THE USE OF ANTI-OXIDANTS IN THE TREATMENT OF PERSISTENT, NON-RESPONSIVE ORAL LICHEN PLANUS: A RANDOMIZED CONTROL CLINICAL TRIAL

A Thesis

by

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ABSTRACT

Oral lichen planus (OLP) is a chronic inflammatory disease of unknown etiology. Typical management of OLP involves topical corticosteroids. Recent literature shows an association between high levels of various oxidative stress markers, such as malondialdehyde (MDA), and OLP. A combination antioxidant gel consisting of phloretin and ferulic acid has been shown to have beneficial effects. In order to test the efficacy of this particular combination of antioxidants in managing OLP and to contribute to the literature linking oxidative stress to signs and symptoms of OLP, we conducted a double-blinded, placebo-controlled randomized clinical trial. A total of 33 patients with biopsy-confirmed OLP being treated for at least 6 weeks and presenting with persistent or non-responsive symptoms and lesions were given either a placebo (PLC, n = 16) or a test gel (AO, n = 17) and instructed to use three times a day for 4 weeks. Symptom scoring using a VAS, lesional scoring using an OLP scoring system, and salivary levels of oxidative stress markers, 8-hydroxy-deoxyguanosine (8-OH-dG) and malondialdehyde (MDA) were measured at baseline, 2 weeks, and 4 weeks.

VAS for the AO group decreased to 14.25 ± 14.05 at 2 weeks and 16.75 ± 22.14 at 4 weeks from 33.25 ± 28.82 at baseline, OLP lesional scores decreased to 6.26 ± 4.10 at 2 weeks and was 6.53 ± 4.63 at 4 weeks from 7.79 ± 5.18 at baseline, 8-OH-dG decreased 17.9% from 216.88 ± 132.01 at baseline to 178 ± 116.56 at 4 weeks, and MDA increased from 3.24 ± 1.07 to 4.63 ± 1.82 at 4 weeks. The changes were not statistically different from the PLC group in terms of VAS, OLP lesion score, salivary 8-

OH-dG, and salivary MDA at any time point (p >0.05) except for at 4 weeks for MDA (p <0.05. The study revealed that a topical combination antioxidant gel did not differ from a placebo in any of the parameters measured. However, patients did not report any severe flare-ups and had better patient acceptance to topical steroids. To our knowledge, this is the first study to report on salivary 8-OH-dG and MDA levels in patients with oral lichen planus undergoing treatment.

DEDICATION

This manuscript is dedicated to my parents who sacrificed so much to bring our family to this nation.

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INTRODUCTION AND LITERATURE REVIEW

Oral Lichen Planus

Definitions and Demographics

Lichen planus (LP) is a disorder of T-cell-mediated chronic inflammation of stratified squamous epithelium. First described by Erasmus Wilson in 1869, it has a wide range of clinical manifestations with oral lichen planus (OLP) being the second most common manifestation after cutaneous lichen planus. About 1-2% of the general adult population has OLP and 35% of patients with LP have oral lesions exclusively.[1] McCartan & Healy reported an overall prevalence of 1.5% in the general population and a 2.3% prevalence in women.[2] OLP affects women more than men at a ratio of approximately 1.4:1 in some studies.[3] OLP occurs predominantly in adults over 40[1] although younger adults and children may be affected.[4]

Clinical Presentations

LP manifests in a variety of clinical forms. Concomitant skin lesions occur in about 15% of patients[5] and typically present as purple, flat-topped papules about 2-4 mm in diameter on the wrist, ankles, and genitalia. Involvement of nails results in pitting, pterygium formation, and permanent nail loss. Occasional scalp involvement leads to alopecia, which is termed lichen planus planopilaris or Graham-Little syndrome.[6]

Intraorally, OLP can manifest anywhere in the mouth, but is most commonly found on the buccal mucosa followed by the tongue, gingiva, labial mucosa, and vermillion border of the lower lip.[7] Scully and El-Kom reported that roughly 10% of

patients have OLP confined to the gingiva,[6] Eisen reported that 8.6% of patients had gingival OLP,[8] and Mignogna reported the prevalence of gingival OLP to be 7.4%.[9] Lesions can be either unilateral or bilateral, but most report bilateral involvement.[6]

There are 6 clinical variants: reticular, papular, plaque-like, erosive (ulcerative), atrophic (erythematous) and bullous.[10] The reticular form is the most common type and presents as slightly raised, fine, whitish lines in an interlocking lace-like pattern coined "Wickham's striae." The striae are often present bilaterally and occur mostly on the buccal mucosa and most patients with reticular lesions are asymptomatic.[8] The erosive form is the second most common type and is often extensive, irregular, and affects mainly the lingual and buccal mucosa. The erosive lesions are usually red and involvement of the gingiva usually leads to desquamative gingivitis. Erosive lesions usually do not resolve and may make differential diagnosis from other autoimmune mucosal diseases difficult.[8] The atrophic form presents as a diffuse red lesion, often surrounded by white striae around the border of the lesion, and usually appears as a mixture of subtypes.[7] This lesion frequently manifests with "Nikolsky's sign" in which the epithelium easily sloughs under slight, rubbing pressure. This sloughing frequently involves the gingiva which is commonly referred to as a "chronic desquamative gingivitis."[11] There appears to be a significant correlation between patients $\geq$ 60 years of age and the presence of atrophic lesions.[12] Both the erosive and atrophic forms result in pain and a burning sensation.[8] The papular form consists of small 0.5-1 mm-wide white, raised papules which are easy to overlook. They also usually occur in association with another subtype.[11] The plaque-like form closely resembles leukoplakia but sometimes

presents with reticular borders. The primary sites for the plaque-like form are the dorsum of the tongue and the buccal mucosa. Thorn reported that these lesions are often found in smokers.[12] Finally, the bullous (blister) form is rare and may range from only a few millimeters to several centimeters. They are usually surrounded by a reticular border and may easily rupture resulting in a painful ulcerated surface.[12]

Histology

Histological features of OLP were first described by Dubreuill in 1906[13] and subsequently by Shklar.[14] Histologically, OLP is characterized by three classic features: 1) a dense band-like layer of chronic inflammatory infiltrate in the connective tissue region, liquefactive degeneration of the basal cell layer of the epithelium, and overlying hyperkeratinization in the reticular, papular or plaque-like forms.[14] Degenerating basal keratinocytes form colloid bodies called Civatte or hyaline bodies which often appear in the epithelium or underlying connective tissue. Recent studies have shown that these Civatte bodies are comprised of apoptotic keratinocytes. Sometimes cleft formations can be seen histologically due to weaknesses at the epithelial-connective tissue interface; this clefting is called a Max-Joseph space. Some other elements of OLP include acanthosis and the presence of "saw-tooth" rete ridge formations.[1] The histological criteria for the definitive diagnosis of OLP include liquefactive degeneration of the basal cell layer and a dense, band-like inflammatory infiltrate consisting of lymphocytes. Supportive findings include: saw-tooth rete ridges, Civatte bodies, and hyperkeratosis.[15] Exclusionary histological criteria include: absence of liquefactive degeneration of the basal cell layer; mixed inflammatory infiltrate; atypical cell morphologies suggestive of

dysplasia; blunted rete ridges; absence of Civatte bodies and absence of hyperkeratinization.[16]

Oral lichenoid reactions have similar histological characteristics to idiopathic OLP, and the World Health Organization (WHO) does not differentiate between the two since they cannot be further substantiated by clinical findings. Although no reliable histological features that can differentiate lichenoid reactions from idiopathic OLP, some authors reported that features such as a deeper-laying inflammatory infiltrate, usually around vascular structures, and plasma cells and neutrophils in the connective tissue infiltrate could help distinguish it from idiopathic OLP.[17] In 2003, van der Meij and van der Waal proposed a modification to the WHO diagnostic criteria to include OLL, or "oral lichenoid lesion," as a separate entity as well as adding "absence of epithelial dysplasia" as a diagnostic criterion for both OLL and OLP.[18]

Immunofluorescence is not necessary for the diagnosis of OLP but sometimes is used to differentiate OLP from other diseases, such as mucous membrane pemphigoid and pemphigus vulgaris, that show clinical features similar to OLP. Direct immunofluorescence (DIF) is used to detect antibodies in the tissues. Studies utilizing DIF have shown a linear pattern and intense fluorescence with anti-fibrinogen outlining the basement membrane for OLP. Sometimes deposits if IgM, IgA, IgG, and C3 can be found in Civatte bodies.[19]

Etiopathogenesis

It is clear that the degradation of the basal keratinocytes by the activation of T lymphocytes leads to the disease signs and symptoms. However, the etiology for the

activation of this immune response is still unknown. In order for the T-cells to be activated, there must be an antigen that is presented by the MHC class I molecules. The exact antigen responsible for the activation of T-cells is still unknown, but Sugerman *et al* suggest that it is expressed by keratinocytes after some extrinsic event, such as exposure to certain drugs, contact allergens, trauma, bacterial or viral invasions, or an intrinsically yet-to-be identified agent. The event subsequently triggers T-cell exposure to MHC class I molecules.[1] A variety of extrinsic factors may induce the onset of signs and symptoms. They include dental restorations and drugs such as antimalarials, ACE inhibitors, β-blockers, NSAIDs, gold salts, and hypoglycemics. In cases triggered by extrinsic factors, the term "oral lichenoid reactions" is preferred although clinical and histologic examination would reveal similar features to classic OLP.[20]

Another potential pathogenic agent may be viral infection. Herpes simplex,[21] Epstein-Barr,[21, 22] Cytomegalovirus,[23, 24] and herpes virus 6[23, 25] have all been implicated in oral manifestations of lichen planus. Lodi *et al* reported that the literature is still inconclusive as to whether or not these viral agents are actually associated with signs and symptoms or if they are simply superimposed upon lesions already present.[26] Of these viruses, human papilloma virus (HPV) is one of the most studied and strongly linked to OLP. Syrjanen *et al* reported in a systematic review that HPV prevalence among patients with OLP ranged from 7.7% to 32.8%.[27] Also, it appeared that more severe presentations were associated with a higher prevalence of HPV.[20] However, Syrjanen *et al* stated that ulcerative OLP lesions increase the susceptibility to HPV infections and that the use of steroids for treatment may actually enhance HPV

replication.[27] Therefore, the direction of influence between HPV and OLP is still unclear.

Hepatitis C virus (HCV) has garnered considerable interest since prevalence of associated HCV infections with OLP range from 2 % to 67.8%[20]. Several recent meta-analyses[26, 28, 29] found strong correlations between HCV and OLP in distinctly different populations. HCV has the ability to infect other cells besides hepatocytes, and the constant immune response associated with the chronic presence of the virus may cause genetic mutations leading to development of autoimmune issues such as OLP. Another possible interaction between HCV and OLP is that gamma-interferon (INF-γ) treatment, which is one of the most common treatments for HCV, can cause oral lichenoid reactions.[28] A unique characteristic sets HCV-associated OLP apart from classic OLP and other OLRs. Carrazzo *et al* found that patients with OLP and HCV have a higher frequency of a specific HLA (human leukocyte antigens) class II allele, HLA-DR6, than compared to patients with OLP and no HCV infection.[30] Although HCV may have the strongest case as a contributing etiologic agent to OLP, many questions remain as to what extent HCV and other viral infections are involved in the etiopathogenesis of OLP.

Heat shock proteins may play a major role in the pathogenesis of OLP. HSPs, which are expressed by all cell types, are involved in cell communication, proliferation, growth, signal transduction, and apoptosis. Increased HSP expression is a result of some form of exogenous insult such as dramatic temperature changes, medications, viruses, inadequate nutrition, and certain growth factors. Sugerman *et al* have suggested that heat shock proteins may be auto-antigens since they are highly expressed in the keratinocytes

of OLP patients as well as patients with classic autoimmune diseases. HSPs are overly expressed in diseases which are associated with chronicity, a preference for female patients, mediation via T lymphocytes, and good response to steroidal therapy. Since all of these traits also apply to OLP, there may be an argument to classify OLP as an autoimmune disease although the autoimmune component seems to be activated after epithelial basal cell degeneration.[31]

The inflammatory infiltrate in OLP lesions is predominantly composed of T-cells, the majority of which are activated CD8+ lymphocytes within the epithelium and adjacent to damaged basal keratinocytes. The CD8+ subset of T-cells, called cytotoxic T-cells, are responsible for recruitment of other inflammatory cells to the area and for the induction of keratinocyte apoptosis. Keratinocytes contribute to the structure of the epithelial basement membrane by secreting collagen IV and laminin V into the basement membrane. There is also evidence that keratinocytes require a basement membrane-derived cell survival signal to prevent the onset of apoptosis. Therefore, the basement membrane is required for keratinocyte survival and vice versa. Apoptotic keratinocytes are not able to perform this function. Hence, keratinocyte apoptosis triggered by CD8+ cytotoxic T cells may result in epithelial basement membrane disruption in OLP which allows the non-specific T lymphocytes present in the sub-epithelial zone to migrate into the epithelium.[32]

CD4+ T-cells may also play a role in the apoptosis of keratinocytes. Sugerman *et al* hypothesized that there may be a secondary antigen which complexes with MHC

Class II molecules to activate T helper cells. These active T helper cells may then "reconfirm" the CD8+ T-cell's "request" for cytotoxic activity.[1]

In addition to the antigen-specific pathways presented above, Zhou *et al* have suggested that several non-specific mechanisms contribute to the breakdown of the keratinocytes of the basal layer.[32] Matrix metalloproteinases are a family of nearly 20 zinc-containing proteins which are responsible for the breakdown of connective tissue proteins. Each MMP usually has distinct substrates and are regulated by tissue inhibitors of metalloproteinase (TIMPs). Zhou *et al* found that there were higher levels of MMP-9 and TIMP-1 from OLP lesional T-cell lysates than peripheral T cells from either OLP or healthy patients. MMP-9 is also known as gelatinase B and cleaves type IV collagen. They also observed that TNF-α stimulation resulted in activation of only the MMP-9 and not TIMP-1. Their results suggested that TNF-α released from T-cells in OLP lesions may upregulate MMP-9s to degrade the epithelial basement membrane. As stated before, this disruption in the basement membrane also deprives the keratinocytes of the keratinocyte survival signal, thereby inducing apoptosis of the keratinocytes.[33]

Another non-specific mechanism of basal cell degeneration involves hyperactivation of mast cells. In two studies, Zhao *et al* showed that there was greater mast cell density as well as a higher percentage of mast cell degranulation in OLP lesional cells. Among the mast cell lysates are MMP-9, which was described above, and chymase, which is a potent mast cell protease capable of activating MMP-9. Also present in mast cell lysate is TNF-α which promotes endothelial adhesion of T lymphocytes to the lesional area. Therefore, Zhao *et al* concluded that the upregulated

mast cell degranulation directly, and indirectly through TNF-α mediated recruitment of T-cells, causes a local increase in MMP-9.[34, 35]

Zhao *et al* also found that the chemokine RANTES (regulated on activation, normal T-cell expressed and secreted) is released by lesional T-cells. RANTES recruits and promotes degranulation of mast cells. TNF-α is an activator of RANTES. Since TNF-α is also released by RANTES-induced mast cell degranulation, this results in cyclic propagation of inflammation and may contribute to the chronicity of OLP.[36]

Sugerman *et al* also suggest a unifying hypothesis on the pathogenesis of OLP which integrates both antigen-specific and non-specific mechanisms. He suggests this model: the initiation of OLP is by the OLP antigen binding with an MHC Class I molecule on keratinocytes. CD8+ cytotoxic T-cells are thus activated, possibly with help from Th1 CD4+ cells induced by a currently unknown secondary OLP antigen, and secrete TNF-α to begin keratinocyte apoptosis. These T-cells undergo clonal expansion and release RANTES to upregulate mast cell presence and degranulation which also increases TNF-α levels. Increased TNF-α levels promote further migration of T-cells and MMP-9 activation to induce a cyclic inflammatory response. This culminates in the apoptosis of the basal keratinocytes.[1] It should be reiterated that a specific OLP antigen has not been identified.

Course of Disease

OLP lesions may be present for years and undergo phases of exacerbation and quiescence. Exacerbations are accompanied by pain and often times erosive or atrophic

lesions. For gingival lesions, exacerbations may also be attributed to the low-grade chronic inflammation caused by dental plaque.[37]

The course of OLP has been studied by several authors. Silverman *et al* treated 570 patients for a mean period of 5.5 years. 75% of patients were treated with topical corticosteroids. They found that of the treated patients, 29% experience complete remission and 63% had partial remission. Only 3% experienced spontaneous remission without treatment. The rate of malignant transformation was 1.2% and occurred in 7 patients in a mean time 3.4 years after onset of the OLP.[7]

In another similar study conducted years later, Silverman *et al* treated 214 patients over the course of 5 years. The results from his study found that OLP was mainly found in women and on the buccal mucosa. Spontaneous remission was found only in 6.5% of patients. The rate of malignant transformation was 2.3% after a mean of 7.5 years after onset of OLP. The erosive form was always associated with pain.[38]

Bicker also reported rates of spontaneous remission of the various forms of OLP. Reticular lichen planus has a remission rate of 41%, plaque-like LP resolves in 7% of cases, and atrophic LP resolves in 12% of cases.[11]

Thorn *et al* followed 611 OLP patients in Denmark seen at least once a year (twice if the patients had atrophic and/or erosive lesions) for 26 years. Most patients presented with reticular lesions (92%) and of these, 28% (26% of the total pool) had complete remission by the end of their last examination. One-third of the patients with plaque-like lesions, 50% of patients with atrophic lesions, and two-thirds of patients with ulcerative lesions experienced complete remission upon final examination. In total 17%

(104) of patients experienced complete remission. Their analysis of various factors and their association with complete remission found that the presence of plaque-like lesions trended towards complete remission. Other factors such as age, sex, presence of systemic diseases, medications, smoking, and other clinical forms other than plaque-like lesions at initial presentation did not influence the probability of having complete remission.[12]

In another study evaluating the course of the disease as well the efficacy of topical/systemic corticosteroids, Chainani-Wu *et al* retrospectively followed 229 patients treated for OLP at a tertiary referral center at UCSF from 1996 to 2000. They scored signs of the disease based on increasing severity: 1 = reticular, 2 = atrophic and 3 = erosive. Symptoms were graded as: 0 = no symptoms, 1 = mild symptoms that do not affect quality of life, 2 = moderate symptoms that were bothersome to the patients and needed medical attention, and 3 = severe symptoms that significantly interfered with quality of life. Treatment at the center consisted of topical corticosteroids such as 0.05% fluocinonide gel, 0.05% fluocinonide ointment mixed with equal parts Orabase paste, 0.05% clobetasol gel, 0.05% clobetasol ointment mixed with equal parts Orabase paste, or systemic corticosteroids. In refractory cases or very severe cases, systemic corticosteroids were combined with azathioprine. Treatment responses were scored at the first week and long term response was measured by subtracting the 1 week follow-up score from the last follow-up visit. The average duration of the disease was 76 months. The buccal mucosa was the most common site. Follow-up data were available for 163 patients, of whom 85 (52%) remained constant in their clinical presentation while 21 (13%) experienced worsened clinical presentation. Only 41 (25%) experienced

improvement, and only 8 (5%) of patients experience complete remission. The overall oral squamous cell carcinoma transformation rate was 1.7%.[4]

Carbone *et al* investigated the course of OLP in 808 northern Italian patients in a retrospective study of data from 1987 to 2004. They included patients with bilateral clinical signs of OLP alone or in association with atrophic or erosive lesions, a positive biopsy confirming OLP, and an absence of suspicion that the lesions were associated with drugs or oral restorations. They followed the patients and evaluated them for clinical improvement, (defined as a transition of observable lesions from red to white) exacerbations (defined as changing from asymptomatic to symptomatic or worsening of a symptomatic form), changes in the morphology of the lesions, and partial or complete remission. Patients were generally seen twice a year, but those undergoing treatment were seen every 2 months. Any treatment was done with a mixture of 0.05% clobetasol propionate, 3% cyclosporine, and 0.05% fluocinonide. They found that 421 (52.1%) of patients did not have any changes in their white lesions. Of these, only 71 were using a form of medication for treatment. One hundred ninety-six patients had no changes in their red lesions. In total there were only 20 patients (2.47%) that experienced complete remission, 13 of whom had white lesions without treatment, and seven from red lesions. Forty-nine patients (6%) had exacerbations of their white lesions into atrophic-erosive lesions. The results from their study confirmed the chronicity of the disease with only a mere 2.47% of patients achieving complete remission. A majority of patients were stable (76.6%) and only a handful became worse off (6%). A little over half of the patients who

were responsive to therapy showed acute flare-ups at some point in time, again highlighting the chronicity of the disease.[39]

Malignant Transformation

In addition to being a chronic, often painful disease, one of the most important sequela for patients and clinicians to be aware of is the potential of OLP lesions to transform into squamous cell carcinoma. In fact, the WHO categorizes OLP as a precancerous condition which is "a generalized state associated with a significant increased risk of cancer." Krutchkoff *et al* evaluated the literature from 1950 to 1976 to assess the risk of malignant transformation by reviewing 223 reported cases. They concluded that the data was inconclusive since many follow-up studies did not use the same diagnostic criteria for the initial lesion.[40] In 1999, van der Meij used the same criteria used by Krutchkoff to examine the evidence from 1977-1999. They found that only 33 (34%) of the 98 reported cases were acceptable as showing clear evidence of malignant transformation of OLP. However, they still lamented the lack of uniformity in diagnosis of OLP which contributed to the difficulty in fully assessing risk.[18]

Several studies investigating the course of the disease have reported on malignant transformation rates. Silverman *et al* reported 7 out of 570 patients (1.2%),[7] Holmstrup *et al* reported 9 out of 611 patients (1.5%),[37] Silverman *et al* reported 5 out of 214 patients (2.3%),[38] Lo Muzio *et al* reported 14 out of 263 patients (5.3%),[41] Carbone *et al* reported 15 of 808 patients (1.86%),[39] and Chainani-Wu *et al* reported 4 out of 154 (1.7%) patients.[4] In a recent meta-analysis, the average malignant transformation rate was 1.09%.[42]

The mechanism of malignant transformation has been studied extensively. One possible mechanism may relate to the macrophage migration inhibitory factor (MIF) which was shown to stimulate tumor formation.[43-45] MIF is released by T-cells of the OLP lesion and also acts to block keratinocyte tumor protein p53, an important cell cycle regulator known as "protector of the genome". Impairment of the p53 gene combined with the chronic inflammatory state may allow gene mutations to go unchecked within OLP lesions. Another player may be TGF-β1 as studies have shown that varying levels of TGF-β1 results in different effects on cell growth, differentiation, and tumor suppression. These studies imply that low levels of TGF-β1 may cause hypo-immunosuppression that exacerbates OLP[46] but that abnormally high levels may suppress antitumor defenses.[47]

Treatment

Corticosteroids

Just as the disease manifests itself in various ways, the methods studied to treat OLP are also varied. Tyldesley and Harding studied aerosolized betamethasone compared to a placebo in 23 OLP patients over the course of 8 weeks treatment. They found that most patients taking the betamethasone showed marked improvement at 2 weeks and further improved to near complete remission at 8 weeks. However, only 2 of the placebo patients showed slight improvement at the end of 8 weeks.[48]

Lozada and Silverman examined the efficacy of 0.05% fluocinonide in an adhesive (orabase) used topically to treat oral lichen planus in an effort to find a more suitable alternative to systemic steroids and their associated side effects. Eighty-nine

patients with various forms of vesiculobullous diseases (OLP, EM, MMP, pemphigus vulgaris) were studied in two phases. In the first phase, 15 patients with various diseases were tested in a double-blind, crossover experiment with a placebo. Patients were instructed to use their active or placebo adhesive material 5 to 6 times per day for 2 weeks with the first gel and then used the other gel for another two weeks. After the crossover period, 6 of the OLP patients experienced complete remission and the remaining 5 experienced partial remission while all but one of the patients experienced no significant remission of lesions.[49]

In another study examining fluocinonide, Voute *et al* studied and compared the visual analog score (VAS) and treatment responses in 20 patients using 0.025% fluocinonide to 20 patients using a placebo after 9 weeks. With regard to clinical signs, 4 patients experienced complete remission and 12 showed good or partial response in the fluocinonide group compared to no patients with complete remission and only 6 with partial remission in the placebo group. The same trends were found regarding symptoms, and both parameters (signs and symptoms) were statistically different between the two groups in favor of the test group.[50]

Two studies compared the previously mentioned fluocinonide with another topical corticosteroid: clobetasol. In the first, Carbone *et al* compared 0.05% clobetasol ointment to 0.05% fluocinonide ointment for a period of 6 months. Twenty-five patients in the clobetasol group, 24 in the fluocinonide group, and 11 in the placebo group were examined every 2 months and assessed using a VAS and clinical response scores. Plasma cortisol levels were measured in half of the patients in the topical steroids groups

to ensure safety of the gels. After treatment, all patients in the clobetasol and 90% of patients in the fluocinonide group experienced some form of relief. However, only 20% of the placebo patients experienced some sort of relief. When the investigators looked at the ability of either ointment to completely resolve the lesions, 75% of the clobetasol-treated lesions achieved complete responses compared to only 25% of the cases treated with fluocinonide. At the final follow-up at six months, roughly 50-60% of the patients in either group were stable, and none of the plasma samples showed an adverse increase in cortisol levels. Another interesting finding was that none of the patients developed oral candidiasis from use of either gel since all were given miconazole gel and 0.12% chlorhexidine to use prophylactically.[51]

In a second comparison, Lozada-Nur *et al* evaluated the efficacy of 0.05% clobetasol propionate ointment in orabase compared to 0.05% fluocinonide ointment in orabase for the treatment of what they termed oral vesiculoerosive diseases (OVED). They treated 60 patients with biopsy-confirmed OVED. There were 43 female and 17 male patients; 35 were diagnosed with OLP, 3 had BMMP, 3 had PV, and 19 patients suffered from EM. 50 patients completed the study. The patients were instructed to use whichever adhesive they were given three times a day for 14 days. At the end of 14 days, they were instructed to discontinue the medication and were re-examined at 28 days. Patients who used clobetasol tended to show greater improvement than fluocinonide users at 1 week, but the difference was not significant. Clobetasol users also reported increased pain reduction compared to fluocinonide users.[52]

Since clobetasol had been established as having similar or even better response than fluocinonide, Carbone *et al* compared different concentrations of clobetasol in a randomized, controlled, double-blind trial to investigate whether the strength mattered. Thirty patients were divided into two equal groups which received either 0.025% or 0.05% clobetasol and instructed to use the gel twice a day for 2 months. Patients recorded symptom scores using a VAS from 0 to 10 and clinical lesions were scored using the following scale: Score 0: no lesions, Score 1: hyperkeratotic lesions, Score 2: atrophic area ≤ 1 cm^2, Score 3: atrophic area >1 cm^2, Score 4: erosive area ≤ 1 cm^2, and Score 5: erosive area >1 cm^2. At the end of 2 months of therapy, 14 of 15 patients in the 0.025% group experienced improvement in their symptoms and 13 of 15 patients in the 0.05% group experienced symptomatic relief. As for clinical assessment, 13 of the 15 0.025% group patients and 11 of the 15 0.05% group patients experienced clinical improvement after 2 months of therapy. Both parameters were significantly improved from baseline measurements, but were not different between either concentrations of clobetasol. The authors suggest that an increased dosage of clobetasol may not necessarily be better.[39]

Another study by Carbone *et al* compared long-term results and effects of systemic steroids plus topical steroids versus topical steroid alone. Their study looked at 49 patients with erosive and/or atrophic OLP lesions. The test group ($n = 26$) received systemic corticosteroids (prednisone) in addition to topical steroids. The control group ($n = 23$) received only topical steroids. To assess any adrenal suppression, blood samples were taken before and after treatment to measure levels of serum cortisol, glucose,

electrolytes, and creatinine. The initial dosage of prednisone was 50 mg/day until 50% of the lesion size was reduced. After this point, the dose was tapered to 25 mg/day for 1 week, 12.5 mg/day for the next week, and ended at 6 mg/day for the last week. Topical treatment comprised of 0.05% clobetasol propionate applied twice a day. Miconazole rinse was prescribed to prevent candidiasis in both groups. At the end of the 6 month follow-up, both the test and control patients experienced significant reduction in signs and symptoms. Also, there was no difference between the percentages of patients experiencing complete remission of signs and symptoms between either treatment modalities. Seven total patients in the test group experience some form of systemic side-effect such as hypertension, abdominal pain, and water retention, but no other negative side-effect of treatment was observed. The authors suggested that high-potency topical steroids should remain the treatment of choice and that systemic steroids should be reserved for more recalcitrant severe erosive or atrophic cases or cases with diffuse systemic involvement.[53]

The efficacy of topical corticosteroids has been very well documented and established. They remain the first choice in most clinical situations for the treatment and control of OLP. One of the potential drawbacks from the use of corticosteroids, especially systemic, may include potential HPA axis suppression, but the literature has not supported this as a common risk. Plemons *et al* studied the levels of urinary and serum levels of cortisol to assess whether or not use of topical 0.05% fluocinonide gel three times a day for 3 weeks would cause adrenal suppression. Ten patients were given fluocinonide to use and another 8 were given a placebo. They found that at day 3 and 21,

no differences in cortisol could be detected between the fluocinonide and placebo groups and that there was no difference within the subject groups at different time points. They suggested that the use of topical steroids does not suppress the HPA axis.[54] In fact, none of the studies mentioned above reported HPA axis suppression as a side effect of topical steroids administered as a gel, ointment, or cream. However, Gonzalez-Miles and Scully reported substantial hypothalamus-pituitary-adrenal suppression when aqueous clobetaol was used as a rinse 3 times daily.[55]

Another potential side effect is the occurrence of oral candidal infections secondary to oral immune suppression. Krogh *et al* reported that around 37% of OLP lesions contain *Candida albicans*[56] and according to several studies, oral candidiasis is a common side-effect of topical steroid application. Although Lozada *et al* reported that none of their patients developed symptoms of candidiasis, 3 patients developed pseudomembranes.[49] Another investigation reported a significant relation between being a carrier of candidal species and having candidiasis during the course of treatment. That study reported that 18 (35%) were normal carriers and of these, 5 of 8 patients using clobetasol and 8 of 10 patients using fluocinonide developed candidiasis during the course of treatment.[52] Fortunately, the efficacy of using antimycotics during treatment with systemic or topical steroids to prevent oral candidiasis was validated by several different studies.[39, 51, 53]

Cyclosporine

Alternative avenues of treatment for OLP instead of corticosteroids have been widely studied. Among the other popular alternatives is topical cyclosporine.

Cyclosporine is a calcineurin inhibitor and acts reversibly to inhibit the effect of immune cells during the G0 or G1 phase of the cell cycle. T-cells, mainly T-helper cells, are the main target, and cyclosporine also inhibits lymphokine production and the release of IL-2, a T-cell growth factor.

Epstein and Truelove evaluated the benefits of a formulation of cyclosporine 100mg/ml compounded with Zilactin, which was a topical film comprised of hydrocellulose, salicylic, boric, and tannic acids. The study population consisted of 14 patients with confirmed OLP and patients were instructed to use the gel for 1 month with follow-ups at 2 and 4 weeks to assess pain using a VAS and clinical response via area measurements of the erosive lesions. At the end of the study, only 8 patients out of 14 experienced relief, none of whom achieved complete remission, and only 7 patients experienced clinical improvement.[57]

Harpenau *et al* studied the potential benefit of low-dose cyclosporine to manage erosive OLP patients. Fourteen patients diagnosed with the erosive form of OLP were instructed to either rinse with a placebo or 5 ml (500 mg) cyclosporine rinse for 5 minutes each day over the course of 28 days. Patients were then seen weekly to record lesion size and features as well as to assess healing, which was defined as a transition from ulceration to erythema to reticulation or to complete remission. A VAS was also done to assess pain. At 28 days, all experimental group patients experienced significant improvement in healing when compared to the placebo patients with no significant side effects noted.[58]

Thongprasom *et al* compared cyclosporine solution in an adhesive base to 0.1% triamcinolone acetonide in a group of 13 Thai patients: Six were assigned to the cyclosporine group and the remaining seven were assigned to the triamcinolone group. Patients were instructed to use their gel TID for 8 weeks with follow-up at 2 and 8 weeks to assess pain using a 100 mm VAS and clinical response via the scale developed by the primary author. Further follow-ups were conducted every 3 months up to one year. After treatment, both groups experienced benefit, but there were no statistically significant differences in symptoms or clinical response at any time point between either treatment. Their results indicated that topical cyclosporine was not better than triamcinolone in treating OLP.[59]

Tacrolimus

Tacrolimus, like cyclosporine, is also a calcineurin inhibitor and has been proposed as a potential treatment for OLP. Hodgson *et al* studied whether or not tacrolimus would be a successful treatment in OLP lesions resistant to traditional topical steroid therapy. Fifty patients with erosive or atrophic OLP were given 0.1% tacrolimus to use topically twice a day for an average treatment time of 19.8 months. Most of the patients (80%) experienced partial resolution, 14% achieved complete remission, and only 6% had no beneficial effect. During usage, 16% of patients reported burning sensations that were transient and even fewer (8%) reported taste disturbance.[60]

Swift *et al* examined the efficacy of pimecrolimus, which has a similar structure and mechanism of action to tacrolimus. Pimecrolimus is derived from *Streptomyces hygroscopicus var.* ascomyceticus. In their study, 20 patients with erosive OLP split into

2 equal groups were treated for 4 weeks with either a placebo or 1% pimecrolimus ointment. Photographs were taken and analyzed and discomfort scores using a VAS were recorded bi-weekly. At the end of the study, the test group had significant reduction in overall lesion size; the control group experienced increased lesion size as well as minimal change or increase in pain scores. Ulcerative lesions trended towards a decrease in the test group and increase in the control group, but neither was significantly different at the mid-point nor at the end of the study from baseline measures. For erythematous lesions, the test group experienced a significant decrease in size at the mid-point compared to baseline whereas the control group did not. For the reticular lesions, the authors noted a significant increase in area of the reticular lesions at mid-point and final evaluation when compared to baseline for the control group. No statistical significance was found for the test group in regards to reticular lesions. When the weighted sums of all lesions were calculated, there was no statistically significant reduction in the size of the lesions between the test and control groups. For pain, the test group experienced a significant decrease in symptoms compared to the control. They concluded that based on the results, 1% pimecrolimus is a safe and effective treatment alternative for erosive symptomatic OLP.[61]

Laeijendecker *et al* sought to compare 0.1% tacrolimus with 0.1% triamcinolone acetonide in a group of 40 patients treated for 4 weeks. Group I (n = 20) received triamcinolone and Group II (n = 20) received tacrolimus. Both groups were instructed to use the gel four times a day for 4 weeks; examination of clinical efficacy was scored as healed if no visual signs of OLP lesions remained, improved if the extent and severity

was reduced by more than 30%, and unchanged if the extent and severity was reduced by less than 30%. At 6 weeks, a higher percentage (90%) of patients had either healed or improved scores in the tacrolimus group compared to the triamcinolone group (45%). However, 40% of the tacrolimus users experienced burning sensations associated with the site of application.[62]

Retinoids

Retinoids are a class of compounds chemically similar to Vitamin A and have regulatory effects on cell proliferation, growth of bone tissue, immune functions, and activation of tumor suppressor genes. Buajeeb *et al* compared patients using 0.05% retinoic acid gel to patients using 0.1% fluocinonide in an oral base. Eighteen patients received 0.1% fluocinonide acetonide and another 15 patients received 0.05% retinoic acid and were instructed to use either gel for 4 weeks. Results were assessed using a 10 cm VAS as well as clinical response based on the scoring system by Thongprasom *et al*. At the end of the follow-up period, 83% of patients in the fluocinonide group experienced improvement in clinical signs compared to only 13% in the retinoic acid group. There was a clear general downward trend in the VAS for the fluocinonide users and the retinoic acid users as well but the latter was much more varied. This study suggests that although retinoic acid may be beneficial, it does not appear to be superior to topical steroids in treatment of OLP.[63]

Piatelli *et al* studied the efficacy of 13-*cis*-retinoic acid (isotretinoin) for the treatment of biopsy-confirmed OLP. Ten patients were given 0.1% isotretinoin gel and another 10 were given a placebo gel formulation to apply four times a day for 4 months.

At the end of the initial 4 months, the placebo patients were given isotretinoin for a further 4 months. They assessed the prevalence of complete and partial response as disappearance of the lesion and 50% or more reduction in lesion sizes respectively. At the end of the initial 4 months, 4 of the 10 test patients had complete remission and another 4 had partial response compared to none in the placebo group. After the placebo patients used isotretinoin for a further 4 months, 6 of these patients had complete remission and the other 4 had partial responses. At the 3-year follow up, the lesions had recurred in 6 of the original 20 patients. The authors suggested that isotretinoin may be helpful in combating OLP.[64]

Others

Wu *et al* looked at the plausibility of thalidomide as a topical medication for the treatment of OLP since thalidomide decreases TNF-α secretion and promotes T-cell suppression. Sixty-nine patients with biopsy-confirmed erosive oral lichen planus were divided into a group using 1% thalidomide rinse (Group A, $n = 37$) and another using 0.043% dexamethasone (Group B, $n = 32$). Both patients and researchers were blinded to the treatment medication. Patients were instructed to rinse three times a day for 1 week. Patients who did not achieve complete remission, defined in this study as VAS of 0 and the resolution of erosive lesions, were instructed to use the gel another 3 weeks. After accounting for dropouts, the 33 patients receiving topical thalidomide experienced a significant average reduction in lesion size, erosive area, and VAS at 1 week as did the dexamethasone group. Eighteen of the 33 thalidomide users experienced complete healing of the erosive lesions compared to 17 of the 30 dexamethasone patients. It

appeared that thalidomide was equally as effective as dexamethasone in reducing VAS and clinical signs of erosive OLP. As for adverse effects, 4 patients complained of transient burning and tingling at the sites of administration of the thalidomide. Otherwise, no undue side effects were noted and no adverse outcomes associated with thalidomide were observed in either group at 1 year.[65]

Salazar-Sanchez *et al* evaluated *Aloe vera* as a topical drug. 70% *Aloe vera* was given to 32 patients and a placebo was given to 32 patients in the control group. All patients were instructed to use the gel three times a day for 12 weeks. Pain was rated using the VAS, and clinical efficacy of treatment was classified by a scale developed by Thongprasom *et al*. The results indicated that *Aloe vera* was not statistically different in alleviating pain based on the VAS. However, the *Aloe vera* group achieved a statistically higher number of patients with complete remission as compared the placebo group at 6 week although this difference disappeared at 12 weeks. The authors suggested that 70% *Aloe vera* may quicken the healing time of patients with OLP in the short term.[66]

Oxidative Stress

Reactive Oxygen Species

Free radicals are defined as any chemical species which are able to exist with one or more unpaired electrons. They are highly reactive species and are capable of oxidizing, or causing loss of electrons, other substances. The first radical described in organic chemistry was the triphenylmethyl radical by Gomberg in 1900 at the University of Michigan. In 1956, Harman proposed the idea of free radicals playing a role in aging which ushered in an era of interest in free radical effects on biologic systems.[67] This idea was inspired by Gerschman's observation that both radiation and hyperbaric oxygen toxicity could be attributed to oxygen free radicals.[68] Later in 1969, McCord and Fridovich from Duke University discovered superoxide dismutase (SOD), which is an important antioxidant enzyme responsible for partitioning superoxide radicals. Their discovery supported the concept of free radicals in living systems.[69] In 1977, even more interest was given to free radicals after Mittal and Murad discovered that hydroxyl radicals were capable of initiating the cGMP messaging system.[70]

Reactive oxygen species (ROS) are generally able to form other potentially damaging radicals with actions similar to true free radicals. Central to the action of ROS is the concept of redox potentials, which is the measure (in volts) of the affinity of a substrate for electrons measured in relation to hydrogen. Substances that have the ability to oxidize hydrogen are more electronegative than hydrogen and have positive redox potentials. Conversely, substances capable of reducing hydrogen are less electronegative than hydrogen and have a negative redox potential. Free radicals in the body can be

found from exogenous sources such as, but not limited to heat, trauma, ultrasound, UV radiation, ozone, smoking, exhaust fumes, radiation, infection, excessive exercise, and some drugs.[72] Endogenous sources include superoxide leakage from the mitochondrial electron transport chain[73] and from phagocytes during host immune responses as well as other connective tissue cells (osteoclasts and fibroblasts). Mitochondrial DNA is most susceptible to damage from free radicals due to the proximity to the electron transport chain as well as a lack of histones protecting genetic material despite the activity of superoxide dismutase 2.[72] Phagocytic cell production of superoxide comes from the "respiratory burst" seen within PMNs in response to mitogens. This "burst" occurs when glucose-6-phosphate is shunted from glycolysis and forms superoxide radicals from an interaction with molecular oxygen and NADPH.[74]

ROS can interact with and damage all types of physiologic systems. Dean *et al* described the effects of free radicals on proteins. These effects include: protein folding or unfolding, protein fragmentation and polymerization reactions, protease degradation of the modified protein, formation of protein radicals, formation of protein-bound ROS, or formation of stable end products.[75]

ROS can also interact with lipids. Lipid peroxidation is one of the major reactions involving free radicals. Halliwell and Gutteridge described the main stages in this process. In the initiation step, a hydroxyl radical removes a hydrogen atom from a polyunsaturated fatty acid, such as arachidonic acid, and forms a carbon-centered radical. This carbon-centered radical can combine with another polyunsaturated fatty acid side-chain radical to link and disrupt the membrane structure via a covalent bond.

This interruption in the membrane structure causes Ca^{2+} influx and subsequent increase in Ca^{2+}-dependent proteases disrupts cellular function. Most commonly, however, the carbon-centered radicals initiate a chain reaction by combing with oxygen to form a lipid peroxyl radical. These radicals can then bind to another polyunsaturated fatty acid to form another carbon-centered radical and a lipid hydroperoxidase. The latter two compounds propagate the same series of reactions creating an overwhelming accumulation of lipid hydroperoxidases which ultimately collapses the cell membrane. The final products of the lipid peroxidation are mainly malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE). MDA is mutagenic in mammalian cells as well as carcinogenic in murine models.[76]

DNA damage from ROS is usually a result of strand breakage, base pair mutations, deletions, insertions, nicking, DNA cross-links, and sequence amplification by free radicals.[77] Free radicals and ROS can also activate gene transcription resulting in induction of cellular apoptosis[78] , activation of NF-κB and activator protein-1 (AP-1),[79] and activation of heat-shock proteins.[80] NF-κB is important since its transcription is linked with production of proinflammatory cytokines such as IL-1, -6, and -8, MHC class I antigens, and TNF-α.

The human body has an innate ability to keep the balance between ROS activity and antioxidant defense to curtail detrimental cellular damage. However, sometimes the delicate and dynamic equilibrium shifts in favor of too much ROS. This results in oxidative stress, which Sies defined as "a disturbance in the pro-oxidant–antioxidant balance in favor of the former, leading to potential damage."[81]

Taken together, the resulting damage to cellular and genetic tissues from ROS and the increase in oxidative stress contributes to various pathologic conditions which can be divided into two major groups. The first group of diseases is caused by a pro-oxidation shift in the redox state and is associated with cancer and diabetes mellitus. The second group of diseases are caused by an increase in either NAD(P)H oxidase (leading to atherosclerosis and chronic inflammation) or xanthine oxidase-induced formation of ROS (leading to ischemia and reperfusion injuries)[82] Aging is attributed mainly to the damage from free radicals to lipids, DNA, and proteins.[67]

Particular interest has been given to the role of ROS in carcinogenesis and several mechanisms and pathways have been studied. Aside from the DNA damage described above and exogenous sources of free radicals such as iron, cadmium, hexavalent chromium, arsenic, and tobacco smoke, there are intracellular factors which may induce cancer cell formation. One possible mechanism is mutation in various mitochondrial genes encoding complexes I, III, IV, and V. Another is via the production of MDA after lipid peroxidation since MDA can attach to guanosine, adenine, and cytosine bases of DNA and HNE can increase transduction of signals involved in cell phenotype.[83] Another pathway of carcinogenesis is through the interference of signal transduction pathways. For example, ROS can promote expression of *c-fos* and *c-Jun* genes, which in turn promote cell proliferation. Activation of NF-κB indirectly by ROS via TNF-α and IL-6 induction promotes cell growth, proliferation, and inflammation.[77] Hollstein *et al* found that genes encoding for tumor suppressor p53, which prevents cells with damaged-DNA from dividing, can be directly damaged by ROS.[84]

With relation to the development of cardiovascular disease, ROS can induce damage in cardiac and vascular myocytes through the disruption in cell membrane structure by lipid peroxidation which allows for an overload of Ca^{2+}. The influx and resultant hyperplasia of the intima contributes to atherosclerosis, vasoconstriction, hypertension, and cardiac hypertrophy in heart failure. Another association between ROS and cardiovascular disease is through the relation between ROS and Angiotensin II. It has been shown that Angiotensin II increased superoxide production by vascular smooth muscle cells.[85] Kasporova *et al* showed that during reperfusion after ischemic cardiac events, there is a massive burst of ROS from a currently unknown source on the cellular level that causes massive amounts of damage to tissues and complicates organ transplantation, myocardial infarcts, and strokes.[86]

Another large body of evidence of the role of ROS in systemic diseases involves type 2 diabetes mellitus (DM). Type 2 DM is a chronic disease in which the β-cells of the pancreas lose the ability to produce insulin. Evans *et al* observed that pancreatic cells, particularly β-cells, are sensitive to ROS as they have low levels of intrinsic antioxidants such as catalase, glutathione peroxidase, and SOD.[87] ROS can also be produced in a diabetic state. In diabetes, the major source of ROS is from mitochondrial membrane complex II.[88] Another source of ROS in diabetes is from NAD(P)H.[89]

Anti-Oxidants

To combat the damage from a variety of ROS in the body, a diverse and equally dynamic antioxidant defense system exists. Halliwell defined an antioxidant as "those substances which, present at low concentrations compared to those of an oxidizable

substrate, will significantly delay or inhibit oxidation of that substrate."[90] Chapple and Matthews presented 5 possible ways to categorize differences among antioxidants: 1) mode of action, 2) location, 3) solubility, 4) structures they protect, and 5) by their origin.[72] Classifying by mode of action, there are preventative and scavenging antioxidants. Preventative antioxidants remove superoxide and hydrogen peroxide or prevent hydroxyl radical formation by sequestering divalent metal ions. These include superoxide dismutase enzymes 1 and 2, catalase, glutathione peroxidase, and DNA repair enzymes.[76] Scavenging antioxidants, which are also known as chain-breaking scavengers, include Vitamin C, carotenoids, uric acid, flavonoids, ubiquinone, albumin, and bilirubin.[72]

Of particular interest in this study are the class of scavenging antioxidants known as flavonoids which are absorbed from dietary wines, fruits, vegetables, and tea. Flavonoids function through many different mechanisms such as radical scavenging, terminating lipid peroxidation, iron chelation, sparing vitamin E, and restoration of vitamin C. Among flavonoids, two are of particular interest: ferulic acid and phloretin.

Ferulic Acid

Ferulic acid (FA) is a polyphenolic compound found in all plants and is formed from metabolism of phenylalanine and tyrosine. It was discovered by Hlasiwetz and Barth in 1866 in Innsbruck, Austria from the resin of the *Ferula foetida* plant. Major fruit and vegetable sources include oranges, tomatoes, carrots, and sweet corn. The source of its antioxidant capability comes from its chemical structure. The phenolic nucleus and unsaturated C-C double bond side chain allows FA to readily accept a

hydrogen atom to form a phenoxy radical. This phenoxy radical is stabilized due to the chemical structure and is incapable of propagating another free radical chain reaction. Compared to other antioxidants such as Vitamin C, FA tends to remain in plasmatic circulation for a longer time period and is more bioavailable. Once FA is absorbed by enterocytes, only about 51% is excreted whole the other 49% is available to diffuse into the peripheral tissues.[91] In addition to its antioxidant actions, many other beneficial effects of FA have been studied such as antidiabetic, antiatherogenic, hepatoprotective, and UV-protective benefits.[92] Another key benefit relates to suppression of chronic inflammation. Hosada *et al* reported that FA blocks COX-2 induction[93] and Sakai *et al* reported that it can also block murine chemokine (C-X-C motif) ligand 2, which is a chemotactic for PMNs.[94]

Some studies have looked into the anti-carcinogenic properties of FA. Balakrishnan *et al* induced oral squamous cell carcinoma (SCC) in male hamsters by painting the buccal mucosa with 7,12-dimethylbenz[*a*]anthracene (DMBA) in a paraffin vehicle. The hamsters were divided into 4 groups of ten: Group I was painted with paraffin only and served as a sham control; Group II and III were both induced by DMBA but Group III was given FA orally on alternating days with DMBA painting; Group IV was not induced and was given ferulic acid only. After 14 weeks, the incidence of tumor formation, tumor burden, and tumor volume was assessed. They found that 100% of the hamsters had SCC in Group II, but none of the hamsters in the other three groups developed any tumors. Tissue levels of TBARS (thiobarbituric acid-reactive substances), which is a marker of lipid peroxidation, and several antioxidants

(glutathione, superoxide dismutase, catalase, Vitamin A and C) were measured using histological assays. They found that TBARS was highly elevated and antioxidant levels were significantly decreased in Group II and that antioxidant levels in Group III, although significantly less than Group I or IV, was markedly higher than Group II.[95]

Kampa *et al* studied the effects of FA and several other polyphenol antioxidants on the proliferation and apoptosis of human breast cancer cells. Treatment of plated T47D breast cancer cells with the different antioxidants significantly reduced cell proliferation in a dose-dependent manner and apoptosis was induced.[96]

Lee *et al* studied the anticarcinogenic effects of FA and caffeic acid on human liver cancer cells. They assessed intracellular levels of ROS and cell viability of HepG2 hepatoblastoma cells via MTT staining after treatment with either caffeic acid or FA. They observed that both CA and FA were cytotoxic and reduced cell viability to about 50% from baseline. Flow cytometry indicated that this decrease in cell viability was a result of increased apoptosis induced by CA and FA in a dose-dependent manner.[97]

Phloretin

Phloretin is a major constituent of apple polyphenols. Its antioxidant capacity, like FA, also arises from its chemical structure and is attributed to the carbonyl side group since hydrogen atoms can be delocalized over the three oxygen atoms.[98]

Devi and Das studied the effect of eleven different plant polyphenols on the growth and cytokine profile of normal human lymphocytes as well as two lines of malignant human leukemic lines (IM-9 and Molt-4). Their results showed that several polyphenols inhibited lymphocyte growth. The most potent, in order, were tannic acid,

phloretin, taxifolin, and fustin. These four were subsequently tested on leukemic cell lines to assess their ability to inhibit growth and were able to significantly inhibit cell proliferation of the leukemic cells as well as the proliferation of IL-2. Based on the results, the authors suggested that further research on plant polyphenols, particularly tannic acid, phloretin, taxifolin, and fustin.[99]

Jung *et al* studied 21 apple polyphenols and analyzed their ability to reduce proinflammatory gene expression by several immunorelevant cell lines induced with specific stimuli. They also looked at the effect on NF-κB-dependent signal transduction. Quantitative real-time PCR and DNA microarray analysis revealed that apple polyphenols significantly inhibited the expression of NF-κB regulated proinflammatory genes (TNF-α, IL-1β, CXCL9, CXCL10), inflammatory enzymes (COX-2, CYP3A4), and transcription factors (STAT1, IRF1). The effects did not carry over to healthy, homeostatic genes. Phloretin compounds, in particular, inhibited the NF-kB signal transduction cascade. The authors suggested that phloretins may play a role in inhibiting proinflammatory cytokine released via blocking of gene expression.[100]

Combinations

After the many beneficial effects of these individual antioxidants had been extensively studied, San Miguel *et al* studied different antioxidants (AO) and evaluated their ability to promote cell migration in the presence of nicotine. They looked specifically at the ability of resveratrol, phloretin, ferulic acid, and curcumin in activating RacGTPases. Rac is an enzyme which participates in cell signaling during cell migration. Rac, which is a member of the Rho-family small GTPase, cycles between an

active state (GTP-bound) and an inactive state (GDP-bound). The investigators took human gingival tissues from healthy, non-smokers as well as HDPL fibroblasts from freshly extracted human teeth. The cells were plated on a special 35-mm culture dish that was divided into quarters which allowed for high-quality phase-contrast observation. The cultured cells received varying doses of nicotine (6, 8, or 10 mM) for 2 hours and were then treated with single, double, or triple AOs. Cell migration as observed every 15 minutes for 10 hours under a live-cell imaging system. To assess RacGTP activity, a scratch-wound assay was performed using a pipette tip. Antibodies for RacGTP and IgG were added and the level of RacGTP was assessed using confocal microscopy. The results showed that nicotine reduced cell viability by 40% to 50%. For all single AO combinations, FA was the most effective in increasing migration rates compared to the controls and nicotine-treated HGF cells and resveratrol was the most effective in increasing cell migration compared to the controls and nicotine-treated HPDL cells. For double combinations, RF and PF increased migrations rates significantly better than any single AO dose and the controls. Finally, all triple combinations improved cell migration significantly more than any double combination. The authors concluded that although triple combinations of AO were the best in improving cell migrations of HGF and HPDL cells after treatment with nicotine, any singly-administered AOs significantly improved cell migration compared to the controls. As for the RacGTP assay, any combination of AO significantly enhanced the level of RacGTP expression when compared to the controls in HGF cells treated with nicotine. However, the HPDL cells treated with nicotine did not improve in terms of RacGTP expression with any combination of AO.

Indeed, there was a significant difference in RacGTP expression after AO treatment between the HGF and HPDL cells.[101]

San Miguel *et al* followed up their previous research by looking at the effects of specific AO combinations on the level of cell viability, DNA synthesis, and ROS activity after treatment of HGF and HPDL cells treated with different stressors (H_2O_2, EtOH, or nicotine). Both types of cells were cultured and exposed to varying doses of irritants (6 mM and 8 mM of nicotine, 5% and 10% EtOH, and 0.0005% and 0.00075% of H_2O_2). Afterwards, the cells were treated with either a combination of RFT (resveratrol, ferulic acid, and tetrahydrocurcuminoids CG in a 1:1:1 ratio), PFR (phloretin, ferulic acid, and resveratrol in a 1:1:1 ratio), or PFT (phloretin, ferulic acid, and tetrahydrocurcuminoids CG). Cell viability was assessed using MTS colorimetric assay, DNA synthesis was assessed using the BrdU assay, and ROS was assessed using the dichlorodihydrofluorescein diacetate (H2DCFDA) reagent. Treatment with any combination of AOs had a marked positive effect on HGF cell viability after treatment with EtOH; HGF cell viability increased to over 100% of the control after treatment. After treatment with any of the AO combinations, the HGF cells exposed to nicotine exhibited 2.5-3 fold increase in cell viability. HGF cells treated with any of the AO combinations after exposure to 0.00075% H_2O_2 showed a significant increase in cell viability, but only the PFR increased cell viability of cells exposed to 0.0005% H_2O_2. After treatment of HGF cells with 0.0005% H2O2, RFT and PFR significantly increased DNA synthesis from baseline. For the cells treated with 0.00075% H_2O_2, only the RFT significantly increased DNA synthesis from baseline. Only HGF cells treated with PFR

were able to increase DNA synthesis after exposure to either 5% or 10% EtOH, and only HGF cells treated with PFT were able to increase DNA synthesis after exposure to nicotine. HPDL cells treated with PFR were able to recover in terms of DNA synthesis after they had been exposed to nicotine. Similar results could be seen in decreasing the levels of ROS after each one of the stressors had significantly increased the levels of ROS.[102]

In another study, San Miguel *et al* examined the potential of certain antioxidant combinations to protect fibroblasts against metal-induced toxicity. Metal ions released from metals such as Zn, Cu and Ni in restorative materials can induce fibroblast and osteoblast apoptosis. They took human gingival fibroblast cultures and human periodontal ligament cell cultures from donors and exposed them to copper (Cu), zinc (Zn), or nickel (Ni) in various doses. Afterwards, the cells were treated to one of three combinations of AOs: 1) "RFT," a 1:1:1 by weight ratio composition of resveratrol, ferulic acid, and tetrahydrocurcuminoids, 2) "PFR," a 1:1:1 by weight ratio composition of phloretin, ferulic acid, and resveratrol, or 3) "PFT," a 1:1:1 by weight ratio composition of phloretin, ferulic acid, and tetrahydrocurcuminoids. Cell viability was tested via MTS calorimetric assay, the ability of the remaining live cells to synthesize DNA was tested with the BrdU assay, and reactive oxygen species was assessed using dichlorodihydrofluorescein diacetate (H2DCFDA) reagent. They found that all combinations of AO significantly increased the viability of HGF and HPDL cells after treatment of 4×10^{-4} M Cu, and increased the viability of HPDL cells after treatment with 5×10^{-4} M Cu. All combinations of AO significantly increased viability of HGF

cells after treatment with 2 x 10^{-3} M Ni. All combinations of AO significantly increased the viability of HGF cells after treatment with 3 x 10^{-4} M Zn and also increased after treatment with 2 x 10^{-4} M Zn, but this was not significant. However, none of the HPDL cells responded to any AO combinations after treatment with any dose Zn or Ni. Only the combination PFT increased HGF DNA synthesis significantly after Cu exposure had reduced DNA synthesis, but all AO combinations significantly increased HPDL DNA synthesis. All AO combinations significantly increased HGF and HPDL cell DNA synthesis after exposure to Ni. PFR and PFT combinations were able to rescue HGF cell DNA synthesis after treatment with Zn, and all combinations were able to rescue HPDL cell DNA synthesis after exposure to Zn. Only the combination of PFR decreased the ROS after treatment with Cu, Ni, and Zn. The authors concluded that various combinations of AO helped HGF and HPDL cells recover in terms of viability and DNA synthesis and reduced ROS after exposure to various concentrations of metal ions. The combination PFR seemed to be the most beneficial.[103]

Oxidative Stress and Oral Lichen Planus

Although many etiologic mechanisms have been explored as presented above, only recently has attention been given to the potential role of oxidative stress in the pathogenic process of oral lichen planus. One of the first key investigations in the search for a potential relationship between oxidative stress and OLP was a cross-sectional study by Sezer *et al*. They studied serum levels of nitric oxide (NO), SOD, MDA, and catalase (CAT) in 40 patients with untreated lichen planus with onset of symptoms within 6 weeks and compared them to 40 healthy volunteers matched for sex and age. All patients presented with cutaneous lesions and 9 patients had additional oral lesions. Their results showed that NO, MDA, and SOD were present in significantly higher levels in serum of test patients compared to controls and serum CAT was significantly lower in test patients compared to controls.[104]

Battino *et al* evaluated levels of serum uric acid, albumin, glucose, total cholesterol, HDL-cholesterol, triglycerides, aspartate transaminase (AST), alananine transaminase (ALT), γ-glutamyltransferase (GTT), and total antioxidant capacity (TAC) as well as salivary levels of uric acid, albumin, and total antioxidant activity from 20 oral lichen planus patients compared to 20 healthy controls. Their results showed that salivary uric acid levels and TAC were significantly lower in the OLP group compared to the controls while serum GTT was significantly increased in the OLP group.[105]

In a similar study to Sezer *et al*, Aly and Shahin studied 45 Egyptian patients with different forms of lichen planus and compared levels of serum superoxide dismutase (SOD), nitric oxide (NO), malondialdehyde (MDA), and catalase (CAT) to

those of 45 healthy volunteers matched for sex and age. Blood was collected after 12 hours of fasting and analyzed. Cutaneous lesions were present in all test patients and oral lesions were present in 26 patients (57.7%) with the reticular form being the exclusive type of oral lesion. Their results showed that serum levels of NO, MDA, and SOD were all significantly higher compared to control patients. They also found that NO, MDA, and SOD levels increased significantly and CAT levels decreased significantly in patients with oral manifestations and skin lesions. They conclude that their evidence supports investigating the use of antioxidants in the treatment of oral lichen planus.[106]

Upadhyay *et al* examined levels of MDA, thiol levels, and TAC in the serum of 22 untreated OLP patients, 10 untreated patients with oral lichenoid reactions and 15 healthy controls matched for age and gender. They found that the level of MDA was 0.7595 ± 0.536 mM/L in OLP patients, 0.4890 ± 0.216 mM/L in the OLR group, and 0.2187 ± 0.054 mM/L in the control group. The difference between the OLP patients and the OLR patients to the control group was significant. For thiol levels, OLP patients had 378.26 ± 1.50 mM/L compared to 472.13 ± 54.27 mM/L in the controls. This difference was also significant. As for TAC, both the OLP and OLR samples had much lower levels of TAC (1.054 ± 0.3013 mM/L and 1.019 ± 0.2435 mM/L respectively) compared to the controls (2.037 ± 0.1382 mM/L).[107]

In another study, Ergun *et al* evaluated the total antioxidant activity (TAA) and levels of malondialdehyde in whole saliva and serum of 21 recently diagnosed, untreated OLP patients and 20 healthy control patients matched for periodontal status. Serum and saliva samples were taken after a midnight fast, centrifuged, and tested for TAA and

MDA levels. Univariate analyses included independent samples *t*-test, Mann-Whitney *U*-test, and Spearman's rho correlation coefficient. The results showed that patients with OLP had significantly higher salivary MDA levels ($p = 0.03$) and lower serum TAA levels ($p = 0.01$) compared to the control patients. The results also indicated a significant inverse relationship between MDA and TAA levels in saliva ($r = -0.598$, $p = 0.005$). The authors suggest that levels of antioxidants and oxidative stress markers can be measured accurately in saliva and that patients with OLP have higher salivary levels of lipid peroxidation.[108]

Furthermore, Scrobotă *et al* studied the levels of MDA and GSH (glutathione, a marker of antioxidant defense) in tissue obtained from biopsies of 9 patients with OLP and 4 healthy volunteers using a fluorometric method. They found the median level of MDA in OLP tissue samples was 2.67 (0.26–3.40) compared to 0.44 (0.19–0.70) for the control tissues and the levels of GSH in OLP tissue samples was 2.3 (1.25–5.70) compared to 9.56 (6.5–12.5) for the control tissues. Both differences were statistically significant.[109]

Aziz *et al* examined the levels of total antioxidant status (TAS) in the saliva and serum of 48 erosive OLP patients and 44 healthy controls matched for age and gender. They found that the level of TAS was 0.98 ± 0.12 mM in erosive OLP patients and 1.32 ± 0.18 mM in the control group.[110]

Most recently, Agha-Hosseini *et al* examined salivary levels of 8-OHdG, MDA, and TAC in patients with squamous cell carcinoma (SCC) and oral lichen planus (OLP). Twenty-six SCC patients and 32 OLP patients were recruited after confirmation by

biopsy. Of the OLP patients, 20 had the erosive form and 12 had reticular lesions. Thirty healthy controls with no signs of inflammation were used as controls. Unstimulated whole saliva was collected after at least 2 hours of fasting and analyzed for levels of 8-hydroxy-deoxyguanosine (8-OHdG), MDA, and TAC. They also calculated the balance of oxidant and antioxidant status by dividing the TAC by the level of MDA. Their results showed that SCC patients had a significantly higher salivary levels of MDA and 8-OHdG and lower levels of TAC compared to the control group. OLP patients had significantly higher levels of salivary 8-OHdG compared to the controls, but levels of MDA and TAC were not statistically different from the controls. They also found that the TAC/MDA ratio was significantly lower in both SCC and OLP patients compared to the controls, but SCC was also significantly lower compared to OLP patients. The authors suggest that an imbalance between oxidative stress and antioxidant capacity plays an important role in OLP and that 8-OHdG may be a better marker for oxidative stress in OLP and SCC patients, and it may also be useful in indicating cancer risk.[111]

Antioxidants and Oral Lichen Planus

Although there is a moderate amount of literature to link oral lichen planus to oxidative stress, most of the literature has been relational and not interventional. One of the early interventional, prospective studies that looked at the use of antioxidants was performed by Chainani-Wu *et al.* Their group studied the efficacy of curcuminoids to treat OLP in a randomized, controlled, double-blind trial. Curcuminoids are similar to curcumin in chemical structure and have antioxidative benefits. The investigators enrolled 17 patients in the placebo group and 16 patients in the curcuminoid group. Patients were given prednisone for 1 week prior to using either the placebo or test gel. Those in the latter group received a formulation of "Curcumin C3 Complex" which consisted of curcumin in a range of 70 to 80%, demethoxycurcumin between 15% and 25%, and bisdemethoxycurcumin between 2.5% and 6.5%. Despite a power analysis that suggested the need for 50 subjects per group, interim analysis after 33 patients were recruited and undergoing treatment revealed that there was no difference in reduction of symptoms or signs between the two groups. Futility analysis was performed and revealed that the probability of finding a significant result if the study was continued to the prescribed number of subjects was only 0.014; therefore the study was ended for futility. However, the authors still recommended future research.[112]

More recently, the only other prospective clinical trial for use of an antioxidant was by Saawarn *et al*. Their group evaluated the efficacy of lycopene in the treatment of oral lichen planus. Lycopene is a fat-soluble carotenoid which provides for the red color of tomatoes and some other fruits and is the most efficient singlet oxygen quenching

carotenoid. In their prospective, randomized, placebo-controlled, double-blind study, they treated 15 patients with 8 mg total daily of lycopene softgel capsules (Group A) and another 15 patients with a placebo (Group B) for 8 weeks. A VAS was used to record burning sensations at baseline, at 2 week intervals during treatment, as well as 30 and 60 days after the completion of therapy. The treatment response was recorded using the Tel-Aviv-San Francisco scale. The results showed that individuals taking the lycopene capsules experienced a significantly reduced VAS at 8 weeks (7.6 ± 9.2) compared to baseline (47.0 ± 22.9) with no difference in the VAS between the experimental and control groups after completion of the treatment. In the placebo group, there was a reduced VAS from baseline (49.0 ± 22.9) to 8 weeks (16.3 ± 18.3) with no difference in the VAS after completion of the treatment. Although there was a marked difference in the average reduction of the VAS between the control and test group, the difference was not statistically significant. The authors commented that this reduction in the placebo group correlates to the spontaneous remission often seen in OLP. However, when the examiners looked at the percentage of complete remission cases in either group, they found that 73.3% of patients in the lycopene group experienced remission whereas only 26.7% of the placebo group experienced remission. Based on these findings, the authors surmised that lycopene, which carries antioxidant properties, is effective at reducing the signs and symptoms of OLP.[113]

As reported in the literature presented above, there appears to be a lack of clinical research relative to the use of antioxidants in the management of patients with oral lichen planus. Adequate research has linked a state of oxidative stress to the intraoral

manifestations of OLP, but stronger interventional studies employing antioxidants is critical to establish the strength of the relationship. Should antioxidants improve the clinical condition of patients afflicted with this chronic, cyclical disease, more research is indicated at elucidating the mechanism of action by which reactive oxygen species and free radicals promote signs and symptoms. Although topical steroids remain an effective and safe choice for treatment, they are not without potential problems such as oral candidiasis. Based on today's healthcare trends, patients often seek all-natural alternatives and remedies. Antioxidants may fit the bill as most are derived from different fruits, vegetables, and other plants.

The purpose of this study is to test the efficacy of a combination topical antioxidant formulation containing ferulic acid and phloretin in treating patients with signs and symptoms of oral lichen planus. The primary outcomes include VAS for symptoms and assessment of clinical improvement based on a scoring system developed by Piboonniyom *et al*.[114] Secondary measures include salivary MDA and 8OH-dG levels taken before and after treatment since no prospective interventional studies have studied the levels of oxidative stress after treatment. This study will be undertaken with the hopes of contributing to growing literature linking oral lichen planus to oxidative stress as well as offering clinicians and patients an alternative therapy capable of treating and controlling this disease.

METHODS AND MATERIALS

Protocol Approval

The research study was submitted to and approved by the Institutional Review Committee at Baylor College of Dentistry, Texas A&M University (Dallas, Texas, United States).

Subject Population

A total of 40 patients (8 males, 32 females with an age range of 32-86) with oral lichen planus were recruited from the Stomotology Clinic, Baylor College of Dentistry (Dallas, Texas, United States).

Inclusion criteria included: (1) at least 18 years of age, (2) must speak and understand English, (3) documented diagnosis of OLP or lichenoid mucositis via biopsy, (4) active with signs and/or symptoms of OLP intraorally, (5) refractory to conventional therapy or incomplete response to conventional therapy after 6 or more weeks of therapy, (6) must be able provide verbal and written informed consent. "Unresponsiveness" is defined as no alleviation in signs and/or symptoms, and "incomplete" response is defined as an improvement in signs and symptoms but not to an acceptable level to the patient.

Exclusion criteria were: (1) females who are pregnant, who are planning on becoming pregnant , or believe they may be pregnant or lactating females, (2) allergy to any ingredients in AO ProVantage Gel (phloretin, ferulic acid, menthol, peppermint oil, thyme, sage oil, clove flower oil, xylitol), (3) past or current use of any topical

antioxidant therapy applied intraorally, (4) history of oral malignancy or active oral infections, (5) having a diagnosis of hepatitis C or HIV, (6) bone marrow and/or kidney transplant recipients, (7) current smoker as defined by the WHO (reports smoking at least 100 cigarettes in their lifetime and, at the time of survey, smokes either every day or some days) or have used or are using smokeless tobacco, (8) uncontrolled diabetes mellitus with a hemoglobin A1c score greater than 7% (53 mmol/mol) (American Diabetes Association) using a chairside test (A1c Now +), (9) and having a known disease resulting in immunodeficiency.

Experimental Design

Patients who agreed to participate in the study were placed into two groups: PLC (placebo, n = 20) or AO (test, n = 20). The placebo formulation was designed without the use of phloretin and ferulic acid, which are the two main anti-oxidant ingredients in the active product, and was of the same color, taste, viscosity, and smell. The company provided certification that the placebo formulations were indeed lacking in both ingredients. All tubes of the placebo and test gels were labeled by the company with either an A or B accompanied by a value from 1 to 20, and a decryption grid was kept by the company until all patients completed the study.

Patients were seen at baseline, 2 weeks, and 4 weeks. At the baseline evaluation, patients were asked to fast and refrain from using any form of topical medications from midnight until their appointment. The morning of their appointment, patients rinsed with sterile water for 30 seconds before saliva collection. Un-stimulated whole saliva was collected by having the patients tilt their heads down and drool saliva into a graded

sampling tube until about 5 ml was collected. The collected saliva was immediately placed into a centrifuge (Hermle Z300K, Labnet International, Inc., Edison, NJ, USA) at 1,250g for 10 minutes at 4°C and the supernatant was stored in 1 ml aliquots at -80°C.

Intraoral lesions were evaluated and scored based on the scoring system described by Piboonniyom *et al*[114] with the aid of a transparency paper with rectangular and round shapes equaling 1 cm^2 and 3 cm^2. The mouth was divided into several areas and given a score for the type of lesion present. Reticular lesions were scored with either a "0" for the absence of lesions or a "1" for the presence of lesions. With the help of transparency paper (Fig. 1), erythematous and ulcerative lesions were scored as: "0" – no lesions present, "1" – lesions $\leq$1 cm^2, "2" – lesions >1 cm but $\leq$ 3 cm^2, and "3" - lesions >3 cm^2. The final whole mouth composite score was the sum of the three types of lesions with the erythematous score multiplied by a factor of 1.5 and the ulcerative lesions factored by a factor of 2 (Fig. 2). Patients indicated what current therapy they were using, lesions were photographed, and patients were asked to indicate their current symptoms using a 100 mm VAS scale with marks at every 5 mm (0 = no pain whatsoever, 100 = worst pain ever).

Figure 1. *Example of measuring clinical lesions. Clear plastic outlines of circles or polygons (not shown) with areas of 1 cm^2 and 3 cm^2 were used to score erythematous and ulcerative lesions.*

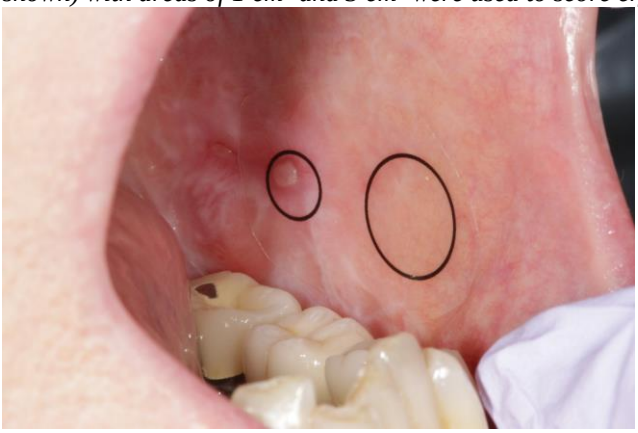


Figure 2. *Oral lichen planus scoring system*

Site	*Reticular Area*		*Erythematous Area*				*Ulcerative Area*			
U/L Labial Mucosa	0	1	0	1	2	3	0	1	2	3
R Buccal Mucosa	0	1	0	1	2	3	0	1	2	3
L Buccal Mucosa	0	1	0	1	2	3	0	1	2	3
Dorsal Tongue	0	1	0	1	2	3	0	1	2	3
Ventral Tongue	0	1	0	1	2	3	0	1	2	3
Floor of Mouth	0	1	0	1	2	3	0	1	2	3
Hard Palate Mucosa	0	1	0	1	2	3	0	1	2	3
Soft Palate/ tonsils	0	1	0	1	2	3	0	1	2	3
Max. Gingiva	0	1	0	1	2	3	0	1	2	3
Mn. Gingiva	0	1	0	1	2	3	0	1	2	3
TOTAL										

After examination, patients were given a tube of either the placebo or test gel. Randomization was achieved by writing all corresponding codes onto small squares of paper and placed into an unlabeled manila envelope. Patients selected one small square of paper and were given the tube with the corresponding code. The patients were

instructed to treat each area with symptoms or lesions with a pea-size amount of product 3 times a day in a gentle dabbing motion and refrain from eating or drinking for 30 minutes after each use. Patients were instructed to continue their current therapy for lichen planus during the duration of their participation, even if it included no treatment at all, to ensure equal baseline and final oral conditions. To ensure compliance, each tube was weighed in ounces before and after demonstration and at each subsequent recall in order to assess if the weight fell within a reasonable range (±20% of expected weight) which would indicate adequate usage.

Repeat photographs, VAS assessment, and OLP scoring were obtained again at 2 weeks and 4 weeks, and saliva collection was repeated again at 4 weeks.

Analysis of 8-OH-dG and MDA

Saliva 8-OH-dG levels were assayed using a commercially available competitive enzyme immunoassay, DNA/RNA Oxidative Damage EIA Kit (Cayman Chemical, Ann Arbor, Michigan, USA), according to the manufacturer's instructions. The enzyme immunoassay buffer consisting of 1 M phosphate solution containing 1% BSA, 4 M sodium chloride, 10 mM EDTA and 0.1% sodium azide was diluted with 90 ml of deionized water and the wash buffer consisting of 4 M phosphate solution was diluted to a volume of 2 liters of deionized water and 1 ml of polysorbate 20. Saliva samples were diluted with the enzyme immunoassay buffer to a 1 to 50 µl dilution and each sample was plated on a 96-well plate coated with goat polyclonal anti-mouse IgG. Fifty microliters of a tracer comprised of acetylcholinesterase linked to an 8-OH-dG and 50 µl of the mouse anti-human 8-OH-dG antibody were added to each sample. Each plate was

then covered with a plastic film and incubated for 18 hours at 4°C. The plate was then emptied and washed with the wash buffer and 200 µl of Ellman's Reagent (5,5'-dithiobis-(2-nitrobenzoic acid)) was added to each well. The plate was again covered with a plastic film and allowed to develop for 90-120 minutes. Afterwards, the film was removed and the plates were read at 450 nm with a spectrophotometer (FLUOStar Optima, BMG Labtech, Cary, NC, USA) and reported as pg/ml.

Saliva MDA levels were assayed using a commercially available kit, NWLSS Malondialdehyde Assay (Northwest Life Science Specialties, Vancouver, Washington, USA), to measure adducts formed by the reaction of MDA with thiobarbituric acid (TBA). Thiobarbituric acid was reconstituted with 10.5 ml deionized water and 10 µl butylated hydroxytoluene in ethanol was added to microcentrifuge vials. The saliva samples were added to the vials and then subsequently combined with 250 µl 1M phosphoric acid and 250 µl TBA reagent. The vials were then vortexed, incubated at 60°C for 60 minutes, and then centrifuged (Spectrafuge 24D, Labnet International, Inc., Edison, NJ, USA) at 10,000x g for 150 seconds. The reaction mixtures were transferred to semi-micro cuvettes, analyzed at 523 nm with a spectrophotometer (FLUOStar Optima, BMG Labtech, Cary, NC, USA), measured based on comparison with a calibrated standard, and reported as µM.

Statistical Analysis

For the VAS, a 20% reduction was considered to be clinically significant[115]. However, there were 12 total individual data sets with a baseline VAS of less than 20. We felt it was reasonable to keep these data sets since it might provide valuable clinical

results. One patient in the AO group dropped out after 1 week of usage due to frustration from a lack to symptomatic improvement. One patient in the PLC group dropped out after 3 weeks of usage due to similar frustrations. For intent-to-treat analysis, the data missing for the remaining time points was assumed to remain the same as the last recorded value for VAS, OLP, MDA, and 8-OH-dG.

In addition to the incomplete data on the drop-outs, 2 additional patients had incomplete data for salivary MDA at 4 weeks due to laboratory error and a resultant loss of remaining sample from those patients. The MDA level from baseline was assumed to have remained the same in these cases.

The final data was further stratified into groups of the patients with the highest value for each variable. The cut-off for ranking in these "high" groups was determined after data collection. If the sample size permitted, statistical analysis was performed. If not, descriptive statistics and observations for trends were used to describe any potential correlations between the different variables and clinical parameters such as lesion type and areas of involvement.

All variables were treated as interval data and since the sample was not normally-distributed, non-parametric tests were used. A Friedman test was used to test for universal differences among the repeated measures within each group. *Post hoc* Wilcoxon signed-rank tests were used to analyze within group differences from baseline to 2 weeks, 2 weeks to 4 weeks, and baseline to 4 weeks. Bonferroni corrections were used to adjust the α value for multiple comparisons ($\alpha = 0.05/3 = 0.0167$). A Mann-

Whitney *U* test was used to examine differences between the two groups at each time point. The significance value was set at $\alpha = 0.05$.

RESULTS

Of the 40 patients screened, 7 were disqualified. One patient had an Hba1c value > 7.0%, 3 had no clinical signs of OLP, and 3 others never made their baseline appointment. A total of 33 patients were included in the study. There were 17 patients in the PLC group (13 females and 4males, ages between 32 and 80, with an average age of 61 ± 12.03 years) and 16 patients in the AO group (12 females and 4 males, ages between 44 and 86, with an average age of 65.69 ± 9.84 years). None were smokers and 2 patients had type 2 diabetes mellitus, one with an Hba1c of 6%, and the other, 6.2% (Table 1). During the course of the study there were 3 drop-outs, 1 from the PLC group, and 2 from the AO group. Complete data was obtained up to the 2 week follow-up for the patients in the AO group, and while only baseline data was available for the PLC group drop-out.

In terms of patient compliance, only one patient was disqualified from the study because he had used 32.28% more than the expected weight of the tube and ran out of the medication at 2 weeks. All other patients were within the arbitrary 20% range (average of -0.43% ± 6.87 for the PLC group, average of 5.51% ± 10.61% for the AO group, data not shown) of their expected tube weight at the 2 week visit. During the 4 week visit, the average difference from the expected tube weight was 2.58% ± 16.69 for the PLC group and -0.10% ± 10.94 for the AO group; 5 patients were outside of the 20% range (average of 26.99% ± 4.58). However, the data was kept so the sample size would not be compromised during analysis.

Table 1. *Patient characteristics*

Parameter	PLC	AO
No. of patients	17	16
Age	61.00 ± 12.03 years	65.69 ± 9.84 years
No. of smokers	0	0
No. of diabetic patients	1	1
Hba1c	6.9%	6.7%
Current therapy		
Clobetasol	10	9
Fluocinonide	3	2
None	3	4
Other	1	1

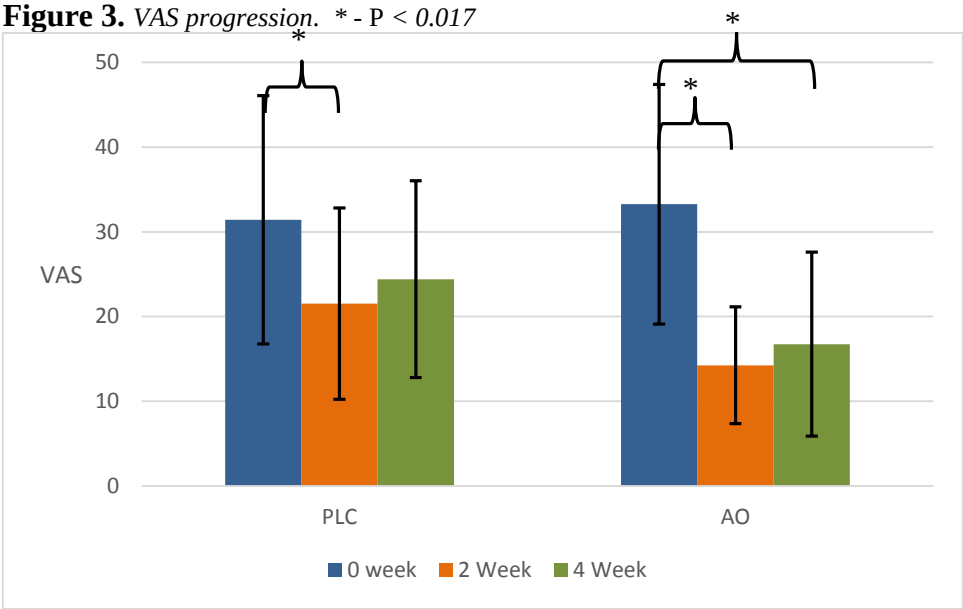
VAS

The mean values for the VAS at each time point are listed in Table 2. At baseline, the mean VAS for the PLC group was 31.41 ± 30.82 and 33.25 ± 28.82 for the AO group. At the 2 week follow-up, the PLC group had a mean VAS of 21.53 ± 23.75, and the AO group had a mean VAS of 14.25 ± 14.05. At 4 weeks, the PLC group had a mean VAS of 24.41 ± 24.44, and the AO group had a mean VAS of 16.75 ± 22.14. From baseline to 2 weeks, both groups had a statistically significant reduction in VAS (P <0.05). From 2 weeks to 4 weeks, neither group had further statistically significant reductions in VAS (P >0.05). Relative to the baseline, there was no difference at 4 weeks for the PLC group, but the VAS for the AO group was statistically lower at 4 weeks than at baseline (Table 2). However, there were no statistically significant differences between the PLC and AO groups at any of the time points (P >0.05). (Fig. 3)

Table 2. *Summary of VAS*

VAS	PLC (n = 17)	AO (n = 16)	*P* values
Baseline	31.41 ± 30.82	33.25 ± 28.82	0.678
2 Weeks	21.53 ± 23.75	14.25 ± 14.05	0.612
4 Weeks	24.41 ± 24.44	16.75 ± 22.14	0.336

Figure 3. *VAS progression.* * - P < *0.017*



Lesion Scoring

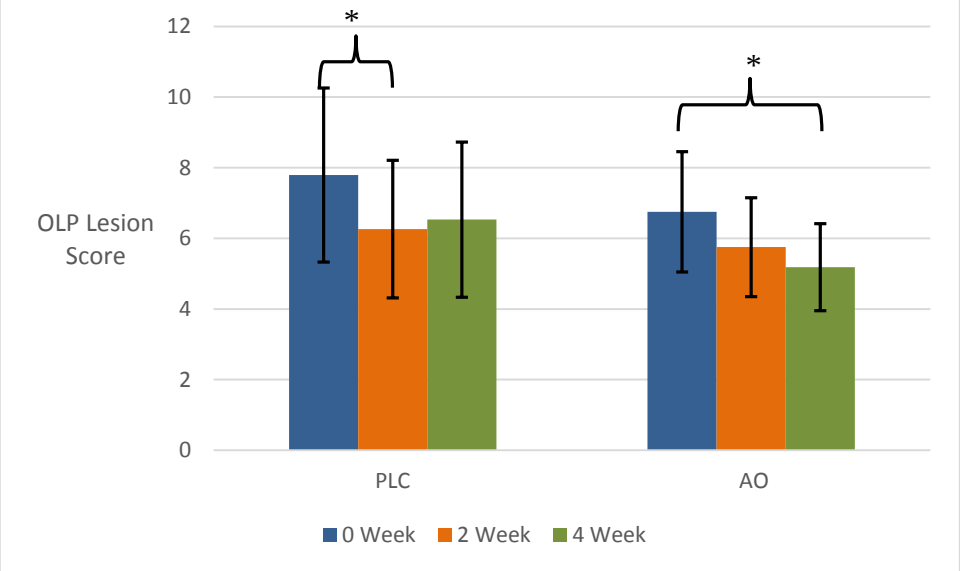
The mean values for the lesion scores at each visit are listed in Table 3. At baseline, the mean lesion score for the PLC group was 6.75 ± 3.48 and 7.79 ± 5.18 for the AO group. At the 2 week follow-up, the PLC group had a mean lesion score of 5.75 ± 2.86, and the AO group had a mean lesion score of 6.26 ± 4.10. At 4 weeks, the PLC group had a mean lesion score of 5.19 ± 2.52, and the AO group had a mean lesion score of 6.53 ± 4.63. The reduction in OLP lesion score from baseline to 2 weeks and from baseline to 2 weeks was statistically significant for the PLC group (*P* <0.05), and the

reduction of the OLP lesion score from baseline to 4 weeks was statistically significant for the AO group (P <0.05) (Table 3). However, after Bonferroni corrections, the reduction in OLP lesion score from baseline to 4 weeks for the PLC group failed to remain statistically significant. There were no statistically significant differences between the groups at any time point in terms of OLP lesion score (P >0.05) (Fig. 4).

Table 3. *Summary of OLP lesion scores*

OLP lesion score	PLC (n = 17)	AO (n = 16)	*P* values
Baseline	6.75 ± 3.48	7.79 ± 5.18	0.759
2 Weeks	5.75 ± 2.86	6.26 ± 4.10	0.885
4 Weeks	5.19 ± 2.52	6.53 ± 4.63	0.588

Figure 4. *OLP lesion score progression. * - P <0.017*



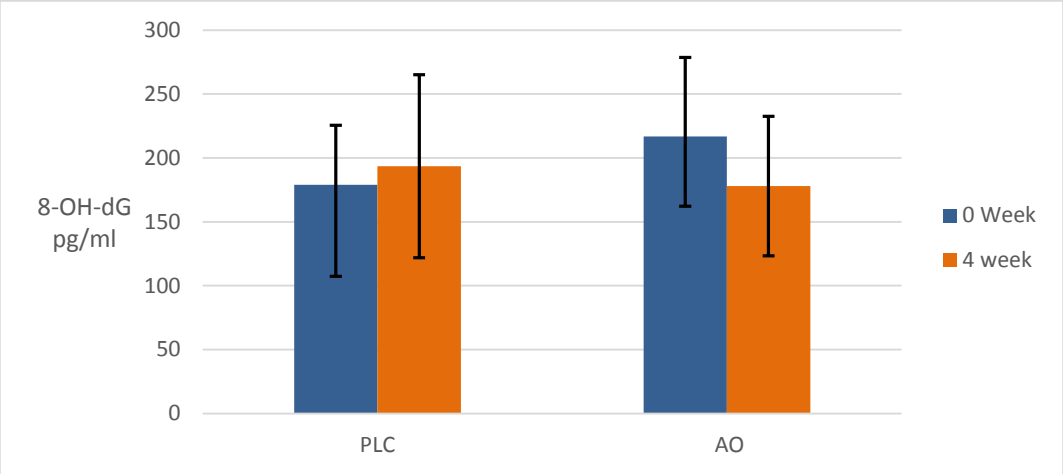
Salivary 8-OH-dG

The mean values for salivary 8-OH-dG at each visit are listed in Table 4. At baseline, the mean salivary 8-OH-dG (in pg/ml) for the PLC group was 179.01 ± 99.47 and 216.88 ± 132.01 for the AO group. At 4 weeks, the PLC group had a mean salivary 8-OH-dG of 193.58 ± 158.23 which was an 8.14% increase from baseline. At 4 weeks, the AO group had a reduced mean salivary 8-OH-dG level of 178 ± 116.56. This represented a 17.93% reduction in 8-OH-dG levels. Neither changes were statistically significant (P >0.05) (Table 4). When comparing both groups at baseline and 4 weeks, there were no statistically significant differences between the PLC and AO group at any time point (P >0.05) (Fig. 5).

Table 4. Summary of salivary 8-OH-dG levels

8-OH-dG (pg/ml)	PLC (n = 17)	AO (n = 16)	*P* values
Baseline	179.01 ± 99.47	216.88 ± 132.01	0.428
4 Weeks	193.58 ± 158.23	178 ± 116.56	0.801

Figure 5. *8-OH-dG progression*



Salivary MDA

The mean values for salivary MDA at each visit are listed in Table 5. At baseline, the mean salivary MDA (in μM) for the PLC group was 3.39 ± 1.07 and 3.24 ± 1.07 for the AO group. At 4 weeks, the PLC group had a mean salivary MDA level of 3.52 ± 1.28 and 4.63 ± 1.82 for the AO group. There was no statistically significant change from baseline to 4 weeks for the PLC group (P >0.05), but the increase in the AO group was statistically significant (P <0.05) (Table 5). Although there was no statistically significant difference at baseline between the AO and PLC group (P >0.05), the AO group had a significantly higher level of MDA at 4 weeks compared to the PLC group (P <0.05) (Fig. 6).

Table 5. *Summary of salivary MDA levels*

MDA (µM)	PLC (n = 17)	AO (n = 16)	*P* values
Baseline	3.39 ± 1.07	3.24 ± 1.07	0.471
4 Weeks	3.52 ± 1.28	4.63 ± 1.82	**0.019**

Figure 6. *MDA progression; * - P < 0.05*

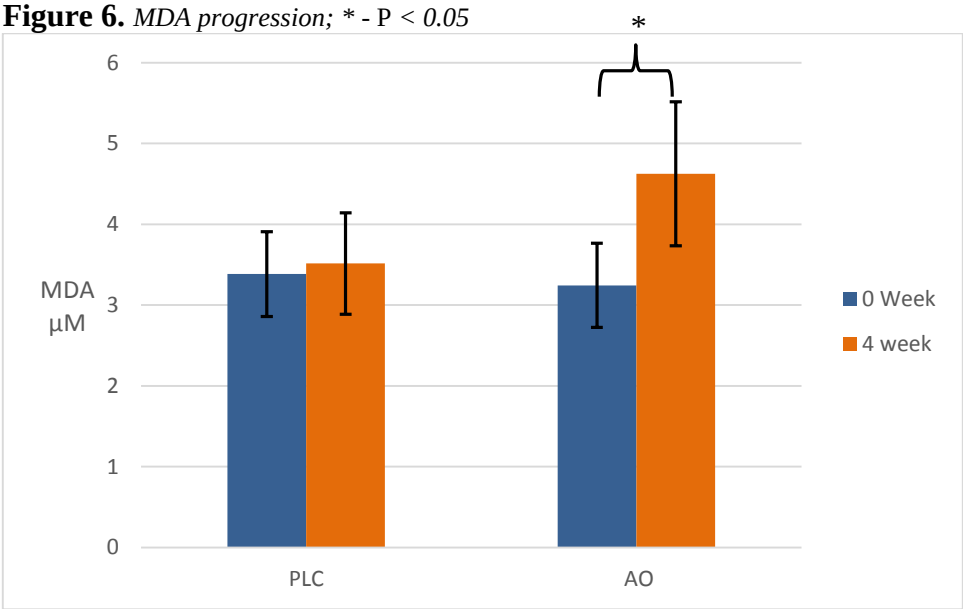


Figure 7. *Patient in the PLC group at (A) baseline, (B) 2 weeks, and (C) 4 weeks.*

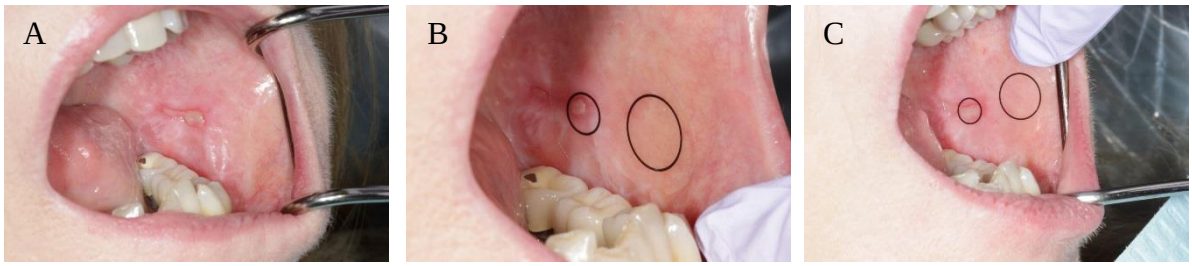


Figure 8. *Patient in the AO group at (A) baseline, (B) 2 weeks, and (C) 4 weeks.*

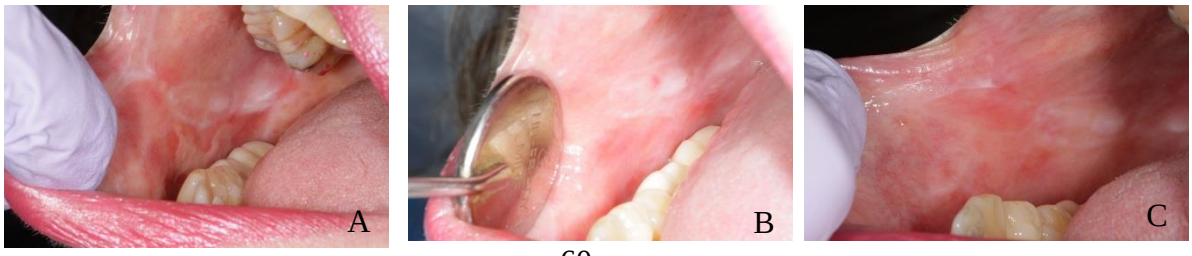
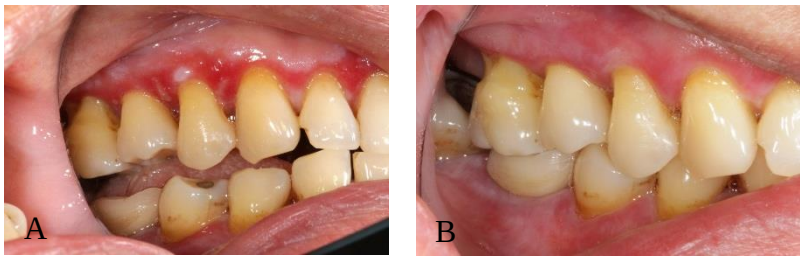


Figure 9. *Drop-out in the AO group at (A) baseline and (B) 2 weeks. Marked improvement in the gingival lesions were noted.*



Descriptive Analysis

Further investigation looked at the trends and possible relations between the variables. To simplify the data and focus on more severe symptoms and clinical presentations, we looked at the data which was the highest for all categories at baseline using arbitrary cutoffs. "High" scores for VAS were considered to a VAS ≥ 50, an OLP lesion score ≥ 9, 8-OH-dG levels ≥ 210 pg/ml, and MDA levels ≥ 3.900 μM. The "high" VAS group consisted of 8 patients with an average baseline VAS of 78.25 ± 13.25, the "high" OLP group consisted of 10 patients with an average baseline OLP score of 12.7 ± 3.32, the "high" 8-OH-dG group consisted of 10 patients with an average baseline 8-OH-dG level of 319.87 ± 103.56, and the "high" MDA group consisted of 10 patients with an average baseline MDA score of 4.23 ± 0.30. The patients in each group were ranked in order of highest to lowest and patients with multiple rankings in these "high" categories were identified to describe any correlation between VAS, OLP, 8-OH-dG, and MDA.

Relationships between variables

The patient with the highest VAS (100) at baseline had the 2nd highest OLP score (16), but only 2 other patients in the top VAS grouping were also among the top 10

OLP lesion scores. In fact, the patient with the highest OLP lesion score (20.5) had a baseline VAS of 15. In addition, only 3 of the 8 "high" VAS patients were among the 10 "high" 8-OH-dG patients. The patient with the highest VAS patient (100) had a baseline 8-OH-dG level of 147.233 pg/ml which was below the overall average of 179.008 ± 99.470 pg/ml. As for VAS and MDA, 4 of the 8 high VAS patients were among the 10 "high" MDA patients. The patient with the highest VAS (100) had an MDA level of 3.586 μM which was only slightly above the overall average of 3.385 ± 0.789 μM. When examining if an increase in 8-OH-dG and MDA levels from baseline to 4 weeks corresponded with an increase in VAS, the results showed 7 patients in the PLC and 4 in the AO group had an increase in 8-OH-dG at 4 weeks compared to baseline, and 8 patients in the PLC group and 12 in the AO group had an increased MDA at 4 weeks. However, none of these patients had a corresponding increase in VAS from baseline to 4 weeks.

For OLP scores, only 1 of the 10 "high" OLP score patients were among the 10 "high" 8-OH-dG patients. In fact, the patient with the highest OLP score at baseline (20.5) had an 8-OH-dG level of 156.882 pg/ml which was below the overall average of 179.008 ± 99.470 pg/ml. Also, 4 of the patients with the 10 highest OLP lesions scores were among the 10 "high" MDA patients. The patient with the highest OLP score at baseline (20.5) had a baseline MDA level of 3.586 μM which was only slightly above the overall average of 3.385 ± 0.789 μM. When examining if an increase in 8-OH-dG and MDA levels corresponded to an increase in OLP scores, the results showed that none of the patients with increased 8-OH-dG at 4 weeks had a corresponding increase in

OLP score, and only 1 patient with an increase in MDA at 4 weeks had a corresponding increase in OLP score.

In addition, only 3 of the 10 "high" 8-OH-dG patients ranked among the 10 high MDA patients. Table 6 shows the patients who were among the "high" group in each category. In total, 4 patients were among the "high" groups in 3 categories while most ranked in 2 or fewer categories (Table 6).

OLP presentation

A majority of patients presented with reticular lesions: 31 out of 33 patients presented with the reticular form of OLP (93.9%). Twenty-three patients presented with erythematous lesions (69.7%), and 8 patients presented with ulcerative lesions (24.24%). Of the 10 with no baseline erythematous lesions, 4 remained free of erythematous lesions but 6 (3 each in the AO and PLC groups) developed erythematous lesions at one point or another during the remainder of the study. Half of these patients had no erythematous at the final visit and of the 3 who did not have resolution of erythematous lesions at 4 weeks, 2 had an increase in both MDA and 8-OH-dG. No other relations could be explored in regards to patients with erythematous lesions versus those without since a majority of patients presented with erythematous lesions.

As for ulcerative lesions, 5 of the 8 patients in the "high" VAS group had ulcerative lesions, 5 of 10 patients in the high OLP score group had ulcerative lesions, 4 of 10 patients in the high 8-OH-dG group had ulcerative lesions, and 3 of 10 patients in the high MDA group had ulcerative lesions.

Table 6. *Highest scores for each variable. This table represents the "high" patients categorized by variable and ranked from highest to lowest. The patient numbers coded with warm colors indicate 3 top ranks, cool colors represent 2 top ranks, and no color represents no additional top ranks.*

Patient	VAS	Patient	OLP score	Patient	8-OH-dG	Patient	MDA
1B	100	13B	20.5	12B	524.4485	13A	4.9105
10A	90	1B	16.0	5A	444.656	18B	4.501
5A	83	3B	13.0	19A	366.653	3B	4.4365
2A	80	9A	12.5	2A	366.156	8A	4.186
17B	76	17B	12.0	18B	307.679	10B	4.128
4B	70	14A	11.5	10A	254.086	10A	4.1025
3B	70	12A	11	14A	245.124	13B	4.100
14B	57	2B	10.5	7B	244.9865	4B	4.062
		4B	10.0	13A	230.1445	4A	3.9835
		15B	9.5	6A	214.775	17B	3.911

Lesion location

The breakdown of areas of involvement at baseline and percentages are presented in Table 7. Gingival lesions were the most prevalent in the study with 23 out of 33 patients (69.7%) presenting with maxillary gingival lesions and 24 out of 33 patients (72.7%) presenting with mandibular gingival lesions. Only 5 patients (15.2%) had no gingival involvement. Three patients, all in the PLC group, developed gingival lesions at the time of the 4 week recall, but only 1 patient had a corresponding increase of MDA at 4 weeks and none had a corresponding increase in 8-OH-dG at 4 weeks. The second most common location was on the buccal mucosa (60.6% for right and left buccal mucosa). The least prevalent locations in this population of patients were the floor of the mouth and the ventral tongue (3%). Out of all the areas of involvement, the buccal and labial mucosal sites had the highest incidence of remission. 2 out of the 3 initial patients

with upper/labial mucosal lesions had complete remission by 4 weeks and 3 out of the 20 patients with right buccal mucosal lesions and 3 out of the 20 patients with left buccal mucosal lesions achieved complete remission by 4 weeks. Only 1 patient with mandibular gingival lesions achieved complete remission of that site by 4 weeks (Table 7).

Table 7. *Summary of lesion locations*

Lesion location	No. patients Baseline	% patients Baseline	No. patients 4 Weeks	% patients 4 Weeks
Upper/lower labial mucosa	3	9.1	1	3.0
Right buccal mucosa	20	60.6	17	51.5
Left buccal mucosa	20	60.6	17	51.5
Hard palate	2	6.1	2	6.1
Dorsum of tongue	2	6.1	2	6.1
Ventral tongue	1	3.0	1	3.0
Floor of mouth	1	3.0	1	3.0
Maxillary gingiva	23	69.7	23	69.7
Mandibular gingiva	24	72.7	23	69.7

Effects of adjunctive therapy

As part of the protocol, patients were allowed to use their current therapy for OLP in addition to either the placebo or active formula. However, at various time points in the study, a total of 17 patients (8 in the PLC group, 9 in the AO group) had decided on their own accord to quit using their current therapy and use only the placebo/active gel. To investigate if the concurrent use of other therapies, or absence of it, affected the

results, patients were stratified into four groups: 1) a placebo only group (PLC Only), 2) an active only group (AO Only), 3) a placebo plus their current therapy group (PLC+TX), or 4) an active plus current therapy group (AO+TX). Statistical analysis was completed to examine for any within group differences at each time point for each variable, and to examine for any differences among the 4 groups at each time point for each variable. Table 8 shows the result of analyses which revealed that asides from the significant difference in baseline VAS and OLP score between the PLC Only and PLC+TX group, there were no differences at any time point between any of the 4 groups for any of the variables.

Table 8. ***Effects of use of topical steroids; †* - P *<0.05 between PLC Only and PLC+TX groups***

VAS	PLC Only (n = 8)	AO Only (n = 9)	PLC+TX (n = 7)	AO+TX (n = 6)	*P* values
Baseline	12.63 ± 11.33†	33.25 ± 28.82	49.00 ± 32.89†	50.67 ± 36.13	>0.05
2 Weeks	7.13 ± 8.17	14.25 ± 14.05	37.86 ± 28.12	20.50 ± 18.41	>0.05
4 Weeks	13.75 ± 9.18	16.75 ± 22.14	30.00 ± 27.87	23.67 ± 34.33	>0.05
P values	>0.017	>0.017	>0.017	>0.017	
OLP lesion score					
Baseline	5.44 ± 3.10†	7.79 ± 5.18	11.43 ± 5.82†	7.08 ± 4.83	>0.05
2 Weeks	4.63 ± 3.64	6.26 ± 4.10	8.50 ± 4.37	5.75 ± 4.22	>0.05
4 Weeks	4.44 ± 3.67	6.53 ± 4.63	8.57 ± 5.05	5.83 ± 3.47	>0.05
P values	>0.017	>0.017	>0.017	>0.017	
8-OH-dG (pg/ml)					
Baseline	170.17 ± 79.72	216.88 ± 132.01	162.83 ± 108.95	193.48 ± 138.05	>0.05
4 Weeks	183.81 ± 152.36	178 ± 116.56	212.78 ± 179.78	141.62 ± 80.12	>0.05
P values	>0.017	>0.017	>0.017	>0.017	
MDA (µM)					
Baseline	3.52 ± 0.73	3.24 ± 1.07	3.34 ± 0.96	3.21 ± 0.88	>0.05
4 Weeks	3.09 ± 0.93	4.63 ± 1.82	3.97 ± 1.40	5.03 ± 1.01	<0.05
P values	>0.017	>0.017	>0.017	>0.017	

DISCUSSION

This was the first study to investigate the efficacy of a topical antioxidant combination gel in treating patients with OLP and also the first interventional study analyzing salivary biomarkers of oxidative stress before and after treatment. Alleviation of symptoms and clinical signs of the disease are important variables in determining the efficacy of any potential treatment for patients with chronic, often painful diseases such as OLP. VAS is usually the standard method to subjectively measure symptoms in patients. Chainani-Wu *et al* studied the efficacy of antioxidant curcuminoids to treat OLP in a randomized, placebo-controlled, double-blind clinical study. They found no difference between 12 patients receiving curcuminoid capsules and 16 patients taking the placebo in terms of VAS after 49 days[112]. Saawarn *et al* studied the efficacy of lycopene softgel capsules in a placebo-controlled clinical study. At 8 weeks, they found no difference in VAS scores between the 15 patients taking the lycopene capsules compared to the 15 patients in the placebo[113]. In our study, although there was a statistically significant reduction in VAS from baseline to 2 weeks for the PLC group and from baseline to 2 and 4 weeks for the AO group, there were no statistically significant differences between the two groups at any time point. Two other studies have looked at antioxidants given in capsule form as an intervention. Inherent bias exists in the VAS as patients were told to indicate with a single mark on a standardized 10 cm line which best represented their current symptoms. In our study, having "symptoms" was defined as having any unpleasant sensation such as, but not limited to, pain, discomfort, soreness,

and burning sensations. To facilitate uniformity, patients were instructed to evaluate their symptoms that day, and for the 2 week and 4 week follow-ups, they were told to view the VAS from their previous visits as reference. Despite this, wide variability was noted in the responses. The error inherent in the VAS is well documented[115, 116] and it was no surprise that the only subjective measure in this study was perhaps the weakest.

The measure for clinical efficacy was the lesion scoring. Statistically significant reductions in OLP lesion score was observed from baseline to 2 weeks in the PLC group and from baseline to 4 weeks in the AO group. Again, as with the VAS, no differences were found between the two groups at any time point. This was similar to the study by Chainani-Wu *et al* in that there were no difference between the active and placebo groups in terms of lesion scoring[112]. Saawarn *et al* recorded clinical response using the Tel Aviv-San Francisco scale and found that at 4 weeks through 8 weeks, the score for clinical response in the lycopene group was statistically better than the response in the placebo group[113]. A possible reason for a lack of statistically significant differences between the two groups in this study may be related to the scoring system used. Although the overall score is reported and analyzed as interval data, the subset scores are ordinal. Erythematous and ulcerative lesions are graded based on lesion area: 0 = no lesion, 1 = lesion present but $\leq$1 cm^2, 2 = lesion >1 cm^2 but $\leq$3 cm^2, and 3 = lesion >3 cm^2. The area difference between a score of 1 and 2 is 2 cm^2, which is twice the interval between a score of 0 and 1. Some lesions, which were scored a 2 on initial examination, actually showed regression in size (Fig. 7, A-C), but if the regression in size was not large enough, the lesion would have still been scored a 2. Obviously, the score would not

reflect more subtle, but noticeable, clinical changes. Indeed, clinical observations and retrospective analysis of photographs suggest there was some improvement in most cases in the AO group, particularly in regards to the intensity of the erythematous lesions. Unfortunately, the scoring system used does not account for changes in intensity of erythematous lesions. We attempted retrospective analysis of erythematous lesions using an erythema index[117], but due to different angulation and lighting conditions of the photographs, we were unsuccessful at our attempt. Regardless, several lesions in the AO group did seem to pass the "eye test" in terms of subtle improvement. Only one group of investigators have used this scoring system to analyze post-treatment oral lesion size. Cutler *et al* found no significant difference between baseline and 1 year oral lesion scores and they only briefly mention that perhaps their "clinical measurement tools were inadequate to detect improvement[118]." Retrospectively, perhaps supplementing the scoring system by Pibooniyom *et al*[114] with one in which the transition from one lesion score to another was linear and better able to detect smaller, but visually noticeable changes, may have been beneficial.

This is the second study to evaluate levels of salivary MDA in OLP patients and the first to do so after treatment. MDA is the main byproduct of lipid peroxidation by free radical interaction with cellular membranes. This study found a total average level of 3.69 μM ± 1.43 in all patients. Ergun *et al* reported MDA levels of 2.03 ± 0.81 nmol/ml (μM) in the saliva of 21 untreated OLP patients compared to 1.37 ± 0.37 μM in the saliva of 20 healthy controls[108]. Other studies reported on serum levels of MDA. Sezer et al reported 18.24 μM ± 5.21 in untreated OLP patients compared to 15.66 ±

5.23 μM in healthy controls, Aly & Shahin reported 18.09 μM ± 3.02 compared to 15.46 ± 3.32 μM in healthy controls, and Upadhyay et al reported 0.7595 μM ± 0.536 compared to 0.2187 ± 0.054 μM in healthy controls[104, 106, 107]. The salivary MDA levels observed in this study are similar in range to that of the OLP patients and higher than the controls observed by Ergun *et al*[108]. Most previous studies measured MDA levels in serum whereas this study looked at salivary levels which are appropriate in the context of studying free radical damage to cell membrane of the keratinocytes in the local environment of the oral cavity. It was a peculiar observation that MDA levels actually increased to a statistically significant level after 4 weeks of usage of the active formulation. Additional investigation found the MDA increase was not associated with any of the other clinical parameters such as lesion type and lesion location, and these changes appeared to be opposite of direction of the VAS and OLP lesion scores from baseline to 4 weeks. A reasonable explanation for this observation is elusive at this time but a small sample size makes it difficult to predict whether or not this would be true in a larger sample.

To the best of our knowledge, this is the first study to examine the level of salivary 8-OH-dG as a marker of oxidative stress in oral lichen planus patients. 8-OH-dG is a by-product of DNA damage by free radicals and has been found to be much higher in patients with chronic periodontitis. Dede *et al* used the same commercially available kit for salivary analysis of 8-OH-dG in patients with chronic periodontitis. They found patients with chronic periodontitis had 605.5 pg/ml ± 139.1 and healthy patients had 550.52 pg/ml ± 150.28[119]. Takane *et al* used a different method of

immunoassay and observed an average level of 3.29 ng/ml ± 0.21 in the saliva of chronic periodontitis patients before treatment compared to 1.56 ± 0.10 ng/ml in healthy patients[120]. In this study, the average level of salivary 8-OH-dG for all patients at baseline was 197.37 ± 115.42 pg/ml or 0.197 ± 0.115 ng/ml. We observed lower levels of 8-OH-dG in our study population compared to both diseased and healthy patients in these other studies. However, chronic periodontitis may have different oxidative stress-mediated mechanisms for tissue destruction and disease progression than that of OLP, and this may have accounted for the differences. Regardless, our data showed that there was no statistically significant differences between the groups in the salivary levels of 8-OH-dG from baseline to 4 weeks. This finding appeared to be related to the lack of statistically significant differences in VAS and OLP. Also, the levels of salivary 8-OH-dG did not appear to be correlated to changes in VAS or OLP. None of the patients who had an increase in 8-OH-dG at 4 weeks from baseline had a higher VAS nor OLP score at 4 weeks from baseline. In addition, only 3 patients with the top 10 highest 8-OH-dG levels also ranked in the top 10 for MDA levels. Based on these observations, despite an almost 18% decrease in levels of 8-OH-dG in the AO group, 8-OH-dG did not appear in this study to be associated strongly with any clinical features of OLP. This finding is rather disappointing when trying to contribute to the scarce literature linking 8-OH-dG and oxidative stress to OLP. Another confounding factor in this study may have been that levels of 8-OH-dG may have been from periodontal disease found in some of our patients. The presence of periodontitis was not an exclusion criteria in this study, and the previous cited studies, Dede *et a*l and Takane *et al*, have shown that salivary levels of 8-

OH-dG are undoubtedly higher in patients with periodontitis than healthy controls[119, 120]. Coupled with the lack of significant change in MDA levels, it appears that the use of this regimen of the antioxidant gel may not result in a statistically significant change in the patients' levels of either of these oxidative stress markers.

Several reasons may account for the lack of statistical difference in all parameters studied excluding the increased salivary MDA at 4 weeks in the AO group. First, the number and characteristics of the patients in the study may not have been ideal. Recruitment was difficult in that many patients were asymptomatic and were not willing to participate. Reasons for weak recruitment include the lack of transportation, inability to contact, and driving distances. Even within the study population, some parameters were not ideal at baseline. For example, numerous patients had a VAS less than 20 (12 total). Given that the literature has shown variation of 20% on a repeated VAS[115], inclusion of the data sets which had a baseline VAS of less than 20 may have masked effects of the test gel since these patients limited their future responses to staying the same or increasing the VAS. Also, the majority of the patients had reticular lesions either alone or in combination with erythematous and ulcerative forms. The literature has shown a majority of patients with reticular lesions are asymptomatic. Since nearly all (94%) of patients in this study presented with reticular lesions, and some with reticular lesions alone, it would be expected that the VAS may tend to be skewed towards the low end. In this study, patients who presented with ulcerative lesions, which the scoring system presumes to be the most painful, tended to have higher VAS, much like what the literature reports[38]. However, many of the patients with higher OLP scores did not

necessarily have the highest VAS scores at baseline. A possible reason is that many of the patients in the study had been in long term management (> 12 months) of their OLP and had reached a level of acceptance. Second, the placebo may have had some inherent antioxidant properties. Although no ferulic acid or phloretin was utilized in the placebo, secondary ingredients include menthol, peppermint oil, thyme, sage oil, clove flower oil, and xylitol. The literature has shown that all of these secondary ingredients do have some level of antioxidant function[121-124]. It may not have been feasible to provide a placebo in the same consistency, color, taste, and smell as the test without the use of the ingredients. However, since all of these secondary ingredients are found in both the placebo and the test, it would be assumed that any differences would be attributed to phloretin and ferulic acid only.

Another possible reason for the lack of difference between the AO and PLC groups may be attributed to the area of involvement in the patients. Most patients had gingival lesions either on the maxilla (69.7%) or mandible (72.7%). This is higher than what the classic literature reports[6, 8, 9]. Gingival lesions did provide a unique set of challenges in the measurement of the OLP score. We found reticular and ulcerative scores to be easily measured if found on the gingiva, but erythematous scores were difficult for several reasons. Involvement of papillae made measuring with a calibrated polygon on transparency paper difficult to do. Perhaps adjunctive use of a grid method used by Plemons *et al* would have been beneficial[54]. Another interesting finding is that only 1 patient had remission of their gingival lesions at 4 weeks while more patients with labial or buccal mucosal lesions were able to achieve complete remission. This finding

that most gingival lesions in this study failed to achieve remission may be attributed to an inability of the scoring system to identify qualitative changes in erythema in the gingiva. This is an important fact since most of the gingival lesions present in this study were erythematous lesions. Also, as mentioned above, patients were not excluded for periodontitis, and it is possible that some of the lack of recorded improvement may have been from overlying plaque-induced gingival inflammation. Other than these issues, observations of the trends associated with lesion location did not reveal any relationship between the intensity of the VAS and OLP score nor did the location affect either levels of salivary biomarkers.

Some weaknesses of the study include small samples size, study duration, incomplete data for salivary MDA at 4 weeks, and wide variability in the clinical parameters and their associated measuring instruments. Also, some ambiguity may have existed in demonstration and instruction for use. One patient was not compliant at all and was dismissed from the study. The patients were instructed not to eat or drink for 30 minutes after each use, but instruction was not given on how long the patients should wait before spitting the excess amounts out. The time that the gel is left within the mouth before being expectorated may have potentially been a confounder in this study. Also, despite informing patients they were to continue their current therapy concurrently with the use of either the placebo or active gel, a large number of patients (17 total) had decided to stop use of their current therapy. This decision was made on the patient's own accord. Although this may have been a confounder, analysis showed that whether or not

patients used another therapy at the same time during the study probably did not have any effect on the final results (Table 8).

Despite the absence of differences in VAS, OLP score, and salivary 8-OH-dG and MDA levels from baseline to completion of the study between the placebo and test group, there are some findings which support further investigation of the use of combination antioxidants in the treatment of OLP. Although the study duration was only 4 weeks, the fact that none of the patients reported flare-ups associated with OLP signs and symptoms during use of either the test or placebo suggest that the combination antioxidant gel as a maintenance treatment may be warranted. This would be helpful to patients whom the bad taste of topical steroids tend to limit compliance. It is worth noting that 2 patients (1 in each group) developed candidiasis-like signs and symptoms such as pseudomembranous lesions and burning sensations. Candidiasis was confirmed with a salivary candida culture. Both patients had reported a previous history of frequent bouts of candidiasis and both had used clobetasol during the duration of the study. It would be of interest to investigate the frequency of oral candidiasis in susceptible patients using a combination antioxidant rather than topical steroids. Furthermore, although not statistically significant, the trend towards more reduction in the VAS, OLP, and 8-OH-dG in the AO group suggests that a study with a longer duration and more intensive application regimen may result in significant clinical and statistical findings. Within the limits of this study, the use of a topical combination antioxidant gel in management of patients with persistent and non-responsive OLP was not statistically different from a placebo formulation in terms of reducing VAS, OLP score, and salivary

8-OH-dG and MDA levels. However, future investigation is warranted in evaluating the efficacy of a longer duration and perhaps a more intensive regimen on these clinical parameters.

CONCLUSIONS

Within the limits of this study, the patients receiving the active AO gel had significant improvement in VAS throughout the 4 weeks and an improved OLP lesion score from baseline to 4 weeks. Patients receiving the AO gel had lower levels of salivary 8-OH-dG before than after treatment. Interestingly, MDA levels increased significantly at 4 weeks in the AO group, and this observation cannot currently be explained. Overall, the differences between the groups were not statistically significant at any point in time except for salivary MDA levels at 4 weeks. Despite the fact that there were limitations to this study such as a small sample size and large variability in the data, there may be a place clinically for the use of this topical combination antioxidant gel in the maintenance of patients with oral lichen planus who have not received further benefit from conventional topical therapy. Future research should further investigate the efficacy of longer use and perhaps a heavier dosage of antioxidants.

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