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The Immunohistochemical Expression of COX-2 and Clinical Parameters in Oral Lichen Planus

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ABSTRACT

Oral lichen planus (OLP) is a chronic inflammatory condition with an unclear etiology. Even though the World Health Organization (WHO) defines OLP as a precancerous condition, the reasons that trigger cancer progression in OLP lesions remain unknown. Cyclooxygenase-2 (COX-2) is an essential enzyme for inflammatory processes and cell proliferation that has been discovered to be overexpressed in OLP lesions in this chronic inflammatory state. The purpose of present research was to determine COX-2 expression levels in histologically identified OLP samples and to correlate it with clinical and histological characteristics. Present study included 30 histopathologically confirmed Oral Lichen Planus lesions. Immunohistochemistry was used to detect COX-2 protein expression. COX-2 expression was compared to certain clinical and histological parameters. The (SPSS) program version (20.0) was utilized to find the relationships between COX-2 expression and these parameters. A Pearson's correlation between age and sex groups revealed that studies of various age groups revealed a comparable gender distribution with a significant link (P = 0.017). OLP lesions are most commonly found in the buccal mucosa. There is a significant decrease in COX-2 expression was detected in all studied cases of the study. The level of the COX-2 expression rise in correlation with clinical manifestations and cancer development. Through inflammatory reactions, COX-2 is associated with epithelium damage and carcinogenesis pathways.

1. INTRODUCTION

Oral lichen planus is an autoimmune disorder that is chronically inflamed. In this situation, cytotoxic T lymphocytes have an effect on epithelial cells. Oral lesions are more robust to cancer development than skin lesions, and they have a higher likelihood of malignant transformation into oral squamous cell carcinoma (OSCC) [1]. OLP's pathogenesis and etiology are not completely understood. The primary stimuli include immunological responses, genetic history, and infections such as bacterial diseases as well as viral illnesses [2], [3]. A precancerous lesion of the oral cavity is identified as any disease or disorder of the oral mucosa that has the capacity to change malignantly. At a 2005 workshop, the phrase "possibly malignant oral

disorders" was introduced by the World Health Organization. Furthermore, the newly recognized term "possibly premalignant oral epithelial lesion (PPOEL)" refers to lesions having carcinogenic potential, like OLP [4]. Presence of oral dysplasia of the oral mucosa is one of the risk variables for cancer development [1], [5]. An investigation conducted recently indicated that the probability of cancer formation in the lesions of OLP is 1.1%, with the likelihood of developing OSCC being higher in individuals who have behaviors such as tobacco smoking, drinking alcohol, and having hepatitis C virus infection [6]. In order to detect cancer at its earliest possible stage, the patient must have a tissue biopsy. There are several types of detection procedures available, including histology, direct immunofluorescence (DIF), serology, hematological studies, cytology, and the investigation of markers [7], [8]. Early detection of malignant transformation of OLP is essential in preventing the disease from progressing. According to certain studies, the likelihood of occurrence is higher among females and in people in their fourth to seventh decades of life [7].

According to [9], detailed clinical examinations, particularly biopsies and histopathological investigations, are required. When traditional oral examinations are too limited, a number of extra tests have been used, like staining with toluidine blue and using light-based detection methods, as well as markers in body fluids like saliva, to get around these limitations [10], [11]. The authors showed that biopsy and histological investigation are the gold standards for determining precancerous lesions.9 However, biopsy may have certain negatives, including anxiety, worry, and pain, which may keep people from getting treatment, as well as the risk of infection, irritation, short-term disability, and cosmetic concerns [12].

In accordance with the evidence, the chronic inflammatory process may result in the formation of a cytokine-based environment that influences cellular proliferation, development, and cancer progression [13]. Several studies established that when the basal-cell layer expands rapidly as a consequence of the action of mediators released by the stratum basale, the malignant process begins. The mediators that are generated by the inflammatory cells have the potential to activate a variety of mechanisms, including COX expression [14], [15]. Specifically; the current idea proposes that epithelial cells are subjected to signals that disrupt their development. They are made as a result of persistent activation of the system of immunity by inflammatory and stromal cells. A lot of oxidative and long-lasting things cause DNA damage, which leads to neoplastic changes [16].

Cyclooxygenase-2(COX-2) is a membrane-bound regulatory enzyme involved in the conversion of arachidonic acid to prostaglandins (PGs) that can be activated by certain stimuli [17]. Additionally, PGs have a role in inflammatory conditions, immunological changes, and apoptotic tissue damage [18]. According to the findings, high COX-2 expression is seen in a variety of autoimmune illnesses, including bullous pemphigoid, pemphigus vulgaris and others [19], [20].

Several studies have shown an increase in COX-2 in premalignant and malignant oral lesions and COX-2 is now regarded as a marker for malignant transformation since it functions as an apoptotic inhibitor as well as a stimulator of cell growth and angiogenesis [21], [22]. The upregulation of COX-2 in cancerous tumors, particularly OSCC, has been demonstrated in different investigations [23], [24]. COX-2 is actually involved in a lot of important things that happen in the development of cancer, like revascularization and cell death [25].

2. Materials and methods

Thirty cases of OLP were obtained from the archives of Baghdad University's oral diagnostic department's oral pathology laboratory. Tissue sections from each example were formalin-fixed and paraffin-embedded which were gathered together with the patient's health clinical evidence (age, sex, location, and type) and

histological factor of dysplasia as reported in the oral and maxillofacial findings. Two expert pathologists analyzed tissue sections stained with hematoxylin and eosin (H&E) from each patient were shown to verify the diagnosis. Colon cancer tissue was chosen and included as a positive tissue control in each immunohistochemistry run according to the manufacturer's data sheets for the COX-2 marker. Primary monoclonal antibodies were made and used in the investigation by Pathnsitu/USA. The PolyExcel detection system is designed for use with primary antibodies produced against mouse and rabbit to qualitatively detect antigens in normal and pathological paraffin-embedded tissues, cryostat tissues, or cellular preparations using light microscopy. PathnSitu's sensitivity and specificity are unmatched. The PolyExcel two-step detection system is a non-biotin, micropolymer-based detection technology that considerably reduces or eliminates background consisting of high levels of avidin or biotin. It is based on a polymer that has been tagged with HRP and linked with secondary antibodies.

2.1 Immunohistochemical procedure

Control and study tissue sections were maintained in 10% formalin solution and produced using the conventional paraffin block process. Each formalin-fixed, paraffin-embedded tissue sample was sliced into two pieces. One section with a thickness of 5 µm was mounted on a pre-cleaned microscope slide for routine Haematoxylin and Eosin dyeing (H&E) for histopathological assessment, while another section with a thickness of 5 µm was fixed on positively charged microscopic slides for improved tissue attachment during immunohistochemical staining. Additionally, as a control sample, tissue slices with a thickness of 5 µ were put on positively charged surfaces. The antibody is adjusted to the appropriate dilution (1:50), then applied to the tissue samples and kept at room temperature in humid conditions for 30–60 minutes. The sections were thoroughly washed with PBS and dried before being gently rinsed with three changes of PBS (5 min. for each). Each slide had a second tissue segment stained with PBS in addition to the main antibody as a negative control. Each segment was incubated for 15 minutes at 37 °C with 1-2 drops of unconjugated rabbit anti-mouse antibody, then washed twice with PBS and dried. A HRP-labeled polymer that has been reacted with secondary antibodies and incubated at 37 °C for 15 minutes before being rinsed twice with PBS and allowed to dry in centrifuge tubes, 20m of DAB chromogen was dissolved in 1 ml of DAB substrate and combined before being administered to the tissue slice overnight at 37 °C for 5-10 mins before being washed with tap water and then dried. After soaking the slides for 1-2 minutes in a bath containing Mayer's Haematoxylin, they were gently rinsed with distilled water. The slides were serially dipped in ethanol and xylene-containing containers, then fixed with 1-2 drops of DPX fixing media and quickly covered with glass slides before being inspected the following day under a light microscope.

2.2 Evaluation of immunohistochemical staining

According to the datasheet for the product, on the positive control tissue slides, the presence of a brown granular DAB dye template inside a particular cellular or structural section for a specific antibody and the lack of staining on negative control tissue samples revealed immunohistochemical signal selectivity. the appearance of a brown granular DAB dye template within the specific cellular or tissue compartment for a specific antibody on positive control tissue slides and the absence of staining on negative control tissue samples indicated immunohistochemical signal selectivity. The COX-2 expression was quantified semi-quantitatively. Without previous knowledge of any other characteristics, all research slides were assessed blindly. Separately, an experienced pathologist evaluated the slides to recalibrate the findings.

2.3 Cyclooxegenase-2 (COX-2) scoring

The immunohistochemistry score of COX-2 was used to assess its presence in the cytoplasm and membranous tissues (HIS). According to the formula below, this number was calculated by multiplying the predicted fraction of positive cells (quantity score) by the coloring intensity score:

No staining received a score of 0, 1-10% of positive cells received a score of 1, 11-50% of positive cells received a score of 2, 51-80% of positive cells received a score of 3, and 81-100% of positive cells received a score of 4.

The strength of the staining was evaluated from 0 to 3, with 0 indicating no staining, 1 indicating mild staining, 2 indicating moderate staining, and 3 indicating strong staining. The score might be anything from 0 to 12. Immunoreactivity was measured using the HIS score system. A HIS score of 9–12 indicated high immunoreactivity, 5-8 moderate immunoreactivity, 1-4 mild immunoreactivity, and 0 no immunoreactivity. It was determined that COX-2 was overexpressed if the HIS score ranged from moderate to strong. All of the photomicrographs that were obtained were sorted and saved on the computer in folders that were properly labeled. Photomicrographs were analyzed using Imagej® software, which is a free and open-source image processing application written in Java that runs on a computer's operating system.

2.4 Statistical Analysis

This study employed the statistical package (SPSS) version (20.0) for descriptive data analysis (mean and standard deviation), as well as graphical presentation (bar charts and histograms) in order to analyze and assess the findings. The relevant data and statistical approaches were used to investigate and evaluate the study's results. The Spearman's (rho) correlation coefficient and the Pearson correlation are employed for inferential data analysis, respectively. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1 Clinicopathological Findings

In this retrospective investigation, thirty cases of oral lichen planus were histologically detected. It showed the distribution of age groups and gender factors, as well as significant comparisons.

According to this result, the age group distribution of the studied patients has no significant differences, indicating that the probability of recorded disorder does not differ according to the distribution of age groups, as well as age pivoted at the sixth decade with mean value and standard deviation (53.2312.409).

In terms of gender, the investigated samples show no significant differences, and based on this, it can be concluded that the likelihood of the recorded examined disorder does not change established on the gender of the patients.

The correlation between age and gender classes proved that studied of different age groups has recorded a similar distribution regarding different gender with significant relationship at P= 0.017 (Table 1).

According to distribution of the "Site, Type and dysplastic" variables concerning of the diagnosed subjects with oral lichen planus, as well as comparisons significant, to be sure that these two variables regarding of studied patients has randomly distributed among their different classes or not (Table 2).

Sits group's distribution of the studied patients has significant different at $P<0.05$, and accordance with this result, it could be indicating that the probability of recorded studied disordered patients has recorded meaningful differences according to the distribution of site classes, and that was clearly demonstrated by the high number of patients with "Buccal Mucosa" site, since they are accounted 23(76.7%), then followed with "Tongue" site, and accounted 4 (13.3%), then followed with "Palate, Gingiva and Floor of the mouth" sites, and accounted 1(3.3%) for each one of them.

In terms of clinical type, there is a very significant difference at P<0.01 between the individuals investigated and accordance with this result, it could be conclude that probability of recorded studied disordered patients has recorded meaningful differences according to distribution of clinical type classes, and that was clearly demonstrated by the high number of patients with "Reticular" type, since they are accounted 16(53.3%), then followed with "Erosive" type, and accounted 8(26.7%), and then followed with "Atrophic" type, and accounted 4(13.3%) and finally followed with" Plaque and Bullous " types and accounted 1(3.3) for each of them.

A possibility distribution between sites and clinical types classes proved that studied of different sites has recorded a similar distribution regarding different clinical types classes with significant relationship at P= 0.002.

Regarding to the presence of dysplasia, studied oral lichen planus lesions showed 6 (20%) with dysplasia and 24 (80%) without any dysplastic features (Table 2).

3.2 Immunohistochemical Findings

The results of immunohistochemical staining revealed brown cytoplasmic and/or membrane positivity of Cox-2 in all the cases of the oral lichen planus which showed 13 (43.3%) instances with a weak score (+1), 12 (40%) cases with a moderate score (+2), and 5 (16.7%) cases with a high score (+3) were found in the basal to deep spinous layers of the conspicuous region and distributed in the deep spinous layer at the location close to the lesion (Fig.1). COX-2 expression was found to be significantly lower in the analyzed OLP patients (P value = 0.023). (Fig. 2).

Table (3) shows relationships among immunohistochemical expression of Cox-2, age groups, gender and clinical types by using “Pearson Correlation" with their significant levels.

4. Discussion

It is important to understand that oral lichen planus is a mucocutaneous, chronic inflammatory condition with a significantly increased risk of malignant transformation in the erosive type [26- 28]. It is a chronic autoimmune illness characterized by CD8+ cells damaging the basal layer. Because of this, degeneration of basal keratinocytes results in the formation of colloid entities that are visible as homogenous eosinophilic globules [29].

The etiology of OLP is unknown, but its development might be influenced by both antigen-specific and non-specific mechanisms [30]. According to [31] results, the pathogenesis and basic pathophysiological processes underlying these lesions are diverse. Thus, basement membrane breakdown may result in T lymphocyte infiltration into the OLP epithelia results in a close cell-to-cell interaction between the epithelium and the T lymphocytes, which may result in OLP epithelial cell death [32], [33]. T lymphocytes proliferate in the band-like inflammatory infiltrates associated with OLP diseases [34]. The degradation of the basal layer by matrix metalloproteinase production and release is one of the negative effects of T lymphocyte infiltration [30], [35].

Regarding to age groups and gender, the results of present study showed that the peak incidence of OLP at the 5^{th} and 6^{th} decades with a mean of age (53.23) years and the most affected patients were women, with no significant differences in both variables. This is consistent with other numerous studies conducting OLP [36], [37]. However; other researchers revealed that the maximum incidence was at the 3^{th} and 4^{th} decades of life [38], [39]. In Unbar's study of 50 individuals, it was discovered that the majority of patients with lichen planus were between the ages of 20 and 50 [40]. This difference occurred due to using small sample size in these

conducted studies that involve the possibility of ignoring significant differences. Regarding to anatomical site, the current study showed that the most affected area was the buccal mucosa. the buccal mucosa was the most affected site by studied disease. This finding is concordance with the majority of authors [41], [42]. Moreover, according to Carrozo and Thrope, bilateral OLP lesions were the most prevalent [41].

During inflammation, the COX-2 enzyme is frequently expressed. It is involved in both carcinogenesis and inflammation. While inflammation is necessary for tissue regeneration, revascularization, cell growth, and development, chronic, persistent inflammation, as described in OLP, may have deleterious consequences. COX-2 overexpression is required for tumor growth, metastasis, angiogenesis, and the suppression of cell death [43]. The results of our study presented that all study cases expressed positive Cox-2 expression in a significant decrease with the distribution of age groups. The existence of COX-2 in OLP lesions may indicate a biological mechanism in chronic inflammation and probable cancer development, giving support to the concept that chronic inflammation is related to the formation of oral squamous cell carcinoma in OLP. COX-2 may therefore be a reliable predictor of cancer development in OLP; therefore COX-2 may be a probable indicator of the risk of cancer progression in OLP [44- 46]. As COX-2 shows a special role in the disease mechanism of OLP, COX-2 inhibitors may diminish the inflammatory reaction causing immunological dysregulation in OLP. There should be more research into how COX-2 inhibitors are effective treatments for oral lichen planus. This would be good [47]. Furthermore; several forms of lesions in the oral cavity, such as OLP, oral leukoplakia, and submucous fibrosis are all conditions that have the potential to become cancerous. However, the variables that promote the change of OLP to SCC are yet unknown [43]. In respect to the clinical type of OLP, present study showed that reticular type was the most frequent type and the expression of COX-2 was independent of the kind of OLP, the severity of inflammation, the site, or the presence of dysplasia. These findings were in accordance with earlier studies [48]. COX-2 upregulation is linked to an increase in clinical symptoms as well as malignant transformation [49]. COX-2 expression is also associated with the destruction of epithelial cells and the activation of carcinogenesis processes through the persistence of inflammatory responses [39]. Our results showed that there are considerable discrepancies in the COX-2 expression of the OLP lesions, this finding consistence with other previous studies stated that the malignant progression of lesions is accompanied by considerable alterations in the expression of apoptotic and proliferation-related proteins [50]. However, the malignant transformation of OLP has been a subject of controversy. The development of epithelial dysplasia and OSCC in individuals with OLP lesions emphasizes the need to assess possible risk factors in OLP patients. Numerous efforts have been made to locate such markers, but no actual marker has yet to satisfy the requirements [51]. The COX-2 expression in the various OLP subtypes revealed to be slightly different. Other subtype of OLP lesions, the erosive form of OLP was shown to have higher COX-2 expression, a decrease in basement membrane integrity, this agreed with other study conducted COX-2 in OLP [52]. While other study showed that none of the clinical OLP subtypes differed in COX-2 expression [50]. Therapeutic study using particular COX-2 inhibitors to prevent cancer progression of these lesions might be an attractive study topic in the future [53]. By suppressing COX2 enzymes and reducing inflammation, non-steroidal anti-inflammatory medications (NSAIDs) may decrease the likelihood of dysplastic alterations and development of cancer in these lesions. A large group of dysplastic specimens must be studied in more depth to verify this hypothesis [54].

5. Conclusions

An upregulation in COX-2 level has been linked to the increased clinical manifestations and the development of malignancy. Presence of COX-2 is related to epithelium damage and the stimulation of carcinogenesis pathways through the maintenance of inflammatory responses. It is possible to do more researches to determine the involvement of COX-2 in the etiology of OLP. There is a need for more investigations to determine the pathogenesis of OLP. COX-2 can be used to determine OLP lesions that are at a high risk of

carcinogenesis.

6. Declaration

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Table (1): Distribution of age groups and gender variables for the studied samples with oral lichen planus.

Parameters	Age groups	Frequencies	(%)	P- Value
Age	(20-29)	2	6.7	P= 0.216 Non Sig.
	(30-39)	4	13.3	
	(40-49)	3	10	
	(50-59)	9	30	
	(60-69)	10	33.3	
	(70-79)	2	6.7	
	Total	30	100	
	Mean ± SD	53.23±12.409		
Gender	Male	14	46.7	P = 0.855 Non Sig.
	Female	16	53.3	
Age +Gender	Pearson Correlation		0.434	P= 0.017 Sig.

Table (2): Distribution of Site, Type and dysplastic variables for studied subjects with Oral lichen planus and comparison's significant.

Parameters	Classes	No.	%	Pearson Correlation P value
Site	Buccal mucosa	23	76.7	0.547 P = 0.002 Sig.
	Tongue	4	13.3	
	Palate	1	3.3	
	Gingiva	1	3.3	
	Floor of mouth	1	3.3	
Type	Reticular	16	53.3	
	Erosive	8	26.7	
	Atrophic	4	13.3	
	Plaque	1	3.3	
	Bullous	1	3.3	

Dysplastic cases	Yes	6	20
	No	24	80

Table (3): Relationships of the immunohistochemical expression of COX-2 with age, gender and type of the studied patients with OLP and testing their significant levels.

Correlations		Age	Gender	Type
COX-2	Pearson Correlation	0.037	0.300	0.230
	Sig. (2-tailed)	0.846	0.107	0.221
	N	30	30	30
Age	Pearson Correlation		0.434	-0.025
	Sig. (2-tailed)		0.017	0.896
	N		30	30
Gender	Pearson Correlation			-0.213
	Sig. (2-tailed)			0.257
	N			30

Testing are based on Pearson Correlation test, NS: Non Sig. at P>0.05; S: Sig. at P<0.05.

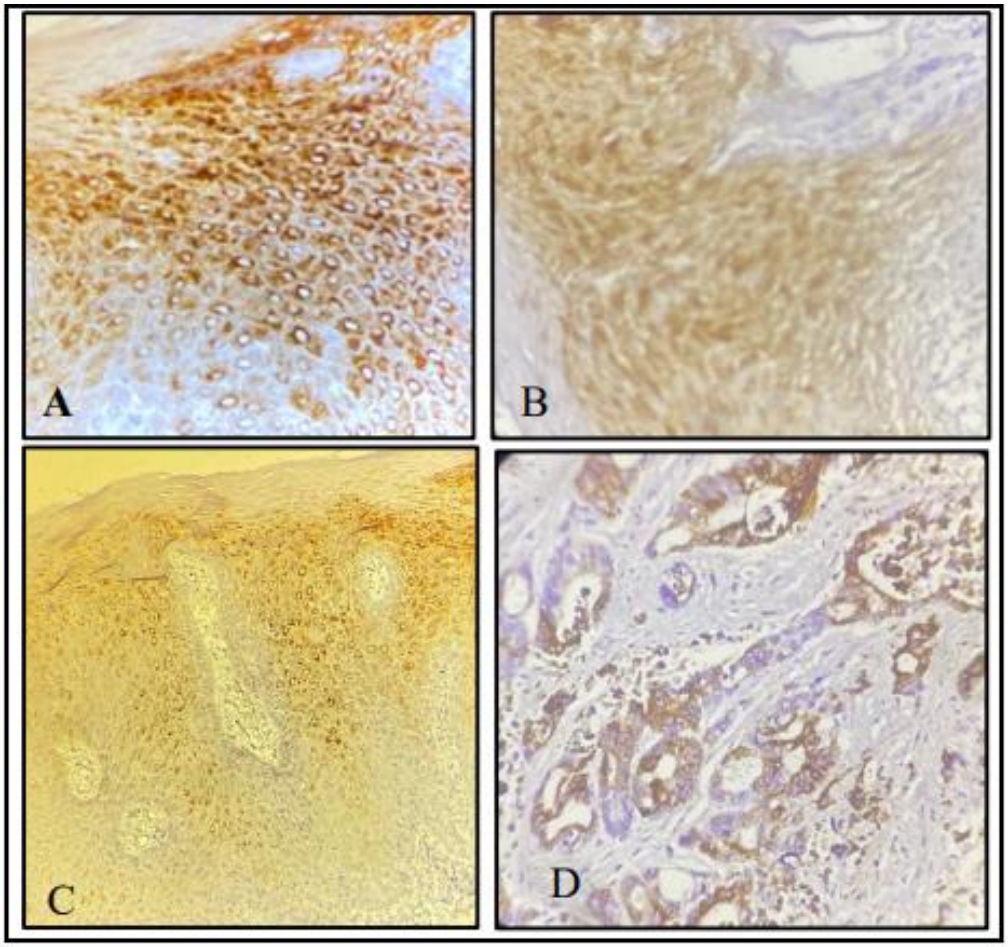

Figure (1): diffuse cytoplasmic and/or membrane immunohistochemical expression of COX-2 in OLP lesions: A- Strong (+3) (magnification 40X), B- Moderate (+2) (magnification 40X), C- Mild (+1) (magnification 20X), D- Positive staining of Cox-2 in the colon cancer (magnification 20X).

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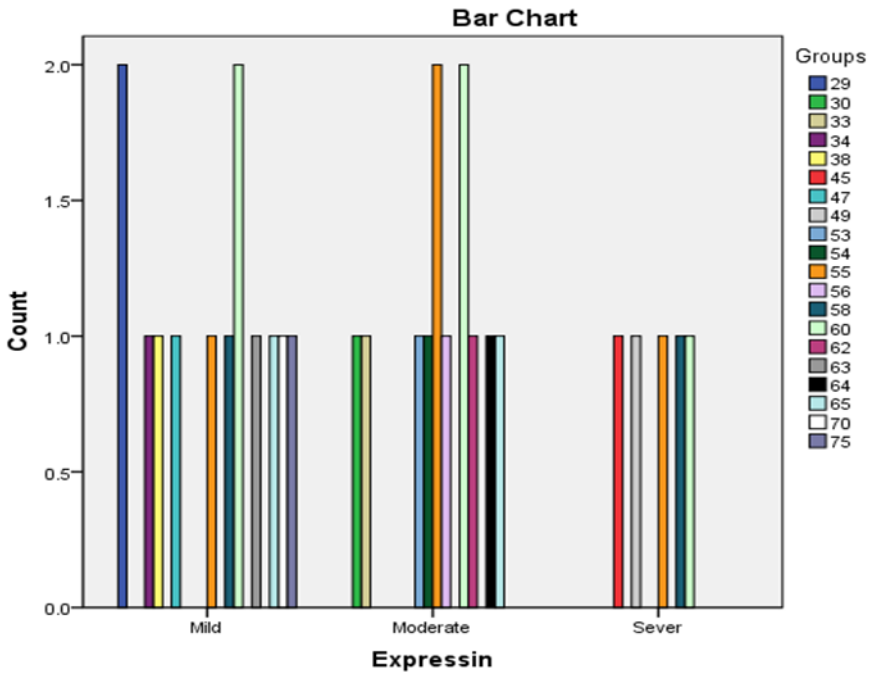

Figure (2): Bar chart presents the distribution of Cox-2 expression with the age groups for the studied lesions of OLP patients.