Evaluation of salivary tumour necrosis factor–alpha in patients with recurrent aphthous stomatitis

Purpose
Present study was undertaken to evaluate and compare the salivary levels of tumour necrosis factor alpha (TNF-α) in subjects with RAS, traumatic ulcers (TUs) in the oral mucosa and in healthy controls.

Materials and Methods
Present study involved 90 participants of which 30 subjects were diagnosed with RAS, 30 subjects with TUs and 30 healthy controls grouped as group 1, group 2 and group 3 respectively. Unstimulated saliva was collected from the subjects through ‘Spit Technique’ and the estimation of TNF-α was done by enzyme linked immunosorbent assay. The data collected was statistically analysed.

Results
Salivary level of TNF-α was significantly higher in RAS patients than in patients with TUs and healthy controls. Difference between the Salivary TNF-α level in our study groups were statistically significant (p<0.001).

Conclusion
Present study suggests that saliva is a convenient and ideal medium for the detection of TNF-α. Statistically significant difference in the level of salivary TNF-α between the RAS and TUs subjects as well as controls suggests the significant contribution of TNF-α in pathogenesis of RAS.

Keywords: Recurrent aphthous stomatitis; tumour necrosis factor-alpha; saliva; oral mucosa; traumatic ulcer
immune response, mainly T-lymphocyte activation which leads to the formation of cytokines such as tumour necrosis factor-α (TNF-α) (9). Among the cytokines implicated in the development of new lesions in RAS patients, TNF-α is considered to be one of the most important. Several studies have reported about the possible role of TNF-α in the active phase of the disease (8, 10-13). Currently the management of RAS is symptomatic due to its unclear aetiology. Effective treatment for RAS is possible only by discovering the underlying factors (14). Previous studies focusing on genetic as well as immunologic background showed inconsistent results. Limited research on Indian population with this regard indicated the need of performing such study in this region. Present study evaluated the salivary levels of TNF-α in RAS patients of in and around Mangalore and compared the salivary levels of tumour necrosis factor alpha (TNF-α) in subjects with RAS, TUs in the oral mucosa and in healthy controls. The null hypothesis is that there are no differences among any variable.

Materials and methods

Study population

The study involved patients visiting a private dental hospital in Mangalore, India between 2015 and 2017. Institutional Ethical clearance was obtained on 3.10.2014. The project was funded by the NITTE University vide Research Project Number NURG/STF/05/11-2014. Study objectives and procedures were explained to the subjects and informed consent was obtained from all the participants. Detailed case history recording and clinical examination was carried out by trained professionals. Patients diagnosed with RAS, traumatic ulcer and healthy controls were recruited for the study. Subjects with history of medication, subjects with systemic diseases were excluded from the study.

Study groups

Selected patients were grouped as follows: Group 1: 30 patients diagnosed with recurrent aphthous stomatitis, Group 2: 30 patients diagnosed with traumatic ulcers in the oral mucosa, Group 3: 30 healthy control subjects.

Saliva collection

Unstimulated saliva was collected from the subjects through ‘Spit Technique’. The subjects were instructed to spit into a sterile graduated container every minute for 8-10 minutes. The collected sample was centrifuged at 3000 rpm for 10 minutes and the supernatant collected was stored at -20°C. The estimation of salivary tumour necrosis factor-α was done by commercially available enzyme linked immunosorbent assay (ELISA) (Hu TNFα Us Elisa Kit, Thermo Fisher Scientific, Waltham, MA USA) at the central research laboratory of the university as per the procedure by Beutler et al. (15).

Statistical analysis

The data collected was analysed using IBM Statistical Package for the Social Sciences Statistics, Version 22 (SPSS IBM Corp.; Armonk, NY, USA). Descriptive data were presented in the form of mean and standard deviation. One Way analysis of variance (ANOVA) test was used to compare TNF-α values between multiple study groups. Comparison of age between the study groups was also done by using ANOVA. Pairwise comparison of age between the study groups was done with post-hoc Tukey’s HSD test. Distribution of study participants according to gender evaluated by chi-square test. Confidence interval was set to 95% and p<0.05 was considered as statistically significant.

Results

The present study involved 3 groups. Group 1 involved 30 patients clinically diagnosed with recurrent aphthous stomatitis. The subjects were between 12 to 54 years of age and the mean age was 29.30 years. The study group 2 involved 30 patients diagnosed with traumatic ulcers of oral mucosa. The subjects were between 22 to 66 years of age and the mean age of the study group was 38.67 years. The group 3 involved 30 healthy controls. These subjects were between the ages of 18 to 63 with the mean age of 32.53. Data is shown in Table 1. In the group 1 there were 14 males (46.7%) and 16 females (53.3%). Among group 2 subjects 17 (56.7%) were males and 13 (43.3%) were females. Group 3 involved 12 (40.0%) males and 18 (60.0%) females. Details shown in Figure 1. Comparison of salivary TNF-α level between the study groups was done. Difference between the salivary TNF-α level in our study groups were statistically significant (p<0.001). Details given in Table 2 and Figure 2.

Study group 1 included 30 RAS patients, out of which 26 subjects were diagnosed with minor RAS and 4 subjects were diagnosed with major RAS. Among the RAS patient group no subjects were diagnosed with herpetiform RAS. Comparison of type of RAS with salivary level of TNF-α is shown in Table 3. Correlation analysis between the age and TNF-α variables in each study group was shown in Table 4.

Discussion

Our study subjects were between 12 to 54 years of age and the mean age was 29.30 years in group 1. RAS was predominant in adults in the present sample. Female predominance was noticed in our study which is in accordance with previous work (16-18). Prevalence of RAS is lower in this region compared to other populations which was showed in our previous study 1.9% (16). Accurate aetiology of RAS still remains unknown (13). Controversy exists among different authors about the pathogenesis of RAS (8). Various factors predispose for its occurrence. Genetic background, stress, anxiety, food allergens, local trauma, smoking cessation, menstrual cycle, chemicals and microbes were identified as predisposing factors (5, 7, 19). A Cochrane analysis mentioned that bacterial or viral aetiology was unlikely (20). Local and systemic factors lead to the targeting of oral mucosal cells by lymphocytes,
monocytes and neutrophils. This leads to the oral mucosal cell destruction, and accumulation of acute inflammatory mediators followed by the development of an aphthous ulcer (11). The process is initiated by antigenic stimulation of the mucosal keratinocytes which leads to stimulation of T-lymphocytes, cytokine release as well as migration of other lymphocytes, neutrophils and Langerhans cells. Cytotoxic trigger causes ulceration of the mucosa. At present, TNF α is considered as the most significant cytokine implied in the development of new RAS lesions (21-23).

Even though the exact role of TNF in the aetiology of RAS has not been determined yet, its possible contribution was explained by the facts such as high levels of TNF in oral ulcerations and high efficacy of anti-TNF drugs like levamisole, thalidomide or pentoxifylline in the treatment of RAS (8). In the present study we evaluated salivary TNF-α in patients with RAS in our region. Several studies have reported an increase in salivary and serological TNF-α, especially during the active phase of the disease (10, 11). However specific cause of this increase has not been established. Most of the studies have compared salivary TNF-α in RAS patients and healthy controls (8, 11). As existing studies shown the role of TNF-α role in the ulceration of mucosa, in the present study we investigated salivary TNF-α in patients with traumatic ulcer and compared with that of controls and RAS. We observed that salivary TNF-α levels was lower in patients with TUs compared to RAS patients, indicating the significant role of TNF-α in RAS than in TUs.

One study used biopsy samples to compare the TNF-α and its cellular distribution in RAS and in induced oral TUs (14). Present study results were in accordance with their findings. We examined the salivary TNF-α and statistically significant difference was noticed in the study groups. This result underlines the importance of this cytokine in the development of RAS. Our study results were consistent with Sun et al. (12) study. Present study used saliva samples since obtaining saliva is easy and non invasive. Patient acceptance of saliva sample collection is much higher when compared to obtaining serum or biopsy samples. We found higher salivary TNF-α in RAS patients compared to TUs and controls. Previous studies have shown significantly higher serum levels of TNF-α in patients with of major, minor or herpetiform RAU (14). In our study equal distribution among the types of RAS was not present, hence analysis of TNF-α in various clinical types of RAS was not carried out. Inflammation and metabolism of free radicals in RAS patients and healthy controls was evaluated by Avci et al. (23). They investigated TNF-α, interleukin-2 (IL-2), IL-10, and IL-12 using ELISA and emphasized their importance in the occurrence of RAS. Similar pattern was observed in our study, however we only evaluated TNF-α.

The use of TNF-α inhibitors or rituximab in ulcerative oral conditions was reviewed by previous researchers. They also

### Table 1. Comparison of age between the study groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean (SD)</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>30</td>
<td>29.30 (10.70)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td>30</td>
<td>38.67 (12.95)</td>
<td>5.03</td>
<td>0.009*</td>
</tr>
<tr>
<td>Group 3</td>
<td>30</td>
<td>32.53 (11.08)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p<0.05 statistically significant; ANOVA: analysis of variance; SD: standard deviation

### Table 2. Comparison of Salivary TNF-α level between the study groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean (SD)</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>30</td>
<td>58.82 (15.24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td>30</td>
<td>35.76 (11.11)</td>
<td>73.10</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Group 3</td>
<td>30</td>
<td>23.09 (6.95)</td>
<td></td>
<td></td>
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</tbody>
</table>

*p<0.05 statistically significant; ANOVA: analysis of variance; SD: standard deviation

### Table 3. Comparison of the type of RAS with Salivary level of TNF-α

<table>
<thead>
<tr>
<th>Type of RAS</th>
<th>Number of patients</th>
<th>Mean TNF-α in pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minor</td>
<td>26</td>
<td>57.71</td>
</tr>
<tr>
<td>Major</td>
<td>4</td>
<td>66.04</td>
</tr>
</tbody>
</table>

### Table 4. Correlation between age and TNF-α in each study group

<table>
<thead>
<tr>
<th>Group</th>
<th>Age</th>
<th>TNF-α in pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>R</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.08 (NS)</td>
</tr>
<tr>
<td>Group 2</td>
<td>R</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.37 (NS)</td>
</tr>
<tr>
<td>Group 3</td>
<td>R</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.15 (NS)</td>
</tr>
</tbody>
</table>

NS: not significant; R: Pearson correlation test
analysed the potential benefits, adverse effect, principles of use and future developments. They concluded that TNF-α inhibitors such as infliximab can be effective in RAS and indicated in patients with severe refractory disease (24). Pentoxifylline, a methylxanthine derivative with immune modulatory and anti-inflammatory properties, is beneficial in the treatment of RAS. This effect was reported to be due to the inhibition on the production of TNF-α and other inflammatory cytokines (11). However, the use of these drugs is restricted due to their side effects. New formulations which provide TNF-α inhibition with less side effects still need to be evaluated.

Conclusion

Detection of higher level of TNF-α in RAS compared to TUs and controls indicates the role of TNF-α in the pathogenesis of RAS. This suggests the significant contribution of TNF-α in the pathogenesis of RAS which can be detected by using saliva as a convenient medium. Thus, future management of RAS should be directed towards newer treatment modalities for better outcome.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of A B Shetty Memorial Institute of Dental Sciences dated 30-09-2014 Cert No. ABSM/EC/114/2014.

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: SH designed the study, SH, VA, SB, SK, HU and AM generated the data. SH gathered the data for the study. SH analyzed the data. SH wrote the majority of the original draft. SH, VA, SB, SK, HU and AM contributed towards writing the paper. SH, VA and SB analyzed the raw data of the study. All authors approved the final version of the paper.

Conflict of Interest: The authors have no conflicts of interest to declare.

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