

## Salivary VEGF-R3, TNF- $\alpha$ , TGF- $\beta$ and IL-17A/F Levels in Patients with Minor Aphthous

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### Abstract

**Background:** Recurrent aphthous stomatitis (RAS) is one of the most common mucosal ulcerative of oral cavity. The role of immune system, especially cytokines in immunopathogenesis of aphthous stomatitis is not highly considered. The aim of this study was to evaluate the levels of salivary cytokines, including VEGF-R3, TGF- $\beta$ 1, and TNF- $\alpha$  and IL-17A/F in patients with RAS in two clinical stages, ulcerative and healing period.

**Materials and Methods:** In this case –control study, 18 patients with RAS (case group) and 18 healthy individuals (control groups) who were matched for age and sex, were selected. In both ulcerative and healing stages, unstimulated saliva of patients with RAS and healthy controls were collected. Levels of salivary cytokines, including VEGF-R3, TGF- $\beta$ , TNF- $\alpha$ , and IL-17A/F at each stage was determined by ELISA procedure and results were compared with the control group.

**Results:** The levels of salivary VEGF-R3 in the ulcerative (5.92  $\pm$  1.87ng/ml) and healing (7.14  $\pm$  3.1 ng/ml) stages significantly decreased compared with those of the control group (9.71 $\pm$ 2.24 ng/ml). Moreover, the level of salivary TGF- $\beta$ 1 in ulcerative (142.21 $\pm$ 18.7 pg/ml) and healing (167.02 $\pm$ 28.1 pg/ml) stages significantly reduced compared with control group (178.35 $\pm$ 55.67 pg/ml). In addition, the case group showed significant increase in both inflammatory cytokines including TNF- $\alpha$  and IL-17 A/F. The level of salivary TNF- $\alpha$  in ulcerative (34.9 $\pm$ 11.35pg/ml) and healing (28.09 $\pm$ 9.07pg/ml) stages demonstrated significant increase when compared with control group (10.76 $\pm$ 1.83 pg/ml). Also, the IL-17 A/F level in the ulcerative (96.44 $\pm$ 25.74 pg/ml) and healing (79.17 $\pm$ 24.96 pg/ml) stages significantly increased compared with the control group (53.47 $\pm$ 13 pg/ml).

**Conclusion:** Our findings showed that the reduction of VEGF-R3 and TGF- $\beta$ 1 cytokines and increase inflammatory cytokines such as TNF- $\alpha$  and IL-17 A/F are effective in the pathogenesis of minor aphthous particularly in ulcerative stage.

**Keywords:** IL-17A/F; Minor Aphthous; TGF- $\beta$ 1; TNF $\alpha$ ; Saliva; VEGF-R3

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### Introduction

Recurrent aphthous stomatitis (RAS) is one of the most common diseases of the oral mucosa. The condition usually starts in childhood or adulthood as recurrent small round or ovoid ulcers with

circumscribed margins, erythematous halo, and yellow or gray floor. RAS has three clinical types: Minor, Major, and Herpetiform ulcers, but the most common type is minor aphthous which is less than

10mm in size. Ulcers with similar clinical features (aphthous –like ulcers) may be because of some systemic conditions such as behcet’s syndrome, auto –inflammatory syndromes, gastrointestinal disease or immune defects. The etiology of RAS is not entirely clear yet. A genetic basis exist for some RAS and involvement of the cell-mediated mechanisms is possible, but the precise immunopathogenesis remains unclear (1, 2). Recent studies have shown the role of genetically mediated disturbances of the innate and acquired immunity in the disease development (3). In addition, phagocytic and cytotoxic T cells probably aid in destruction of oral epithelium that is directed and sustained by local cytokine release (4). Vascular endothelial growth factor (VEGF) is a multifunctional angiogenetic cytokine involved in angiogenesis and wound healing (5). VEGF regulate blood and lymphatic vessel development and homeostasis. VEGF specially interact with one or several receptor tyrosine kinases, VEGF-R1, 2, 3 (6).

On the other hand, VEGF-R3 was the first cloned lymphatic marker, and is predominantly expressed on lymphatic endothelium in adult tissues. On binding to its ligands, VEGF-C and VEGF-D, VEGF-R3 signals for tumor lymphangiogenesis mediating tumor metastasis to lymph nodes (7, 8). VEGF-R3 is suppressed by IFN- $\gamma$  which is a major producer of interleukin -12 in tumor vessels, implying a central role for IFN- $\gamma$  in VEGF-R3 inhibition in tumor models (9). However the role of VEGF-R3 on RAS lymphangiogenesis has not been reported yet. Transforming growth factor beta-1 (TGF- $\beta$ 1) as immune-modulators regulates the immune response through lymphocyte and phagocyte inhibition. TGF- $\beta$ 1 has anti-inflammatory and pro-inflammatory effects, depending on expression systemic or local and the time or amount of production (10). TGF- $\beta$ 1 inhibits proliferation and differentiation of T cells, activation of macrophages and secretion of pro-inflammatory cytokines. It is also responsible for regulating various processes including apoptosis of extracellular matrix homeostasis, embryonic development, wound healing and angiogenesis (11). TGF- $\beta$ 1 can modulate the development of both T regs and Th17 by balancing the stimulatory and inhibitory components of signaling pathways that drive specific cell differentiation (12). In the first stage of disease, TNF- $\alpha$  is one of the cytokines that is important in the initiation of inflammation and stimulates the activity of cytotoxic T cells and neutrophils, necrosis of the epithelium tissue and finally, leads to development of aphthous ulcers (13). But the most important functions of TNF- $\alpha$  are recruiting the leukocytes into infection site and inflammation (14). Pro-inflammatory cytokines such as IL-17 can stimulate

epithelial cells to the produce TNF- $\alpha$ . IL-8, TNF - $\alpha$  and IL-17, producing by oral epithelium, increases significantly in aphthous patients in compared to the controls (15). Therefore, the aim of this study was to measure the levels of salivary cytokines, including TNF- $\alpha$ , TGF- $\beta$ 1, VEGF-R3, and IL-17 A/F in two clinical stages to investigate the role of above cytokines in pathogenesis of minor aphthous (ulcerative and healing period) compared to controls.

### Materials and methods

This case control study included 18 patients with RAS, who were referred to the Oral Medicine Department, Babol University of Medical Sciences. Prior to any procedure, informed written consent was obtained from all subjects. Afterwards a questionnaire was filled out for each patient, recording the following information: type of RAS according to Lehner (16), and the number and duration of RAS (occurring on the first or second week of the disease). There were also 18 individuals as control group without any history of RAS who were matched for age and sex. Smokers and patients with systemic disease such as colitis ulcerative, Behcet’s syndrome, anemia (all confirmed by laboratory tests), periodontal disease, and patients undergoing medical treatment were excluded from the study. All subjects were evaluated during two clinical stages of RAS; ulcerative stage (first week) and healing period (clinical healthy appearance of mucosa). Unstimulated saliva was collected between 9:00 and 11:00 AM. The participants were asked not to drink or eat two hours prior to saliva collection. All patients were asked to retain mixed saliva in their mouth for 1 to 3 minutes without swallowing and then expectorate it into clean plastic containers. The samples were immediately stored at -80 c in the laboratory of cellular and molecular biology research center until analysis. In order to salivary concentrations of TGF- $\beta$ 1 and TNF- $\alpha$ , (Koma Biotech kit, South Korea), VEGF-R3 (Cusabio Kit, China) and IL-17A/F (Diaclone Kit, France) were determined by sandwich ELISA. The concentration of above cytokines was reported in Pg/ml except VEGF-R3 which was presented in (ng/ml). Results were expressed as mean (standard deviation) and analyzed by SPSS for windows version 20. Comparison of above cytokines between case and control groups was done in two clinical stages, ulcerative and healing stages. Each stage was compared with control group using T-Test and finally, the data was analyzed by ANOVA and significance was set at  $P < 0.05$ .

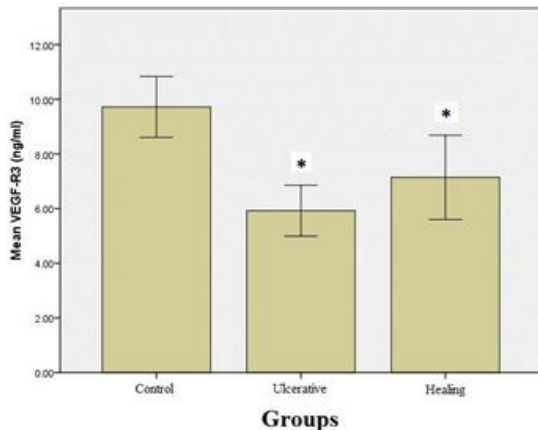
### Ethics statement

The study protocol was approved by the ethics

committee of the Babol University of medical sciences.

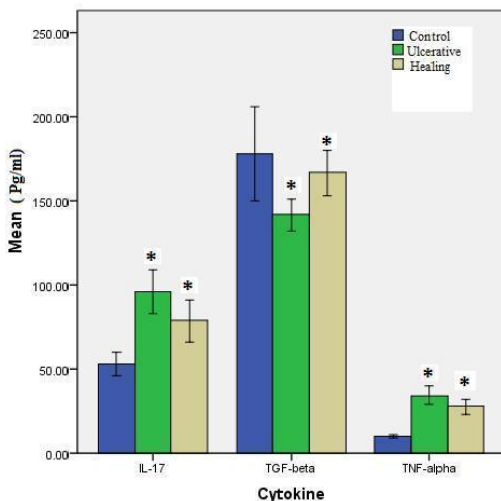
## Results

In this study, 18 cases (with a minor aphthous) and 18 controls (no aphthous and no history of this disease) were selected. The case group included 13 females and 5 males aged between 20-41 years (mean age  $31.5 \pm 10.7$ ).



**Figure 1.** The mean concentrations of VEGF-R3 in saliva in three groups. \*  $P < 0.05$

The control groups were similar to case group in age and sex. The mean levels of salivary VEGF-R3 in case group in ulcerative stage and healing period were  $5.92 \pm 1.87$  ng/ml, and  $7.14 \pm 3.1$ , respectively which was significantly lower compared to the control group ( $9.71 \pm 2.24$  ng/ml) ( $P < 0.001$ ).



**Figure 2.** The mean concentrations of cytokines in saliva in three groups. \*  $P < 0.05$

However, the decrease in the salivary VEGF-R3 compared to the control group was significant in

ulcerative stage. As shown in Figure 1 no significant difference was seen in the level of VEGF-R3 in ulcerative and healing period ( $P = 0.093$ ). The mean levels of TGF- $\beta$ 1 in patients in ulcerative and healing period was  $142.21 \pm 18.7$  Pg/ml and  $167.02 \pm 28.1$ , respectively which reduced to and in comparison to the control group reduced to  $178.35 \pm 55.67$ , but this reduction was significant only in the ulcerative stage ( $P = 0.016$ ). As illustrated in Figure 2 a significant difference was seen in the level of TGF- $\beta$ 1 in ulcerative and healing period ( $P = 0.004$ ).

TNF- $\alpha$  levels in patients with minor aphthous in ulcerative stage and healing period were  $34.9 \pm 11.35$  Pg/ml and  $28.09 \pm 9.07$ , respectively and had an increasing by the amount of  $10.76 \pm 1.83$  in comparison to control group. Both clinical groups compared to the control statistically was significant ( $P < 0.001$ ). Although, TNF- $\alpha$  have increased in ulcerative and healing stages, but when comparing the two levels, the average showed no significant difference ( $P = 0.092$ ) Figure 2.

The average levels of IL-17 A/F in ulcerative stage and healing period were  $96.44 \pm 25.74$  and  $79.17 \pm 24.96$ , respectively, indicating significant compared to the control  $53.47 \pm 13.6$  ( $P < 0.001$ ) Figure 2.

## Discussion

Recent studies showed that RAS is driven by unnatural cytokine responses associated with cellular immunity in oral mucosa. The aim of this study was to evaluate the levels of salivary cytokines such as IL-17A/F, TNF- $\alpha$ , TGF- $\beta$ 1, and VEGF-R3 in minor aphthous patients and comparing them with healthy controls and cases in two clinical stages, ulcerative and healing period. The level of VEGF-R3 in ulcerative stage was significantly less than that in the control group, therefore, changes in VEGF-R3 may have occurred during the apathies process. In other words aphthous ulcer formation was along with a reduction in the amount of VEGF-R3. In fact, when ulcer occur some parts of the epithelium and connective tissue and its components including blood vessels damage. Thus reduction in the number of blood vessels at ulcerative stage seems to be logical but healing initiates along with the destruction in the area synchronously. Healing is a changing dynamic process. According to the results, the level of VEGF-R3 in case group was lower than the control group in aphthous healing period. It seems that a relative increase in the expression of VEGF-R3 were adequate in healing period. Agha Hosseini et al had reported that VEGF increases in remission stage toward acute phase of minor aphthae, but found no difference in VEGF level in the active and remission period of the disease (17). Although in our study, VEGF-R3 level in healing period was more than that

in ulcerative stage, but this difference was not statistically significant between ulcerative and healing period. These results endorse similar roles of salivary VEGF and VEGF-R3 in the pathogenesis of aphthae. Our study revealed that the mean of salivary TGF- $\beta$ 1 levels in patients with aphthae was lower than that in healthy people in two stages. In another study performed in 10 patients with aphthae, TGF- $\beta$  level was lower compared to the controls which is consistent with current research (18). Anti-inflammatory cytokines such as TGF- $\beta$  and IL-10, in aphthae disease compared to the control significantly reduced (19). Some evidences show that imbalance of anti-inflammatory cytokines may be predisposed to autoimmunity disease. TGF- $\beta$  inhibits T cell proliferation and differentiation and activated macrophages to produce pro-inflammatory cytokines (20). Therefore, decreasing the level of TGF- $\beta$  makes the individual susceptible to autoimmune lymphocytic inflammation while its administration in autoimmune disorders, in which T cells have a prominent role, might have a therapeutic effect (21). TNF- $\alpha$  is one of the cytokines which plays an important role in initiation of inflammation. At the onset of the disease it can stimulate cytotoxic T cells and neutrophils, necrosis epithelium tissue and finally causes aphthous ulcer development (13). TNF- $\alpha$  can cause inflammation due to impact on endothelial adherent cells neutrophils chemotaxis during inflammation. IL-2 stimulates pro-inflammatory cytokines secretion such as IL-1 and TNF- $\alpha$ . Enhancement of Th1 genes and systematic cytokines production such as IL-2, TNF- $\alpha$  and IL-6 was observed in patients with recurrent aphthous (22). In this study, the level of TNF- $\alpha$  in aphthous patients has increased significantly compared to healthy controls, which many factors may have been involved. TNF- $\alpha$  is produced by monocytes and macrophages and is involved in neutrophils, endothelial cells and keratinocytes stimulation (15). Therefore increasing the level of TNF- $\alpha$  in aphthous ulcers can stimulate many cells in the local area tissue and peripheral blood (23). In a study conducted by Valle et al, changes in level of salivary TNF- $\alpha$  were measured during active phases by ELISA in 20 individuals suffering minor aphthae and 10 healthy controls in oral recurrent aphthae. They found that the levels of salivary TNF- $\alpha$  was 2 to 5 times higher in patients with active lesions of RAS compared with controls, and also pointed out that TNF- $\alpha$  may play a role in the etiology of disease and considered that as a treatment tool of RAS (14). These results are consistent with results of our study. Al-Ghurabei et al observed a significant increase in the salivary level of TNF- $\alpha$  in patient with RAS by ELISA method. Similar to current study they believed that salivary TNF- $\alpha$  has a main role in pathogenesis and treatment

of these patients (13). Peripheral blood leukocytes in patients with RAS have a high level of TNF- $\alpha$  compared to the control. In addition, the biopsy of aphthous ulcers also showed high levels of TNF- $\alpha$  (15). Taylor et al reported that TNF- $\alpha$  may play an important role in pathogenesis of aphthae disease. According to this study although the level of serum TNF- $\alpha$  in ulcerative stage increased significantly, but there was no significant difference in healing period compared to the control group(23). In our study, increasing TNF- $\alpha$  in healing period, showed that it has an important role in the remission phase and cytotoxic effects on epithelial cells at the ulcerative phase. TNF- $\alpha$  has a synergism effect with IFN- $\gamma$  and especially with IL-10 which can affect various stages of disease and ultimately have a significant impact on the ulcer formation and remission period (24). TNF- $\alpha$  is involved in pathogenesis of aphthae. Anti-TNF drugs like pentoxifylline and thalidomide could be useful in treatment of RAS patients when systemic immune suppression is required (22). In another study, pro-inflammatory cytokine, TNF- $\alpha$ , significantly increased by treatment with IL-17. This important result could introduce a new approach in treatment of RAS, because IL-17 and TNF- $\alpha$  has a synergistic effect on epithelial cells and inflammatory leukocytes (25). In a study which was conducted in 24 patients with RAS, the level of IL-17 had a significant increase compared to healthy controls (26). These findings are compatible with each other. Considering many reports related to etiologic mechanisms which can lead to aphthae, immunological pathogenesis of this disease is known more and acceptable as its etiology (27). Pro-inflammatory cytokines such as IL-17 can stimulate epithelial cells to produce TNF- $\alpha$ , IL-8 and IL-17. In addition, TNF- $\alpha$  excessively produce by oral epithelium in patients with aphthae and significantly increased patients compared to the controls (28). What has mentioned so far is a proof for why pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-17 in patients with RAS has increased compared to the control group. In this study increased expressions of these cytokines in patients with RAS explain the mechanisms and reactions during inflammation phase. IL-17 and other related mediators are effective in treatment of auto inflammatory diseases such as aphthae and behcet's syndrome. To our best knowledge, this study is the first to investigate salivary IL-17A/F and VEGF-R3 levels in patients with RAS and healthy individuals that found higher IL-17A/F levels in patients. These results support the study of Al-samadi et al (28), emphasizing the effect of IL-17A/F in RAS.

## Conclusion

Briefly, based on results, increasing the IL-17 and

TNF- $\alpha$  and reduction of VEGF-R3 and TGF- $\beta$ 1 in aphthous patients compared to the control group, especially at the ulcerative stage, it seems that immunopathologic disorder and antigenic stimulation of oral mucosa keratinocytes led to minor aphthae. In fact, antigenic stimulation disrupts Th1 and Th2 cytokines balance to secretion of cytokines and inflammatory mediators. Then we might speculate that different antigens activate different types of cytokines.

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### Authors' Contributions

E M, SS and H B Kh performed the experiments. Eh M, Gh M and AA wrote the draft. E M, M M, M K S, SS, RF and AA designed the study and supervised study protocol. AA and E M reviewed the manuscript.

### Conflicts of Interest

The authors declare that no conflict of interest.

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