 mg. This is the highest documented uptake of a non occupational carcinogen (Idris AM and Ibrahim, 1989).

Epidemiological evidence suggests that toombak is a risk factor for cancer of the oral cavity and possibly of the oesophagus in the Sudan (Petti S, 2009).

Data from 1,916 cases of oral neoplasms occurring in the Sudan in a 16 year period, from January 1970 to December 1985, were retrieved and analyzed. The study revealed a relatively high frequency of oral neoplasms in comparison with neighbouring countries. Squamous-cell carcinoma was the most common oral malignancy (66.5%), followed by tumours of the salivary gland (14.7%), neoplasms of non-odontogenic and non-epithelial origin (9.6%) and odontogenic neoplasms (8.6%). Men had a higher frequency than women (Idris AM and Ibrahim, 1989). Female toombak use is considered social stigma in Sudan, consequently the 95% of toombak users were males, which supports its etiological effects.

In Sweden, snus (locally known as snus), was introduced since the year 1637.

Clinical and histopathological characteristics of toombak-associated oral mucosal lesions detected in an epidemiological study in northern Sudan in 1992/93 found Parakeratosis, pale surface staining of the epithelium and basal cell hyperplasia were commonly observed, but epithelial dysplasia was infrequent (10/141) (Babekir AR,1989). Ultra structural features oral toombak dipper’s lesions with distinctive sub epithelial hyaline deposits their bulk is made up of collagen, as typical cross-striated fibrils. The pathogenesis of this deposit could therefore be interpreted as over-production and/or reduced turnover of collagen by resident fibroblasts, which is further altered by the ingredients of toombak (Djordjevic MV, 1993).

In a study investigated the effects of toombak on primary normal human oral keratinocytes, fibroblasts, and a dysplastic oral keratinocytic cell line, compare to Swedish snuff, a potential for toombak, higher than for Swedish snuff, to damage human oral epithelium (Furthermore, In OSCC, apoptosis was associated with bad expression and was unaffected by p53 gene status) (M.Collins 2013).
The study by Idris AM and Ibrahim (1998), analyzed 14 oral squamous-cell carcinomas (OSCCs) and 8 pre-malignant oral lesions from different Sudanese patients for prevalence of mutations in exons 5 to 9 of the p53 gene in relation to toombak-dipping status. OSCCs (14 from Sudan, 28 from Scandinavia), and 3 pre-malignant oral lesions from Sudanese non-dippers were used as controls. A statistically significant (P < 0.05) increased incidence in mutations of the p53 gene was found in OSCCs from toombak dippers (93%; 13/14), as compared with those from non-dippers in Sudan (57%; 8/14) and in Scandinavia (61%; 17/28) respectively (Idris AM, Ibrahim 1998).

Several studies from Sudan have proved that toombak use is a major risk factor that is responsible of high frequencies of potential malignant oral lesions and oral cancers and in particular OSCCs in Sudan. Most of tumours were observed at the site of dip application (lower lip). Oral cancer seems to be gender-specific, as the majority of cases were males (petti S, 2009). However, all of the preceded discussed literatures support the criminal role of toombak in the aetiology of oral cancer in the Sudan. Probably toombak has a major role but it is not alone responsible of oral cancer in the Sudan, particularly in the recent years with dramatic increase in overall cancer risk. only partially undergo keratinisation (parakeratinisation) (petti S, 2009).

2.3 Epidemiology of Oral Mucosal Changes caused by tobacco use:

The oral changes from tobacco ranges from harmless soft tissue changes to a life threatening cancer. Worldwide, more than 500,000 new cases of oral cancer are diagnosed each year. It has been established that there is a relationship between tobacco use and the development of oral cancer. Each part of the oral cavity is susceptible to cancer from tobacco smoking or chewing (snuffing) including the lip, tongue, palate, gum and cheek. The mouth offers non-invasive and repetitive examinations compared with other body sites. Thirty to eighty percent of the malignancies of the oral cavity arise from pre-malignant lesions, such as leukoplakia, erythroplakia and oral sub mucous fibrosis. It has been established that there is a relationship between tobacco use and the development of oral cancer (Hoffmonn D, 1992).
2.3.1 Risk factors

- Excessive consumption of alcohol. Oral cancers are about six times more common in drinkers than in nondrinkers (Chepaha DB, 2011).
- Family history of cancer.
- Excessive sun exposure, especially at a young age.

2.3.2 Toombak, Snuff and Oral Cancer:
The Impact of Tobacco Use on Systemic Health results in a greater risk of cancer, lung disease, and cardiovascular diseases. The statistics are overwhelming—almost 90% of lung cancer deaths in men and almost 80% in women, as well as between 80% and 90% of chronic obstructive pulmonary disease (COPD) deaths are caused by smoking of tobacco (Chepaha DB, 2011) (AbdAlgaffer, 2011).

Smokeless tobacco also increases the risk of cardiovascular disease; smokeless tobacco users have higher daytime heart rates than nonusers and have twice the risk of dying from cardiovascular disease. Unlike with smoking tobacco, however, no observed increase in atherosclerosis was observed (Jaramillo F, 2009).

A study of 2,840 adult males found that tobacco smokers and heavy smokeless tobacco users were both more than twice as likely as nonusers to suffer from hypercholesterolemia; for mild/moderate smokeless tobacco users, the risk increased 1.5 times. Smokeless tobacco has also been found to be associated with pancreatic cancer, mouth cancer more often than non-users, as well as higher risk for cancers of the esophagus and stomach (Mendenhall WM, 2011).

There are also many published papers in South Africa assessing the effects of Snuff on the oral mucosa among the black South African population plus its other effects. In Sudan many papers were published reporting Oral Epithelial Proliferative Markers among Sudanese Toombak Dippers, published in the Journal of rare tumors in 2013 another paper published in e-Journal of Oral and Maxillofacial Research in 2013 assessing the Aetiology of Oral Cancer in the Sudan.

From the other carcinogenic materials in tobacco is Benzopyrene , Alcohorton, and aldehyde, formaldehyde (element used in Embalming the dead ) Acetydehyed (irritant material) , Hydrazine(chemical poisonous matrial ) Arsenic (poisonous material) , Cadmium(an element
used information of cars battery), Polonium (radioactive element). Tobacco users can develop cancer and different kinds of white and red spots lesions in lining of the mouth which is very dangerous when it develops into receding gums from the teeth, yellowing teeth, often it is placed in the mouth between the gums and lower lip or between the gum and upper lip or any part of mouth. Reuters website published joint study between Sudanese, Norwegians and Swedish researches.

**Figure (2.1) Change in Oral mucosa where toombak is dipped**

2.4 Oral Cytology:

Early diagnosis is great important for oral SCC. Oral exfoliative cytology, a simple, painless and inexpensive method has become a preferred method for both early diagnosis of the lesion and for establishing quantitative techniques. The presence of two or more of the following features were consistent with atypia: nuclear enlargement, associated with the increased nuclear/cytoplasmic ratio, nuclear hyperchromatism, chromatin clumping with prominent nucleation, irregularity of nuclear membranes, bi- or multinucleation, increased keratinisation(D.Mongelluzzo, 2000) (Prout, M.N, sidari, 1997).
2.5 Lab Diagnosis:

To establish a diagnosis of oral mucosal changes (cytological atypia) in tobacco users, in addition to clinical findings if presents, three laboratory diagnostic methods are used, the Haematoxylin and Eosin stain method and papanicolaou technique, and Ag NOR, which is confirmed by silver nitrate technique.

2.5.1 H&E stain

Is one of the principal stains in histology, it is the most widely used stain in medical diagnosis. The staining method involves application of hemalum, a complex formed from aluminium iron, and hematein, an oxidation product of hematoxylin. Hemalum colors nuclei of cells “blue”, the nuclear staining is followed by counterstaining with an aqueous or alcoholic solution of eosin (Y), which colors eosinophilic structures in various shades of red, pink, and orange.

2.5.2 Pap stain

Is a multichromatic staining cytological technique developed by George Papanikolaou. Pap staining is used to differentiate cells in smear preparations of various bodily secretions; the specimens can be gynecological smears (Pap smears), sputum, brushings, washings, urine, cerebrospinal fluid, abdominal fluid, pleural fluid, synovial fluid, seminal fluid, fine needle aspiration material, tumor touch samples, or other materials containing cells. First OG-6 counterstain (-6 denotes the used concentration of phosphotungstic acid; other variants are OG-5 and OG-8). Orange G is used. It stains keratin. Its original role was to stain the small cells of keratinizing squamous cell carcinoma present in sputum. Second EA (Eosin Azure) counterstain, comprising three dyes; the number denotes the proportion of the dyes, e.g. EA-36, EA-50, EA-65) (Bancroft 2007).

2.5.3 Argyrophilic Nucleolar organizer regions (AgNORs)

NORs are located in the cell nucleoli during interphase. They are loops of DNA in which ribosomal RNA is encoded. 10 Nucleolar organizer regions can be demonstrated with the use of AgNOR technique which is the silver staining technique for showing NORs as black dots inside the nucleus when examined under a light microscope. (Bancrofti 2007)
Chapter Three

Materials and Methods

3.1 Materials:

3.1.1 Study design:

This was cross sectional study for assessment of cytological atypia in epithelial cells of oral mucosa of toombak users, using H&E, PAP stain, and AgNOR.

3.1.2 Study area :

Gezira which state is one of the eighteen states of Sudan and it is one of the biggest states by area (25,549 km2) with a population of (3.796,000 inhabitants according Sudan.gov.sd 2012 census). Study was carried out in Wad medani dental teaching hospital in Wad medani the capital of Gezira state which lies in the western bank of the blue Nile.

3.1.3 Study Duration :

The Study Carried out during the period of November 2014 to July 2017

During the period (March - May 2015).

3.1.4 Study setting:

Wad medani dental teaching hospital is one of the leading teaching hospitals in the Sudan in the field of dentistry, which is located in Wad medani, the capital of Gezira State. The hospital was made initially for providing health care and services for people living in Wad medani city and Gezira state as whole. Latter it grew up to receive patients from all over the middle states of Sudan about 90-150 patient per day. The hospital is rated as one of the best in Sudan as it includes both training and teaching facilities of the faculty of Dentistry, University of Gezira, beside all major departments and specializations in dentistry. Most of the consultants working in the hospital are broadly recognized.
3.1.5 Study population:

Patients who presented to Wad medani dental hospital who have a history of snuff dipping of both genders.

3.1.6 Inclusion criteria:

- Patients who presented to the outpatient and used to snuff of both genders.
- Age above 18 years.
- History of snuff dipping for more than 2 years.

3.1.7 Exclusion criteria:

- Patients with history of radiotherapy or chemotherapy for oral or other system malignancy
- Patients with history of smoking or alcohol consumption.
- Patients with positive history systemic diseases (DM, HTN, RF,....)
- Patients with a history benign or malignant lesions were excluded from this study (to avoid pias)

3.1.8 Sample size

All patients (480 cases) presented to the outpatient of the dental hospital during the period data collection of the study who used to snuff and met the inclusion criteria of the study.

3.1.9 Type of samples:

oral mucosa smear

3.2 Methods of data collection

3.2.1 Pre procedural evaluation:

- Questionnaire contain demographic data (Age, duration of snuffing, and frequency of dipping per day
• Volunter examination :

The users examined in dental chair by dentist using chair light, mirror, examination probe checking mucous membrane of buccal and libal mucosa of upper and lower Jaws areas of snuff dipping.

• A questionnaire include, the age of the participant, the duration of snuffing and frequency of dipping toombak per day.

3.2.2 Sample collection and processing:

Oral smear which was done by brush from the dipping site. Participants were asked to rinse their mouth with saline solution for a while before the sample is taken. The materials were collected by a smooth brush after brushing the floor of the mouth at the dipping site two times, then rinsing and cleaning the brush each time in a saline solution. This was done so as to collect cells from the inner layers of the oral mucosa. The material collected for each specimen was smeared on three slides and immediately fixed in 95% ethyl alcohol for 15 minutes, one slide was stained by H&E, the second slide was stained by PAP stain, and the third slide was stained by Silver nitrate stain for AgNOR.

3.2.3 Staining:

3.2.3.1 H & E staining method

Deparaffinize section, hydrate through graded alcohols to water
Stain Mayers Haematoxylin for 5 minutes
Wash well in running tap water until section( blue) for 5 minutes or less
Differentiate in 1% acid alcohol (1% HCL in 70% alcohol ) for 5 second
Wash in tap water until sections are again (blue) (10 – 15 minutes),
Stain in 1% eosin (y) for 10 minutes
Wash in running tap water for 1 – 10 minute
Dehydrate through alcohol, clear, and mount

Interpretation of the results:

Nucleus; deep blue colour. Cytoplasm and background tissue; pink colour. RBCs; orange colour (John D. Bancrofti et al, (2008)
Figure (3.1) H&E stain normal appearance

Figure (3.2) Atypical cell Using H&E stain
3.2.3.2 Papanicolaou staining method

Ethyl alcohol fixed smears were hydrated in descending concentrations of 95% alcohol through 70% alcohol to distilled water, for two minutes in each stage. Then the smears were treated with Harris’ hematoxylin for five minutes to stain the nuclei, rinsed in distilled water and differentiated in 0.5% aqueous hydrochloric acid for a few seconds, to remove the excess stain. They were then immediately rinsed in distilled water, to stop the action of discoloration. Then the smears were blued in alkaline water for a few seconds and dehydrated in ascending alcoholic concentrations from 70%, through two changes of 95% alcohol for two minutes for each change. The smears were next treated with Eosin Azure 50 for four minutes. For cytoplasmic staining, they were treated with Papanicolaou Orange G6 for two minutes, rinsed in 95% alcohol and then dehydrated in absolute alcohol. The smears were then cleared in Xylene and mounted in DPX (Distrene Polystyrene Xylene) mount.

- All the reagents used were from Thermo Electron Corporation, UK.

Results interpretation:

![Figure (3.3) PAP stain normal appearance](image-url)
Atypia was assessed cytologically by using the criteria described elsewhere. The presence of two or more of the following features were consistent with atypia: nuclear enlargement associated with increased nuclear: cytoplasmic ratio, hyperchromatism, chromatin clumping with moderately prominent nucleoli, irregular nuclear membranes and bi- or multi-nucleation, scant cytoplasm, and variation in size and/or shape of the cells and nuclei. (Ahmed HG, AI Elemirri)

3.2.3 AgNOR stain:

The smears were stained according to the AgNOR staining method. Working solution was freshly prepared by mixing one volume of 2% gelatin in 1% formic acid solution and two volumes of 50% aqueous silver nitrate solution. All smears were incubated with this silver solution for 30 minutes at room temperature in a dark medium and they were protected in the dark until each slide was analyzed.

Results interpretation
AgNORs were visible as black-dark brown dots located within the nuclei of the cells. Normal cells AgNORs appeared as discrete black brown dots present within the nucleus (Figure 3.3). AgNOR sites appear as intranuclear black dots in a pale yellow background. (Crocker J, (argyrophilic nucleolar Boldy DA1989)
Figure (3.5) AgNor; buccal cells from toombak user showing more than 4 dots per nucleus organizer region stain 1000x)

Figure (3.6) Normal AgNor; count buccal cells

3.4 Assessment of slides

Each specimen had three slides, which was reviewed by a cytopathologist according to criteria of benign and malignant.
3.5 **Statistical analysis method:**

Data were processed using Microsoft office SPSS Acess 2007 and Microsoft Excell 2007 to process and analyze the data for clearing the data and check up in variable. chi square test was used for analytic comparison between different variable (duration, frequency of snuff) using it statistically different. the statistically significance of the different was assess by calculate and interpreting the P.value . p.value less than 0.05 interpreted as significant difference.

3.6 **Ethical approval:**

Was taken from the university ethical research board (ERB) and the hospital administration. Each participant was asked to sign a written ethical consent form during the interview, before the specimen was taken. Consents were taken from patients to take samples after agreement of the participant, name of patients were taken but symbolized by numbers instead to guarantee confidentiality.
Chapter Four

Results and Discussion

In this study 100 samples were screened using different techniques; ordinary used H&E stain, PAP stain, and the simple method AgNORs. Other informations include; the age, the duration of snuffing, and frequency of dipping per day.

4.1 Demographic data of the study group:

4.1.1 The age of the study population:
The largest group in the age of more than 50 years (30%), followed by 41-50 years (25%), 31-40 years (23%), and finally 20-30 years (22%) (figure 4.1).

![Age distribution among study group](image)

This figure shows increasing of snuffing users samples raise with increasing of their ages.

These results agree with many authors like Hussain Gadelkarim Ahmed (2013) and J.Jalouli (2012) they said that raise with increasing in age.
4.1.2 The duration of snuffing of the study population:

The largest group that lies in duration of snuffing 6-10 years (35%) followed by 2-5 years (28%), 11-15 years (25%), 16-20 years (9%), and finally 20 years (3%).

Table (4.1) Duration of snuffing among study group

<table>
<thead>
<tr>
<th>Duration per year</th>
<th>Snuff users</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 -5 yrs</td>
<td>28</td>
<td>28.0</td>
</tr>
<tr>
<td>6 - 10 yrs</td>
<td>35</td>
<td>35.0</td>
</tr>
<tr>
<td>11 -15 yrs</td>
<td>25</td>
<td>25.0</td>
</tr>
<tr>
<td>16 - 20 yrs</td>
<td>9</td>
<td>9.0</td>
</tr>
<tr>
<td>above 20 yrs</td>
<td>3</td>
<td>3.0</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100.0</td>
</tr>
</tbody>
</table>

These results agree with Hussain G Ahmed (Sudan 2013) and Ghada Radwan (Egypt 2013) they said that the severity of risk depends on duration of contact of the noxious agent with the tissues.

4.1.3 Frequency of dipping per day:

The largest group in frequency per day 2-5 times (52%) followed by 6-10 times (46%) and 11-15 times (2%) (table 4.2).

Table (4.2) Frequency of dipping per day

<table>
<thead>
<tr>
<th>Frequency / day</th>
<th>Snuff users</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 -5 times / day</td>
<td>52</td>
<td>52.0</td>
</tr>
<tr>
<td>6 -10 times / day</td>
<td>46</td>
<td>46.0</td>
</tr>
<tr>
<td>11 - 15 times / day</td>
<td>2</td>
<td>2.0</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100.0</td>
</tr>
</tbody>
</table>
These results agree with A M Abbas (2013) said that frequency has a role in oral changes. And disagree with Idris AM (2011).

### 4.2 The cytological diagnosis of samples

#### 4.2.1 Comparison between H&E and Pap stain in the diagnosis of samples

H&E and Pap stain had the same cytological diagnosis that; most of samples were normal 49 (49%), the inflamed samples were 45 (45%), and atypical cells in 6 (6%) (table 4.3).

**Table (4.3) The cytological diagnosis of H&E stain and Pap stain**

<table>
<thead>
<tr>
<th>Cytology diagnosis</th>
<th>H &amp; E</th>
<th>Pap stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>49 (49%)</td>
<td>49 (49%)</td>
</tr>
<tr>
<td>Inflammation</td>
<td>45 (45%)</td>
<td>45 (45%)</td>
</tr>
<tr>
<td>Cytological atypia</td>
<td>6 (6%)</td>
<td>6 (6%)</td>
</tr>
<tr>
<td>Total</td>
<td>100 (100%)</td>
<td>100 (100%)</td>
</tr>
</tbody>
</table>

The results are same in diagnosis. Santhosh Kumar (2016), and (R Vezhavendhan 2016), they said that Pap stain high accuracy than H&E. (Back ground – cytological features – inflammatory cells – nuclear details)

Rania SidAhmed (2007), compared between H&E, Pap and MGG in the diagnosis of urine samples, she found that Pap stain was superior to H&E and MGG stain.
4.2.2 The cytological diagnosis of samples using AgNor count

Most of samples were normal 94(94%) and just 6 (6%) samples were abnormal (table 4.4).

**Table (4.4) mean AgNor count among study group**

<table>
<thead>
<tr>
<th>Count</th>
<th>Snuff users and percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (1-3)</td>
<td>94 (94%)</td>
</tr>
<tr>
<td>Abnormal (4 and above)</td>
<td>6 (6%)</td>
</tr>
<tr>
<td>Total</td>
<td>100 (100%)</td>
</tr>
</tbody>
</table>

Argyrophilic nuclear organizer region (AgNor) is a silver staining procedure to observe the nucleolar organizer region (Nor) of the nucleous that is suitable for prognostic assessment. Mean Nor count measured by counting the number of silver stained dots per 50 nucleous for every smear, then the number obtained divided by 50 (Blody D and Croker J1989), the high mean Nor count was found in (6%) in cases.

These results agree with Sandhya Gulia (2010) and Emani sitaramam (2011), they said that the mean AgNor count in atypia smears showed higher than normal oral Epithelium and silver nitrate stain has a major role in staining oral samples.

4.1.5 Comparison of the snuffing duration with cellular appearance using H&E and Pap and AgNor:

The normal cytological appearance were 25 cases in snuffing duration of 2-5 years, 20 cases in snuffing duration of 6-10 years, and 4 cases in 11-15 years of snuffing.

The inflamed samples were 3 cases in snuffing duration of 2-5 years, 15 cases in 6-10 years of snuffing, 16 cases in 11-15 years, in 16-20 years were 8 cases, and 3 cases in above 20 years of snuffing.
The cases of cytological a typical cells appear in 11-15 years of snuffing in 5 cases, and only one case in 16-20 years.

When using AgNor count the samples were normal in snuffing duration of 2-5 years and 6-10 years.

The abnormal AgNor count appear in 11-15 years in 5 cases, and only one case in 16-20 years of snuffing (\textit{P value} 0.01) (Table 4.5).

**Table (4.5) Comparison of the snuffing duration with cellular appearance using H&E and Pap and AgNor count**

<table>
<thead>
<tr>
<th>Duration of snuffing</th>
<th>Cytology appearance</th>
<th>AgNor count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>normal smear</td>
<td>inflammation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 - 5 yrs</td>
<td>25 89.3%</td>
<td>3 10.7%</td>
</tr>
<tr>
<td>6 - 10 yrs</td>
<td>20 57.1%</td>
<td>15 42.9%</td>
</tr>
<tr>
<td>11 - 15 yrs</td>
<td>4 16.0%</td>
<td>16 64.0%</td>
</tr>
<tr>
<td>16 - 20 yrs</td>
<td>0 0.0%</td>
<td>8 88.9%</td>
</tr>
<tr>
<td>above 20 yrs</td>
<td>0 0.0%</td>
<td>3 100.0%</td>
</tr>
</tbody>
</table>
Table (4.6) Comparison of the snuffing Frequency / day with cellular appearance using H&E and Pap and AgNor count:

<table>
<thead>
<tr>
<th>Frequency / day</th>
<th>Cytology appearance</th>
<th>AgNor count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1-3</td>
</tr>
<tr>
<td>2 -5 times / day</td>
<td>Normal smear</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>68%</td>
</tr>
<tr>
<td></td>
<td>40%</td>
<td></td>
</tr>
<tr>
<td>6 -10 times / day</td>
<td>Inflammatio</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>15%</td>
<td></td>
</tr>
<tr>
<td>11 - 15 times / day</td>
<td>cell a typia</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>6%</td>
</tr>
<tr>
<td></td>
<td>0%</td>
<td></td>
</tr>
</tbody>
</table>

The normal cases decrease with increasing of snuffing duration.
The cases of inflammation increase with increasing of snuffing duration, and the overall study population decreases after 16-20 years of snuffing.
The cases of a typical cells appear with increasing of snuffing duration after 11-15 years of snuffing and frequency per day.
The AgNor count like the cytological diagnosis of H&E and Pap stain that the normal count found in short snuffing duration and the abnormal count appears with the increasing of snuffing duration after 11-15 years of snuffing, the difference was statistically significant (P value 0.01).

These results agree with many authors like Derenzini et al. (1998), said that NORs have been shown to be the site of rDNA which are transcribed to rRNA, they can routinely highlight by virtue of argyrophilia of their associated proteins.

It has been reported that, particularly human somatic cells could contain demonstrable NORs in nuclei, but many resting cells contain only one particle (Crocker and Egan 1988). Due to increased proliferative activity of neoplastic cells, higher number of NOR particles in cancerous cells might be expected, this in addition to the presence of a reasonable of cytological atypia among cases compared to controls (Boldy et al. 1989).
De Paula AMB (2000), in the part of the development of a screening procedure for oral cancer and precancerous cellular activity, exfoliative cytology was applied to a retrospective cohort study to assess the presence and severity of oral epithelial atypia in 200 volunteers snuffing toombak and 100 without prior exposure to tobacco. He found that the micronuclei frequencies increase with an increase in the duration and frequency of Toombak use and this was found statistically significant. Atypical changes in results of AgNOR, micronuclei and cytological examination show that cellular proliferation is significantly higher in Toombak users and this might be attributed to the fact that production of a malignant cell requires cell proliferation and DNA activity.

Numerous studies have shown that the number of AgNORs are significantly higher in malignant tumors than in physiological reactive and benign processes, also toombak dippers more susceptible to inflammation (Paiva KR, 2004), (De Paula, 2000), (ploton D, 1986).

Oral cancer is one of the most common cancers worldwide. In Sudan, the use of Toombak plays a significant role in the etiology of squamous cell carcinoma. Specifically, nitrosamines present in Toombak possibly act as principal carcinogen (Mehrotra and Yadav 2006).

Ankle and Kale (2007), found that smear is useful in evaluation of epithelial atypia that is frequently encountered in pre-malignant and early malignant oral lesions. There is a need to standardize the cytological proliferative marker methods to provide rapid, inexpensive, quantitative, reproducible, technologically simple, and applicable monitoring and screening procedures for subjects who have been identified as being at high risk for developing oral cancer.

Ahmed and Mahgoub (2007), published that in the Sudan snuff locally known as Toombak, Furthermore, study by Idris et al. (1994), revealed that Toombak contains at least 100-fold higher concentration of tobacco-specific N-amine TSNA.

(Ahmed and Babiker, 2009) established that, chemical carcinogenesis is a prolonged process and progressed with increasing of exposure, the duration of tobacco exposure seemed to have effects on increasing the risk of oral cancer.
Chapter Five

Conclusion and Recommendations

5.1 Conclusions

Oral exfoliative cytology using Pap and H&E stain is useful in evaluation of epithelial atypia that is frequently encountered in pre-malignant and early malignant oral lesions.

The results of AgNor count like the cytological diagnosis of H&E and Pap stain that the normal count found in short snuffing duration and the abnormal count appears with the increasing of snuffing duration, the difference was statistically significant ($P$ value 0.01).

Increasing of snuffing population with increasing of their ages.

The prolonged exposure to toombak is significant factor in increasing cellular atypia.

5.2 Recommendations

- There is a need to standardize the cytological proliferative marker methods to provide rapid, inexpensive, simple and applicable screening test for subjects who have been identified as being at high risk for developing oral cancer, therefore the micronuclei frequency and AgNOR counts recommended to be used with Pap and H&E stain.

- Establishing effective Health education program to educate the population about the health problems associated with using of tobacco.

- Further studies using another markers to increase the quality of the diagnosis.
Chapter Six

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