A study on serum levels of malondialdehyde, ascorbic acid and α-tocopherol in recurrent aphthous stomatitis

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Abstract

Introduction: Recurrent Aphthous Stomatitis (RAS) is a chronic, relapsing inflammatory disorder of the oral mucosa. It is one of the commonest oral mucosal disorders affecting all races, age groups, geographical regions. The imbalance between production of oxidants and body's antioxidant defences is termed as oxidative stress. The role of systemic oxidative stress in the pathogenesis of RAS needs to be fully elucidated to understand its pathogenesis.

Aim: This study aims to determine the role of oxidative stress and antioxidant status in RAS subjects by comparing the serum levels of Malondialdehyde (MDA), Ascorbic acid and α-Tocopherol between RAS patients and healthy controls.

Materials and Methods: The study comprised of 30 RAS subjects along with 30, age and gender matched healthy volunteers. Estimation of MDA, Ascorbic acid and α-Tocopherol was undertaken in the serum samples collected from all the subjects.

Results: Serum MDA levels were significantly higher in the RAS subjects (4.25 $\pm$ 0.94nmol/ml) compared to controls (2.52 $\pm$ 0.67nmol/ml, $p<0.001$). Mean ascorbic acid levels were significantly lower in RAS subjects (10.47 $\pm$ 2.88mg/L) compared to controls (12.75 $\pm$ 1.69mg/L, $p = 0.002$). α-Tocopherol levels were also significantly lower in RAS subjects (7.75 $\pm$ 1.50mg/L) compared to controls (9.87 $\pm$ 1.06mg/L, $p<0.001$).

Conclusion: Elevated MDA levels and low antioxidant levels suggest increased oxidative stress in RAS subjects.

Keywords: Antioxidants, Malondialdehyde, Oxidative stress, RAS.

Received: 20th July, 2017 **Accepted:** 29th September, 2017

Introduction

Recurrent Aphthous Stomatitis (RAS) is a chronic relapsing inflammatory disorder of the oral mucosa characterized by multiple, recurrent, small, round or ovoid, well circumscribed ulcers with erythematous haloes and yellow to grey floors. It tends to occur at intervals of few months to few days and heals spontaneously. It is most common oral mucosal disorder and has a worldwide distribution, affecting all races, age groups, geographical regions. The prevalence across different populations worldwide is estimated to be between 20-25%. The range of discomfort due to RAS lies between annoying painful episodes to intensely painful periods disturbing normal oral activities.[1-4]

Classically described as minor, major and herpetiform ulcers, the exact aetiology of the disorder remains unclear. Many factors including trauma, stress, genetic predisposition, hypersensitivity, nutritional and immune disturbance, viral or bacterial infections, stress and hormonal imbalance have been postulated as predisposing factors for development of RAS. As these factors play a role in the oxidant-antioxidant status of the body, the role of systemic oxidative stress in the pathogenesis of RAS needs investigation.[3,5-7]

The imbalance between the production of oxidants and body's antioxidant defences is termed as oxidative stress. The production of oxidants exceeds the body's capacity to eliminate or scavenge them leading to oxidative stress. Free radicals are the body's most important oxidants. Free radicals include the Reactive oxygen species (ROS) and Reactive Nitrogen species (RNS).[8]

Important ROS include the hydroxyl radical, superoxide radical, hydrogen peroxide and singlet oxygen species. Nitric oxide is the important member of the RNS that can cause free radical damage.[8,9]

Free radicals are generated in the body during enzymatic reactions of the respiratory chain, phagocytosis, prostaglandin synthesis and cytochrome P450 system. Non-enzymatic reactions generating free radicals involve the effects of ionizing radiation.[8,9]

Being highly unstable, they react with various biomolecules such as lipids, proteins and deoxyribonucleic acid (DNA). Hydroxyl radicals and peroxynitrite damage cell membranes and lipoproteins by a process called lipid peroxidation. This reaction leads to formation of the product - Malondialdehyde (MDA).[8]

The body has its own antioxidant mechanisms to scavenge free radicals. They include non-enzymatic antioxidants like Vitamin A, Vitamin C (Ascorbic acid), beta-carotene, glutathione, Vitamin E (α-Tocopherol), melatonin, uric acid and selenium. Enzymatic antioxidants include enzymes like Superoxide dismutase, Catalase and Glutathione peroxidase.[2-5]

Antioxidant status being an important determinant in the development and recurrence of symptoms of RAS, this study aims to determine the role of oxidative stress and antioxidant status in RAS subjects by comparing the serum levels of MDA, Ascorbic acid and α-Tocopherol between RAS patients and healthy controls.

Ethics

Ethical clearance was obtained from the Ethical Committee of Vokkaligara Sangha Dental College and Hospital (VSDCH) to conduct the study. Informed consent was taken from all the subjects included in the study.

Materials and Methods

Subjects

The study was conducted between September 2015 to July 2016. It comprised of 30 RAS subjects who presented to the out-patient department of Oral Medicine and Radiology, VSDCH along with 30, age and gender matched healthy volunteers. The diagnosis of RAS was made clinically by an oral physician. Subjects with a history of three or more episodes of aphthous stomatitis in the past year, presenting with active lesions, were included in the study. Medical history and general physical examination were undertaken in all RAS subjects as well as controls, by a physician, to rule out any systemic illness. Exclusion criteria involved subjects with history suggestive of concomitant systemic illness including diabetes, hypertension, gastrointestinal, endocrine and autoimmune diseases, consumption of alcohol or tobacco products and any therapeutic regimen in the last three months.

Measurement

Estimation of MDA, Ascorbic acid and α-Tocopherol were undertaken in the serum samples collected from all the subjects.

Collection of serum samples

6 ml of venous blood was collected from all subjects. The samples were kept for clotting and later centrifuged at 3500 rpm for 10 minutes to separate the serum. The serum was stored at -20^{o}C until analysis.

Estimation of MDA[10]

MDA was estimated by Thiobarbituric acid (TBA) method described by Kei Satoh. It is based on the principle that MDA reacts with TBA in the presence of heat in weak acidic medium, resulting in a chromogen that is extracted with n-butyl alcohol. The absorbance of the organic phase is read at 530 nm. TBA is dissolved in 2M sodium sulphate solution to avoid interference due to sialic acids that are known to affect lipid peroxide determination.

The reaction involves a nucleophilic attack by the fifth carbon atom of TBA on to the first carbon atom of MDA followed by the loss of a water molecule. The intermediate MDA-TBA adduct reacts with a second molecule of TBA forming a pink colored MDA $(TBA)_2$ adduct.

Estimation of Ascorbic acid[11]

Ascorbic is acid was estimated by 2, 4-Dinitro phenyl hydrazine method.

Ascorbic acid is oxidised to form dehydroascorbic acid and diketogulonic acid in the presence of copper. When treated with 2, 4-dinitrophenyl hydrazine (DNPH), these compounds form bis-2, 4-dinitrophenylhydrazone which undergoes rearrangement in the presence of strong sulphuric acid to form a product with an absorption band that can be measured at 520nm. Thiourea provides a mild reducing medium that prevents interference with non-ascorbic acid chromogens.

Estimation of α-Tocopherol[12]

α-Tocopherol was estimated by the method described by Baker and Frank.

The principle is based on the Emmerie and Engel colour reaction. Tocopherols in the serum reduce ferric ions to ferrous ions that react with α, α' dipyridyl to give a red-coloured complex. Initially, tocopherols as well as carotenes are extracted into Xylene. The absorbance is read at 460 nm to measure the carotenes. Ferric chloride is then added and absorbance measured at 520 nm. The absorbance of carotenes at 520 nm is

29% of their absorbance at 460 nm; hence this fraction is then subtracted from the absorbance at 520 nm to obtain the concentration of Tocopherol in serum.

Statistical Analysis

The data collected was analysed statistically using IBM SPSS v22 statistical software. Statistics namely mean, standard deviation, range and any significant difference between the mean values of the study group and the control group was tested using independent sample student t-test. A p value of $\leq$ 0.05 was considered statistically significant.

Results

RAS group comprised of 15 male and 15 female subjects with a mean age of 27.8$\pm$ 7.9 years. The controls comprised of 14 males and 16 female subjects with a mean age of 28$\pm$ 6.8 years (Table 1).

Table 1: Gender and Age distribution of study subjects

	Males	Females	Total	Mean Age (years)
Cases	15	15	30	27.8 $\pm$ 7.9
Controls	14	16	30	28 $\pm$ 6.8

As shown in Table 2, serum MDA levels were significantly higher in the RAS subjects (4.25 $\pm$ 0.94nmol/ml) compared to controls (2.52 $\pm$ 0.67nmol/ml, p<0.001). The mean ascorbic acid levels were significantly lower in RAS subjects (10.47 $\pm$ 2.88mg/L) compared to controls (12.75 $\pm$ 1.69mg/L, p = 0.002). α-Tocopherol levels were also significantly lower in RAS subjects (7.75 $\pm$ 1.50mg/L) compared to controls (9.87 $\pm$ 1.06mg/L, p<0.001)

Table 2: Comparison of serum MDA, Ascorbic acid and α-Tocopherol levels in cases and controls

Group	MDA (nmol/ml)		Ascorbic acid(mg/L)		α- Tocopherol (mg/L)	
	Mean	SD	Mean	SD	Mean	SD
Cases (N=30)	4.25	0.94	10.47	2.88	7.75	1.55
Controls (N=30)	2.52	0.67	12.75	1.69	9.87	1.06
P value	<0.001		0.002		<0.001	

Discussion

RAS is the most common oral mucosal disorder with a worldwide prevalence. The management of the disorder being largely symptomatic, a better understanding of disease etiology may help explore more specific treatment options, relieving its patients from repeated episodes of pain and discomfort.

An imbalance between free radical production and antioxidant defenses leads to oxidative stress, one of causes for which can be lower antioxidant levels in the body. Free radicals generated during metabolic reactions may not be efficiently eliminated in the absence of adequate antioxidant activity.

Significantly elevated MDA levels in RAS subjects compared to controls were noted in our study. MDA is the end product of free radical mediated lipid peroxidation. Peroxidation of membrane lipids alters membrane integrity, causing cell membrane damage. This may play a role in recurrent episodes of mucosal ulceration. This suggests a greater imbalance between ROS and antioxidant defenses among RAS patients. Similar results have been obtained in studies by Cimen et al, Saral et al and Bagan et al. Avci et al, however, did not obtain a statistically significant elevation of MDA in their RAS subjects.

Ascorbic acid is an exogenous aqueous phase antioxidant that effectively scavenges peroxyl and superoxide radicals. It also plays a role in regeneration of α-Tocopherol as well as glutathione, an endogenous antioxidant. A similar study by Saral et al also revealed lower ascorbic acid levels among RAS subjects in comparison to healthy controls. Increased consumption of ascorbic acid in the setting of free radical mediated mucosal injury may explain the lower levels in RAS subjects. Ascorbic acid is an exogenous antioxidant and can be taken in the diet or as a vitamin supplement.

α-Tocopherol is a powerful exogenous lipid phase antioxidant that is highly effective in against lipid peroxidation. It is the most important exogenous antioxidant that protects

cell membranes from free radical injury. As a result, increased utilization the setting of oxidative stress explains lower levels of the antioxidant. Further, reduced ascorbic acid levels may lead to a reduction in the active form of α-Tocopherol. Saral et al have obtained similar results in their study.

Many authors have studied various antioxidant enzyme levels in oxidative stress and have obtained results showing lower antioxidant enzyme activities in RAS subjects compared to healthy controls.[2, 5, 6]

Both Ascorbic acid and α-Tocopherol can be supplemented in the diet. Studies on the association of a diet rich in vitamins with the prevalence of RAS, as well as the role of supplementation of these vitamins in the relation to recurrence and severity of the symptoms, will be useful to supplement our findings.

Conclusion

Elevated MDA levels suggest increased free radical mediated lipid peroxidation in RAS subjects. Evidence of oxidative stress is reflected by accompanying lower antioxidant levels. This may be the result of increased utilization of antioxidants in RAS patients. Studies with a larger sample size, including the effect of antioxidant supplementation on the severity and recurrence of symptoms in RAS can be undertaken to substantiate the therapeutic role antioxidants in RAS.

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