



**EVALUATION OF THE THERAPEUTIC EFFECT OF TOPICAL
CALCIPOTRIOL VERSUS TOPICAL TRETINOIN IN TREATMENT
OF ORAL LEUKOPLAKIA AND THEIR EFFECT ON CLINICAL
IMPROVEMENT AND SALIVARY LEVEL OF MMP-9, IL-6 AND TGF- β :
A RANDOMIZED CLINICAL TRIAL**

Dalia M. Ghalwash*, Enji A. Mahmoud** and Olfat G. Shaker***

ABSTRACT

Background: Leukoplakia is the most common potentially malignant lesion of the oral cavity. The main objective in treatment of oral leukoplakia is to prevent malignant transformation. Retinoids and vitamin D analogues have anti-keratinizing and immunomodulatory properties and proven effectiveness in the treatment of leukoplakia.

Aim of the study: the aim of this clinical trial was to compare the effectiveness of topical calcipotriol versus topical tretinoin in treatment of oral leukoplakia regarding changes in clinical score as well as salivary IL-6, TGF- β and MMP-9 levels.

Methodology: 40 patients with oral leukoplakia were randomly assigned into two groups. **Group 1:** (n=20) patients were treated with topical calcipotriol gel applied twice daily for 4 weeks. **Group 2:** (n=20) patients were treated with topical tretinoin cream applied twice daily for 4 weeks. Clinical improvement as well as salivary level of TGF- β , IL-6 and MMP-9 was evaluated 4 weeks after treatment and compared to baseline values.

Results: Both treatments resulted in clinical improvement with no significant differences between groups. However, calcipotriol produced a highly statistically significant reduction in salivary IL-6 and MMP-9 compared to tretinoin while both treatments caused significant reduction in salivary TGF- β with no significant difference.

Conclusion: Calcipotriol could be considered a promising therapeutic alternative with fewer side effects for the treatment of oral leukoplakia.

KEYWORDS: Leukoplakia, Calcipotriol, Tretinoin, MMP-9, IL-6, TGF- β .

* Associate Professor of Oral Medicine and Periodontology, Faculty of Dentistry. The British University in Egypt.

** Associate Professor of Oral Medicine and Periodontology, Faculty of Dentistry. Cairo University.

*** Professor of Biochemistry, Faculty of Medicine. Cairo University.

INTRODUCTION

Oral leukoplakia is one of the most common potentially malignant lesions⁽¹⁾. Leukoplakia was defined by WHO in (2005) as “a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer⁽²⁾. According to the systematic review conducted by **Petti**⁽³⁾, the estimated prevalence rate of leukoplakia was found to be 2% worldwide. Leukoplakia lesions are most common in the buccal mucosa, floor of the mouth, tongue, lip and vermilion border and have a high risk of malignant potential⁽⁴⁾.

Rate of malignant transformation of oral leukoplakia has an average of 1% of cases per year⁽⁵⁾. Factors blamed to be responsible for higher risk of malignant transformation are: female gender, non-smokers, speckled or proliferative leukoplakia and presence of epithelial dysplasia. However, cases of malignant transformation of leukoplakia have been reported in the absence of the above mentioned factors⁽⁶⁻⁸⁾.

It's well recognized that numerous proinflammatory cytokines besides being regulators of the inflammatory response, have an important role in oral diseases and their level was reported to be increased in patients with potentially malignant lesions and oral cancer, one of such cytokines is Interleukin-6 (IL-6)⁽⁹⁾.

Interleukin-6 (IL-6) is a multifunctional cytokine that was reported to be involved together with its downstream targets in the regulation of cell proliferation, survival, and metabolism, IL-6 signalling has also been implicated in malignant transformation and tumorigenesis⁽¹⁰⁾. IL-6 expression has shown to be induced by the transcription factor NF- κ B under hypoxic conditions which is correlated with aggressive tumor growth and poor patient prognosis⁽¹¹⁾. This detrimental effect of hypoxia has been attributed to an increase in the level of hypoxia inducible factor-1 α . The latter

one is mediated through the downstream signalling element of IL-6 pathway—the STAT3⁽¹²⁾. STAT3 regulates G1 to S cell-cycle progression as well as the prevention of apoptosis. Thus, the IL-6/gp130/STAT3 axis has been implicated in playing a crucial role in the sequential change from hyperplasia to neoplasia^(13,14).

Brailo et al.,⁽¹⁵⁾ reported that patients with oral leukoplakia have significantly higher level of salivary IL-6 compared to healthy control. They suggested that this increase might be due to local production of IL-6 that may have two sources: either from the lesional epithelium itself or from lymphocytes of a discrete chronic inflammatory infiltrate present in tissues affected with leukoplakia.

The expression of growth factors and their receptors are thought to play a crucial role in the malignant transformation and tumor progression⁽¹⁶⁾. Transforming growth factors (TGFs) are involved in normal growth and differentiation of oral keratinocytes⁽¹⁷⁾. Transforming growth factor β 1 (TGF- β 1) is a member of a highly pleiotropic family of growth factors. Their signalling pathway has multiple and diverse roles in epithelial-type cells. In precancerous lesions and during the early stages of tumor progression, TGF- β 1 was found to exert tumor-suppressive effects. While during the later stages of cancer, TGF- β 1 signalling was reported to promote tumor growth and invasion⁽¹⁸⁾. Understanding the mechanisms behind the role of TGF- β 1 in oral cancer is vital for identifying novel targets for pharmacological intervention⁽¹⁹⁾.

Matrix metallo-proteinases (MMPs) a group of enzymes secreted by macrophages, neutrophils and fibroblasts in response to the stimulus from TGF- β and IL-8 and are responsible for the degradation of most extracellular matrix proteins during organogenesis, growth and normal tissue turnover. The secreted MMPs maintain the growth factors bioavailable which results in cancer proliferation⁽²⁰⁾. Among many factors included in etiopathogenesis

of potentially malignant disorders as leukoplakia, MMP-9 plays a main role in tumorigenesis ranging from initiation/promotion to angiogenesis, dissemination, immunological surveillance and metastatic growth. Tissue damage due to excessive proteolytic activity is a common consequence of sustained expression of MMP-9^(21,22).

Generally, the main objective in treatment of oral leukoplakia is to prevent malignant transformation which requires adequate knowledge about nature of the disease and careful evaluation of the case⁽²³⁾.

In cases with moderate to severe epithelial dysplasia where there is high risk for malignancy, surgical removal is recommended⁽²⁴⁾. While in low to moderate epithelial dysplasia, non-surgical treatment should be considered with the advantage of less adverse reactions, less invasion, ease of application and relative low cost, especially, when oral leukoplakia affects large area or in patients with medical condition contraindicating surgical management^(6,25,24).

Retinoic acid (Vitamin A) and Vitamin D analogue both were examined in treatment of oral leukoplakia and has shown efficacy^(23,26-29).

Topical retinoids such as tretinoin are used as off-label drugs in some oral pathologies after addition of a mucosal adhesive paste (Orabase) to improve their adherence and retention to the oral mucosa⁽³⁰⁾. The ability of retinoids to regulate growth, differentiation, proliferation and apoptosis of cells together with their effect in modulating MMP-9 expression explains their efficacy in suppressing tumor formation in many cancers⁽³¹⁻³³⁾.

Calcitriol or vitamin D3 is a well-known pro-differentiating hormone that regulates the activity of more than 60 genes involved in cell differentiation and inducing the expression of adhesion proteins. These effects are potentially therapeutic as these adhesion proteins have the ability to maintain cell bonding preserving tissue integrity⁽³⁴⁾. Calcipotriol

or calcipotriene is a synthetic analogue of calcitriol or vitamin D3⁽³⁵⁾.

Calcipotriol has proved efficacy in treatment of hyperkeratotic skin lesions such as psoriasis. The therapeutic potential of topical calcipotriol in hyperkeratotic lesions rely on its antiproliferative and anti-inflammatory effect. The use of calcipotriol in treatment of precancerous and cancerous lesions has gained interest since many tissues express vitamin D receptor as well as vitamin D metabolizing enzymes. Thus, it inhibits proliferation and stimulates differentiation of normal as well as malignant cells⁽³⁶⁾.

Modulation of cellular differentiation and proliferation by compounds such as some of the newer retinoids and calcipotriol offers the possibility for the therapeutic prevention, reversal, or arrest of carcinogenesis⁽²³⁾.

Therefore, the aim of this clinical trial was to compare the effectiveness of topical calcipotriol versus topical tretinoin in treatment of oral leukoplakia regarding changes in clinical score as well as salivary IL-6, TGF- β and MMP-9 levels.

SUBJECTS AND METHODS:

Study design: This study is a parallel group, two arms, Randomized Controlled Trial (RCT) with 1:1 allocation ratio.

Patient selection: The present study was performed on a total of 40 subjects having oral leukoplakia (OL) selected from the outpatient clinic of oral medicine & periodontology Department Faculty of Oral and Dental Medicine, Cairo University, Cairo-Egypt.

Eligibility criteria for patients' selection:

Inclusion criteria included:

1. Systemically free patients as evaluated by the aid of dental modification of the Cornell Medical Index to standardize their systemic condition⁽³⁷⁾.

2. Patients with oral leukoplakia, histologically confirmed, with only mild or moderate epithelial dysplasia.
3. Lesions located at sites where the patients could readily apply a topical drug and anticipate that it could remain localized for several minutes ⁽²³⁾.
4. Patients willing and able to return for multiple follow up visits.

Exclusion criteria included:

1. Presence of any visible oral lesions other than leukoplakia.
2. Leukoplakia on soft palate due to difficult accessibility in topical drug application.
3. Periodontitis patients: since chronic periodontitis may affect salivary IL-6, TGF- β and MMP-9 level ^(38,39).
4. Contraindications for the use of calcipotriol ⁽⁴⁰⁾.
5. History of topical and/or systemic therapy for 2 months before the start of study ⁽⁴¹⁾.

Ethical procedures: The study was approved by the Research Ethics Committee of Faculty of Dentistry, Cairo University. Each patient was informed about the detailed procedure and both benefits of the treatment and the known side effects and follow-up appointments needed. Each subject participating in the study signed an informed written consent form.

Preoperative patient evaluation: was done as follows:

(i) **Case history:** Comprehensive oral diagnosis was carried out using the department's oral diagnosis chart. For each patient reviewed, the age at presentation, gender, chief complaint, if there was previous treatments for the chief complaint in more than a month, current medications if any.

Complete medical history was taken to exclude: Concomitant use of vitamin D or calcium or any other drug that can affect calcium homeostasis, conditions contraindicated for the use of calcipotriol⁽⁴⁰⁾ such as: a. Hypercalcemia and hypercalciuria, b.

Urolithiasis, c. Parathyroid disease, d. Disorders of calcium metabolism, and e. Pregnancy and lactation

Patients were asked to perform the following laboratory tests: Urea and electrolytes; liver function and haematological status (Complete Blood Count).

(ii) **Clinical examination:** included examinations of intraoral soft tissue.

A provisional diagnosis of leukoplakia is made as oral leukoplakia implies only the clinical feature of a persistent, adherent white plaque that cannot be rubbed off, that needs to be distinguished from other predominantly white keratotic lesions including frictional keratosis and stomatitis nicotina, which do not have malignant potential ⁽⁵⁾.

Histopathological examination: The definitive diagnosis of oral leukoplakia was established by histopathological exclusion of other keratotic oral lesions that are recognized as specific entities, and by exclusion of any aetiological agents other than tobacco/areca nut use ^(42-45,5). Microscopically, there is simple orthokeratosis, or parakeratosis with epithelial hyperplasia and minimal inflammation, or hyperkeratosis with varying degrees of dysplasia ⁽⁴⁶⁾.

Assignment of interventions:

Allocation; Sequence generation, Allocation concealment mechanism and implementation:

Patients of each of study groups were randomly assigned to one of two parallel groups, in 1:1 ratio, to receive either topical calcipotriol (group1) or topical tretinoin (group 2). The method used to generate the random allocation sequence of the participants is a computer-generated list of random numbers. Allocation was done using simple randomization procedure using randomization list sequentially numbered, patient were given serial numbers every patient added to list occupied serial number.

Blinding: Single blinded (Data analyst) while operator and patient were not blinded.

Interventions:

Participants in this study were divided into 2 groups as follow: **Group 1:** (n=20) patients were treated with 50 mcg/g calcipotriol in an adhesive vehicle (carboxymethyl-cellulose) applied twice daily for 4 weeks ^(23, 40). **Group 2:** (n=20) patients were treated with 0.05% Tretinoin cream applied twice daily for 4 weeks (Rouses point pharmaceuticals, LLC by DBT laboratories, San Antonio, USA) ^(47, 23, 48).

Calcipotriol (0.005%) preparation:

Topical calcipotriol gel was prepared by a specialist in Faculty of Pharmacy, Cairo University as follows: Using balance 4 digit 10 mg of pure calcipotriol powder (TOCRIS Bioscience Company, UK) was added to 10 mL ethanol + water using hot magnetic stir. This was diluted by taking 2 ml from (1 ml solution+1mg pure drug) and dilute in 10 ml solution thus gives (200 µg/ml). 1 ml of this dilution (200 µg/ml) added to 40 gm of 2% carboxymethyl cellulose powder (Research-lab company, India) this gives dilution of 5µg/g.

Patients were instructed to: 1. Dry the lesion site with sterile gauze, and apply the therapeutic agent as a cream with a cotton bud. 2. Leave the agent in situ for at least 15 mins. 3. Try to avoid swallowing the drug. All beverages were avoided for at least 30 min after the drug application. The treatment was for 4 weeks in each case.

Criteria for discontinuing or modifying intervention: If there was any sign of adverse effects or toxicity other than mild hypercalcaemic or hypercalciuric action of calcipotriol.

Clinical Evaluation:

Lesions were evaluated, recorded photographically and given scores at baseline with regular clinical assessments at 0, 2 and 4 weeks for groups 1 and 2 as follows: **Clinical score: Clinical scores for roughness & intensity of whiteness**

was estimated visually according to **Femiano et al.**, ⁽²³⁾ to which the following values were given: 0: Total resolution of lesions. 1: Moderate resolution=reduction of consistence with leveling of the lesion. 2: Minimal change=reduction of consistence without leveling of the lesion. 3: No visible change.

Collection of Salivary Samples: Whole saliva was collected at baseline for groups A, B and C and 4weeks follow up after therapy for groups A and B, under resting conditions in a quiet room between 8 A.M. and noon, at least 1 h after food intake. Patients were asked to generate saliva in their mouths and to spit into a wide test tube for 10 min, as previously described Following the collection, the saliva was stored at 4_C for up to 2 hours, immediately centrifuged at 800g at 4°C for 10 min and the resulting supernatant was used for further biochemical analysis ⁽⁴⁹⁾.

Detection of human IL-6 levels in salivary samples:

The AssayMax Human IL-6 ELISA kit is designed for detection of IL-6 in human plasma, serum, saliva or cell culture supernatants. This assay employs a quantitative sandwich enzyme immunoassay technique that measures IL-6 in 5 hours. A murine monoclonal antibody specific for human IL-6 has been pre-coated onto a microplate. IL-6 in standards and samples is sandwiched by the immobilized antibody and a biotinylated polyclonal antibody specific for human IL-6, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured. The minimum detectable dose of IL-6 is typically < 10 pg/ml. This assay recognizes both natural and recombinant human IL-6

Detection of human TGF-β levels in salivary samples:

TGF-β1 was measured in the sera of all patients using Invitrogen Multispecies TGF-β1 kit (catalog #

KACL1688/ KAC1689) provided from Biosource, California. The Invitrogen Multispecies TGF- β 1 kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for TGF- β 1 has been coated onto the wells of the microtiter strips provided. Samples, including standards of known TGF- β 1 content, control specimens, and extracted unknowns, are pipetted into these wells, followed by the addition of a biotinylated second antibody. During the first incubation, TGF- β 1 antibody binds simultaneously to the immobilized (capture) antibody on one site, and to the solution phase biotinylated antibody on a second site. After removal of excess detection antibody, Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove all the unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of TGF- β 1 present in the original specimen.

Detection of human MMP-9 levels in salivary samples: Quantitation of MMP-9 levels in saliva was done using human MMP-9 ELISA kit provided by affymetrix. Bioscience. This assay is a quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for MMP-9 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any MMP-9 present is bound by the immobilized antibody. The unbound substances were removed by washing. The enzyme-linked monoclonal antibody specific for MMP-9 is added to all wells. A substrate solution is added to the wells after washing to remove any unbound antibody-enzyme reagent. Color was developed in proportion to the amount of MMP-9 bound in the initial step. The color development is stopped and the intensity of the color is measured by ELISA reader.

Statistical Analysis

All Data were collected, tabulated and subjected to statistical analysis. Statistical analysis is performed by SPSS in general (version 17), also Microsoft office Excel is used for data handling and graphical presentation. **Quantitative variables:** were described by the Mean, Standard Deviation (SD), the Range (Maximum – Minimum) and **qualitative categorical variables** were described by proportions and Percentages. Kolmogorov-Smirnova and Shapiro-Wilk tests of normality are used to test normality hypothesis of all quantitative variables for further choice of appropriate parametric and non parametric tests. Paired sample t test and Wilcoxon Signed Ranks Test are used for testing Pre –post measurements within the same group while independent sample t test and Mann and Whitney U test are used for comparing the mean changes between the two groups. The z statistics is used for hypothesis testing of two proportions, while chi-squared test is used for categorical variables in contingency tables. Significance level is considered at $P < 0.05$ (S); while for $P < 0.01$ is considered highly significant (HS). Two Tailed tests are assumed throughout the analysis for all statistical tests.

RESULTS

Results for Clinical Score

In calcipotriol group patients showing total resolution of lesions were (0%) at 2 weeks vs (25 %) at 4 weeks, while patients having moderate resolution were (20 %) at 2 weeks vs (30%) at 4 weeks. Finally, cases with no visible changes were 30% at 2 weeks vs 10 % at 4 weeks. Accordingly, there was statistically significant higher clinical resolution ($P < 0.05$) at 4 weeks than 2 weeks for scores 0 and 1 and lower for scores 2 and 3.

Regarding tretinoin group patients showing total resolution of lesions were (0%) at 2 weeks vs

(10%) at 4 weeks, while patients having moderate resolution were (10 %) at 2 weeks vs (30%) at 4 weeks. Finally, cases with no visible changes were (45%) at 2 weeks vs (30%) at 4 weeks. Accordingly, there was statistically significant higher clinical resolution ($P < 0.05$) at 4 weeks than 2 weeks for scores 0 and 1 and lower for scores 2 and 3.

When clinical scores were compared between the two groups it was evident that at 2 weeks of treatment no patient showed total resolution of lesion neither in group I nor in group II. However, patients with moderate resolution of lesions were 20 % in group I vs 10 % in group II. Then, the percentage was higher in calcipotriol than in tretinoin treated group but doesn't reach significance ($P\text{-value} = 0.943$). Additionally, patients with minimal change were 50 % in calcipotriol group vs 45 % in tretinoin group. Then, the percentage of improved cases is slightly higher in calcipotriol group than tretinoin however it doesn't reach significance ($P\text{-value} = 0.8808$). Moreover, there was no visible change in 30 % of calcipotriol vs 45 % in tretinoin treated group which indicates higher percentage of patients with no visible improvement in tretinoin group. However, it is also statistically non-significant ($P\text{-value} = 0.8808$). At 4 weeks of treatment, the percentage of improved cases was slightly higher in calcipotriol than tretinoin group however it didn't reach significance as well ($P\text{-value} 0.2119$) as shown in **Table (1)**.

Comparing cases with any improvement whether total, moderate or minimal improvement versus cases with no visible changes at 4 weeks which is the total follow up period. The improved cases in calcipotriol group were 16 out of 20 representing 80% and those of tretinoin group were 14 out of 20 equals to 70%. However, cases with no visible changes in calcipotriol group were 2 out of 20 cases representing 10 % compared to 2 out of 20 patients which are 10 % of the tretinoin cases. Accordingly, there is a higher percentage of improved cases compared to non-improved cases in both groups. Generally, there was no statistically significant difference between the two groups with $P\text{-value} (0.854)$ **Figure (1)**

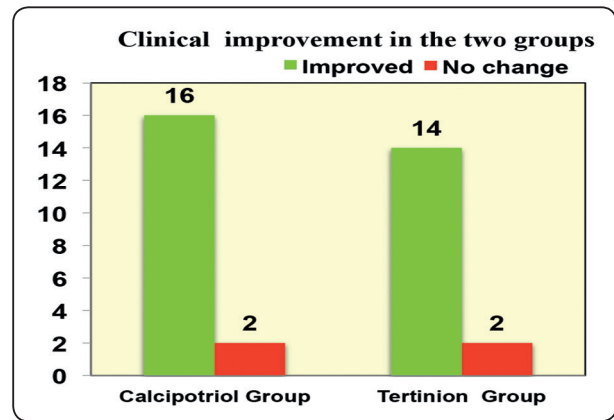


Fig. (1): Comparison between the two studied groups in number of cases improved and those with no visible change

TABLE (1) Comparison in improvement in clinical score between groups I and II at 4 weeks

4 weeks	Calcipotriol Group		Tretinoin Group		Z	P-Value
	Number	%	Number	%		
0: Total resolution	5	25%	2	10%	1.25	0.2119
1: Moderate resolution	6	30%	6	30%	Equal Percentage	
2: Minimal change	5	25%	6	30%	0.35	0.7233
3: No visible change.	2	10%	2	10%	Equal Percentage	

P>0.05 Non significant

Results for biochemical markers

On comparing pre and post treatment values of each variable within Group I (Calcipotriol), this comparison revealed that the mean and SD values of MMP-9 level ($\mu\text{g/L}$) pre-and post-treatment was $7.50(\pm 0.88)$ and $4.71(\pm 0.91)$ respectively with a mean difference of (-2.79) . Therefore, there is a statistically highly significant reduction in MMP-9 level post-treatment with P-value (< 0.001). The mean and SD values of TGF- β (ng/ml) level pre-and post-treatment was $18.89(\pm 2.44)$ and $14.30(\pm 2.91)$ respectively with a mean difference of (-4.59) . Therefore, there is also a statistically highly significant reduction in TGF- β level post-treatment with P-value (< 0.001). Regarding IL-6 (pg/ml) the mean and SD values for pre and post-treatment level was $51.64(\pm 9.71)$, and $27.19(\pm 7.24)$ respectively with a mean difference of (-24.45) . Therefore, there is also a highly significant reduction statistically in IL-6 level post-treatment with P-value (< 0.001).

Table (2)

On comparing pre and post treatment values of each variable within Group II (Tretinoin) this comparison revealed that the mean and SD values of MMP-9 ($\mu\text{g/L}$) level pre- and post-treatment level

was $7.72(\pm 0.60)$ and $87(\pm 0.76)$ respectively with a mean difference of (-1.86) . This shows a statistically highly significant reduction in MMP-9 level post-treatment with P-value (< 0.001). Same for the mean and SD values of TGF- β (ng/ml) level pre-and post-treatment was $25.16(\pm 4.84)$ and $19.72(\pm 4.80)$ respectively with the mean difference was (-5.44) . This denotes that there is also a highly significant reduction in TGF- β level post-treatment with P-value (< 0.01). Regarding IL-6 (pg/ml) the mean and SD values for its pre- and post-treatment level was $50.62(\pm 10.99)$ and $33.31(\pm 8.31)$ respectively with the mean difference was (-17.31) . Therefore, there is also a significant reduction in IL-6 level post-treatment with P-value (< 0.05). **Table (2)**

Table (2) shows comparison in pre-and post-treatment values of the three biological markers (MMP-9, TGF- β , and IL-6) between the two groups.

Group I (calipotriol) revealed highly significant reduction ($P < 0.001$) in post-treatment level of MMP-9 and IL-6 compared to Group II (tretinoin). On the other hand, there was non-significant difference ($P > 0.05$) between the two groups post-treatment values of TGF- β .

TABLE (2) Comparison in pre-and post-treatment values of the three biological markers (MMP-9, TGF- β and IL-6) between the two studied groups.

		N	Mean	Std. Deviation	Mean Difference	Std. Error Difference	t	df	Probability	
MMP-9 (ug/L)	Group I (Calcipotriol)	20	-2.79	0.92	-0.93	0.26	-3.65	38	0.00078	P < 0.001 Highly Significant
	Group II (Tretinoin)	20	-1.86	0.67						
TGF- β (ng/ml)	Group I (Calcipotriol)	20	-4.59	0.98	0.84	0.57	1.49	38	0.14414	P > 0.05 Non Significant
	Group II (Tretinoin)	20	-5.44	2.33						
IL-6 (pg/ml)	Group I (Calcipotriol)	20	-24.45	6.67	-7.14	2.20	-3.25	38	0.00245	P < 0.01 Highly Significant
	Group II (Tretinoin)	20	-17.31	7.23						



Clinical Photograph of 47 years old male patient with oral leukoplakia before and after treatment with topical calcipotriol



Clinical photograph showing a case of 49 years male patient with oral leukoplakia before and after treatment with topical tretinoin

DISCUSSION

The aim of this clinical trial was to compare the effectiveness of topical calcipotriol versus topical tretinoin in treatment of oral leukoplakia regarding changes in clinical score as well as salivary IL-6, TGF- β and MMP-9 level.

Patients were chosen to be systemically healthy according to modified Cornell index⁽³⁷⁾. Periodontitis patients were excluded since IL-6, TGF- β and MMP-9 have shown to play role in the pathogenesis of periodontitis. **Rai et al.**,^(50,51) concluded that MMP-9 may serve as biomarkers of periodontal disease and aid in early detection of periodontitis. Diabetic patients and those with coronary heart diseases were

also excluded from the study. **Maxwell et al.**,⁽⁵²⁾ demonstrated that MMP-9 and their inhibitors are integral to the vascular changes of atheroma and MMP-9 level is raised in diabetes. Furthermore, hepatic patient were also excluded since the use of vitamin A related compounds have demonstrated liver damage^(53,50,51,39,38).

Additionally, cases with hypercalcemia, hypercalciuria, urolithiasis, parathyroid disease, disorders of calcium metabolism, concomitant use of vitamin D or calcium or any other drug that can affect calcium homeostasis were all excluded since they are contraindicated for treatment with calcipotriol⁽⁴⁰⁾. Although the risk of embryopathies associated to topical retinoids is considered low,

their use during pregnancy is not recommended because their risk/benefit ratio remains questionable. Therefore, Pregnancy and lactation were also excluded⁽⁵⁴⁾.

Location of lesions in this study was readily accessible for topical application of drug since lesions in inaccessible areas like soft palate were excluded.

Unfortunately, though many leukoplakias in previous studies have regressed or resolved during systemic treatment with vitamin A analogues, toxicity and risk of teratogenicity has been the main limiting factors in the use of systemic retinoids. It is possible that the local application of retinoic acid results in a higher concentration of the substance directly in the target tissue. For this reason, in this study the use of topical preparations was preferred^(23, 55). Topical preparation could adhere well to oral mucosa and provide transport medium of active drug together with reasonable exposure time since it was prepared in adhesive vehicle (carboxymethylcellulose).

Dosage of tretinoin cream (0.05%) used as twice daily was in accordance with other studies that used tretinoin in treatment of oral leukoplakia^(47, 23, 56, 48). Dosage of calcipotriol preparation (5 μ g/g) (0.005%) twice daily was similar to dosage reported by **Shahzad et al.**⁽⁴⁰⁾ reviewing the use of topical calcipotriol in dermatology.

In the present study the follow up period was chosen to be 4 weeks in accordance with **Günther**,⁽⁵⁷⁾ who reported improvement in lesions in this period of time. This period probably correspond to the time required for the renewal of the epithelium.

Salivary testing has the advantage of being a non-invasive alternative to serum testing, can be an effective modality for diagnosis and prognosis predicting of oral cancer and premalignant lesions as well as for monitoring the patient's post-therapy status⁽⁵⁸⁾.

This study utilized ELISA technique in order to assess the level of IL-6, TGF- β and MMP-9 in whole unstimulated saliva. The ELISA system is cited as the most sensitive, well-established, and widely available protein-based testing platform for the detection of specific proteins in body fluids or tissue⁽⁵⁹⁾. ELISA-based techniques seem more cost-effective because they are simple to perform and inexpensive. It also allows for antigen detection using extremely small samples⁽⁶⁰⁾.

Result of the present study showed that there was a statistically significant improvement in clinical scores within tretinoin group with total resolution in 30% of cases, moderate resolution in 30% of patients and minimal change in 10%. So patients showing any improvement constitutes 70% while those with no visible change are only 10%. These percentages were significantly higher at 4 weeks than those at 2 weeks of treatment period.

The clinical results in the tretinoin group were in line with those of **Shah et al.**⁽⁶¹⁾ who performed a 6-month trial using isotretinoin lozenges in patients with oral leukoplakia. They evaluated clinically thinning of the leukoplakia and a reduction in the white surface. In patients who completed trial, (27%) showed complete visible clinical response, while (80%) of patients documented remission.

Epstein and Gorsky,⁽⁴⁷⁾ used 0.05% tretinoin gel that was applied topically 4 times per day for the management of non-malignant oral white lesions. Complete clinical remission was reported in (27%) of patients. However, partial response was noted in 54% of patients. Their results were in accordance with results of the present study were they used same concentration but in the form of gel.

Piattelli et al.,⁽²⁷⁾ during the assessment of 0.1% isotretinoin gel applied 3 times per day in oral leukoplakia for 4 months a complete response was noted in (10%) of patients, although (90%) of patients experienced a reduction in size of at least 50%, representing a partial response. **Tete et al.**,⁽⁶²⁾

noted complete remission in (21.5%) of patients with topical 0.1% isotretinoin, a marked improvement (a reduction in size 50%) was achieved in (78.5%) of patients in 4 months of treatment.

Considering the assumption that topical tretinoin could be a potential alternative for surgical removal in some cases of oral leukoplakia, **Starzyńska et al.** ⁽⁴⁸⁾ performed a study which included patients having oral leukoplakia, compared the effectiveness of 0.05% topical tretinoin cream with surgical and cryosurgical treatment. In over 77% of patients treated with tretinoin, total improvement was observed after 4 weeks of treatment time. This percentage is consistent with results in the present study in which 70% of patients treated with tretinoin showed total improvement in same period of time (4weeks).

Data from the current study showed that there was a statistically significant improvement in clinical scores within calcipotriol group with total resolution in 25% of cases, moderate resolution in 30% of patients and minimal change in 25%. So patients showing any improvement constitutes 80% while those with no visible change were only 10%. These percentages were significantly higher at 4 weeks than those of 2 weeks of treatment period.

Moreover, in the present clinical trial by 4 weeks, the percentage of improved cases was slightly higher in calcipotriol than tretinoin group with non-significant difference between groups.

These results were in accordance with an open trial conducted by **Femiano et al.**, ⁽²³⁾ to compare the clinical efficacy of topical calcipotriol (50mg/g) with tretinoin cream (0.05%) in the therapy of hyperkeratotic oral lesions (leukoplakia). Their results showed that in 2 weeks, keratotic lesions in both study groups had substantially regressed, with leveling of hyperkeratotic surfaces, softening and an attenuation of the whiteness. By the fifth week, 80% of patients in both groups showed complete clinical resolution of lesions in both calcipotriol and

tretinoin groups that was consistent with results in the present study.

Femiano et al.,⁽²³⁾ suggested that topical calcipotriol is as effective in the treatment of oral leukoplakia as is topical tretinoin. These suggestion was confirmed by results of the present study. Modulation of cellular differentiation and proliferation by compounds such as some of the newer retinoids and calipotriol offers the possibility for the therapeutic prevention, reversal, or arrest of carcinogenesis.

Among many factors included in etiopathogenesis of PMDs as leukoplakia, MMP-9 plays a fundamental role in tumor biology. **Stott-Miller et al.** ⁽⁶³⁾ stated that salivary MMP-9 is a robust diagnostic biomarker of malignant transformation. Detection of MMP-9 proteins in saliva in particular may provide a promising means to detect and monitor PMDs post-therapy status non-invasively.

Results of the present study indicated highly significant reduction in MMP-9 ($\mu\text{g/L}$) level ($P < 0.001$) in tretinoin treated group at the end of the study period. Results were in accordance with **Nguyen et al.**⁽⁶⁴⁾, **Papi et al.**⁽⁶⁵⁾; **Jalian et al.**⁽⁶⁶⁾; **Yang et al.**⁽⁶⁷⁾. In their in vitro investigations, they found that ATRA (tretinoin) might down regulate the expression of MMP-9. Contrarily, **Darmanin et al.**,⁽⁶⁸⁾ reported that ATRA could increase the expression of MMP-9. Therefore, available evidences were controversial due to disagreement between studies ⁽⁶⁹⁾.

Results of the present study indicated highly significant reduction in salivary MMP-9 ($\mu\text{g/L}$) level in calcipotriol treated group ($P < 0.001$). Results were in accordance with **Meephansan et al.**⁽⁷⁰⁾ who investigated whether calcipotriol could suppress the expression of MMP-9 in a human squamous cell carcinoma (SCC) cell line. They found that calcipotriol suppressed the production of MMP-9 mRNA and proteins significantly, in a dose-dependent manner.

In the present study, there was highly significant reduction in MMP-9 level after both tretinoin and calcipotriol treatment. However, there was a statistically significant higher reduction of salivary MMP-9 level in calcipotriol than tretinoin treated group. This supports the suggestion that calcipotriol could be a potential alternative to tretinoin in management of premalignant lesions.

Results of the present study indicated a highly significant reduction in TGF- β level ($P < 0.001$) in the calcipotriol treated group. Results of the present study also indicated a highly significant reduction in salivary TGF- β level in the tretinoin treated group ($P < 0.01$) at the end of the study period. When the reduction in TGF- β level was compared between the two groups, no significant difference was found ($P > 0.05$).

Wagner et al.⁽⁷¹⁾ analysed TGF- β expression in a spectrum of samples that included normal mucosa, leukoplakia with or without dysplasia, and OSCC and reported that OSCC exhibited higher levels of expression of TGF- β than leukoplakia and normal mucosa samples and suggested that TGF- β modifications are associated with the progression of oral carcinogenesis, based on the gradual increase in the expression of TGF- β between the normal mucosa, leukoplakia, and then the carcinoma cases.

Moreover, previous reports have demonstrated that TGF- β stimulation induces severe epithelial hyperplasia in oral mucosa and increases the proliferation rate of oral cancer cells⁽¹⁹⁾. In the same line, another study of TGF- β receptor II has indicated that its down-regulation is an early event in oral carcinogenesis, which may occur in the loss of TGF- β mediated inhibition, thereby facilitating progression of precancerous lesions to SCC⁽⁷²⁾.

All these data support the hypothesis that during the process of oral carcinogenesis, the TGF- β signalling pathway loses the capacity to inhibit cell growth and begins to enhance tumor progression, indicating that TGF- β is highly active in this type of

oral lesions and may represent a valuable therapeutic target, and that the development of TGF- β inhibitors for cancer therapy is under progress⁽⁷¹⁾.

Taking that under consideration together with the results of the present study showing the effect of topical application of both calcipotriol and tretinoin that has led to a highly significant reduction in TGF- β level at the end of the treatment period, illustrating the inhibitory effect they have on TGF- β which could in turn contribute to the possible arrest or slowing of the progression of tissue change that may occur in leukoplakia lesions, which is further confirmed with the recorded clinical improvement in most cases. This also provides more evidence that using TGF- β as a therapeutic target in leukoplakia is a promising strategy not only for improving the case clinically but also to reduce the possibility of occurrence of malignant transformation of one of the most common PMDs of the oral cavity.

IL-6 is an NF- κ B dependent cytokine produced by inflammatory cells as well as tumor cells⁽⁷³⁾. As an NF- κ B-dependent cytokine, IL-6 levels were found to be significantly elevated in saliva of patients with potentially malignant lesions and oral squamous cell carcinoma⁽⁷⁴⁾. It has been found that the IL-6 level in saliva was three to four times more than that in serum. For all the previously mentioned facts, the potential value of IL-6 as a diagnostic marker of malignant transformation was considered⁽⁷⁵⁾.

The results of the present study showed that IL-6 salivary level was significantly reduced after topical application of calcipotriol. To the best of our knowledge, this is the first clinical trial to evaluate the effect of topical calcipotriol and topical tretinoin used for the treatment of oral leukoplakia on salivary IL-6 level, this makes comparison with similar studies not possible. Calcipotriol shares the anti-inflammatory attributes of vitamin D, and is used as a topical treatment in many cutaneous lesions, particularly psoriasis. The effects of calcipotriol are likely due primarily to its anti-proliferative effects on human keratinocytes^(76,77), with the drug's ability

to suppress secretion of pro-inflammatory cytokines such as IL-1, IL-6, IL-8, and IL-18 from both human and murine keratinocyte⁽⁷⁸⁾. This explains the highly significant reduction in IL-6 level in calcipotriol treated group.

There was also a significant reduction in salivary IL-6 in tretinoin- treated group. Studies showed that tretinoin has a direct and indirect inhibitory effect on IL-6. The human epidermoid carcinoma cell line A431 was cultured with phorbol 12-myristate 13-acetate, which stimulates release of the proinflammatory cytokine IL-6 from this cell line. Addition of tretinoin to this system inhibited very potently the release of IL-6⁽⁷⁹⁾.

TNF- α , which is multifunctional cytokine that can induce a broad range of secondary proinflammatory effects is produced in part by human keratinocyte⁽⁸⁰⁾. It can activate cell adhesion molecules; upregulates prostaglandins, and can induce the release of other cytokines, such as IL-1, IL-6, IL-8, and granulocyte macrophage colony-stimulating factor⁽⁸¹⁾. **Brailo et al.**⁽¹⁵⁾ found that patients with oral leukoplakia has significantly higher levels of salivary TNF- α compared to healthy individuals. Several studies have shown that tretinoin is a potent inhibitor of TNF- α production^(82,83, 44) which leads to the reduction in IL-6.

Results of the present study showed that topical calcipotriol produced more significant reduction in salivary IL-6 than do topical tretinoin. Therefore, IL-6 has shown to be an important biomarker for predicting therapeutic responses⁽⁷⁵⁾.

From the previously mentioned results we could conclude that topical calcipotriol is as effective as topical tretinoin in the clinical improvement of oral leukoplakia. Moreover, both treatments produced significant reduction in salivary levels of MMP-9, IL-6 and TGF- β , which may help monitoring the progression of potentially malignant lesions into carcinoma. Furthermore, this inhibitory effect of both therapeutic agents on the three important cytokines indicated that they can

reduce the possibility of occurrence of malignant transformation in oral potentially malignant lesions. Taking into consideration that in the present study calcipotriol treatment resulted in more cases with clinical improvement than tretinoin and that it also resulted in greater reduction of MMP-9, IL-6 levels than tretinoin indicating that calcipotriol is not only as effective as tretinoin but could even be considered a promising therapeutic alternative with fewer side effects.

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