

Estimation of serum, salivary immunoglobulin G, immunoglobulin A levels and total protein, hemoglobin in smokeless tobacco chewers and oral submucous fibrosis patients

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Abstract

Background: Oral submucous fibrosis (OSMF) is a debilitating, potentially cancerous oral condition. Although areca nut is the most important causative agent, it is also considered that the disease is immunologically mediated. **Aim of the Study:** To establish that autoimmunity and nutritional deficiency play a role in the etiopathogenesis of OSMF. **Objectives of the Study:** To show that serum immunoglobulin markers (immunoglobulin-G [IgG], immunoglobulin-A [IgA]) and nutritional parameters such as total serum protein (TSP), Hemoglobin (Hb) play a role in causing OSMF and also to correlate serum, salivary IgG, IgA levels in OSMF patients. **Settings and Design:** A case-control study was done with 50 patients (25 patients who were provisionally diagnosed as OSMF - Group I, and 25 patients who were chronic smokeless tobacco chewers and who did not have any intraoral lesion - Group II). **Materials and Methods:** Five milliliters of blood and saliva were collected from both the groups. Quantitative analysis of serum, and salivary IgG, IgA was done by turbidometric immunoassay. TSP and Hemoglobin (Hb) were estimated by spectrophotometry. **Statistical Analysis:** Results were analyzed by independent samples *t*-test and one-way analysis of variance (ANOVA). **Results:** All patients of OSMF showed significant ($P < 0.01$) increase in serum IgG, IgA, and salivary IgG levels as compared to smokeless tobacco chewers. The salivary IgA levels showed a significant decrease in OSMF patients ($P < 0.05$). TSP and Hb levels showed significant ($P < 0.01$) decrease in OSMF patients as compared to smokeless tobacco chewers. **Conclusion:** The elevation of immunoglobulin levels supports the concept of autoimmunity. The decrease in TSP and Hb suggests that nutritional deficiency plays a defined role in the occurrence as well as a further progression of OSMF.

Keywords: Hemoglobin, immunoglobulin A, oral submucous fibrosis, serum and salivary immunoglobulin G, total serum protein

Introduction

Oral submucous fibrosis (OSMF) is a chronic disease of oral mucosa characterized by inflammation and progressive fibrosis of lamina propria and deeper connective tissues, followed by stiffening of an otherwise yielding mucosa resulting in difficulty in opening the mouth.^[1,2] This condition was described first by Schwartz (1952) while examining five Indian women from Kenya, to which he ascribed the descriptive term

“atrophia idiopathica (tropica) mucosae oris.” Later in 1953, Joshi from Bombay re-designated the condition as OSMF.^[3] The diagnosis of OSMF is based on features such as burning sensation, difficulty in taking spicy foods, paleness, blanching of the mucosa, palpable fibrotic bands in the buccal and labial mucosa and progressive reduction in opening the mouth. OSMF has been reported almost exclusively among Indians living in India and among other Asiatics with a reported prevalence ranging up to 0.4% in Indian rural population.^[4] According to World Health Organization the prevalence of tobacco habits in India is high with 34% using bidis, 31% cigarettes, 19% smokeless tobacco, 9% hookah, and 7% other forms, respectively. The cancer patients aid association of India revealed the prevalence of cigarette usage as 20%, bidis 40%, and chewable tobacco 40%.^[5] The age-adjusted incidence rates of oral cancer vary from over 20/100,000 populations in India.^[6] OSMF also has a significant mortality rate because

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it is a precursor to oral cancer, particularly squamous cell carcinoma, seen in 7.6% of the cases.^[7]

The etiology of OSMF is considered to be multifactorial,^[8] areca nut being the prime etiology.^[9,10] The occurrence of OSMF in cases without any history of using irritants, in teenagers, the idiopathic nature of the disease and various immunological changes have led many researchers to consider OSMF as an autoimmune disorder.^[11,12] Quantitative assay of immunoglobulin is the most frequently performed screening test for humoral immunity.^[13] Usually, it is sufficient to assay the two major immunoglobulin classes (immunoglobulin-G [IgG], immunoglobulin-A [IgA]) since, there is no proof that deficiency of other classes might have pathological consequences. Hyperimmunoglobulinemia is invariably associated with OSMF.^[13] Therefore, serum immunoglobulin levels of classes IgA, IgG are used as parameters to add on to the existing knowledge of the immune profile.

Serum immunoglobulin levels, which are used as parameters to assess humoral immunity, still continue to be an area of intensive investigative research. In the present era, the saliva which is much handy than serum is of utmost importance. IgA is the main component of the adaptive immune system present in the saliva. It is also referred to as secretory IgA. The functional ability of the secretory immune system is the major line of defense against the mucosal pathogens.^[14] As irritants stay longer in saliva, they affect the oral cavity which is bathed in saliva.^[15] Thus, the local immune response also plays an important role in causation of OSMF.^[16] Therefore, our study aimed at estimation of salivary IgG and IgA. If, we could appreciate significant positive correlation between serum and salivary immunoglobulin levels, saliva could be used as an effective diagnostic tool instead of serum in future.

Several investigators have reported iron, vitamin, protein deficiencies in OSMF.^[17] Lack of iron causes improper vascular channel formation and concomitant decrease in vascularity. This makes percolation of esters of arecoline easier. Also for normal epithelium maturation iron-containing enzyme cytochrome oxidase is required. In iron deficiency, levels of this enzyme are low and consequent atrophy of epithelium results, which leads to burning sensation and ulcerations of the oral cavity in areca chewers. This causes difficulty in

consumption of solid food eating to anemia. Thus, it forms a vicious cycle.^[18] To determine this concept, our study aimed at estimation of total serum protein (TSP) and hemoglobin (Hb) as protein and Hb can be considered as broad indicators of anemia and hence the status of nutrition.

Materials and Methods

A total of 50 patients were involved in the study. The study population was divided into two groups. Group I [Figure 1] included 25 patients who were provisionally diagnosed as OSMF, and Group II [Figure 2] included 25 patients who were chronic smokeless tobacco chewers who did not have any intraoral lesion. Permission from the Institutional Ethical Committee was obtained before starting the study. Informed consent was obtained from patients to participate in this study. The inclusion criteria were smokeless tobacco chewers with OSMF as Group I, and smokeless tobacco chewers without any intraoral lesion as Group II. The exclusion criteria were patients who were under medications such as immunomodulators or immunosuppressors, patients with bleeding disorders and patients suffering from autoimmune diseases such as pemphigus, pemphigoid, systemic lupus erythematosus, rheumatoid arthritis, systemic sclerosis, lichen planus, myasthenia gravis, Sjogren's syndrome, rheumatic fever, psoriasis, psoriatic arthritis, and chronic infections for both the groups. Physiological conditions such as pregnancy and menopause were also excluded. Clinical staging of OSMF was based on the classification by Khanna and Andrade.^[19]

Stage I

Very early cases, normal mouth opening, burning sensation, excessive salivation, acute ulceration, and recurrent stomatitis ($n = 7$).

Stage II

Early cases, mouth opening: 26–35 mm (interincisal opening), soft palate, and faucial pillars are areas primarily affected. Buccal mucosa appears mottled and marbled, with dense, pale, depigmented, and fibrosed areas alternating with pink

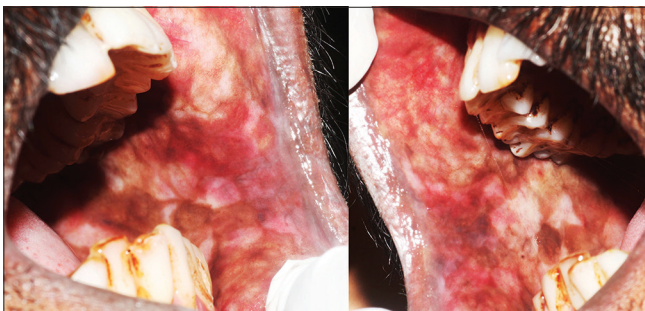


Figure 1: Oral submucous fibrosis involving buccal mucosa

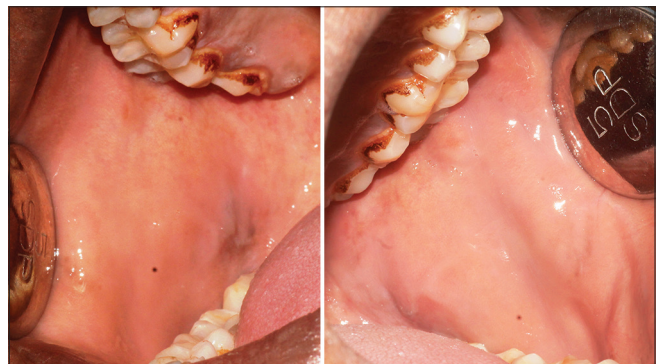


Figure 2: Smokeless tobacco chewer without oral submucous fibrosis

normal mucosa, red erythematous patches, and widespread sheets of fibrosis ($n = 5$).

Stage III

Moderately advanced cases, mouth opening: 15–25 mm (interincisal opening), trismus, vertical fibrous bands can be palpated and are firmly attached to underlying tissue. Patient unable to puff out the cheeks or whistle, soft palate-fibrous bands seen to radiate from the pterygomandibular raphe or anterior faucial pillar in a scar-like appearance, atrophy of vermilion border of the lips, unilateral posterior cheek involvement with only ipsilateral involvement of the faucial pillar, and soft palate, and reduced mouth opening ($n = 10$).

Stage IVa

Advanced cases, stiffness/inelasticity of the oral mucosa, trismus, mouth opening: 2–15 mm (interincisal opening), fauces thickened, shortened and firm on palpation, uvula seen to be involved as a shrunken, small and fibrous bud, tongue movement restricted, papillary atrophy (diffuse), lips-circular band felt around entire mouth, intraoral examination is difficult ($n = 3$).

Stage IVb

Advanced cases with premalignant and malignant changes such as leukoplakia and squamous cell carcinoma ($n = 0$).

The study comprised of 50 subjects. A total of 25 cases of OSMF (Group I), and 25 smokeless tobacco chewers (Group II). Maximum cases of OSMF and smokeless tobacco chewers were males (98%) and remaining (2%) were females. The age range of Group I patients were between 25 and 45 years with a mean age of 30 years and the age range of Group II patients were between 21 and 44 years with a mean age of 31 years. The OSMF group of 25 patients were identified as being in different stages of OSMF. Stage I ($n = 7$), Stage II ($n = 5$), Stage III ($n = 10$), and Stage IVa ($n = 3$). In OSMF group, the duration of chewing habit was more than 10 years in 15 subjects 10–15 years in 10 subjects. In smokeless tobacco chewers (Group II) the duration of chewing habit was <5 years in 11 subjects, 15–10 years in 7 subjects, and more than 10 years in 7 subjects. All the 50 patients from both the groups were using tobacco in a smokeless form such as mawa, gutkha, tobacco, pan, etc., 5–10 packets/day. In the study group (Group I) and control group (Group II), 23 patients were male and two patients were female. In Group I male patients were in the age ranging from 30 to 49 years with the mean age of 39 ± 3.5 years, and females were in the age ranging from 36 to 40 years with the mean age of 38 years. Age and sex matched smokeless tobacco chewers who did not have any intraoral lesion were included as controls (Group II) where the age of males were between 28 and 47 years with the mean age of 36 ± 3 years, and age of females were between 34 and 40 years with the mean age of 36 ± 2 years.

5 ml of peripheral blood was drawn from the two groups using standardized phlebotomy procedures, and 2 ml was used for estimation of TSP and Hb. For Hb Beckman coulter AcT DIFF II hematology analyzer was programmed to automatically pipette out 5 ml of Drabkin's reagent, and 0.02 ml of sample in to cuvette and incubated for 5 min and the value was read at 546 nm (530–550 nm) or green filter against the blank at 25–35°C. From the remaining samples, sera were separated by centrifugation in a centrifuge machine at 2500 rpm for 15 min. For TSP, Beckman coulter AU480 automated chemistry analyzer was programmed to pipette out 20 μ of sample and 1000 μ of reagent (copper [III] acetate 6 mmol/L, potassium iodide 12 mmol/L, sodium hydroxide 1.15 mol/L, detergent). The values were read by spectrophotometer at 545 ± 10 nm. For the collection of saliva each individual were asked to swallow first, tilt their head forward, and expectorate 5 ml of whole saliva into the uricup for 10 min without swallowing under nonstimulatory conditions. Following collection, the saliva was immediately centrifuged in a centrifuge machine at 2500 rpm for 15 min to obtain supernatant saliva. Serum and salivary IgG and IgA were quantified by using a diagnostic kit (IgG and IgA euro diagnostics system kit). The IgG and IgA euro diagnostic system kit contains diluents (R1) and antibody (R2). For estimation of serum or salivary IgG, IgA the assay procedure and the diluent (tris buffer) R1 value was same, but the antibody reagent (R2) used was antihuman IgG and anti-human IgA.

Beckman coulter AU480 was programmed to bring the reagents and photometer (cuvette holder) to 37°C. Assay conditions were maintained at 600 nm wavelength. The machine is programmed to pipette reagent (R1) of 800 μ l and sample of 10 μ l in to a cuvette automatically. Incubation is done for few minutes, after which reagent (R2) of 200 μ l is pipetted immediately in to the cuvette. Two minutes after the addition of R2 the readings were read by spectrophotometric detectors.

Statistical analysis

Independent samples *t*-test were used for statistical comparison between the two groups of OSMF and smokeless tobacco chewers. A one-way analysis of variance was used for statistical comparison between the same group.

Results

Table 1 shows reference range of different parameters. All the patients of OSMF group showed significant ($P < 0.01$) increase in serum IgG, IgA and salivary IgG levels but there was a significant decrease in salivary IgA levels ($P < 0.05$) when compared with smokeless tobacco chewers. TSP and Hb were also decreased in OSMF patients than smokeless tobacco chewers and were statistically significant ($P < 0.01$) [Table 2].

When comparisons were made between stages of OSMF group (Group I) there was an increase in serum IgG, salivary

IgG as the stage advances but was not statistically significant. There was a positive correlation between serum and salivary IgA also. Serum IgA and salivary IgA were found to be elevated as the stage of the disease progresses ($P < 0.05$). However, the Grade I OSMF recorded the lowest salivary IgA levels and even the values of salivary IgA in Grade IVa were only in the lower limits of the normal range. The TSP and Hb levels were also decreased as the stage of the disease advances ($P < 0.05$) [Table 3].

When comparisons were made between smokeless tobacco chewers group, serum IgG and salivary IgG were found elevated as the duration of chewing habit increases ($P < 0.01$). But the increased value of serum IgG and salivary IgG were only in the upper limit of the normal range. However, there was a negative correlation between serum IgA and salivary IgA. Serum IgA was found to be

increased as the duration of chewing habit increases (not statistically significant) but the salivary IgA which was initially in the normal range showed marked decrease as the duration of habit progresses ($P < 0.01$). There was no difference in the TSP and Hb levels when compared between the varying durations of habits [Table 4].

Discussion

Several theories have been put forth to explain the etiology of OSMF, such as the use of betel nut, tobacco and allergy to chilies. However, cases of OSMF with none of these etiological factors and reported occurrence of OSMF in young adults have led to the postulation of immune mechanism as the basis of OSMF.^[9] Immunoglobulins are a heterogeneous group of proteins having three major classes of immunoglobulin G, A, M designated as IgG, IgA, IgM. Increased levels of serum IgG, IgA in OSMF cases ($P < 0.01$) were observed in the present study [Table 2] compared to smokeless tobacco chewers without OSMF. In our study, serum IgG, IgA levels were also elevated as the stage of OSMF advances [Table 3]. The severity of the disease and the levels of immunoglobulins were directly proportional which further emphasizes the possible role of autoimmunity in OSMF.^[20] The role of serum immunoglobulins has been well hypothesized, but little is known about the role of saliva. Salivary immunoglobulins play an important role in

Table 1: Reference values

Parameters	Reference range
Serum and salivary IgG	700-1600 mg/dl
Serum and salivary IgA	70-400 mg/dl
TSP	6.4-8.3 g/dl
Hb	14-17 g%

IgA: Immunoglobulin-A; IgG: Immunoglobulin-G; TSP: Total serum protein; Hb: Hemoglobin

Table 2: Comparison between serum IgG, serum IgA, salivary IgG, salivary IgA and total serum protein, haemoglobin levels in smokeless tobacco chewers and OSMF patients

Parameters	Group								Independent	
	Smokeless tobacco (n=25)				OSMF group (n=25)				Samples t-test	
	Mean	SD	Min.	Max.	Mean	SD	Min.	Max.	T	Sig.
Serum IgG	1444.92	211.28	926	1726	1835.96	178.12	1512	2126	-7.075	0.000
Serum IgA	401.36	101.61	124	561	539.08	87.22	363	709	-5.142	0.000
Salivary IgG	1452.00	266.98	960	1834	1829.76	176.26	1581	2099	-5.904	0.000
Salivary IgA	143.12	71.80	64	314	92.28	31.26	27	145	3.246	0.003
TSP	7.15	0.45	6.4	8.1	6.05	0.26	5	6	10.658	0.000
Hb	14.68	0.58	13.5	16	13.34	1.17	11	15	5.106	0.000

IgA: Immunoglobulin-A; IgG: Immunoglobulin-G; TSP: Total serum protein; Hb: Hemoglobin; OSMF: Oral submucous fibrosis; SD: Standard deviation

Table 3: Comparison of serum IgG, serum IgA, salivary IgG, salivary IgA and total serum protein, haemoglobin between different stages of OSMF

Parameters	Stage								One Way	
	I (n=7)		II (n=5)		III (n=10)		IVa (n=3)		ANOVA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	F	Sig.
Serum IgG	1709.71	154.25	1800.00	143.38	1894.90	172.73	1994.00	141.86	2.986	0.054
Serum IgA	473.71	79.95	517.60	73.31	564.90	71.07	641.33	59.21	4.378	0.015
Salivary IgG	1715.43	134.22	1800.20	181.90	1890.80	180.49	1942.33	142.88	2.085	0.133
Salivary IgA	57.57	24.12	95.40	14.05	105.10	22.72	125.33	23.71	9.335	0.000
TSP	6.21	0.20	6.10	0.16	6.01	0.22	5.73	0.38	3.408	0.036
Hb	14.01	0.69	13.86	0.53	13.05	1.37	11.90	0.26	3.861	0.024

IgA: Immunoglobulin-A; IgG: Immunoglobulin-G; TSP: Total serum protein; Hb: Hemoglobin; SD: Standard deviation; ANOVA: Analysis of variance

Table 4: Comparison of serum IgG, serum IgA, salivary IgG, salivary IgA and total serum protein, haemoglobin between smokeless tobacco chewers having different duration of habit

	Years						One Way	
	≤5 years (n=11)		5-10 years (n=7)		>10 years (n=7)		ANOVA	
	Mean	SD	Mean	SD	Mean	SD	F	Sig.
Serum IgG	1290.73	176.89	1493.14	159.04	1639.00	104.25	11.241	0.000
Serum IgA	369.73	132.90	399.86	65.53	452.57	51.69	1.480	0.250
Salivary IgG	1214.55	211.43	1590.14	100.83	1687.00	101.90	21.996	0.000
Salivary IgA	196.18	77.93	120.14	17.66	82.71	12.51	10.424	0.001
TSP	7.25	0.55	7.1	0.48	7.04	0.14	0.477	0.627
Hb	14.72	0.68	14.66	0.46	14.63	0.61	0.051	0.95

IgA: Immunoglobulin-A; IgG: Immunoglobulin-G; TSP: Total serum protein; Hb: Hemoglobin; SD: Standard deviation; ANOVA: Analysis of variance

OSMF since irritants stay longer in saliva.^[21] In our study, there was a negative correlation between salivary IgG and salivary IgA in OSMF patients. Salivary IgG was increased ($P < 0.01$) whereas a decrease in salivary IgA ($P < 0.05$) was noted in OSMF when compared with smokeless tobacco chewers [Table 2]. The increase in salivary IgG may be due to increased permeability leading to passive diffusion of IgG from the vascular and extravascular compartment into the saliva. This may be suggestive of the active inflammatory process.^[15] Thus, IgG in saliva may originate from serum and be transported by passive transmucosal diffusion. Though OSMF patients recorded lower salivary IgA levels ($P < 0.05$), when comparison were made between the two groups individually the salivary IgA was increased as the stage of the disease progressed in OSMF group [Table 3] and the salivary IgA was decreased as the duration of chewing habit increases in smokeless tobacco chewers group [Table 4]. The decrease in salivary IgA may be due to the reason that tobacco is composed of many pharmacological agents including nicotine and its major metabolite cotinine. Nicotine significantly reduces the secretory component, lactoferrin and lysozyme, also reducing the total cell numbers and their metabolic activity in smokeless tobacco users than nonusers.^[14] Another reason for the reduction could be due to an immunosuppressive effect of the combustion products of tobacco and the possibility of the incidence of the intraoral neoplastic disease being increased.^[22] The reason for decrease in salivary IgA in OSMF more particularly in Stage I OSMF might be due to the fact that OSMF group are also smokeless tobacco chewers and for the reduction of salivary IgA in OSMF the same reason could be applicable^[14,22] and thus the initial decrease of salivary IgA creates deficiency in local mucosal immunity making susceptible for the disease to occur as the IgA present in saliva plays an important role in mucosal immunity. But as the stage of OSMF increases the salivary IgA levels were also increased which gained statistical significance ($P < 0.01$). This might be due to the local changes in the immunological competence might occur due to prolonged exposure to the antigen in the oral cavity.^[23]

Apart from the immunological basis several authors explain that hemoglobin and serum iron have been significantly

reduced in OSMF patients. This raised the suspicion that nutritional deficiency could be precipitated by the effect of defective nutrition due to impaired food intake in advanced cases, and may be the effect, rather than the cause of the disease.^[24] Proteins are physically and functionally complex macromolecules that perform multiple roles.^[25] Levels of protein, Hb, Vitamins B complex, etc., in OSMF are important factors which suggest the role of nutrition in OSMF. The seed and soil theory put forth by Ramanathan states that OSMF is an Asian version of sideropenic dysphagia, where iron deficiency leads to mucosal susceptibility to irritants such as a betel nut.^[26] As the stage of OSMF advances the atrophy, stiffening of the mucosa increases the burning sensation while consuming food and this leads to further reduction in intake of nutrients leading to a decrease in TSP [Table 3]. Further iron is also utilized during the process of fibrosis and hence there is a decrease in serum iron levels as the stage of OSMF advanced [Table 3].^[27] It is well noted that the serum immunoglobulin values are age- and sex-dependent. IgG and IgA concentrations increases with age with slight but significant differences with sexes. However, our study eliminated the confounding influence of age and sex.^[28] There could be other confounding factors such as race, smoking and alcohol intake.

Conclusion

This study has shown a significant difference in immunoglobulin levels, nutritional parameters in OSMF group suggesting that immunology and nutritional deficiency has a definite role in OSMF. A retrospective cohort study of OSMF patients will further strengthen the role of autoimmunity in the etiopathogenesis of OSMF. It is also of key importance that the iron therapy should be instituted parallel with the initial diagnosis along with a proper balanced diet, as a part of the overall management of OSMF with other modes of treatment.

Limitations of the study

The limitations of the study could be that the normal salivary IgG, IgA levels could not be determined precisely as they vary

among different age groups, and also based on the equipment and methodology involved to establish its procedures have to be performed on larger sample.

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Conflicts of interest

There are no conflicts of interest.

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