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Immunohistochemical Expression of Octamer-binding protein-4, in Oral Epithelial Dysplasia Induced in Experimental Rats (Animal Study)

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Abstract: *Octamer-binding protein-4 (OCT4) is an embryonic stem cell marker essential for the maintenance of embryonic stem cell (ESC) pluripotency and was believed to be crucial for progression of various human malignances (Jerabek et al., 2014). However, for the best of our knowledge comprehensive investigation of the role of OCT4 in oral epithelial dysplasia (OED) has not been carried out. That is why; it seemed interesting to study expression of OCT4 in different grades of OED, to point out new marker that could be used for early detection of molecular changes prior to malignant transformation. A total of 84 adult male albino rats were selected. Our results showed a highly significant difference in area percentage of OCT4immunoexpression among the different experimental groups after 6 and 9 weeks of experiment. We have concluded the possible role of OCT4 in early molecular events of tumorigenesis. Therefore, it may represent a promising early biomarker for carcinogenesis. In addition, the prognostic value of OCT4 in prediction of prognosis in OED was elucidated in the current work.*

Keywords: *OSCC, OED, OCT4,IHC*

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I. Introduction

Oral squamous cell carcinoma (OSCC) accounts for about 90% of all oral cancers. Generally, the mortality rate of oral cancer has increased seven times in the last 50 years in-spite of millions worth research going on globally (Yu et al., 2016). Despite several improvements in therapy routines of this disease, unfortunately, only 50% of OSCC cases have shown 5-year survival rate (Ribeiro et al., 2016). This could be attributed to the detection of OSCC at late stages when the cancer has advanced and finally resulted in poor prognosis and survival. Therefore, early disease detection was imperative as it could result in more effective treatment with greater results (Warnakulasuriya et al., 2008).

Although, OSCC might arise de novo in clinically normal mucosa, it was mostly preceded by oral potentially malignant disorders (OPMD). Most of these lesions were diagnosed histopathologically as epithelial dysplasia (Kujan et al., 2016). OED is a histopathological diagnosis made for clinical mucosal lesions which have shown a rate of progression to OSCC, reportedly ranging from 1% to 36% (Kujan et al., 2016). In medical terminology, the word dysplasia means an abnormality in development, however histomorphologically, it expresses cellular and structural changes in the epithelium (Sadiq et al. 2015).

In the last decade, several studies have been conducted to develop biomarkers for identification of dysplasia and prediction of its malignant potential (Pitiyage et al., 2009). Molecular biological markers have been suggested to be of value in the early detection of mucosal changes, diagnosis and prognostic evaluation of OED. These markers would refine our ability in predicting the biologic course of oral cancer and distinguishing individuals at low and/or high risk of oral cancer development (Shah &Kaur, 2014).

In the past few years, many studies shed the light on OCT4. It is an embryonic stem cell marker essential for the maintenance of ESCpluripotency and was believed to be crucial for progression of various human malignances (Jerabek et al., 2014). However, for the best of our knowledge, in English retratures comprehensive investigation of the role of OCT4 in OED has not been carried out. That is why; it seemed interesting to study expression of OCT4 in different grades of OED, as this would enhance to our knowledge about the molecular changes that would take place in OED, prior to frank malignant transformation.

II. Material And Methods

1.A total of 84 adult male albino rats, maintained in the animal house as an inbred colony (obtained from the Faculty of Medicine, Cairo University, Egypt) were used in this study. Rats with an age range of 3 to 4 months and those with a weight range of 130 to200 gm were selected for carrying out this experiment

Study Design: An experimental study

Study Location: Animal house of the Faculty of Medicine, Cairo University, Egypt

Sample size: 84 adult male albino rats

Sample size calculation: A total sample size of 66 rats (22 in each of the 3 groups) will be sufficient to detect a 0.52 effect size with a power of 80% and 5% significant level. The number is increased to a total of 84 to allow for the use of non-parametric test. The sample is further increased to 84 (28 in each of the 3 groups) to allow for about 25% losses. The sample size was calculated using G*power program (University of Dusseldorf, Dusseldorf, Germany).

Subjects & selection method:

• Numbers from 1 to 84 were written on folded papers that were placed in opaque sealed envelopes.

• Matching of threats with the numbers was done blindly through the technician in charge at the animal house.

• Each rat was attached to its number till the end, then the numbers were opened and threats were allocated in their groups according to the program's recommendations

Rats were divided into two groups as following:

Group A: Control group.

Group B: Experimental group

The control group comprised 28 rats, which didn't receive any induction of carcinogenesis. On the other hand, the experimental group comprised 56 rats were anesthetized by ketamine 80-100 mg/kg intraperitonealy (IP) and xylazine 10-12.5 mg/kg (IP). The buccal mucosa was painted once with a number 3 camel hairbrushes according to The INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE GUIDLINES**(IACUC guidelines 2014**). Then, the rats had their buccal mucosa painted (topically) with DMBA and formaldehyde, 0.5% DMBA in acetone 3 days/week, and after 9 days 10% formaldehyde/water was used side by side with DMBA throughout the study period (6 and 9 weeks).

Inclusion criteria:

1. Adult rats
2. White colored rats
3. Male rats
4. Rats with weight from 130 to200 gm
5. Healthy rats

Exclusion criteria:

1. Female rats
2. Rats with oral or systemic disorders

1. Experimental animals

A total of 84 adult male Albino Rats, maintained in the Animal House as an inbred colony. They were obtained from the Faculty of Medicine, Cairo University. Rats with an age range of 3 to 4 months, and a weight range of 100 to 200 mg were selected for carrying out the experiment.

2. Positive control specimens

Three specime

ns of seminoma (a germ cell tumor of the testicle) were obtained from National Center Institute, Cairo University, to be used as positive control specimens for IHC expression of OCT4 antibody as reported by **Chen et al. (2009).**

3. Anti-OCT4 antibody

Anti-OCT4 mouse monoclonal antibody ready to use was purchased from Dako Company*

4. Universal Kit

The immunodetection kit: Mouse and Rabbit Specific HRP/DAB (ABC) detection immunohistochemical kit (ab64264), which comprised the following reagents and materials:

1. Peroxidase block
2. Serum block (5% normal goat serum)
3. Biotinylated secondary antibody
4. Horseradish peroxidase streptavidin reagent
5. Peroxidase substrate
6. Tri buffer saline (TBS)

7. DAB chromogen

5. Drugs:

For induction of epithelial dysplasia two drugs were used:

A. DMBA:

The full name is 7,12-dimethylbenz[a]anthracene is a carcinogenic agent (an initiator). It was purchased from **Sigma Chemical Company*, USA**

B. 10% Formaldehyde

It is the promoter was purchased from **Horizon Chemical Company****

- **Procedure:**

1-Rats were anesthetized intraperitoneally using ketamine 75-100 mg/kg combined with 10 mg/kg xylazine (Wellington et al., (2013).

2- 0.5% DMBA in acetone was painted on the right buccal mucosa every alternate day.

3- The rats were placed in cages without food and water for one hour to allow the drug to stay in direct contact with the buccal mucosa as long as possible.

4- Then after 2 weeks, 10 % formaldehyde was used side by side with DMBA every alternate day throughout the study period till the time of euthanize.

- **Animal Euthanasia**

The rats were euthanized by an overdose of barbiturate anesthetic solution intraperitoneal (100 mg/kg) according to the **Research Animal Guidelines of Euthanasia University of Minnesota (2009).** Half No. of each group was euthanized after 6 and 9 weeks, respectively **(Kasem et al., 2014).**

- **Tissue processing:**

Cheek was immediately dissected and fixed in 10% buffered formalin for 24 hours, dehydrated in ascending grades of ethyl alcohol, cleared in xylene and embedded in paraffin. Sections of 5 microns thickness were cut from paraffin blocks and mounted on glass slides for Hematoxylin and Eosin (H&E) staining and subsequent examination under the ordinary light microscope for detection of dysplastic changes and identify low and high risk groups according to Kujan et al. (2006).

Their scheme was based on the same architectural and cytological criteria reported by the WHO classification (2005). They graded the lesions into either, low risk dysplasia or high risk dysplasia based on scoring the features. All the specimens were examined by two pathologists to avoid bias.

*** Dako, Life Trade – Egypt 9, Al-Obour Bldgs. Salah Salem Rd. Heliopolis Cairo.**

***Sigma-Aldrich-PO Box 14508-St. Louis, MO 63178-USA. Phone:1-800-325-3010-Fax: 1-800-325-5052-Email: DeuExport@sial.com**

****Horizon Chemical for special chemicals Company-10 repeated- MostafalotfyManfalotyst- Stanly, Alexandria, Egypt-http://www.horizon-chemical.com eg1064108994.trustpass.alibaba.com**

Statistical Analysis

Data from the three groups were collected, tabulated and statistically analyzed and illustrated in tables and figures. The data were summarized as means and standard deviations. Collected data were analyzed using a SPSS statistical package*. One way ANOVA followed by pair-wise Tukey's post-hoc tests were performed to detect significance between groups. The P values were considered as follows:

• P value ≥ 0.05, not significant. • P value ≤ 0.05, significant. • P value ≤ 0.001, highly significant

III. Results

I. <u>Clinical Findings:</u>

The only change detected in the experimental rats at 6th week interval was the development of yellowish white plaques on the buccal mucosae of about 40% of them (Fig. 1). On the other hand, at 9^{th} week interval, 8 out of 26 experimental rats developed swellings in their buccal mucosae (Fig. 2). Wrinkled yellowish white plaques were also observed in about 35% of rats (9/26). About 12% of the rats showed extension of the changes to their eyes on the same side of DMBA painting. In spite of all these alterations, the remaining experimental rats didn't show obvious changes. None of the experimental rats showed severe weakness or loss of appetite throughout the experiment. The death rate was inconsiderable, where only 3 rats died, one before 6^{th} week interval and 2 after it.

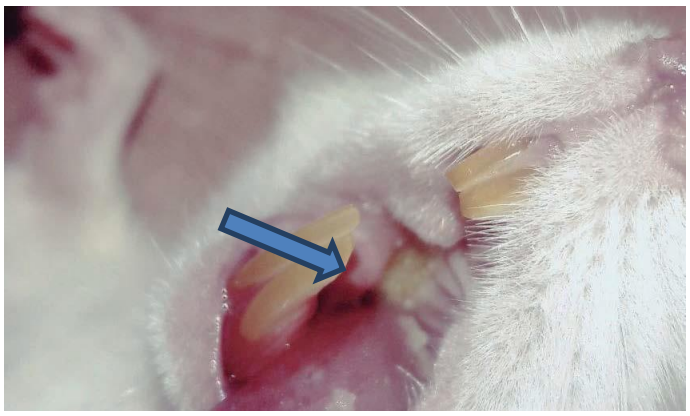

Fig. (1): A photograph showing yellowish white plaque at the buccal mucosa of an experimental rat at 6th week interval

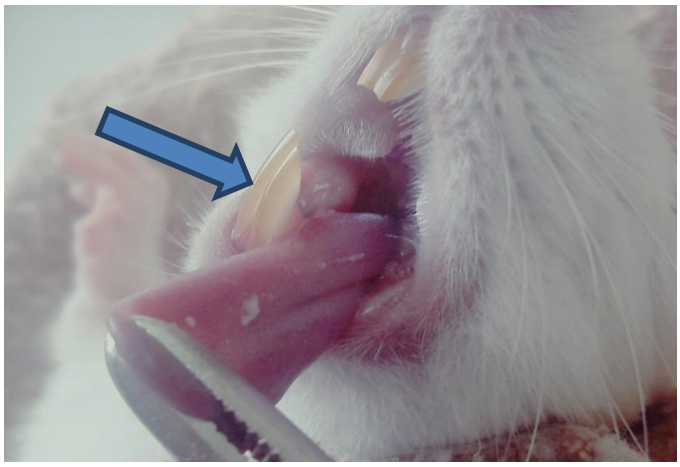

Fig. (2): A photograph showing a swelling in the baccul mucosa of an experimental rat at 9th week interval

II. <u>Histopathological Findings:</u>

A. <u>Control group</u>

Examination of H&E stained sections of control group showed normal histologic appearance of the buccal mucosa of experimental animals. Keratinized stratified squamous epithelium with 6-8 cell layers thickness was detected. Basal cell layer, prickle cell layers as well as granular cell layers were identified. The underlying connective tissue showed collagen fibers and blood vessels (Figs 3-4).

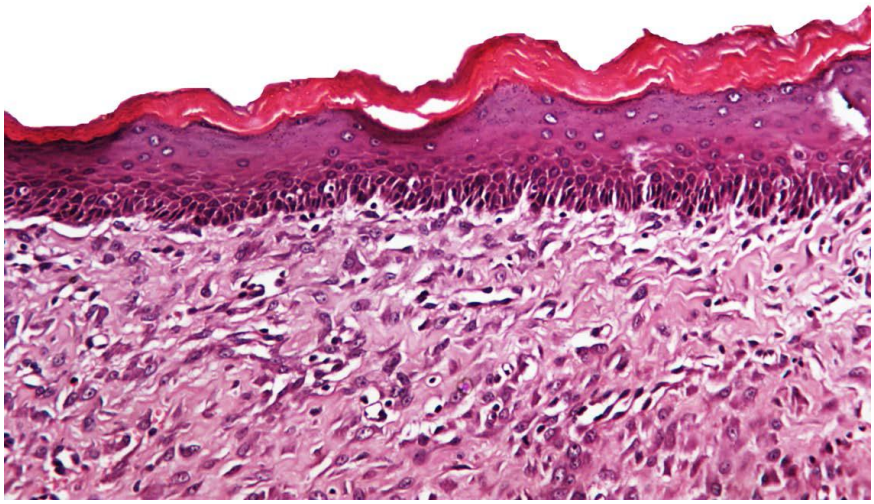

Fig (3): A photomicrograph of normal buccal mucosa of a rat in the control group revealing normal keratinized stratified squamous epithelium overlying normal connective tissue (H&E x200).

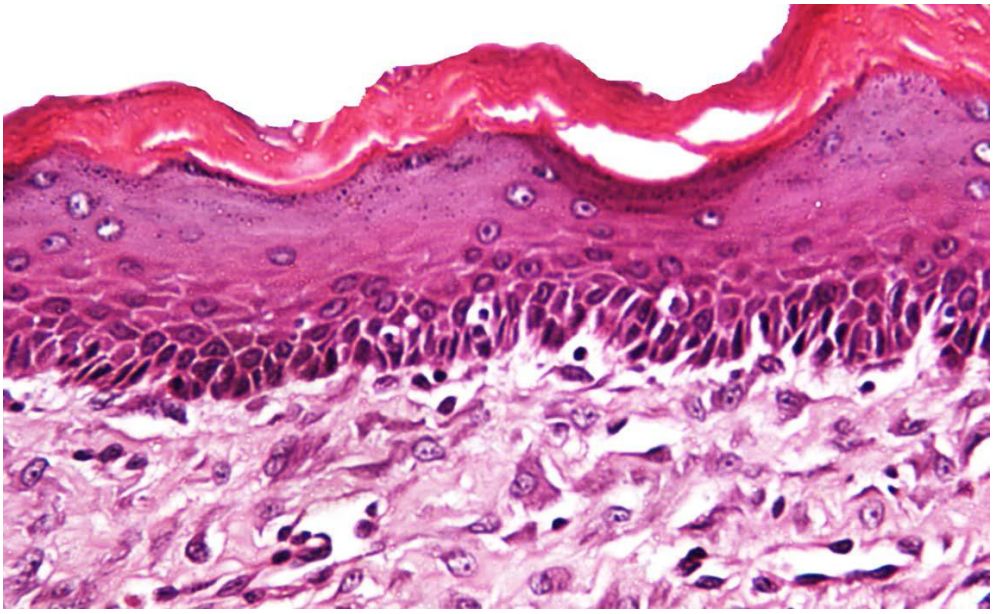

Fig (4): High magnification of previous photomicrograph revealing 6-8 layers of normal keratinized stratified squamous epithelium overlying normal connective tissue (H&E x400).

B.Experimental Groups

About 80% of specimens obtained from sacrificed rats at the 6 week interval showed less than 4 architectural changes and less than 5 cytological changes. That's why, they were considered as low risk group. 6 specimens were excluded as they didn't show any obvious epithelial changes. On the other hand, about 77% of the specimens obtained at the 9 week interval showed about 4 or more than architectural changes and 5 cytological changes. So they were considered as high risk group. The remaining of the specimens was included in the low risk group. Accordingly, the low risk group comprised 27 cases, whereas the high risk group comprised 20 cases.

- **Low risk OED group**

100% of the cases comprising the Low risk OED group showed hyperplastic epithelium with elongated reteridges. Clubbing and acanthosis were also seen in most of the cases. The main architectural changes observed in this group were consistingof loss of basal cell polarity, drop shaped rete ridges and increased number of mitotic figures in basal and parabasal cell layers of the epithelium. Moreover, many cytological changes were detected in the examined cases, and were localized to the basal and parabasal cell layers. These dysplastic criteria included cellular and nuclear pleomorphism, karyomegaly, prominent nucleoli, hyperchromatism and few abnormal mitotic figures (Figs.5-7).

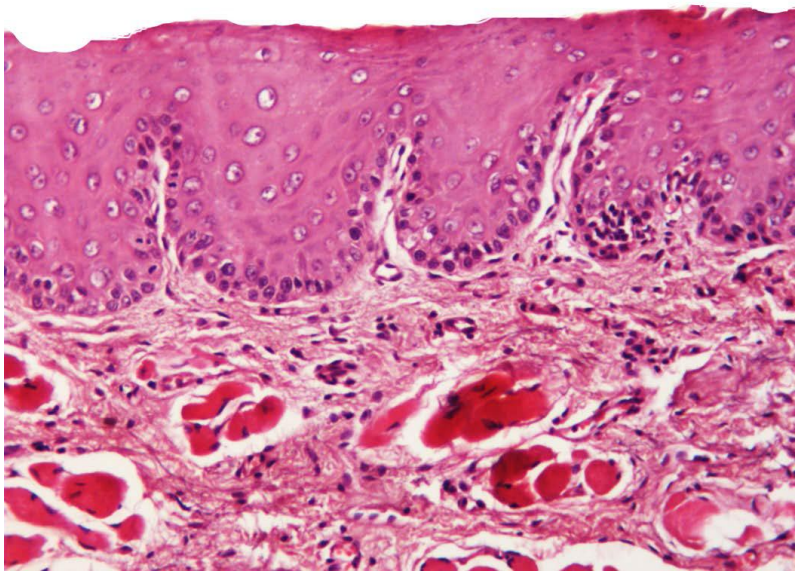

Fig (5): Photomicrograph of low risk OED group showing numerous clubbing of the rete ridges and basal hyperplasia (H&E, x200).

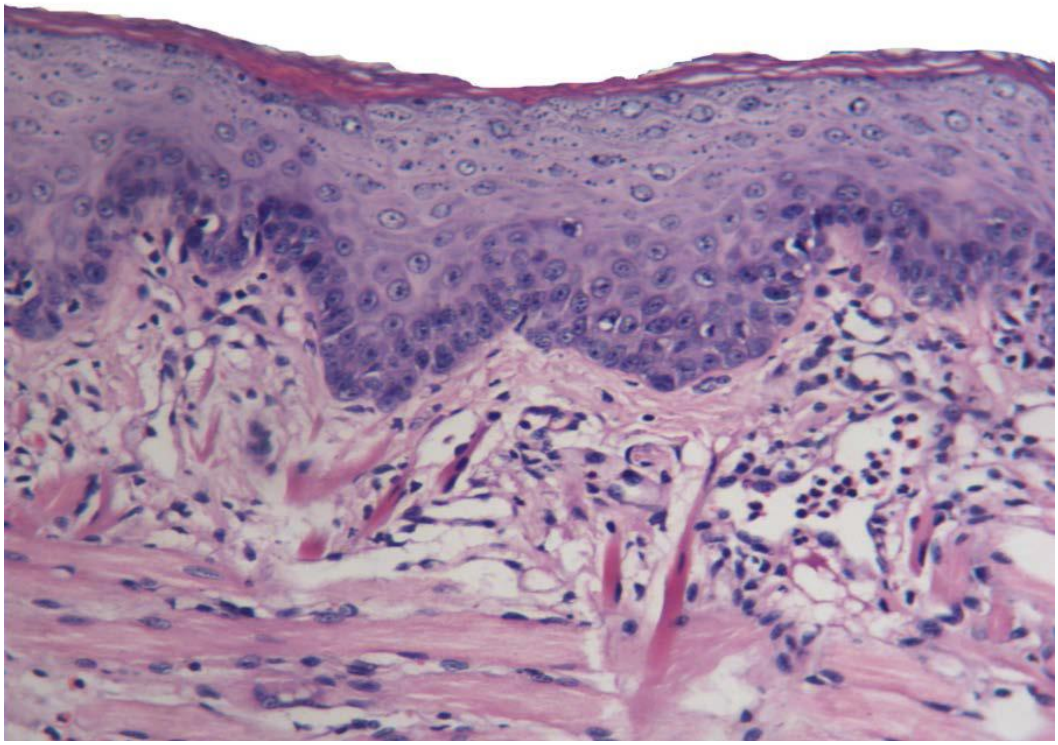

Fig (6): Photomicrograph of low risk OED group showing numerous clubbing of the rete ridges and basal hyperplasia (H&E, x200).

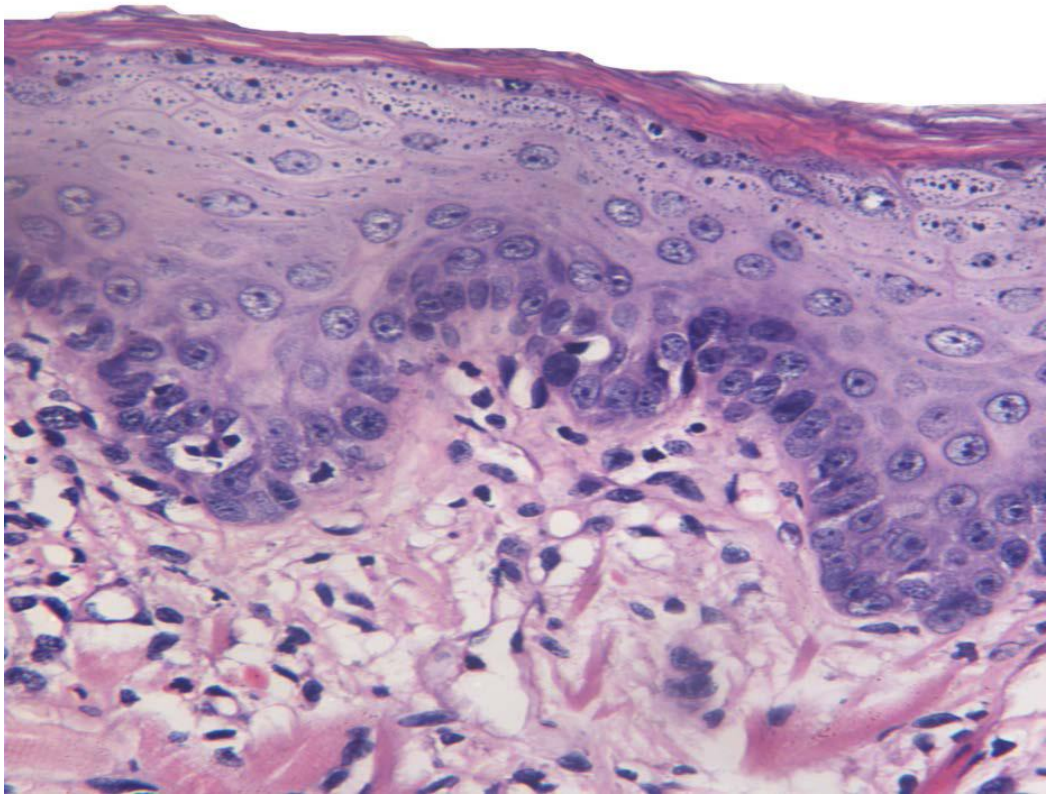

Fig (7): Higher magnification of the previous case showing prominent nucleoli (black arrow), and hyperchromatism (yellow arrows) (H&E, x400)

- **High risk OED group**

H&E stained sections of high risk OED group revealed epithelial hyperplasia and elongated retepegs in all the examined cases. Thickness of epithelium was dramatically increased, exceeding 20 cell layers. Regarding the

architectural changes, many of them were observed such as irregular epithelial stratification, abnormally superficial mitoses, cell nest and keratin pearls formation within reteridges. In addition, similar to low risk OED group, loss of polarity of basal cells, drop shaped reteridges and increased number of mitotic figures were also seen in almost all the cases. Unlike low risk OED group, numerous cytological changes were observed throughout the epithelium or at least extending beyond 1/2 of the epithelial thickening. Among these epithelial alterations, cellular and nuclear pleomorphism, karyomegaly, prominent nucleoli, hyperchromatism, increased nuclear/cytoplasmic ration and many abnormal mitotic figures were easily detected (Figs.8-11).

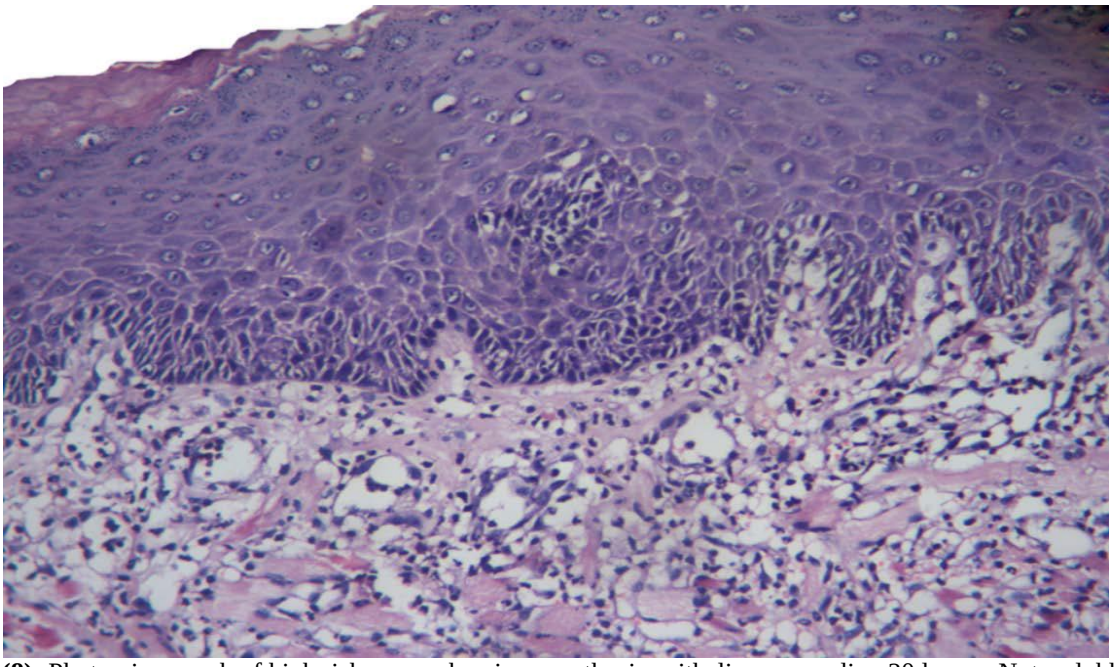

Fig (8): Photomicrograph of high risk group showing acanthosis epithelium exceeding 20 layers. Note, clubbing of rete ridges (H&E, x200).

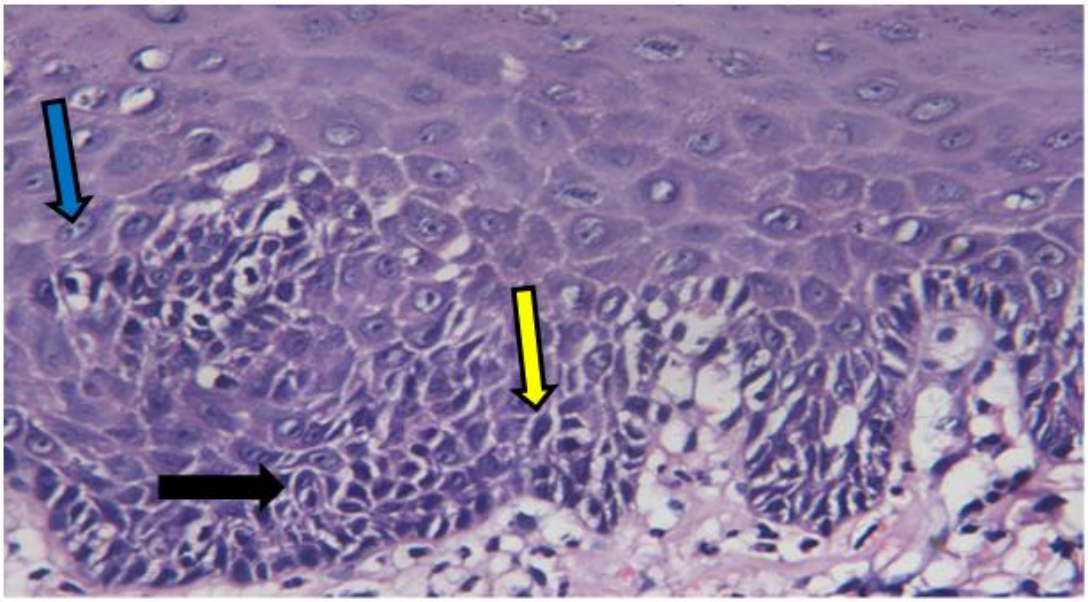

Fig (9): High magnification of previous photomicrograph revealing cellular and nuclear pleomorphism: mitotic figure (black arrow), prominent nucleoli (blue arrow) and hyperchromatism (yellow arrow) are also seen (H&E x400).

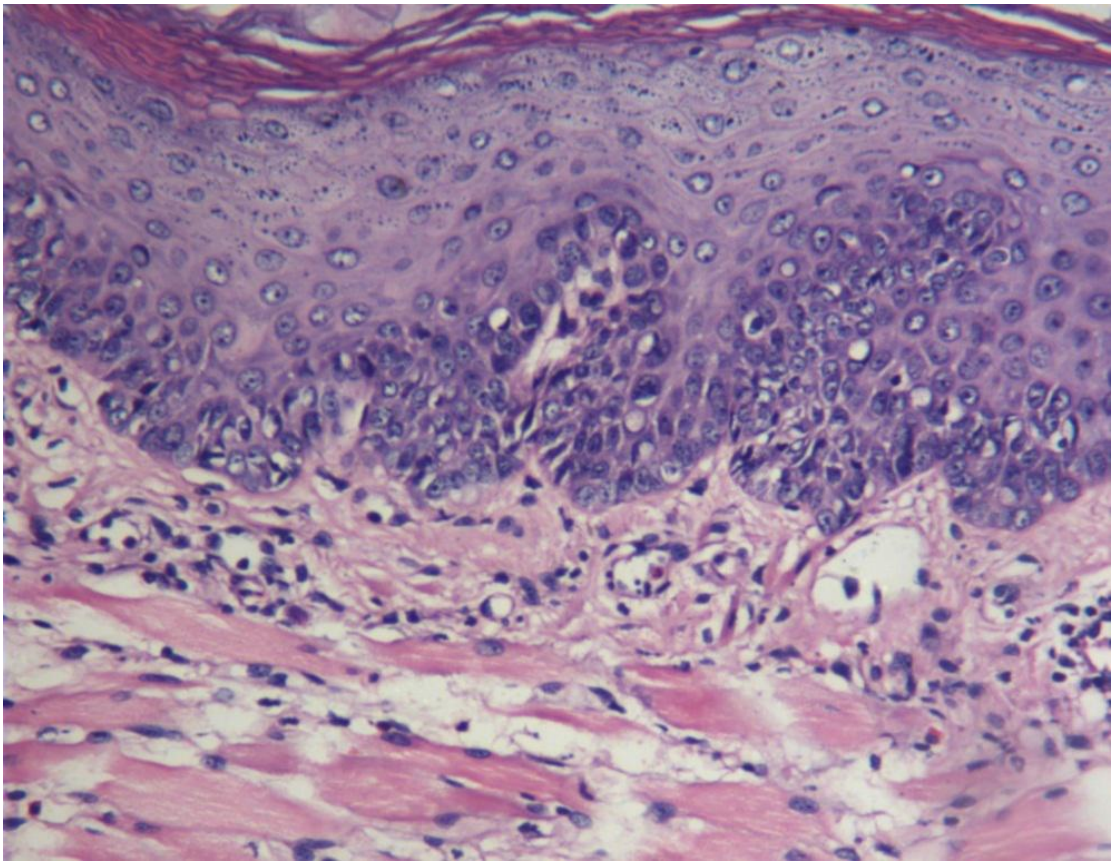

Fig (10): Photomicrograph of high risk group showing loss of polarity and basal hyperplasia (H&E, x200).

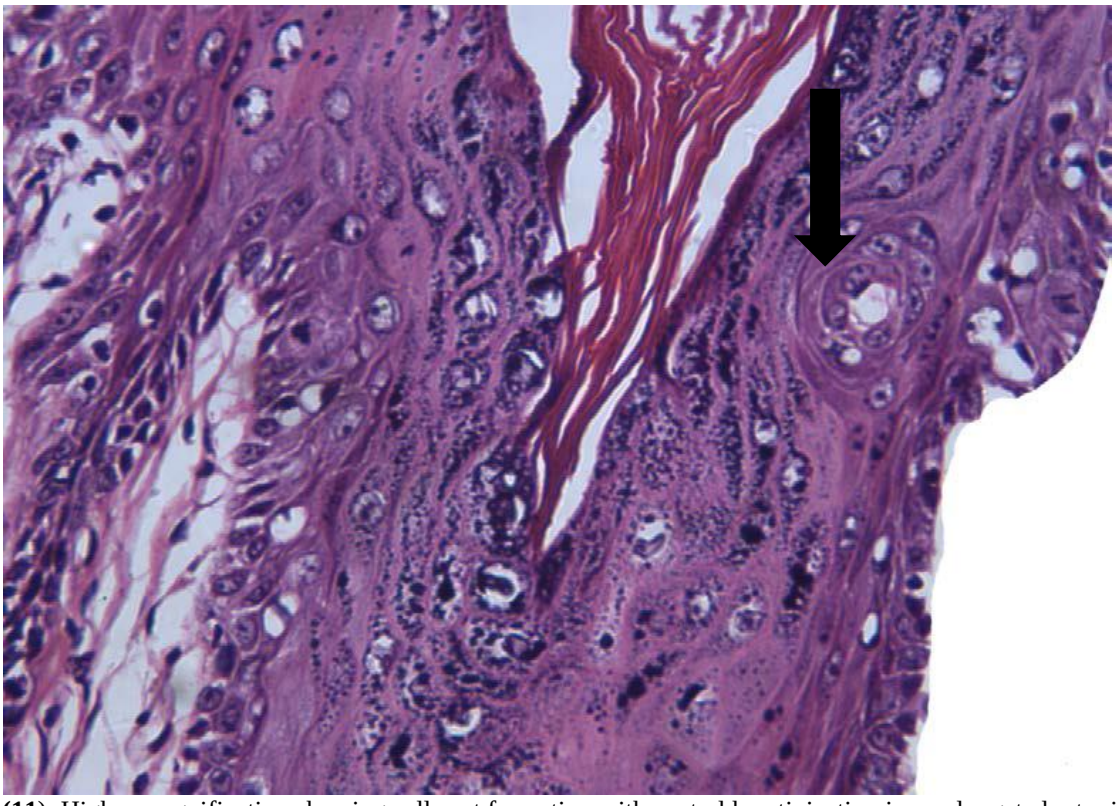

Fig (11): Higher magnification showing cell nest formation with central keratinization in an elongated rete ridge (black arrow) (H&E, x400).

III. Immunohistochemical Finding of OCT4:

A. Control Groups

All the examined seminoma cases, which were used as positive control showed positive nuclear OCT4 immunoreactivity in most of the tumor cells. On the contrary, the negative control specimens were totally negative due to omission of the OCT4 antibody (Figs 12 and13).

All the cases of control group (100%) demonstrated negative OCT4 immunoreactivity throughout epithelium, with no evidence of any immunoreaction (Figs 14 & 15).

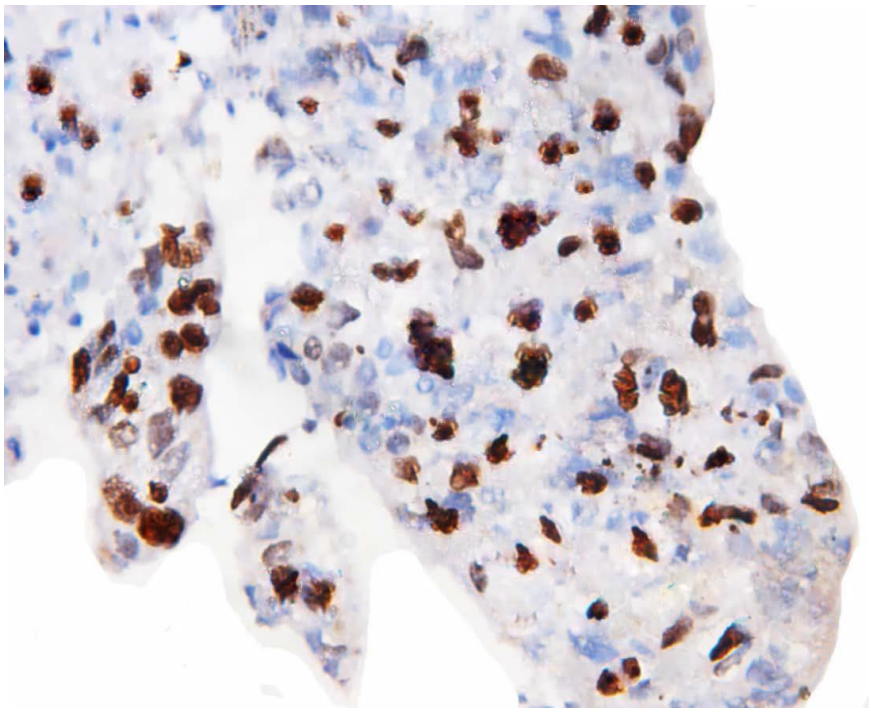

Fig (12): A photomicrograph of seminoma (positive control) showing nuclear and cytoplasmic immunoreactivity for OCT4 in most the tumor cells (anti OCT4 antibody x 400).

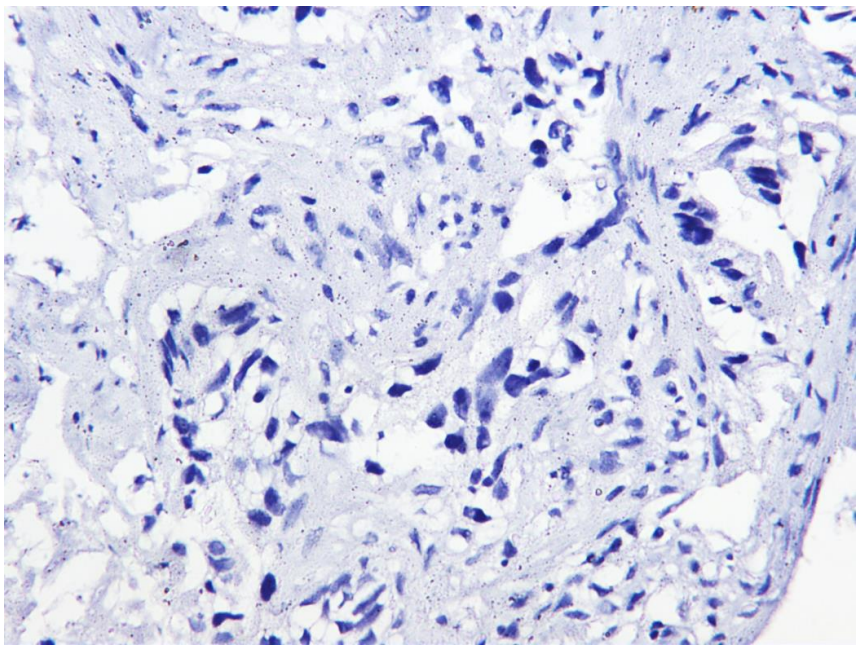

Fig (13): A photomicrograph of the negative control specimen (seminoma) showing negative OCT4 immunostain (anti OCT4 antibody x 400).

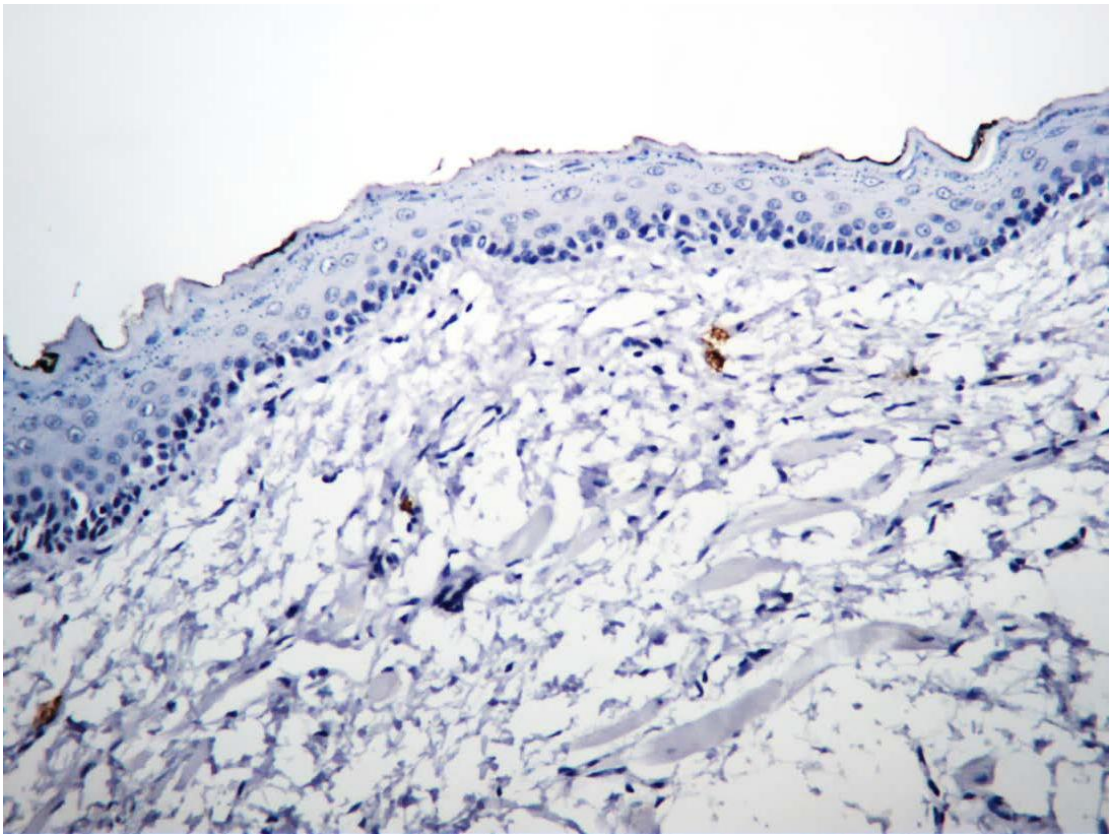

Fig (14): A photomicrograph of normal buccal mucosa of the control group showing negative expression of OCT4 in epithelium (anti OCT4 antibody x 200).

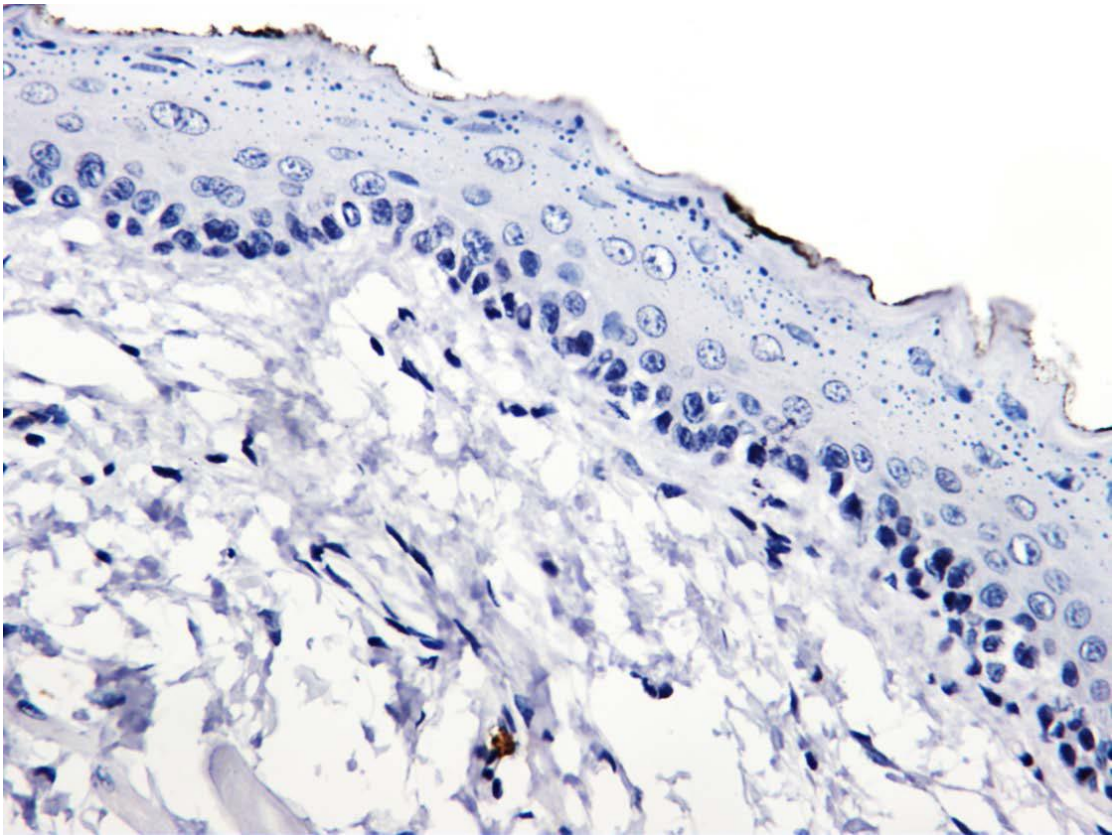

Fig (15): Higher magnification of the previous case showing negative immunostaining of OCT4 in all layers of epithelium (anti OCT4 antibody x 400).

B. Experimental Groups

Low risk OED group

About 2/3 of the examined low risk OED cases showed positive OCT4 immunoreactivity, they showed one of 2 patterns. Eight out of 27 cases showed localization of the immunoreaction to the basal cell layer, and suprabasal cells were less commonly involved. OCT4 expression was chiefly nuclear or perinuclear (Figs 16-18). On the other hand, the other pattern demonstrated extension of OCT4 immunoexpression to nearly ½ the epithelial thickness, with stronger reaction in the basal cells (10/27). Unlike the basal cells, prickle cells mainly exhibited cytoplasmic and membranous expression (Figs 19-20). Contrarily, 9 out of 27 cases showed negative OCT4 immunoexpression.

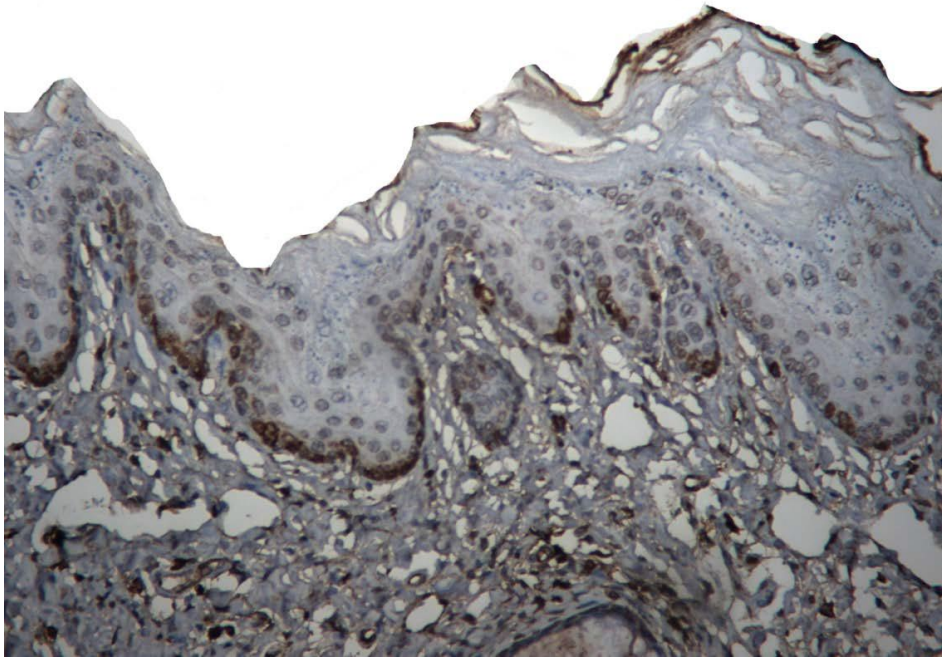

Fig (16): A Photomicrograph of low risk OED showing nuclear immunoexpression of OCT4 localized to the basal cell layer (anti OCT4 antibody x 200).

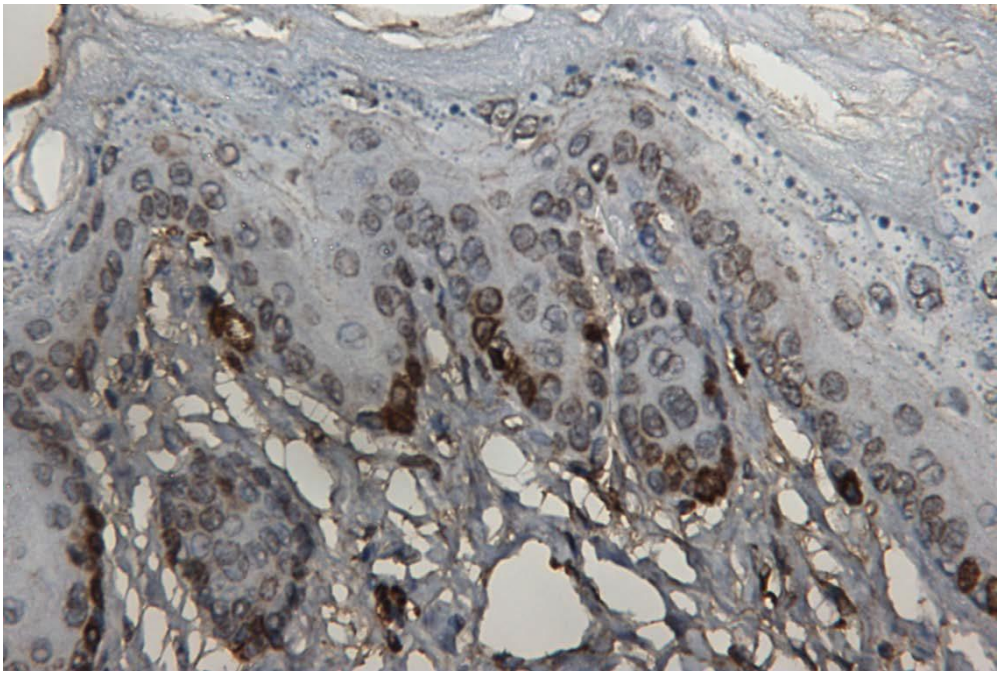

Fig (17): Higher magnification of the previous case showing nuclear and perinuclear expression of OCT4 in most of cells of the basal layer (anti OCT4 antibody x 400).

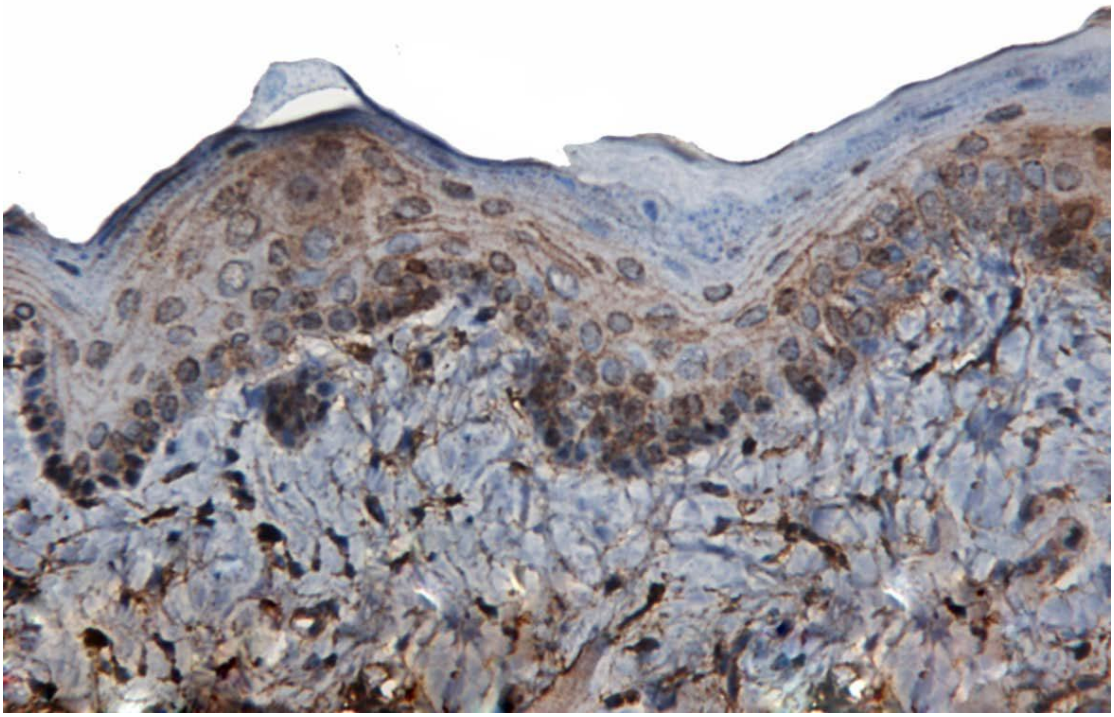

Fig (18): A Photomicrograph of low risk OED showing weak cytoplasmic and membranous expression of OCT4 at the basal and suprabasal cell layers, and extending to half the epithelial thickness (anti OCT4 antibody x 200)

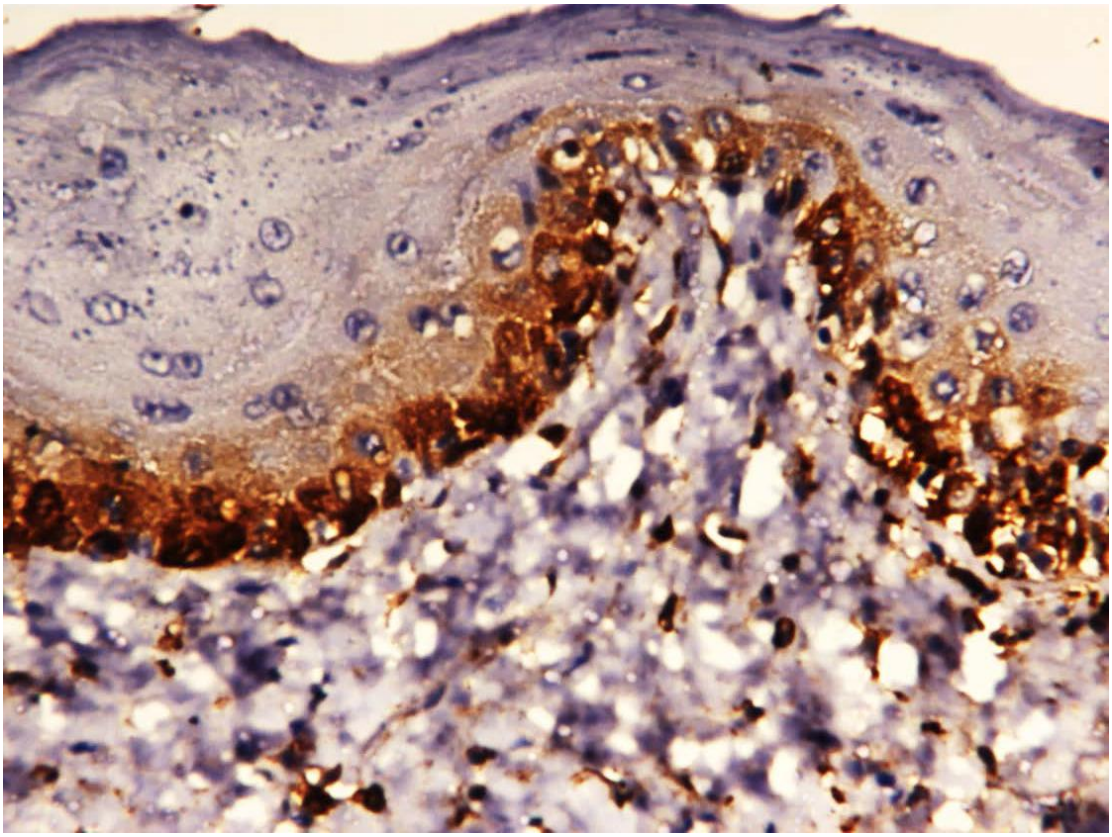

Fig (19): A Photomicrograph of low risk OED showing cytoplasmic expression of OCT4 in basal and suprabasal cells. Note, strong nuclear OCT4 immunostain localized to the basal cell layer (anti OCT4 antibody x 400).

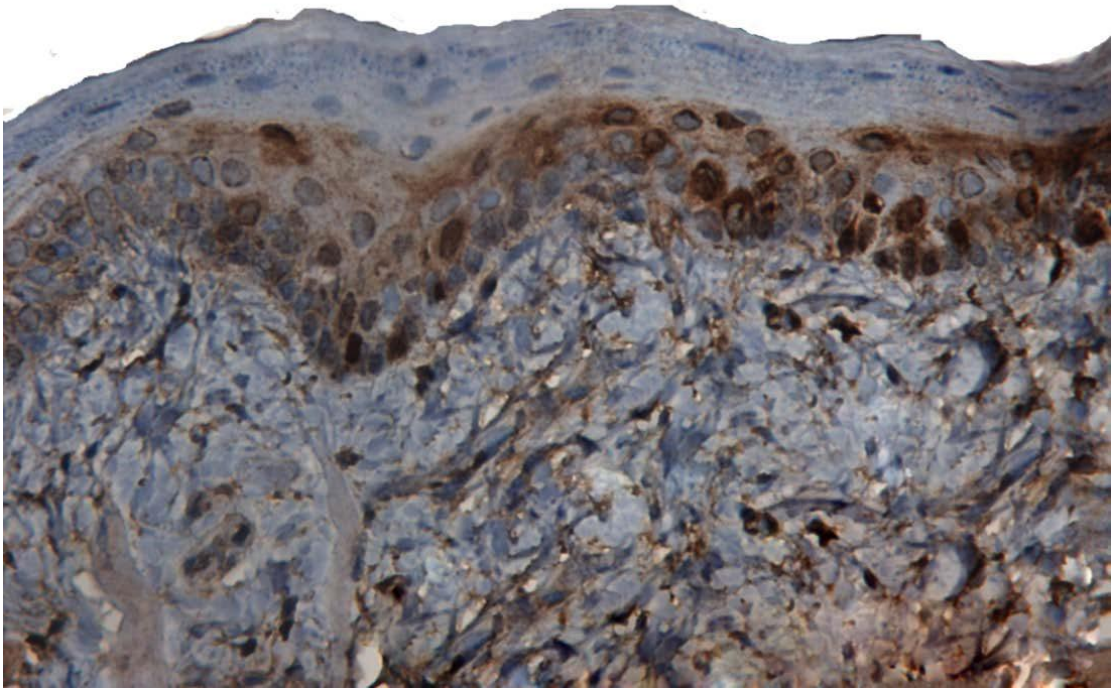

Fig (20): A Photomicrograph of low risk OED showing diffuse cytoplasmic immunoexpression of OCT4 in basal and prickle cell layers. Strong nuclear reaction is seen in many basal and parabasal cells. Note, perinuclear OCT4 immunostaining (arrow) (anti OCT4 antibody x 200).

High risk OED group

About 80% of the examined high risk OED cases showed positive OCT4 immunoreactivity. On the other hand, only 4 cases showed negative expression. OCT4 immunoreaction was consistent and reproducible in all the cases, where they showed strong homogenous cytoplasmic and membranous reaction throughout the epithelial thickness. However, stronger nuclear and perinuclearimmunoreactivity was localized to the basal cell layer (Figs 21-24).

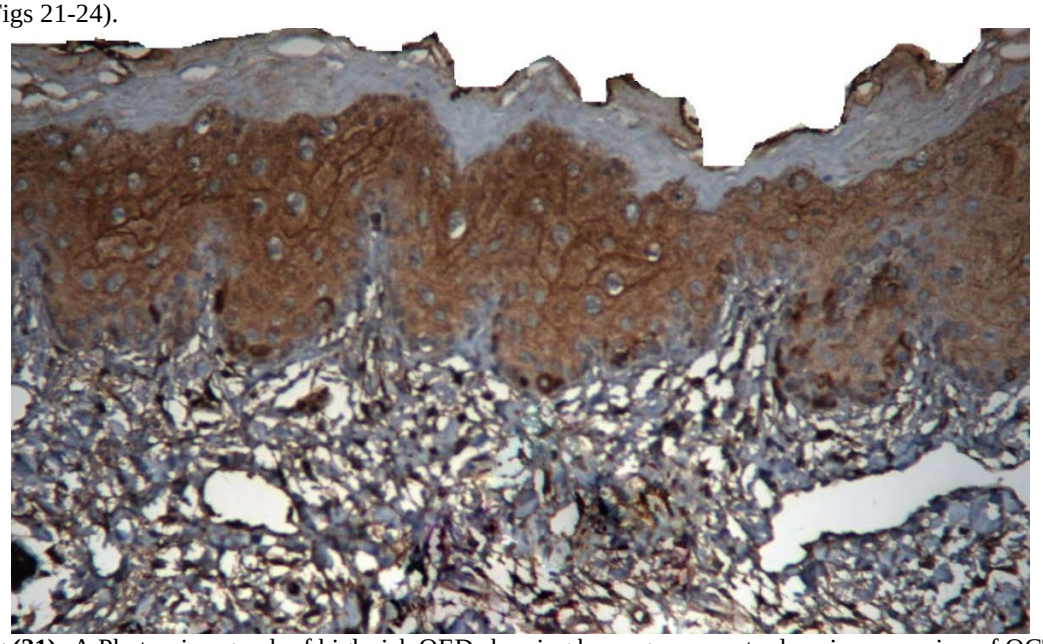

Fig (21): A Photomicrograph of high risk OED showing homogenous cytoplasmic expression of OCT4 throughout the epithelium (anti OCT4 antibody x 200).

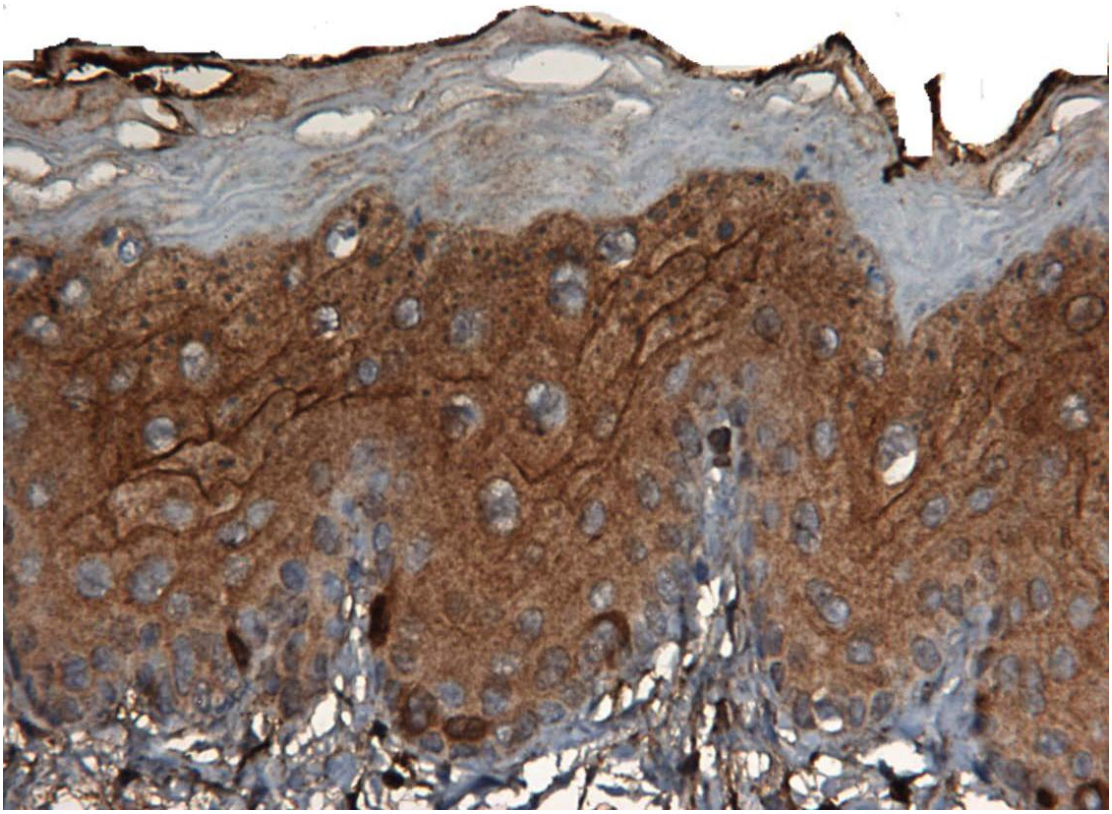

Fig (22): High magnification of previous case showing nuclear and perinuclear OCT4 expression in some basal cells) (anti OCT4 antibody x 400).

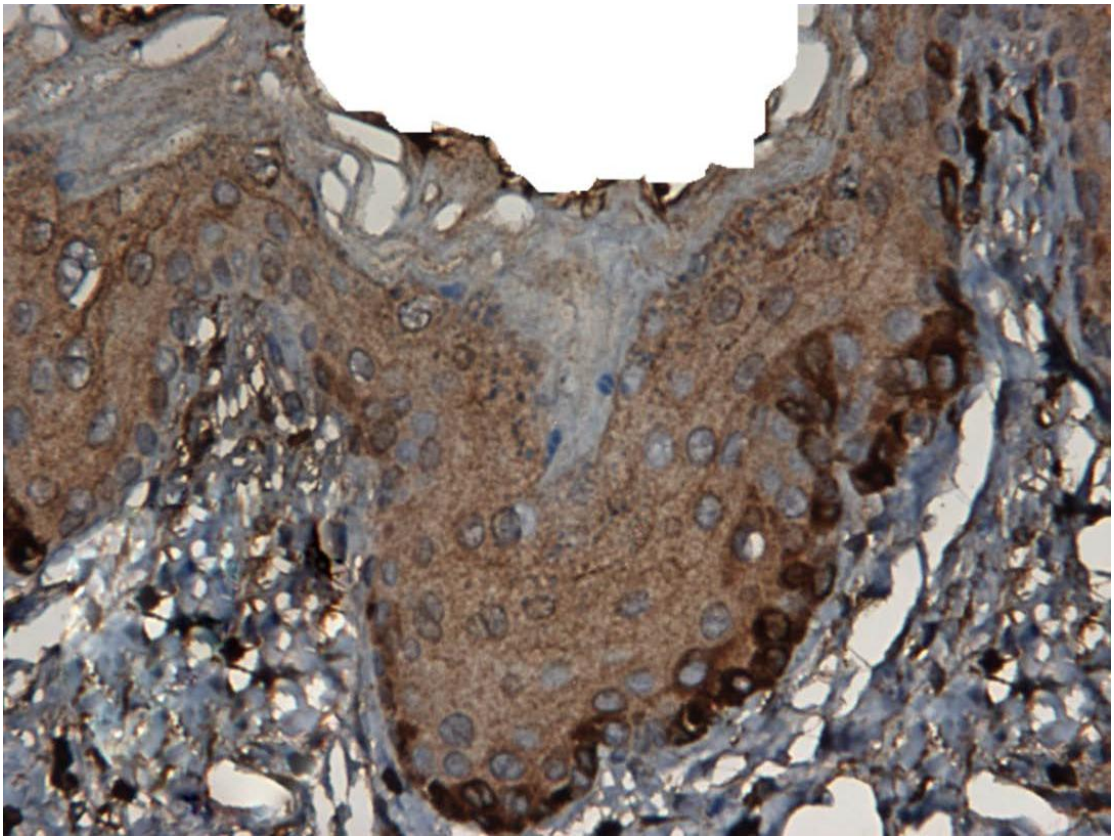

Fig (23): High magnification howing nuclear and perinuclear reaction to OCT4 in most of the cells in basal layer (anti OCT4 antibody x 400).

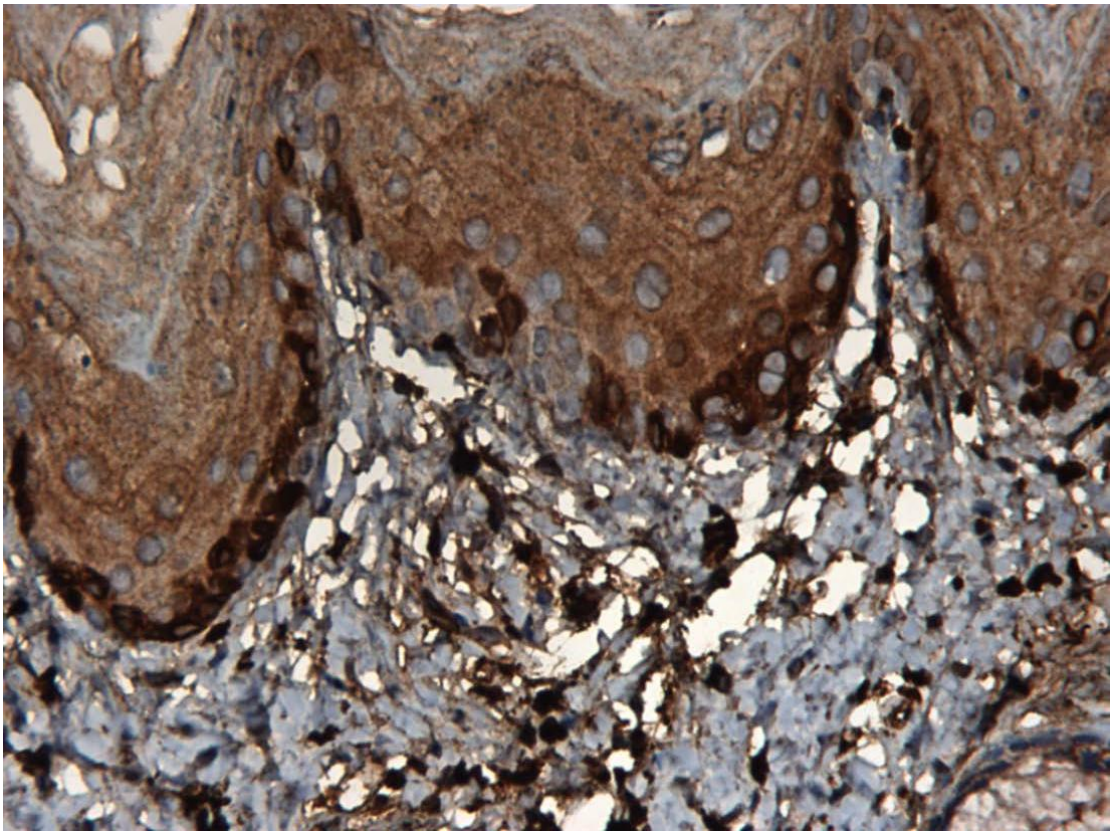

Fig (24): High magnification showing stronger OCT4 immunostaining localized to the nuclei of almost all basal cells (anti OCT4 antibody x 400).

Statistical Analysis

Area percentages of OCT4 immuno-expression

A highly significant difference ($p < 0.0001$) was obtained on comparing the area percentage of OCT4 immunoexpression among the experimental groups. It increased gradually from normal control group, which showed the lowest values, passing by the low risk OED group and finally reached highest values in the high risk group. All the pair-wise comparisons between each 2 groups showed a significant difference. On comparing the control group versus the low risk OED group, the results recorded statistically significant ($P = 0.03$) difference. Moreover, comparing the high risk OED group versus the control group or the low risk group, the results recorded a highly statistically significant ($P<0.0001$) difference. All the values were represented in tables 1 & 2 and graphically drawn in bar chart (Fig 25).

Table (1): Area percentage of OCT4 immunoexpression among the experimental groups

Groups	**Control group**	**Low risk group**	**High risk group**
Mean±SD	51.67±2.91	11.36±0.74	3.12± 0.82
P value		<0.0001	

Table (2): Pair wise comparisons between each 2 groups

Groups	**Significant? (P <0.05)**
Control Versus Low risk OED groups	Yes*
Control Versus High risk OED groups	Yes**
Low risk OED Versus High risk OED groups	Yes**

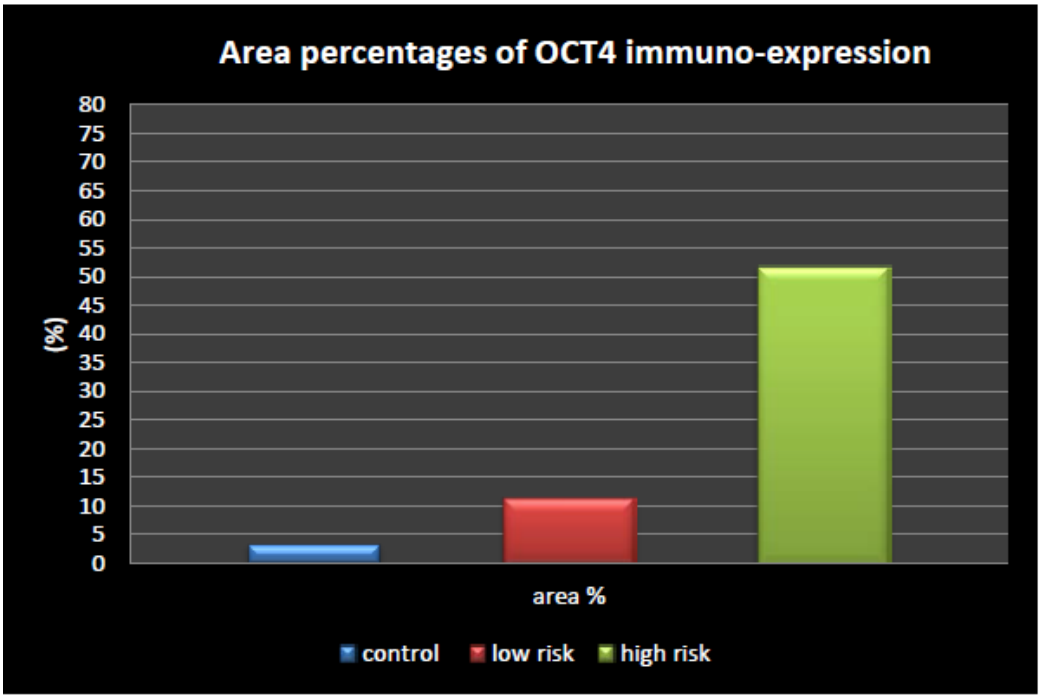

Figure (25): Area percentages of OCT4 immunoexpression among the experimental groups.

IV. Discussion

OSCC, one of the ten most common cancers worldwide, is a debilitating disease often associated with detrimental impact on quality of life. Despite the accomplishments already achieved concerning OSCC diagnosis and therapy, mortality and morbidity rates are still exceedingly high. However, early detection and treatment are key factors, carrying great hope for cancer patients. They are able to limit advances in the cancer outcome, as they may reduce the side effects of the disease and improve prognosis (Gillenwater et al., 2006; Zygogianni et al., 2011and Mahakian et al., 2014).

The development of oral cancer is a multistep process, which includes stepwise accumulations of mutations resulting in transition of normal mucosa to dysplasia and then invasive carcinoma over time (Gillenwater et al., 2006 and Zygogianni et al. 2011). The presence of OED was accepted as one of the most important predictors of malignant development in OPMD (Shirani, 2014 and Watanabe et al., 2015). The nonspecific clinical appearance of these dysplastic lesions in the oral cavity, and its subjective histopathologic diagnosis are true challenges, which have further emphasized a compelling need to develop effective methods for earlier detection and identification of OED lesions with higher potential for malignant transformation (Ribeiro et al., 2016).

Many studies showed that early detection, diagnosis, and treatment of precancerous changes would be imperative to increase survival and improve functional outcomes for persons at risk to develop OSCC. Moreover, recognition of these lesions with proper biomarkercorrelation might circumvent tumor progression, if properly diagnosed and treated (Safadi et al., 2010 and Dutra, 2017).Unfortunately up to date, there are no currently accepted markers that reliably predict the tendency for malignant transformation in epithelial precursor lesions. Thus, unraveling the complex cascade of molecular changes comprehensively participating in this process will further help in understanding the pathogenesis of oral cancer, and limiting the probability of cancer progression (Zygogianni et al., 2011).

Among these molecular changes, the acquisition of genetic or epigenetic predisposition to tumorigenicity by CSCs has been evident in premalignant lesions as well as oral epithelial carcinogenesis (Prince et al., 2007; AbdulMajeed et al., 2013 and Feng et al., 2013). CSCs have been considered as the hot spots in recent cancer research, as they have the ability to self-renew, drive initiation and progression of cancer and affect some aspects of tumor behavior, such as tumor recurrence or resistance to therapies. These aspects have increased the importance of the signaling pathways required for the maintenance of CSCs and stem cell's self-renewal regulator genes, which became candidate targets for recent investigations in this field (Atlasi et al., 2007 and El Deeb&Abdelzaher, 2014).

During the past decade, a large number of markers involved in the regulation of CSCs have been identified as OCT4, CD133, SOX2 and Nanog-1(Koukourakis et al., 2012). Wen et al. (2010) proposed that carcinogenesis recapitulated embryogenesis and the same proteins involved in embryonic development were

also involved in carcinogenesis. Being highly expressed during the embryonic period andinvolved in self-renewal of undifferentiated ESCs, OCT4was considered as a stem cell protein. An accumulating number of studies have demonstrated ectopic or aberrant expression of OCT4 in many tumor types (Atlasi et al., 2007).

Despite the interest in exploring expression of OCT4 in OED, this field is not adequately addressed. Accordingly, our goal was to study the immunoexpression of OCT4 in OED, in an attempt to understand its contribution to the development and progression of these lesions and its potential role as early marker for detection of malignant transformation. For this purpose, immunohistochemical technique was used as this method is sensitive, efficient, and most widely used for localization of a variety of antigens (Manual et al., 2009 and Griffin et al., 2011). OCT4 immunostaining was evaluated by an image analyzer computer system, which is capable of performing accurate determination of expression of biomarkers in tissues. Moreover, it has the advantages of being a sensitive, objective, and quantitative (Rizzardi et al., 2012).

However, the molecular analysis of biomarkers in these multiple steps was hampered by the unavailability of biopsies of all the stages of carcinogenesis, preceding the frank malignancy. One of the challenges in the current study was obtaining OED tissue specimens from human patients. Fortunately, animal models of carcinogenesis allowed the reproducible isolation of all stages, including normal tissues, which are then amenable to pathological, genetic and biochemical analyses (Kanojia&Vaidya 2006).

In an attempt to develop oral carcinogenesis in the buccal mucosa of experimental rats, DMBA was used, as it is one of the most preferred chemical carcinogens, which is able to produce histopathological, biochemical and a molecular abnormalities closely mimic cancer of the human oral cavity (Karthikeyan&Manoharan, 2016). Our findings supported the efficiency of DMBA to induce dysplastic changes in epithelial cells. This is consistent with the findings of Silvan&Manoharan (2013) and Mahakian et al. (2014). The former explained that the pathogenesis of DMBA-induced oral carcinogenesis was mainly attributed to overproduction of reactive oxygen species, impairment in antioxidant defense system and defects in the detoxification cascade, leading to oxidative modification of DNA bases and deregulated expression pattern of molecular markers.

In the current work, the period of 6 weeks for DMBA application was suitable for development of early dysplastic changes, and further changes were observed after 9 weeks. This is in agreement with Andrejevic et al. (1996) and Mahakian et al. (2014) who showed that 5–8 weeks after the start of DMBA application were enough to produce dysplastic changes in male Hamster's cheek pouch, and buccal mucosa of Syrian Golden hamsters, respectively. They agreed that severe dysplasia was detected after 8 weeks.

Although many systems of grading OED have been proposed, most of them were subjective and encountered to considerable inter and intra-observer variations (Geetha et al., 2015). In the current work, a binary system of grading OED into low and high risk groups was used, which was proposed by Kujan et al. (2006). The utility of this systemwas recently tested and has shown better agreement among pathologists (Warnakulasuriya et al., 2008 Geetha et al., 2015).

In the present work, negative IHC reactivity of OCT4 in all normal control specimens is consistent with Hochedlinger et al. (2005), Wen et al. (2010) and El Deeb&Abdelzaher(2014). This finding reflects the differentiation of epithelial cells in normal control group and lack of any evidence of stemness in these cells. In addition, Xi et al. (2011) provided that OCT4 was not detected in the normal esophageal mucosa in comparison to human esophageal SCC. In contradiction to our results, Wen et al. (2010) and Alexander et al. (2013) found positive OCT4 staining in islet cells of human pancreas, and human adrenal gland, respectively. The variability of their results in comparison to ours may be attributed to the use of polyclonal OCT4 antibodies, unlike the current study, in which monoclonal antibody was used.

Positive expression of OCT4 in most of the examined cases of OED is in accordance with Gidekel et al. (2003) and Hochedlinger et al. (2005), who reported postive expression of OCT4 in premalignant lesions as well as frank carcinomas. The former added that OCT4 inactivation induced regression of the malignant component. Furthermore, the concomitant negative OCT4 expression of the control specimens with its positive reaction in OED reflects its possible role in early stages of tumorigenesis, and the feasibility of using OCT4 as an early marker for carcinogenesis. This is in accordance with Bahl et al. (2012) who studied OCT4 expression in human esophageal cell carcinoma, dysplasia and normal esophageal epithelium. In the same context, the studies of Chen et al. (2009), Wen et al. (2010), Gazouli et al. (2012). and Fu et al. (2015) showed that Oct4 expression was higher in adjacent non-cancerous tissues than normal tissues in healthy controls.

The significant increase of OCT4 levels from low risk to high risk OED, not only reflects its role in the multistep process of carcinogenesis, but also its prognostic value. The current investigation provides some data supporting the suitability of OCT4 as a prognostic molecular marker, which is overexpressed in association with poorer prognosis. In the same context, Dong et al. (2012) established that overexpression of OCT4 in hepatocellular carcinoma cell lines was significantly associated with low differentiation and poor behavior. Moreover, Koo et al. (2014) showed that elevated OCT4 levels in head and neck tumors were associated with poor survival of the patients, and more histologic undifferentiation.

Conversely, Cantz et al. (2008) demonstrated expression of OCT4 in normal cells and total absence of both its RNA and protein levels in carcinoma cell lines. Ge et al. (2010) proposed that OCT4 overexpression could be used as an indicator of better prognosis for patient with hypopharyngeal SCC. Fu et al. (2015) also showed that OCT4 expression was significantly associated with early stages of OSCC and better prognosis for patients

Although OCT4 was positively expressed in most of examined OED cases, about 1/3 low risk group and 20% of high risk group showed negative immunoreaction. This may be attributable to decreased tendency of these lesions for malignant transformation. In the same line, Gidekel et al. (2003) and Hochedlinger et al. (2005) agreed that OCT4 high levels increased the malignant potential of premalignant lesions. This may shed the light on the importance of OCT4 as a biomarker that may identify OED lesions with higher malignant potential.

Nuclear localization of OCT4 in tumor cells is widely reported in most of the studies in the available literature, as Chiou et al. (2008), Karoubi et al. (2010), He et al. (2012) and Fu et al. (2015). Nevertheless, our study showed both nuclear and cytoplasmic OCT4 expression in dysplastic cells of OED. This finding is consistent with Iki& Pour (2006), Dong et al. (2012), Koukourakis et al. (2012), Alexander et al. (2013), Ji et al.(2014) and Qiao et al.(2014). This is based on the presence of different OCT4 isoforms: OCT4A and OCT4B. They exhibit identical structures except for exon1, in which self-renewal and pluripotent properties of OCT4 are encoded. This exon is present in OCT4A, which is specifically expressed in the nucleus, whereas OCT4B lacked it, and is preferentially detected in the cytoplasm. Yet, OCT4B has been shown to play a role in the stress response and stemness of cells. In spite of this variability and differences in the biological functions of OCT4 isoforms, most studies in the literature do not discriminate between OCT4A and OCT4B (Atlasi et al., 2008 and Samardzija et al. 2012).

Regarding the nuclear expression of OCT4, it was restricted to a subpopulation of cells, chiefly located in basal and parabasal cell layers. These cells may be probably stem cells, stem cell-like or dedifferntiated cells that gained stemness characteristics, and reside within the tissues. Therefore, our work provides evidence that stem cells, stem cell-like or dedifferntiated cells may be not only a constant finding in cancer but also they comprise a subpopulation of cells in OED. Moreover, this supports the efficiency of OCT4 as a stem cell marker and a key stemness regulator. This claim is in the same line with the reports of (Hatefi et al. (2012) and Sedaghat et al. (2016).

In the current work, perinuclear OCT4 expression was detected in most of the examined cases. Consistent with our finding, Karoubi et al. (2010) and Rolf et al. (2012) reported OCT4 perinuclear immunoreaction. However, this pattern of expression was less encountered in the available literature. The perinuclear space lies between inner and outer nuclear membranes, through which the proteins synthesized by ribosomes are transported to the nuclues (Alberts et al., 2015 and Shaiken&Opekun, 2015). Thus, localization of OCT4 in the perinuclear area denotes active synthesis of OCT4 protein in OED cells.

The results of the present study revealed the possible role of OCT4 in early molecular events of tumorigenesis. Therefore, it may represent a promising early biomarker for carcinogenesis. In addition, the prognostic value of OCT4 in prediction of prognosis in OED was reported. Furthermore, our work shed the light on the importance of OCT4 as a reliable predictor for the tendency of malignant transformation in epithelial precursor lesions, and its feasibility for identification of OED lesions with higher malignant potential.

V. Conclusions

From the present study, it can be concluded that:

- OCT4 may have an important role in early molecular events of tumorigenesis.
- It may serve as a candidate molecular marker for early detection of carcinogenesis.
- OCT4 may be used as potential diagnostic tool for identification of OED at early stages to improve its prognosis
- It may have a prognostic value in prediction of prognosis in OED.

References:

[1]. AbdulMajeed, A. A., Dalley, A.J. and Farah, C.S. (2013). Putative Cancer Stem Cell Marker Expression in Oral Epithelial Dysplasia and Squamous Cell Carcinoma. Journal of Oral Pathology & Medicine, 42(10); 755-760.

[2]. Alberts, B., Alexander, J. and Julian, L. (2015). Molecular Biology of the Cell, Sixth Edition ; Edited Walter. Garland Science : New York and Abingdon , UK , 1464 Pages with 1492 Illustrations.

[3]. Alexander, R.E., Liang, C. and David, J. (2013). Cytoplasmic Staining of OCT4 Is a Highly Sensitive Marker of Adrenal Medullary. The American Journal Of Surgical Pathology, 37(5); 727-733.

[4]. Andrejevic, S., Savary, J. and Fontolliet, C. (1996). Cell carcinoma in the Golden Syrian hamster : Evaluation of an Animal Model and Comparison with Early Forms of Human Squamous Cell Carcinoma in the Upper Aero-digestive Tract. International Journal of Experimental Pathology, 77; 7-14.

[5]. Atlasi, Y., Seyed, J. and Ahmad, B. (2007). Short Report OCT-4 , an embryonic stem cell marker , is highly expressed in bladder cancer. International Journal of Cancer, 602; 1598-1602

[6]. Atlasi, Y., Paul, J. and Gokhale, W. (2008). OCT4 Spliced Variants Are Differentially Expressed in Human Pluripotent and Nonpluripotent Cells. Stem Cells, 26(12); 3068-3074.

[7]. Bahl, K., Saraya, A. and Sharma, R. (2012). Biomarker Insights Increased Levels of Circulating and Tissue mRNAs of Oct-4 , Sox-2 , Bmi-1 and Nanog is ESCC Patients : Potential Tool for Minimally Invasive Cancer Diagnosis. Journal Biomarkers Insights, (7); 27-38.

[8]. Cantz, T., Key, O. and Bleidi, M. (2008). Absence of OCT4 Expression in Somatic Tumor Cell Lines. Stem cells Journal, 26(3); 692-701.

[9]. Chen, Z., Qian, H. and Zhu, W. (2009). Oct4, a novel marker for human gastric cancer. Journal of Surgical Oncology, 99(7); 414-419.

[10]. Chin, D., Boyle G. M., Porceddu, S. and Theile, D. (2006). Head and neck cancer : past , present and future. Journal Expert Review of Anticancer Therapy, 6; 1111-1118.

[11]. Chiou, S.H., Lin, S. H. and Chung, J. L. (2008). Positive correlations of Oct-4 and Nanog in oral cancer stem-like cells and high-grade oral squamous cell carcinoma. Clinical Cancer Research, 14(13); 4085-4095.

[12]. Deep, K., Nanda, S. and Ranganathan, K. (2012). Increased expression of CK8 and CK18 in leukoplakia , oral submucous fibrosis , and oral squamous cell carcinoma : An immunohistochemistry study. Journal Oral Surgry Oral Medine Oral Pathology Oral Radiology,3(2); 245-253

[13]. Dong, Z., Qin, Z., Hesan, L., and Jinjin, Z. (2012). Increased expression of OCT4 is associated with low differentiation and tumor recurrence in human hepatocellular carcinoma. Pathology Research and Practice, 208(9); 527-533.

[14]. Dutra, A. (2017). Expression of E-cadherin and Involucrin in Leukoplakia and Oral Cancer : An Immunocytochemical and Immunohistochemical Study. Barazelian Oral Research, 31; 1-8.

[15]. El Deeb, N.M.F. and Abdelzaher, E. (2014). Stem cell markers OCT4 and nestin in laryngeal squamous cell carcinoma and their relation to survivin expression. Pathology Research and Practice, 210(11); 751-758..

[16]. Feng, J.Q., Zi, J., Xu, Z. and June, L. (2013) Expression of cancer stem cell markers ALDH1 and Bmi1 in oral erythroplakia and the risk of oral cancer. Journal of Oral Pathology &Medicine, 42(2); 148-153.

[17]. Fu, T.Y., Cheng, T. and Tsai, M. (2015). Association of OCT4, SOX2, and NANOG expression with oral squamous cell carcinoma progression. Journal of Oral Pathology & Medicine,

[18]. Gazouli, M., Roubelakis, M., Theodoropoulos, G. and Papailiou, J. (2012). OCT4 spliced variant OCT4B1 is expressed in human colorectal cancer. Molecular Carcinogenesis, 51(2); 165-173.

[19]. Ge, N., Lin, H. X., Xiao, S. and Guo, L. (2010). Prognostic significance of Oct4 and Sox2 expression in hypopharyngeal squamous cell carcinoma. Journal of Translational Medicine, 8(1); 94-101.

[20]. Geetha, K.M., Leeky, M., Narayan, T.V. (2015). Grading of oral epithelial dysplasia: Points to ponder. Juornal Oral Maxillofacial Pathology, 19(2); 198-204.

[21]. Gidekel, S., Pizov, G., Bergman, Y. (2003). Oct-3/4 is a dose-dependent oncogenic fate determinant. Cancer Cell, 4(5), 361-370.

[22]. Gillenwater, A., Papadimitrakopoulou, V. and Richards-Kortum, R. (2006). Oral premalignancy: New methods of detection and treatment. Current Oncology Reports, 8(2); 146-154.

[23]. Greer, R.O. (2006). Pathology of Malignant and Premalignant Oral Epithelial Lesions. Otolaryngol Clinics of North America, 39; 249-257

[24]. Griffin, M.C., Bonelli,V., Daumas, F. (2011). Immunohistochemical expression of p53, p16 and hTERT in oral squamous cell carcinoma and potentially malignant disorders. Journal of Clinical Pathology, 48(1); 801-804.

[25]. Hang, Z. Z. and Al, T.E.T. (2003). Risk Factors For Multible Oral Premalignant Lesions. International Journal of Cancer , 291(April); 285-291.

[26]. Hatefi, N. and Nazila, O. (2012). Evaluating the Expression of Oct4 as a Prognostic Tumor Marker in Bladder Cancer.Iranian Journal of Basic Medical Sciences, 15; 1154-1161.

[27]. He, W., Ke, L., Feng, W. and Qin,Y.R. (2012). Expression of OCT4 in Human Esophageal Squamous Cell Carcinoma is Significantly Associated With Poorer Prognosis. World Journal Gastroenterol. 18(7); 712-719.

[28]. Hochedlinger, K., Yasuhiro, Y. and Caroline, B. (2005). Ectopic expression of Oct-4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues. Stem Cell Journal, 121(3); 465-477

[29]. http://www.ahc.umn.edu/rar/euthanasia.html

[30]. Iki, K. & Pour, P.M. (2006). Expression of Oct4, a stem cell marker, in the hamster pancreatic cancer model. Pancreatology, 6(4); 406-413.

[31]. Jaber, M.A., Portera, S. R., Speightb, P. and Evesonc, J. W. (2003). Oral epithelial dysplasia : clinical characteristics of western European residents. Oral Oncology, 39; 589-596.

[32]. Jerabek, S., Merino, F. and Robert, H.S. (2014). OCT4 : Dynamic DNA binding pioneers stem cell pluripotency. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms, 1839(3); 138-154.

[33]. Ji, J., Wei, X. and Wang, Y. (2014). Embryonic Stem Cell Markers Sox-2 and OCT4 Expression and Their Correlation with WNT Signal Pathway in Cervical Squamous Cell Carcinoma. International Journal of Clinical and Experimental Pathology, 7(5); 2470-2476.

[34]. Kanojia, D. and Vaidya, M.M. (2006). 4-Nitroquinoline-1-oxide induced experimental oral carcinogenesis. Oral Oncology, 42(7); 655-667.

[35]. Karthikeyan, S. and Manoharan, S. (2016). Cromolyn Inhibits 7 , 12-Dimethlbenz (A) Anthrance Induced Oral Cancer Through Apoptotic Induction and Suppression of Cell Proliferation. International Journal of Pharma and Bio Sciences, 7(1); 35-42.

[36]. Karoubi, G., Corte-Dericks, L. and Gugger, M. (2010). Atypical expression and distribution of embryonic stem cell marker, OCT4, in human lung adenocarcinoma. Journal of Surgical Oncology, 102(6); 689-698.

[37]. Kasem, R. F., Hegazy, R. H., Arafa, M. A., and Abdelmohsen, M. M. (2014). Chemopreventive effect of Mentha piperita on dimethylbenz[a]anthracene and formaldehyde-induced tongue carcinogenesis in mice (histological and immunohistochemical study). Journal of Oral Pathology and Medicine, 43(7); 484-491.

[38]. Kashyap, V., Rezende, N.C., Scotland, K.B. and Shaffer, S. M. (2009). Regulation of Stem Cell Pluripotency and Differentiation Involves a Mutual Regulatory Circuit of the Nanog , OCT4 , and SOX2 Pluripotency Transcription Factors With Polycomb Repressive Complexes and Stem Cell microRNAs. Stem Cell and Development, 18(7).

[39]. Khan, Z., Epstein J. B., Shanthi, M. and Gillespi, M. B. (2016). Cetuximab activity in dysplastic lesions of the upper aerodigestive tract . Oral Oncology, 53; 60-66.

[40]. Kim, R. and Nam, J. (2011). OCT4 Expression Enhances Features of Cancer Stem Cells in a Mouse Model of Breast Cancer. Laboratory Animal Research , 27(2); 147-152.

[41]. Korkola, J.E., Jane, H. and Rajendrakumar, S.V. (2006). Down-Regulation of Stem Cell Genes , Including Those in a 200-kb Gene Cluster at 12p13 . 31 , Is Associated with In vivo Differentiation of Human Male Germ Cell Tumors. Amrican Assoctiation for Cancer Research, (2); 820-828.

[42]. Koukourakis, M.I., Giatromanolaki, A., Tsakmaki,V. and Danielidis, S.H. (2012). Cancer stem cell phenotype relates to radio-chemotherapy outcome in locally advanced squamous cell head – neck cancer. British Journal of Cancer, 106(5); 846-853.

[43]. Krishna Prasad, R.B., Sharma, A. and Babu, H.M. (2013). An insight into salivary markers in oral cancer. Dental research journal, 10(3); 287-295.

[44]. Krishnan, L., Karuppiah, K. and Jayalakshmi, K. (2016). Inter and intra observer variability in three grading systems for oral epithelial dysplasia. Journal of Oral and Maxillofacial Pathology, 8 ; 261-268.

[45]. Kujan, O., Richard., J. and Ammar, K. (2006). Evaluation of a new binary system of grading oral epithelial dysplasia for prediction of malignant transformation. Oral Oncology, 42(10); 987-993.

[46]. Kujan, O., Shearston, K. and Farah, C.S.(2016). The role of hypoxia in oral cancer and potentially malignant disorders : a review. Journal of Oral Pathology and Medecine, 4(8).

[47]. Lengner, C.J., Fernando, D. and Camargo, K. G. (2007). Oct4 Expression Is Not Required for Mouse Somatic Stem Cell Self-Renewal. Stem Cell Journal, 1(4); 403-415.

[48]. Madhura, M.G., Lipika, K. B. and Veerendra, K. (2016). Algorithm to reduce subjectivity in grading oral epithelial dysplasia - A preliminary study. Journal of Advanced Clinical & Research Insights, 3; 112-117.

[49]. Mahakian, L.M., Gregory, D., Zhang, H. and Woong, S. (2014). Comparison of PET imaging with 64Cu-liposomes and 18F-FDG in the 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch model of oral dysplasia and squamous cell carcinoma. Molecular Imaging and Biology, 16(2); 284-292.

[50]. Manual, M. T. (2009). Oral Squamous Cell Carcinoma; Role of the Plasminogen Activation System in Tumour Progression. Bagheri Current Therapy,1-5.

[51]. Masthan, K.M.K., Rajesh, U. and Anitha, N. (2016). Grading of Oral Epithelial Dysplasia - A Review. Biomedical & Pharmacology Journal , 9(2); 833-835.

[52]. Nordhoff, V., Karin H., Andrea, B. and Irina, O. (2001). Comparative analysis of human , bovine , and murine Oct-4 upstream promoter sequences. Mammalian Genome, 12; 309-317.

[53]. Pitiyage, G., Tilakaratne, W. M. and Mahvash, T. (2009). Molecular markers in oral epithelial dysplasia : review. Journal of Oral Pathology & Medicine, 737-752.

[54]. Prince, M.E., Sivanandan, R., Kaczorowski, A. and Wolf, G. T. (2007). Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proceedings of the National Academy of Sciences of the United States of America, 104(3); 973-978.

[55]. Qi, X., Michael, V., Sivak, J., and Willis, J. (2011). Computer-aided diagnosis of dysplasia in Barrett's esophagus using endoscopic optical coherence tomography. Journal of Biomedical Optics, 11(4).

[56]. Qiao, B., Baoxia, H., Jinghua, C. and Wenli, Y. (2014). The expression profile of Oct4 and Sox2 in the carcinogenesis of oral mucosa. International Journal of Clinical and Experimental Pathology, 7(1); 28-37.

[57]. Ribeiro, I.P., Leonor, B., Francisco, M. and Joana, B. M. (2016). Early detection and personalized treatment in oral cancer : the impact of omics approaches. Molecular Cytogenetics, 1-7.

[58]. Rizzardi, A.E., Johnson, A. T. and Voge, R. I. (2012). Quantitative comparison of immunohistochemical staining measured by digital image analysis versus pathologist visual scoring. Diagnostic Pathology, 7(1); 42-47.

[59]. Rolf, H.J., Niebert, S., Niebert, M. and Lena Gaus, L. (2012). Intercellular transport of Oct4 in mammalian cells: A basic principle to expand a stem cell niche? PLoS ONE, 7(2).

[60]. Sadiq, H.,Priti, G., Nisha, S. and Sahil, S. T. (2015). Various Grading Systems of the Oral Epithelial Dysplasia: A Review. International Journal of Advance Health Sciences, 1(11); 20-26.

[61]. Safadi, R.A., Priti, G., Nisha, S. and Sahil, S. T. (2010). Analysis of Immunohistochemical Expression of k19 in Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma Using Color Deconvolution-image Analysis Method. Head and Neck Pathology, 4(4); 282-289.

[62]. Samardzija, C., Michael, Q. and Jock, K .F. (2012). Attributes of Oct4 in stem cell biology: perspectives on cancer stem cells of the ovary. Journal of Ovarian Research, 5(1); 37-42.

[63]. Schwartz, J.L. (2000). Biomarkers and Molecular Epidemiology and Chemoprevention of Oral Carcinogenesis. Critical Reviews in Oral Biology & Medicine., 11; 92-122.

[64]. Sedaghat, S., Elmira, G. and Mojgan, A. (2016). Expression of Cancer Stem Cell Markers OCT4 and CD133 in Transitional Cell Carcinomas. Applied Immunohistochemistry & Molecular Morphology,1-12.

[65]. Shah, S. and Kaur, M. (2014). Biomarkers and chemopreventives in oral carcinogenesis and its prevention. Journal of Oral and Maxillofacial Pathology, 18(1); 69-76.

[66]. Shaiken, T.E. and Opekun, A.R. (2015). Dissecting the cell to nucleus, perinucleus and cytosol. Scientific Reports, 4(1); 4923-4932.

[67]. Shirani, S., Kargahi, N. and Mohammad, S. (2014). Epithelial Dysplasia in Oral Cavity. Iranian Journal of Basic Medical Sciences. 39(5); 406-417.

[68]. Silvan, S. and Manoharan, S. (2013). Apigenin prevents deregulation in the expression pattern of cell-proliferative, apoptotic, inflammatory and angiogenic markers during 7,12-dimethylbenz[a]anthracene-induced hamster buccal pouch carcinogenesis. Archives of Oral Biology, 58(1); 94-101.

[69]. Tsai, L.L., Fang, H., Shiuan, S., and Chuan, H. (2014). Oct4 mediates tumor initiating properties in oral squamous cell carcinomas through the regulation of epithelial-mesenchymal transition. Public Library of Science, 9(1); 1-8.

[70]. Wang, X. and Dai, J. (2010). Concise review: Isoforms of OCT4 contribute to the confusing diversity in stem cell biology. Stem Cells, 28(5); 885-893.

[71]. Wang, Z., Jacqueline L. L., Kueh, T. and Lufkin, P. (2007). Oct4 and Sox2 Directly Regulate Expression of Another Pluripotency Transcription Factor , Zfp206 , in Embryonic Stem Cells. Journal of Biological Chemistry, 282; 12822-12830

[72]. Warnakulasuriya, S., J. Reibe, J. and Bouquot, J. (2008). Oral epithelial dysplasia classification systems: predictive value, utility, weaknesses and scope for improvement. Journal of Oral Pathology & Medicine, 37(3); 127-133.

[73]. Watanabe, N., Tsunemasa, O. and Masahito, S. (2015). Preneoplasia and carcinogenesis of the oral cavity. Oncology Discovery , 3; 1-12.

[74]. Wellington, D., Mikaelian, I. & Singer, L., 2013. Comparison of Ketamine – Xylazine and Ketamine – Dexmedetomidine Anesthesia and Intraperitoneal Tolerance in Rats.Journal of the American Association for Laboratory Animal Science , 52(4); 481-487.

[75]. Wen, J., Park, J. Y., Kyung, H. P. and Won, C. (2010). Oct4 and Nanog Expression Is Associated With Early Stages of Pancreatic Carcinogenesis.pancreas journal, 39(5); 622-626.
[76]. Wu, G. and Schöler, H.R. (2014). Role of Oct4 in the early embryo development. Cell Regeneration Journal, 3(7); 1-10.
[77]. Xi, Z., Guang-Rong, H. and Pin, H. (2011). Over-Expression of Oct4 in Human Esophageal Squamous Cell Carcinoma.Molecules and Cells Journal, 32; 39–45.
[78]. Yu, J., Yi, T., Chenb, D., Chiangg, W. F. and Hsiao, Y. G. (2016). Saliva protein biomarkers to detect oral squamous cell carcinoma in a high-risk population in Taiwan. Medical Scinece Journal, 10
[79]. Zygogianni, A.G., George, K., Karakitsos, P. and Amanta, P. (2011). Oral squamous cell cancer: early detection and the role of alcohol and smoking. Head & Neck Oncology, 3(1); 2-11.

Maged Ali." "Immunohistochemical Expression of Octamer-binding protein-4, in Oral Epithelial Dysplasia Induced in Experimental Rats (Animal Study).”."IOSR Journal of Dental and Medical Sciences (IOSR-JDMS), vol. 17, no. 7, 2018, pp 26-46.