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Evaluation of serum and salivary transforming growth factor beta, vascular endothelial growth factor and tumor necrosis factor alpha in oral lichen planus.

Evaluación sérica y salival del factor de crecimiento transformante beta, factor de crecimiento endotelial vascular y factor de necrosis tumoral alfa en liquen plano oral.

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Abstract: **Introduction:** Lichen planus is one of the most common oral mucosal lesions. Transforming growth factor-β (TGF- β) has a marked effect on epithelial–mesenchymal transition and immune cells function. Vascular Endothelial Growth Factor (VEGF) is a key regulator of vasculogenesis and angiogenesis. Tumor necrosis factor-α (TNF-α) mediates T-lymphocyte homing and apoptosis of epithelial cells. **Objetive:** The present study was conducted in order to compare the expression of serum and salivary TGF- β, VEGF, TNF-α between OLP patients and control individuals to investigate if saliva can be used as an alternative to serum for diagnostic purposes and for monitoring disease. **Materials and Methods:** 23 OLP patients and 23 control individuals were included to evaluate serum and salivary TGF-β, VEGF, TNF-α using ELISA kits. Five milliliters of venous blood was collected and unstimulated saliva was collected by the spitting method. **Results:** Serum and salivary levels of TGF- β, VEGF, TNF-α are higher in OLP patients compared to normal controls. Mean difference is higher in saliva than serum. Moreover, there was a significant difference in serum and salivary VEGF and TNF-α between symptomatic and asymptomatic groups. **Conclusions:** Saliva can be a used as a substitute for serum to evaluate levels of the assessed biomarkers.

Keywords: Oral lichen planus; serum, saliva; transforming growth factor beta; vascular endothelial growth factor a; tumor necrosis factor-alpha.

Abstract: **Introducción:** El liquen plano oral es una de las lesiones de la mucosa oral más comunes. El factor de crecimiento transformante β (TGF-β) tiene un efecto marcado sobre la transición epitelial-mesenquimal y la función de las células inmunes. El factor de crecimiento endotelial vascular (VEGF) es un regulador clave de la vasculogénesis y la angiogénesis. El factor de necrosis tumoral α (TNF-α) media la localización de los linfocitos T y la apoptosis de las células epiteliales. **Objetivo:** El presente estudio se realizó con el fin de comparar la expresión en suero y saliva de TGF-β, VEGF, TNF-α entre pacientes con OLP y personas de control para investigar si la saliva se puede utilizar como alternativa al suero para fines de diagnóstico y monitoreo de la enfermedad. **Material y Métodos:** Se incluyeron 23 pacientes con OLP y 23 individuos control para evaluar los niéveles en suero y en saliva de TGF-β, VEGF, TNF-α utilizando kits ELISA. Se recogieron cinco mililitros de sangre venosa y se recogió saliva no estimulada por el método de escupir. **Resultado:** Los niveles séricos y salivales de TGF-β, VEGF, TNF-α son más altos en pacientes con OLP en comparación

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con los controles normales. La diferencia media es mayor en saliva que en suero. Además, hubo una diferencia significativa de VEGF y TNF-α en suero y saliva entre los grupos sintomáticos y asintomáticos. **Conclusion:** La saliva puede usarse como un sustituto del suero para evaluar los niveles de los biomarcadores estudiados.

Palabra Clave: *Liquen plano oral; suero; saliva; factor de crecimiento transformador beta; factor a de crecimiento endotelial vascular; factor de necrosis tumoral alfa.*

INTRODUCTION.

Lichen planus (LP) is a chronic inflammatory mucocutaneous disease and one of the most common oral mucosal lesions. The exact etiology of this disease is still unknown. Trauma, stress, infection and restorative materials have been proposed.[1,2]

Diabetes mellitus and hypertension are associated with LP.[3] A large body of evidence supports a role of immune dysregulation in oral lichen planus (OLP) pathogenesis. Antigen-specific cell-mediated immune response, nonspecific mechanisms, humoral immunity and autoimmunity have been proposed.[4]

Transforming growth factor (TGF-β) has several functions including growth inhibition and apoptosis induction of many cell types, including fibroblasts, epithelial, endothelial and immune cells. It has marked effects on the extracellular matrix (ECM) composition, angiogenesis and induction of epithelial mesenchymal transition (EMT).[5] Therefore, the TGF-β may play an important role in the cancerization of OLP.

TGF-β also modulates differentiation and function of immune cells.[6] Vascular endothelial growth factor (VEGF) is a key regulator of vasculogenesis and lymphangiogenesis, which are of particular importance in the pathogenesis of chronic inflammatory disorders.[7] VEGF has an important role in OLP pathogenesis through activating angiogenesis.[8] Tumor necrosis factor (TNF-α) has also been proposed to be involved in basal cell apoptosis, T-lymphocyte homing and Langerhans' cells activation, which are the typical hallmarks of OLP.[9] The three clinical patterns of OLP that may coexist are papular, atrophic and bullous erosive.[10] Histopathologically, they are characterized by liquefactive degeneration of basal cell layer, hyperkeratosis, shortened saw-toothed rete pegs, and dense subepithelial band of chronic inflammatory cells.[11]

Diagnosis of OLP is often challenging because various oral disorders resemble OLP clinically or pathologically, many microscopic features are not specific and may be influenced by disease activity.[10] Moreover, difficulties in diagnosis may arise if the lesion is superimposed by Candida infection because the microorganism may disturb the characteristic reticular pattern. Biopsy is often necessary to rule out other erosive or ulcerative diseases especially if lesions are confined to the gingiva. Direct, indirect immunofluorescence and direct oral microscopy may be used as alternative diagnostic techniques.[12] Cytological examination, commercially available chemiluminescence kits and photodynamic diagnosis can also be used.[13]

Other diagnostic modalities that are simpler and less expensive are required, particularly in the case of repeated sampling. Diagnostic modalities such as serum and salivary biomarkers are promising. Salivary biomarkers that are easier to be collected than serum can be used in diagnosis, monitoring disease activity and response to treatment.[14] According to our knowledge, few studies have evaluated both serum and salivary TNF-α.[15] or VEGF[16] and there is no available data comparing salivary and serum TGF- β levels.

Therefore, the aim of the current study was to compare the expression of serum and salivary TGF-β, VEGF and TNF-α between OLP patients and control individuals to investigate if saliva can be an alternative to serum in evaluating the levels of these biomarkers. Additionally, their role in monitoring disease activity has been investigated.

MATERIALS AND METHODS.

The current study was carried out at the Oral Medicine and Diagnosis Department, Faculty of Dentistry, Cairo University, Egypt between October 2016 and October 2018. The study included 46 individuals divided into two groups. Group 1 included 23 patients (16 females and 7 males) clinically and histopathologically diagnosed as having OLP. Their mean age was 48.1 years. Group 2 included 23 systemically healthy individuals (14 females and 9 males).

Their mean age was 45.7 years. The inclusion criteria for patient group were: newly diagnosed subjects and previously diagnosed patients who had stopped topical treatment for 2 weeks or systemic treatment for 1 month prior to participating in the study. All types of OLP were included. OLP patients included five papular,

one erythematous and seventeen bullous erosive; all cases involved the buccal mucosa. The tongue was affected in five cases and one case involved cheeks, tongue and labial mucosa. (Figure 1)

Patients with cutaneous lesions were excluded to ensure that serum level changes were caused solely by oral lesions. The exclusion criteria for all individuals included subjects with systemic conditions associated with immune dysfunction, the presence of any oral mucosal lesions, pregnancy or lactation.

Serum and salivary sample collection

Five milliliters of venous blood were collected from all subjects in plain tubes. The collected samples were left to clot, and were then centrifuged and the separated serum samples were stored at -20°C. During the same visit, saliva collection was performed early in the morning under resting conditions. Individuals were instructed to rinse mouth and wait at least 10 minutes after rinsing to avoid sample dilution before collecting saliva.

Before sample collection, patients were instructed to avoid foods high in sugar, acidity, or high in caffeine content since they may compromise the assay by lowering salivary pH. Whole unstimulated salivary samples were collected by spitting into a collecting cup; five milliliters were collected from each participant.

Biochemical analysis

TGF-β levels were measured using ELISA kit provided by ID labs Biotechnology London, ON, Canada. The method is a solid phase sandwich ELISA. It utilizes a monoclonal antibody specific for human TGF-β coated on a 96-well plate. Quantitation of VEGF was performed by VEGF ELISA kit provided with AviBion, Orgenium Laboratories Business Unit, Vantaa FINLAND.

It is an in vitro ELISA assay that employs an antibody specific for human VEGF coated onto a 96-well plate. Quantitation of TNF-α was done using TNF-α ELISA kits provided by AviBion, Helsinki FINLAND. All assays were performed according to each manufacturer's instructions.

Statistical analysis

Comparison of numerical variables between the study groups was done using Student t test and Chi-square (X^2) test for categorical data. Correlation between various variables was done using Pearson moment correlation equation for linear relation of normally distributed variables and Spearman rank correlation equation for non-normal variables/non-linear monotonic relation.

Receiver operator characteristic (ROC) analysis was used to determine the optimum cut off value. All statistical calculations were done using the computer program IBM SPSS.

RESULTS.

A statistically higher mean value of TGF-β, VEGF and TNF-α (pg/ml) in serum and saliva were recorded in OLP group. Mean difference (MD) in saliva was higher than serum for all three investigated biomarkers (Table1). There was a strong correlation between serum and salivary levels for each biomarker.

Moreover, there was a significant difference in serum and salivary VEGF and TNF-α between symptomatic and asymptomatic groups.

However there was no significant difference between serum and salivary TGF-β between both disease groups (Table 2). Diagnostic accuracy of TGF-β, VEGF, and TNF-α was high. Sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) were very high (Table 3).

Figure 1. Clinical photograph showing erosive OLP in a 60 years old male with bilateral involvement of the buccal mucosa and tongue.

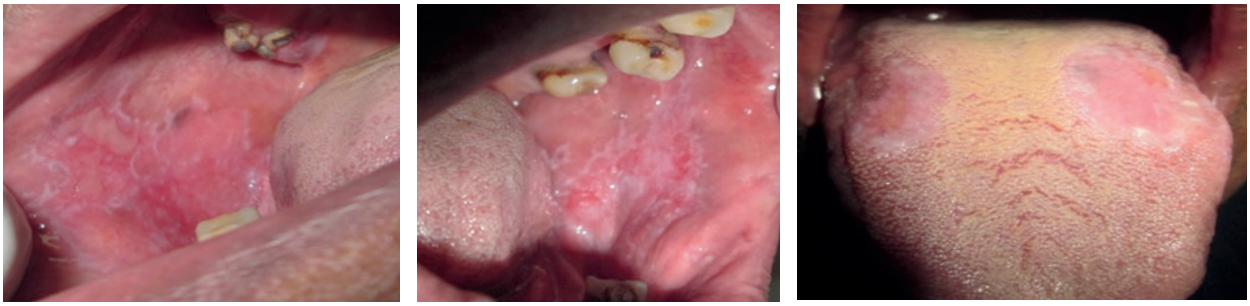

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Table 1. Comparison of serum and salivary TGFβ-1, VEGF and TNF-α levels (pg/ml) between control and OLP (*p*-value <0.001).

		TGF- β		VEGF		TNF-α	
		Controls	OLP	Controls	OLP	Controls	OLP
Serum (pg/ml)	Mean	21.7	134.8	210.9	704.9	15.4	150.3
	Standard Deviation	3.2	50.4	17.5	97.8	4.1	16.9
	Mean difference	113.0963		494.043		134.8913	
Saliva (pg/ml)	Mean	31.8	1,586.2	472.3	1,684.9	42.7	192.6
	Standard Deviation	4.5	298.0	54.9	268.4	11.1	23.5
	Mean difference	1,554.407		1,212.609		149.8913	

S: Statistically significant difference (independent Student t-test)

Table 2. Comparison between serum and salivary TGF-β, VEGF, and TNF-α level in symptomatic and asymptomatic OLP patients.

		Symptomatic (N=18)		Asymptomatic (N=5)
Serum TGFβ	Mean	139		120
	Standard Deviation	57		2.4
	p-value		0.135 (NS)	
Salivary TGFβ	Mean	1641		1388
	Standard Deviation	306		168
	p-value		0.073 (NS)	
Serum TNF-α	Mean	156		131
	Standard Deviation	15		1.2
	p-value		0.007 (S)	
Salivary TNF-	Mean	201		163
	Standard Deviation	19		7.6
	p-value		0.005 (S)	
Serum VEGF	Mean	742		573
	Standard Deviation	74		41
	p-value		0.005 (S)	
Salivary VEGF	Mean	1763		1405
	Standard Deviation	248		95
	p-value		0.011 (S)	

S: Statistically significant difference (independent Student t-test). **N:** Number of cases.

Table 3. Diagnostic accuracy of TGF-β, VEGF, TNF-α in serum and saliva.

		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Cut off value (pg/ml)
TGFβ-1	Serum	91	100	100	92	70.96
	Salivary	100	100	100	100	529.08
TNF	Serum	100	100	100	100	394.00
	Salivary	100	100	100	100	904.00
VEGF	Serum	100	100	100	100	75.85
	Salivary	100	100	100	100	102.80

PPV: Positive predictive value. **NPV:** Negative predictive value.

DISCUSSION.

OLP is a T-cell immune mediated disease in which the cytotoxic $CD8^+$ T-cells trigger apoptosis of the basal epithelial cells.[4] Diagnosis of OLP is based on the history, clinical findings and histopathological examination. Cytokines involved in OLP pathogenesis can be investigated in serum and saliva to monitor disease activity and response to treatment. Biologic biomarkers could facilitate diagnosis, prognosis and prevention of diseases. Saliva has been viewed as an important diagnostic fluid because it offers an easy, inexpensive and painless approach to disease detection that allows repeated sampling over time.[17]

It could be a helpful tool for diagnosis, risk assessment and prognosis. Screening an entire population for a certain type of disease will be possible in the future by employing saliva diagnostics.[18] Unstimulated saliva is preferred over stimulated because the content of stimulated saliva is slightly altered. In the present study, mean serum and salivary TGF-β levels were higher in OLP patients than in normal controls. Saliva was better than serum in evaluating TGF-β levels.

There was no significant difference in serum and salivary TGF-β between symptomatic and asymptomatic groups of patients indicating that monitoring serum and salivary TGFβ is of no value in monitoring disease activity. The results of our study are supported by Zhou *et al.*,[13] who observed slightly increased serum levels of TGF-β in patients with OLP when compared with normal controls. However, in contrast to our study, they found a statistically significant difference between serum levels of TGF-β between erosive and reticular forms of OLP.[19]

TGF-β regulates cell differentiation, proliferation and apoptosis and is also known to have pro-inflammatory and immunosuppressive effects.[5] There is increased expression of SMAD3, a gene involved in TGF- β pathway, in OLP compared to normal oral mucosa which might be due to its probable role in inflammation, apoptosis and EMT and may indicate a higher potential for malignant transformation.[20] The results of the present study were in contrast to those of Zenouz *et al.*,[21] who reported there was a significant decrease in the serum levels of TGF-β in OLP patients, although they didn't correlate it with disease severity. This difference may be attributed to difference in the ethnicities of the study populations. The present study was the first one to investigate TGF-β in saliva in OLP patients.

In the present study, it was observed that both serum and salivary levels of VEGF and TNF-α were significantly higher than controls and saliva was better than serum to monitor VEGF and TNF-α level. Moreover, there was a significant difference in serum and salivary levels between symptomatic and asymptomatic OLP groups indicating that measuring serum and salivary VEGF and TNF-α may help in monitoring disease activity and response to treatment.

As an immune mediated disease with an inflammatory origin and chronic progression, OLP satisfies prerequisites of hypoxia, under which increased angiogenesis and VEGF levels can be expected.[22,23] If angiogenesis is increased, it will lead to recruitment of inflammatory cells, progression of disease and recurrence of the lesions.[24] Moreover, alterations in angiogenesis as a part of chronic inflammation have been implicated in malignant transformation.[25] Zahra concluded that VEGF might be involved in OLP pathogenesis through increasing lymphangiogenesis.[7]

Results of this study were in accordance with the study performed by Mardani *et al.*,[26] who found that serum VEGF level was significantly higher in OLP compared to normal controls, with the erosive/atrophic form showing a particularly increased level.

They reported that the significantly increased serum VEGF may point out that angiogenesis in OLP is a systemically driven process. Moreover, because of its high sensitivity and specificity, measuring serum VEGF levels can be used as a diagnostic tool. Results of the present study were in contrast to those reported by Agha *et al.*,[16] They reported that serum level of VEGF was significantly lower in OLP than controls and there was no significant difference in salivary levels between OLP and controls. They concluded that saliva is not a good biomarker for OLP unlike serum. This difference in results may be due to difference in study populations.

TNF-α induces apoptosis of epithelial cells, reducing the epithelium thickness.[9] Therefore, TNF-α might be considered a factor involved in the incidence of atrophy and ulcerations in OLP.[21] TNF-α has a vital regulatory effect in the onset and progression of OLP.[27] Results of the present study were supported by a systematic review performed by Mozaffari *et al.*[28]

The pooled mean difference showed that serum and salivary TNF- α levels were higher in OLP compared with healthy individuals and this difference was highly significant in saliva. They concluded that measurement of

salivary TNF- α is more useful than serum for diagnostic and prognostic purposes.

Additionally, results of the present study were supported by Kaur *et al*.,[15] and Malarkodi *et al*.,[29] who investigated serum and salivary TNF-α in OLP. They documented that saliva is a better substitute for serum to evaluate TNF-α. Malarkodi *et al*.,[29] concluded that expression of serum and salivary TNF-α was the same in OLP and controls but saliva is a better fluid because it is less invasive to obtain.

Kaur *et al*.,[15] suggested that saliva is better than serum not only because the procedure to obtain it is less invasive but also because salivary levels are significantly higher than serum levels. More studies are required to monitor the level of these biomarkers after treatment and to compare levels in OLP with other lesions that clinically resemble lichen.

CONCLUSION.

Serum and salivary levels of TGF-β, VEGF and TNF-α are significantly elevated in OLP compared with normal individuals. Saliva can be used as a substitute for serum analysis in monitoring TGF-β, VEGF and TNF-α.

There is a statistically significant difference in serum and salivary VEGF and TNF-α levels between symptomatic and asymptomatic groups indicating that they can be used in monitoring disease activity.

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