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Estimation of Iron and Vitamin C Levels In Serum and Saliva: A Clinical and Biochemical Study In Oral Submucous Fibrosis Patients.

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ABSTRACT

Background: OSMF is a well-recognized, potentially malignant condition of the oral cavity. Monitoring the widespread consequences of OSMF requires interventions in at-risk persons ideally earlier the disease becomes aggressive. Iron and vitamin C have been widely studied in recent years to judge whether they have any modifying effects in the etiology of precancer and cancer. Aim of study was to evaluate levels of iron and vitamin C in serum and saliva in patients with OSMF. **Methods**: Study group comprised of 66 patients, out of which 22 cases of clinically diagnosed OSMF patients and 22 cases of betelnut habitual without OSMF and 22 cases of age and sex matched control healthy patients were recruited. Estimation of Iron by Ferrozine method and vitamin C by 2-4 dinitrophenylhydrazine method in serum and saliva was carried out by using Spectrophotometer. The statistical analysis was done. **Results**: Level of iron and vitamin C in serum and saliva was significantly decreased in OSMF patients when compared to betelnut habitual and controls group which were statistically significant. **Conclusion**: The present study, all the cases of OSMF showed decrease in iron and vitamin C levels which suggests that the iron and vitamin C plays an important role in the pathogenesis and progression of OSMF. In group II (Betel nut habituals without OSMF) patients showed decrease in iron and vitamin C levels which suggest that, betel nut quid with or without tobacco consumption may alter the serum and salivary levels of iron and vitamin C and plays an important role in the advancement of OSMF. Therefore regular monitoring of betel nut habituals should also be carried out because they are at higher risk of developing OSMF in future. Study indicates that saliva also may be used as a potential non-invasive diagnostic tool to evaluate the iron and vitamin C in OSMF patients. Estimation of iron and vitamin C in OSMF patients would help in management, in developing therapies based on the trace element expression.

Keywords: OSMF, Iron, Vitamin C, Serum and Saliva.

INTRODUCTION

Oral submucous fibrosis (OSMF) is now accepted globally as an Indian disease, having highest malignant potential than any other oral premalignant lesions.[1] Monitoring the overwhelming, widespread consequences of oral submucous fibrosis requires interventions in at-risk persons ideally earlier the disease becomes aggressive.[2]

Overall prevalence of oral submucous fibrosis in India is about 0.2-0.5% and prevalence by gender varying from 0.2% to 2.3% in males and 1.2% to 4.57% in females in different regions of the country.

The age range of the patients with oral submucous fibrosis is wide ranging between 20 years and 40 years of age.[3-4] Malignant transformation of oral submucous fibrosis varied from 7%-13% in India.[5]

Numerous studies conducted in India advocated that betel-nut products with or without tobacco is responsible for oral submucous fibrosis, but now betel-nut specific nitrosamine have been known which are responsible for the premalignant and malignant oral mucosal lesions/conditions.6 The duration and frequency of betel nut products used, affects the incidence and severity of oral submucous fibrosis.[7]

Iron content can be a predictor for the progression of Oral submucous fibrosis. This condition had been previously described as "sideropenic

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dysphagia." Kapoor S et.al.[8] (2013) recognized the generalized occurrence of anemia in patients with oral submucous fibrosis to predisposition of the condition in patients consuming betel nuts. His "seed and soil theory" was one of the earlier attempts to implicate iron deficiency in the etiopathogenesis of the condition.8The purpose for the depletion of iron could be multifactorial. Factors such as altered epithelial cell turnover rate and tumor cell proliferation leads to the depletion of iron levels and the reserve body iron stores. Additional, reduction in iron indices occurs due to difficulty in consumption of normal diet, thereby leading to poor nutrition.[9]

Vitamin C and iron is consistent as vitamin C plays an imperative role in absorption of iron from the gut. Vitamin C helps the body to absorb non-heme Iron. Vitamin C improves iron absorption by reducing dietary Iron from ferric form to the ferrous form. Therefore, Vitamin C deficiency may lessen the availability of intracellular Iron. This has led to the theory that vitamin C may have been used for the disproportionate collagen production and cross-linking that occurs in OSMF. Thus vitamin C could be an important indicator for the establishment of oral submucous fibrosis.[9]

Considering the multifactorial aetiology of oral submucous fibrosis, more exploration is required to develop sensitive, specific, and faster tests in the diagnosis and prognosis of these diseases. Recently trace element like iron is delivery much attention in the detection of oral precancerous lesions or conditions as it was institute to be significantly rehabilitated in this conditions.[10]

Thus, the present study was undertaken to comprehend the association of serum and salivary iron and vitamin C in oral submucous fibrosis patients, betel nut habitual without oral submucous fibrosis and control healthy subjects. An effort was also made to evaluate the ratio of iron and vitamin C in serum and saliva of OSMF patients and betel nut habituals.

MATERIALS AND METHODS

The study sample comprised of 66 subjects, 22 cases of clinically diagnosed oral submucous fibrosis and 22 cases of betel nut habitual with or without tobacco without OSMF and 22 cases of age and sex matched control healthy individuals attending the outpatient department of Oral Medicine and Radiology, Sharad Pawar Dental College and Hospital, DMIMS (DU), Sawangi, (Meghe) Wardha. After obtaining the clearance from Institutional Ethics committee, an informed written consent was attained from all 66 participants. A detailed case history of all subjects was recorded including history of deleterious habit, systemic diseases and intake of antioxidant/ multivitamin supplementation. A detailed clinical examination was carried out and findings were recorded. The clinical diagnosis of oral submucous fibrosis was made by using the criteria as mentioned according to clinical and functional staging of More C et al (2012).[11]

Inclusion criteria for study group: Clinically newly diagnosed cases of oral submucous fibrosis with habit of betel nut with or without tobacco in any form and both men and women in between the age of 15 to 65 years were included.

Exclusion criteria for study group: Subjects suffering from any systemic diseases like diabetes, renal diseases, liver diseases and malignancies, medically compromised patients, patients less than 15 and more than 65 years of age, patient undergoing treatment or previously treated cases of OSMF were excluded. Inflammatory and infection causes responsible for trismus and burning sensation, temporo-mandibular joint disorders, trauma to maxillofacial region and patient not willing to undergo investigations were also excluded.

Methodology: Five ml of venous blood was obtained from median cubital vein; blood was allowed to clot at room temperature for 1 to 2 hours. The serum was separated by centrifuge machine at 3000 rpm for 10 minutes to get a clear serum sample. The serum thus obtained was pipetted using a micro pipette and transferred into sterile plastic storage vial and was stored at -200 C in a dark container until assay. Five millilitres of unstimulated saliva was obtained by the spit method after following a standard pre-collection protocol.

Materials: Estimation of Vitamin C done by 2-4 dinitrophenylhydrazine method. The Principle of this method is that Dehydro ascorbic acid was coupled with 2, 4 dinitrophenylhydrazine and the resulting derivative is treated with sulphuric acid to produce a newly observed colour which is measured at 545 nm.[12] Iron estimation was done by Ferrozine method and the principle of transferrin bound iron breaks into free ferric ions in an acidic medium. These ferric ions react with Hydroxylamine Hydrochloride reduced into ferrous ions which react with Ferrozine to form a violet coloured complex measured at 560 nm. The difference before and after the addition of ferrozine is proportional to iron concentration reaction in the specimen.[13]

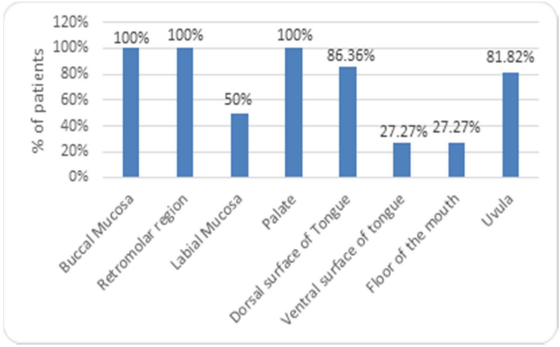
Statistical analysis: Statistical analysis was done by using descriptive and inferential statistics using Chi-square test, Student's unpaired t test, One way ANOVA, Multiple Comparison Tukey Test and Pearson's Correlation Coefficient and software used in the analysis were SPSS 17.0 version, Graph Pad Prism 6.0 version and EPI-INFO 6.0 version and $p<0.05$ is considered as level of significance.

RESULTS

All the subjects studied were between the age group of 15 to 65 years with the mean age in group I(OSMF) was 28.95±7.43 and maximum number of patients i.e. 13 (59.09%) were between the age group of 21-30 years. Out of 22 patients of group I (OSMF), maximum number of patients i.e. 18(81.82%) were males and 4(19.18%) were females and male to female ratio was 4.5:1.

Out of 22 patients of group I (OSMF), 20(90.90%) males had multiple habits (betel nut, Khara, tobacco, betel quid chewing and tobacco smoking) and 2(9.09%) female patients had betel nut chewing habit and in group II (habit without OSMF), 18(81.81%) males had multiple habits (betel nut, Khara, tobacco, betel quid chewing and tobacco smoking) and 4(18.18%) female patients had betel nut chewing habit. In group I (OSMF), the mean duration of betel nut habit in any form was 8.34 ± 5.09 and in group II, the mean duration of betel nut habit in any form was 6.28 ± 4.95. The mean frequency of habit in group I was 7.22 ± 4.20 and in group II was 4.04 ± 2.68. Majority of the patients were using khara or betel nut ≥ 4 to 5 times in a day. The mean of interincisal opening in group I was 23.72±9.27. Out of 22 patients of Group I, 7(31.82%) had mouth opening in range of 16-20 mm.

Most commonly involved mucosal surfaces were buccal mucosa, retromolar region and palate (100%), dorsal surface of tongue (86.36%), uvula (81.82%), labial mucosa (50%) and ventral surface of tongue and floor of the mouth (27.27%).[Graph 1]. Classification of More C et al (2012) for grading of OSMF was used. Out of 22 patients of OSMF (group I) there were 3(13.64%) patients of Grade I OSMF, 13(59.09%) were of Grade II and 6(27.27%) were of Grade III OSMF.



Graph 1: Distribution of subjects according to involvement of number of mucosal surfaces in group I.

[Table 1] shows a mean value of serum iron, serum vitamin C, salivary iron and salivary vitamin C in three groups.

The mean serum iron value of in Group I (OSMF) was 47.16±25.36 µg/dl, Group II was 53.16±19.66 µg/dl and Group III was 72.58±27.85 µg/dl. When the mean serum iron of Group I (OSMF) and Group II compared with group III, there was statistically significant difference. [Table 2 and Graph 2].

The mean serum vitamin C value in Group I was 0.16±0.03 mg/dl, Group II was 1.06±0.71 mg/dl and Group III was 1.33±0.65 mg/dl. When the mean serum vitamin C value of Group I (OSMF) compared with group II and group III, there was statistically significant difference.[Table 3 and Graph 3]

Table 1: Mean serum iron (ug/dl), mean serum vitamin C, mean salivary iron and mean salivary vitamin C levels among the controls, habitual without OSMF and OSMF patients.

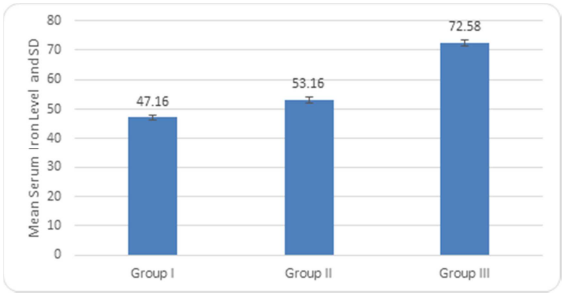
Study Groups	No. of cases	Serum iron (ug/dl) Mean ± S D	Serum vitamin c (mg/dl) Mean ± S D	Salivary iron (ug/dl) Mean ± S D	Salivary vitamin c (mg/ml) Mean ± S D
Controls	22	72.58±27.85	1.33±0.65	13.13±11.83	1.19±0.40
Habitual without OSMF	22	53.16±19.66	1.06±0.71	5.84±3.07	0.82±0.56
OSMF Group	22	47.16±25.36	0.16±0.03	3.43±2.43	0.19±0.04
Grade I	3	39.32±7.60	0.16±0.03	2.65±1.21	0.20±0.03
Grade II	13	39±20.80	0.15±0.03	2.70±1.73	0.19±0.06
Grade III	6	52.74±29.21	0.17±0.03	3.95±2.85	0.19±0.01

Table 2: Comparison of serum iron in three groups

Group		Mean Difference (I-J)	Std. Error	p-value	95% Confidence Interval	
					Lower Bound	Upper Bound
Group I	Group II	-5.99	7.39	0.698,NS	-23.75	11.76
	Group III	-25.41	7.39	0.003,S	-43.17	-7.66
Group II	Group III	19.42	7.39	0.029,S	1.66	37.18

The mean salivary iron value in Group I was 3.43±2.43 µg/dl, Group II was 5.84±3.07 µg/dl and Group III was 13.13±11.83 µg/dl. When the mean salivary iron value of Group I (OSMF) and Group II was compared with group III, there was statistically significant difference. [Table 4 and Graph 4]

The mean salivary vitamin C value in Group I was 0.19±0.04 mg/ml, Group II was 0.82±0.56 mg/ml and Group III was 1.19±0.40 mg/ml. When the mean salivary vitamin C value of Group I (OSMF) was compared with group II and group III and Group II with group III, there was statistically significant difference. [Table 5 and Graph 5].



Graph 2: Comparison of serum iron in three groups

Table 3: Comparison of serum vitamin C in three groups

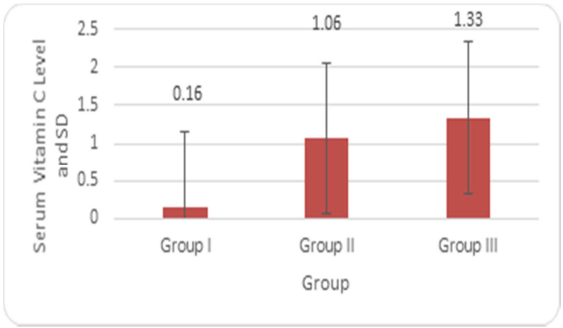
Group		Mean Difference (I-J)	Std. Error	p-value	95% Confidence Interval	
					Lower Bound	Upper Bound
Group I	Group II	-0.90	0.16	0.0001,S	-1.30	-0.49
	Group III	-1.16	0.16	0.0001,S	-1.57	-0.76
Group II	Group III	-0.26	0.16	0.259,NS	-0.67	0.13

Multiple Comparison: Tukey Test

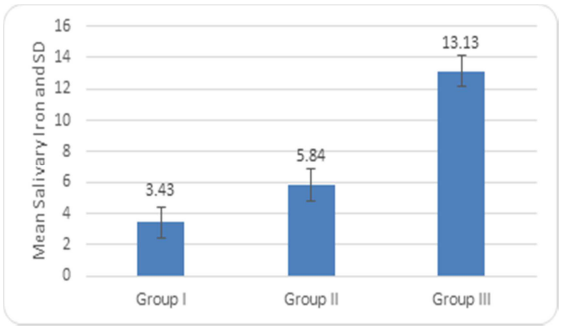
Table 4: Comparison of salivary iron in three groups

Group		Mean Difference (I-J)	Std. Error	p-value	95% Confidence Interval	
					Lower Bound	Upper Bound
Group I	Group II	-2.40	2.17	0.513,NS	-7.61	2.80
	Group III	-9.69	2.17	0.0001,S	-14.90	-4.48
Group II	Group III	7.29	2.17	0.004,S	2.08	12.50

Multiple Comparison: Tukey Test



Graph 3: Comparison of serum vitamin C in three groups



Graph 4: Comparison of salivary iron in three groups

The correlation of serum iron (47.16±25.36 µg/dl) with serum vitamin C (0.16±0.03 mg/dl), in Group I, II and III was not statistically significant, but there was negative correlation seen between serum iron and vitamin C values.

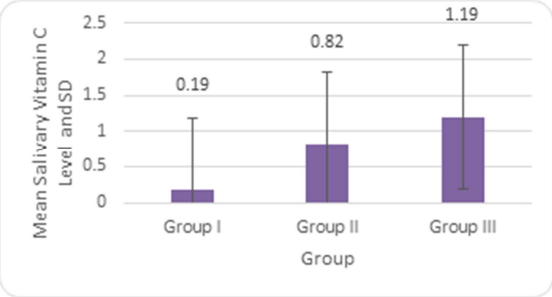
In group I, the ratio of serum vitamin C with serum iron was 3.42:1. In group II, the ratio of serum vitamin C with serum iron was 2.004:1. In group III, the ratio of serum vitamin C with serum iron was 1.83:1.When compares the ratio between the serum vitamin C and serum iron which was showed statistically significant difference between Group I, Group II and Group III ($p < 0.05$). [Table 6 and Graph 6].

The correlation of serum iron (47.16±25.36 µg/dl) with salivary iron (3.43±2.43 µg/dl) in group I and the correlation between serum iron (53.16±19.66µg/dl)and salivary iron (5.84±3.07µg/dl) in Group II was not statistically significant ($p>0.05$) but there was negative correlation in Group I and II. Whereas the correlation between serum iron (72.58±27.85µg/dl) and salivary iron (13.13±11.83µg/dl) value was statistically significant in Group III ($p<0.05$).

Table 5: Comparison of salivary vitamin C in three groups

Group		Mean Difference (I-J)	Std. Error	p-value	95% Confidence Interval	
					Lower Bound	Upper Bound
Group I	Group II	-0.62	0.12	0.0001,S	-0.91	-0.33
	Group III	-0.99	0.12	0.0001,S	-1.28	-0.70
Group II	Group III	-0.36	0.12	0.010,S	-0.65	-0.07

Multiple Comparison: Tukey Test



Graph 5: Comparison of salivary vitamin C in three groups

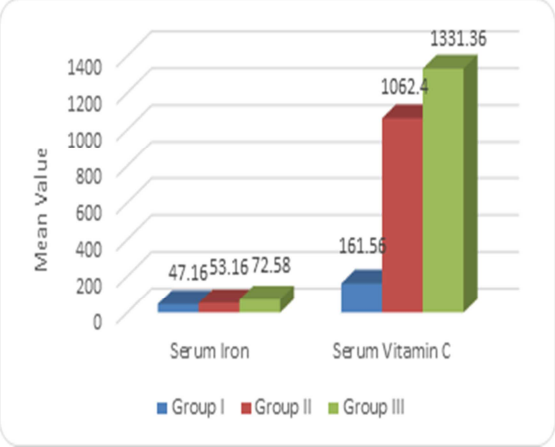
In group I, the ratio of serum iron with salivary iron was 13.74:1, in group II, the ratio of serum iron with salivary iron was 9.10:1 and in group III, the ratio of serum iron with salivary iron was 5.52:1. When compare the ratio between the serum iron with salivary iron which was showed statistically significant difference between Group I and Group II ($p< 0.05$) and Group I and Group III ($p< 0.05$). ($p> 0.05$).[Table 7 and Graph 7].

Table 6: Comparisons of ratio of serum vitamin C and serum iron in 3 groups

Groups	Parameters	Mean (µg/dl)	Ratio	Comparisons of ratio in 3 groups
Group I	Serum iron	47.16±25.36	3.42:1	Group I vs. Group II : p-value=0.001,S
	Serum vitamin C	161.56±35.32		
Group II	Serum iron	53.16±19.66	2.004:1	Group I vs. Group III : p-value=0.001,S
	Serum vitamin C	106.24±716.91		
Group III	Serum iron	72.58±27.85	1.83:1	Group II vs. Group III: p=0.990,NS
	Serum vitamin C	133.13±657.72		

Table 7: Comparisons of ratio of serum iron and salivary iron in three groups

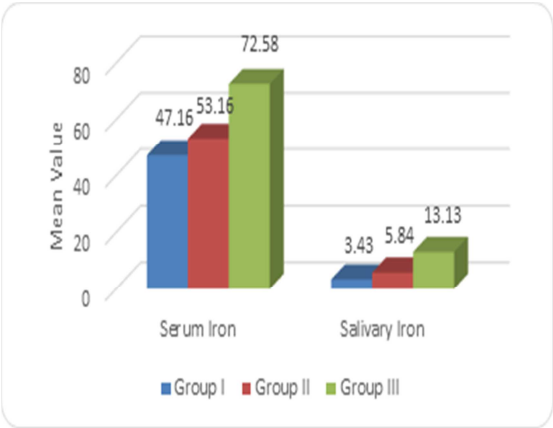
Groups	Parameters	Mean (µg/dl)	Ratio	Comparisons of ratio in 3 groups
Group I	Serum iron	47.16±25.36	13.74:1	Group I vs. Group II : p-value=0.001,S
	salivary iron	3.43±2.43		
Group II	Serum iron	53.16±19.66	9.10:1	Group I vs. Group III : p-value=0.001,S
	salivary iron	5.84±3.07		
Group III	Serum iron	72.58± 27.85	5.52:1	Group II vs. Group III: p=0.806,NS
	salivary iron	13.13±11.83		



Graph 6: Comparisons of ratio of serum vitamin C and serum iron in three groups

The correlation of salivary iron (3.43±2.43 µg/dl) with salivary vitamin C (0.19±0.04 mg/ml) in Group I, which was statistically significant ($p<0.05$). The correlation of salivary iron (5.84±3.07µg/dl) with salivary vitamin C (0.82±0.56mg/ml) in Group II and salivary iron (13.13±11.83µg/dl) with salivary vitamin C (1.19±0.40mg/ml) in Group III ($p>0.05$), there was no statistically significant but there was a negative correlation seen between the three groups.

In group I, the ratio of salivary vitamin C with salivary iron was 57.62:1. In group II, the ratio of salivary vitamin C with salivary iron was 141.13:1. In group III, the ratio of salivary vitamin C with salivary iron was 90.70:1. When compare the ratio between the salivary vitamin C with salivary iron which was showed statistically significant difference between Group I and Group II and Group I and Group III and Group II and Group III ($p< 0.05$). [Table 8 and Graph 8]



Graph 7: Comparisons of ratio of serum iron and salivary iron in three groups

Table 8: Comparisons of ratio of Salivary Vitamin C and Salivary Iron in 3 groups

Groups	Parameters	Mean (µg/dl)	Ratio	Comparisons of ratio in 3 groups
Group I	Salivary Iron	3.43±2.43	57.62:1	Group I vs. Group II : p-value =0.006,S
	Salivary Vitamin C	197.66±47.45		
Group II	Salivary Iron	5.84±3.07	141.13:1	Group I vs. Group III : p-value=0.001,S
	Salivary Vitamin C	824.20±568.37		
Group III	Salivary Iron	13.13±11.83	90.70:1	Group II vs. Group III: p=0.001,S
	Salivary Vitamin C	1190.90±403.62		

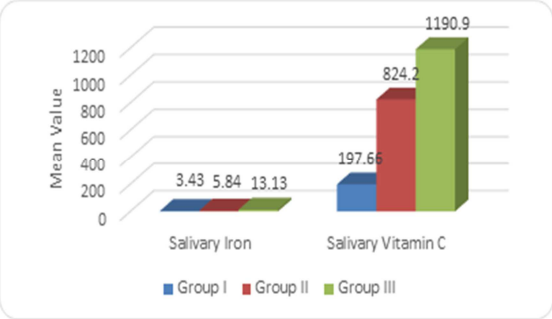
The correlation of salivary vitamin C (0.19±0.04 mg/ml) with serum vitamin C (0.16±0.03 mg/dl) in Group I was not statistically significant (p>0.05), but there was a negative correlation seen. The correlation of salivary vitamin C (0.82±0.56 mg/ml) with serum vitamin C (1.06±0.71 mg/dl) in group II and the correlation of salivary vitamin C (1.19±0.40 mg/ml) and serum vitamin C (1.33±0.65 mg/dl) in group III, which was statistically significant (p>0.05).

In group I, the ratio of salivary vitamin C with serum vitamin C was 1.21:1. In group II, the ratio of salivary vitamin C with serum vitamin C was 7.75:1. In group III, the ratio of salivary vitamin C with serum vitamin C was 8.94:1. When compare the ratio between the salivary vitamin C with serum vitamin C which was shows statistically significant difference between Group I and Group II, Group I and Group III (p< 0.05). (p> 0.05). [Table 9 and Graph 9]

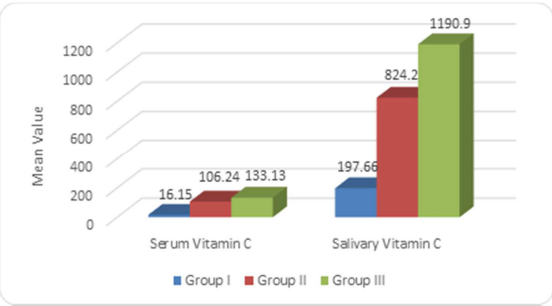
In Group I(OSMF patient), correlation of clinical grading with mean serum iron , serum vitamin C, salivary iron and salivary vitamin C levels was not statistically significant, due to uneven distribution of clinical gradings of OSMF.

Table 9: Comparisons of ratio of Salivary Vitamin C and Serum Vitamin C in three groups

Groups	Parameters	Mean (µg/dl)	Ratio	Comparisons of ratio in 3 groups
Group I	Serum vitamin C	161.5±3.53	1.21:1	Group I vs. Group II : p-value=0.004,S
	Salivary Vitamin C	197.66±47.45		
Group II	Serum vitamin C	106.24±71.69	7.75:1	Group I vs. Group III : p-value=0.002,S
	Salivary Vitamin C	824.20±568.37		
Group III	Serum vitamin C	133.13±65.77	8.94:1	Group II vs. Group III : p=0.895,NS
	Salivary Vitamin C	1190.90±403.62		



Graph 8: Comparisons of ratio of Salivary Vitamin C and Salivary Iron in three groups



Graph 9: Comparisons of ratio of Salivary Vitamin C and Serum Vitamin C in three groups

DISCUSSION

Oral submucous fibrosis is a chronic, insidious oral mucosal condition affecting the most parts of the oral cavity with high malignant transformation rate triggered by areca nut chewing, nutritional deficiencies, immunologic processes and genetic predisposition. It causes significant hematological abnormalities resulting in anemia and a decrease in serum iron levels.[10,14] Biochemical investigations of blood, serum, saliva and tissues have been the initial form of interventions. Such investigations have largely assisted to localize parameters that influence to the development of the condition, modify its behaviour and prognosticate on its malignant transformation potential.

Iron and vitamin C have been studied in recent years to see whether they have any modifying effects in the aetiology of oral precancer and cancer. Immunological and biochemical variations in the serum and saliva of such patients can aid not only in the initial diagnosis, appropriate treatment but also as indicators of prognosis.[15]

In the present study, the mean age of all patients of group I (OSMF) was found to be a 28.98±7.43 year which was in accordance with several studies.[16-19] Maximum number of patients i.e. 13 (59.09%)

were between the age group of 21-30 years. This finding was in accordance with several studies.[16,20-22] Studies conducted before 2003 revealed that maximum number of OSMF cases were in the age group of 30-59 years. However studies conducted after 2003 and in the present study, maximum number of OSMF cases were in the age group of 20-40 years. This can be due to easy availability of various commercial products containing betel nut in attractive sachets which fascinate the young generation and may explain the decrease in the age of OSMF patients.

In the present study, in group I male: female ratio was 4.51:1. This finding was in accordance with several studies.[17,18,20] In the present study and the previous studies mentioned above, it was found that males were exposed more to habit of khara users and other related products than females because conservative culture of the society acts as a major inhibitor in adaptation of these habits in female.

Out of 22 patients of group I, 20(90.90%) males had multiple habits (betel nut, Khara, tobacco, betel quid chewing and tobacco smoking) and 2(9.09%) female patients had betel nut chewing habit. Out of 22 patients of the group II (with habit without OSMF), 18(81.81%) males had multiple habits (betel nut, Khara, tobacco, betel quid chewing and tobacco smoking) and 4(18.18%) female patients had betel nut chewing habit. In Indian mythology betel nut is considered as a divine fruit and its usage has an important place in social and cultural customs and hence not included under adverse habits. This increasing use of betel nut products are a cause for concern. Psycho-social aspects play an important role for maintenance of the habits.[24] Dose-response relationships were observed for both the frequency and duration of betel quid chewing without tobacco on the risk of OSMF.24 Frequency of chewing rather than the total duration of the habit was directly correlated to Oral submucous fibrosis.[25]

In the present study, duration and frequency of betel nut or khara users may act cumulatively on the severity of OSMF. Variation in site involvement is attributed to the habit of the individual as chewing habit varies among individuals. This can be due to unawareness in patients about the relationship between betel nut and OSMF.

In the present study, buccal mucosa was the most common site of involvement. Fibrous bands were most commonly palpated in the posterior buccal mucosa, followed by the anterior buccal mucosa, soft palate, tongue, labial mucosa, floor of the mouth, and uvula. The clinical profile has probably changed in the recent years due to alterations in the patterns of betel nut use; prolonged stacking of commercially prepared fridge dried sweetened betel nut products in the buccal mucosa and probably a greater familiarity among the medical practitioners about the presentation of the condition, leading to higher and early detection rates.[23] The patterns of blanching or formation of fibrous bands depend largely on the style of chewing (whether it is swallowed or spitted out), duration of addiction and the period of contact of the quid with the specific site. The quid remains in contact for a longer time with the buccal mucosa rather than the retromolar area, soft palate or labial mucosa, causing localized irritation, thus amplification the findings.[27] Exposure of the buccal mucosa fibroblasts to betel nut alkaloids in vivo may contribute to the accumulation of collagen in Oral submucous fibrosis.[26,27]

From the present study and various studies mentioned above, it can be inferred that advanced disease in OSMF group could be due to lack of awareness among patients and healthcare professionals. Other social and environmental factors like nutritional deficiency deprived socio-economic status, poor education, duration and frequency of betel nut or khara chewing habit may act cumulatively on the severity of OSMF. Increase in clinical grading in young adults is worrisome as there is a likelihood of malignant transformation.[45]

However when the mean serum iron of Group I (OSMF) compared with group III, there was statistically significant difference. This findings was in accordance with several studies.[2,8-10,14,17,18, 28-37]

In the present study and all the various above studies, the level of serum iron was significantly decreased in OSMF patients when compared to controls. This can be due to cytochrome oxidase, an iron dependent enzyme, is required for the normal maturation of the epithelium. In iron deficiency state, levels of cytochrome oxidase are low, consequently leading to epithelial atrophy. An atrophic epithelium makes the oral mucosa vulnerable to the soluble irritants. Further lack of iron in tissues causes improper vascular channel formation resulting in decreased vascularity. This leads to derangement in the inflammatory reparative response of the lamina propria resulting in defective healing and scarification. Thus, the cumulative effect of these initiating and promoting factors leads to further fibrosis, which is a hallmark of OSMF. Fibrosis dictates that OSMF is basically a disorder of collagen metabolism.[2] Hydroxyproline is an amino acid found only in collagen, which is incorporated in the hydroxylated form. This hydroxylation reaction requires ferrous iron and vitamin C. Utilization of iron, for the hydroxylation of proline and lysine, leads to decreased serum iron level.[2,17] In OSMF patients, there is an increase in the production of highly cross-linked insoluble collagen type I, loss of more soluble procollagen type III and collagen type VI. The crosslinking of collagen due to the up-regulation of lysyl oxidase plays a crucial role in

the development and progression of the condition. It is evident that a significant lower level of serum iron in OSMF patients, indicates that as disease progresses, serum iron levels also depletes.[2] Deficiency of vitamin B complex, iron and other trace elements due to nutritional depletion could possibly initiate anaemia and altered cell-mediated immunity, which in turn acts as a promoting factor to this pre-existing pathologic response of the lamina. After a frank establishment of the lesion, anemia may further perpetuate by inadequate intake of food due to fibrosis and trismus.[2] The betel quid and betel nut chewing habits are associated with the disease state, which may play a role in altering the tissue and serum iron levels.[19] So, serum iron levels are considered as biochemical indicators for nutritional assessment and the evaluation of serum iron levels puts the clinician in better position to determine the stage of this precancerous condition and could advise the patients to stop the habit of chewing nut. The lack of iron in the tissues resulted in decreased vascularity which facilitated percolation of arecoline.[28]

When the mean serum iron of Group II was compared with group III, there was statistically significant difference. This finding was in accordance with several studies.[38,39] When the mean serum iron of Group I (OSMF) was compared with group II, there was not statistically significant difference. Basically Group II individuals were habitual without OSMF and were consuming betel nut in one or the other forms. It is found that betel nut and or betel quid consumption prolongs the hunger leading to overall reduction in quantity of food intake. There is a possibility of creating lack of proper nutrients required for the functioning of body, ultimately causing depletion in serum iron levels. Body iron absorption is controlled by the duodenal mucosa which allows the intake of appropriate quantities of iron to balance exactly the required small daily iron loss. If these iron losses are amplified by the disease or if dietary intake and absorption are impaired, a negative iron balance will result. The effects of this negative balance are counter balanced for a short duration time by mobilization of body iron stores, resulting in depletion of tissue iron and the serum iron falls resulting in failure of iron supply to the bone marrow.[14]

When the mean serum vitamin C value of Group I (OSMF) was compared with group II,III there was statistically significant difference. This finding was in accordance with several studies.[2,40,41] In the present study, there was a decrease in serum vitamin C levels in OSMF patients as compared to control group, which suggests that in OSMF patients, vitamin C might have been used for excessive collagen production and cross-linking occurring in OSMF. OSMF is basically a disorder of collagen metabolism where Vitamin C gets utilized in conversion of proline into hydroxyproline, this hydroxylation reaction requires ferrous Iron and Vitamin C.[2,9] Thus vitamin C might be an important indicator for the establishment of OSMF.[9]

When the mean serum vitamin C value of Group II was compared with group III, there was no statistically significant difference. This can be explained by the fact that, in both the group II and group III, the individuals were free of OSMF therefore there might not be an excessive utilization of vitamin C for collagen synthesis.

When the mean salivary iron value of Group I (OSMF) was compared with group III and Group II (OSMF) with group III, there was statistically significant difference. This finding was in accordance with several studies.[19,38,39,41] According to Shetty SR et.al.[41] (2014) the decrease in the salivary iron level could be predominantly due to reduced iron absorption owing to alterations in dietary factors rather than secretion of iron from the salivary gland itself.[41] The reduction in salivary iron could also be due to overall decrease in serum iron.

It can be concluded by the present study and study of Okade AR et.al.[19] (2015) that estimation of salivary iron could be integrated in decision-making allowing it to individualize the treatment protocol of OSMF. It also indicates that, saliva may be used as a potential diagnostic tool which can be efficiently employed to evaluate the trace elements in potentially malignant disorders of the oral cavity.[41] As less iron is incorporated into saliva from the blood through the trans-cellular and para-cellular routes. When the mean salivary iron value of Group I (OSMF) was compared with group II and III, there was statistically non-significant difference. This can be because, Group II individuals were primarily habituals without OSMF and were consuming betel nut in one or the other forms. Therefore what holds true for mean serum iron also stands true for mean salivary iron values.

When the mean salivary vitamin C value of Group I (OSMF) was compared with group II ,Group I with III, and Group II with Group III, there was statistically significant difference. This finding was in accordance with several studies.[31,42] The decrease in salivary Vitamin C levels can be due to poor nutritional status and oxidative stress in precancerous lesions and conditions.[24]

In present study, correlation of serum iron with serum vitamin C in Group I,II and III was not statistically significant ($p>0.05$). But there was a negative correlation seen between serum iron and vitamin C values in I, II and III groups i.e. as levels of serum iron decreases, the levels of serum vitamin C also decreases in all 3 groups. Vitamin C is a powerful enhancer of non-heme iron

absorption. Vitamin C facilitates iron absorption by forming a chelate with ferric iron at acidic pH that remains soluble at the alkaline pH of the duodenum.[43] In this study, group II & III patients were free of OSMF and surplus utilization of iron and vitamin C in excessive collagen synthesis might not have been taken place.

When compared the ratio between the serum vitamin C and serum iron in group I with group II and group I with group III was statistically significant and group II with group III was not statistically significant. Lynch SR and Stoltzfus RJ,[43] (2003) found that cereal foods that contain polyphenols or weaning foods that have significant quantities of soybean flour or soybean protein products, a molar ratio of ascorbic acid to iron of 4:1 should be used and cow's milk and low phytate or dephytinized cereal foods if ascorbic acid and ferrous sulphate are added in an ascorbic acid to iron molar ratio of 2:1.43 The key role of ascorbic acid for the absorption of dietary non-heme iron is generally accepted. The reasons for its action are twofold: (1) the prevention of the formation of insoluble and unabsorbable iron compounds and (2) the reduction of ferric to ferrous iron, which seems to be a requirement for the uptake of iron into the mucosal cells.[44]

In the present study, the correlation of serum iron with salivary iron in group I and II was not statistically significant ($p>0.05$) but there was negative correlation between serum iron and salivary iron, as the levels of serum iron decreases, levels of salivary iron also decreases. This finding was in accordance with several studies.[9,38,39] The importance of salivary iron estimation in OSMF, offers support for the estimation of salivary iron analysis in OSMF however, the salivary iron levels detection is late as compared to serum iron.

The correlation of serum iron and salivary iron value was statistically significant in Group III ($p<0.05$). This can be because both serum iron and salivary iron in Group III (healthy patients) were within their normal range. Salivary iron correlates well with serum iron and a decrease in the salivary iron levels indicates a severe depletion in body iron stores. This decrease in the salivary iron level could be predominantly due to reduced iron absorption owing to alterations in dietary factors rather than secretion of iron from the salivary gland itself.[41]

In present study the correlation of salivary iron (3.43±2.43 µg/dl) with salivary vitamin C (0.19±0.04 mg/ml) in Group I was statistically significant ($p<0.05$), and there was negative correlation in Group I. The findings of the present study thus validate and reinforce that saliva can be used as an efficient, non-invasive; patients friendly tool to measure the oxidative stress in OSMF patients.40 When the ratio between the salivary vitamin C with salivary iron, in group I was compared with group II and group I with group III and group II with group III, it was statistically significant.

In present study the correlation of serum vitamin C with salivary vitamin C with in Group I was not statistically significant ($p>0.05$), but there was a negative correlation seen. The correlation of serum vitamin C with salivary vitamin C in group II and in group III, was statistically significant ($p<0.05$). When the ratio between the salivary vitamin C was compared with serum vitamin C of group I with group II, group I with group III was statistically significant and group II with group III was not statistically significant.

In present study, compare the various grades of OSMF and the mean serum iron and vitamin C value and the mean salivary iron and vitamin C value showed statistically non-significant difference. This can be due to uneven distribution of number of patients according to grading of OSMF (Grade I, II and III).

Overall the present study shows that, there was statistically significant reduction of serum iron and serum vitamin C levels in OSMF patients compared to controls group and similarly, there was statistically significant reduction of salivary iron and salivary vitamin C levels in OSMF patients compared to controls group.

The evaluation of levels of serum iron and vitamin C and salivary iron and vitamin C, puts the clinician in comprehensive position to determine the grades of OSMF and could advise the patients to discontinue the habit of chewing nut. It will generate awareness between the population chewing betel nuts and people who are in contact with habitual persons not to indulge in this habit and stop if they have previously started.

From the present study, it is evident that by estimation of Serum Vitamin C levels in OSMF patients, one can assess the degree of oxidative damage resulted by this disease.

CONCLUSION

In the present study, all the cases of oral submucous fibrosis showed decrease in mean serum iron and vitamin C as well as mean salivary iron and vitamin C levels which suggest that the iron and vitamin C play an important role in the pathogenesis and progression of OSMF.

In group II (Betel nut habituals without OSMF) patients, betel nut quid with or without tobacco or betel nut consumers also showed decrease in mean serum and salivary iron and vitamin C levels which suggest that, betel nut and or betel nut quid with or without tobacco consumption may alter the serum and salivary levels of iron and vitamin C. Betel nut consumptions plays an important role in the pathogenesis and progression of OSMF. Therefore regular monitoring of betel nut habituals should

also be carried out because they are at higher risk of developing OSMF in future.

The present study indicates that saliva also may be used as a potential non-invasive diagnostic tool to evaluate the iron and vitamin C in OSMF patients.

Estimation of iron and vitamin C in OSMF patients would, therefore, help in management, in developing therapies based on the trace element expression.

Overall, salivary iron and vitamin C analysis can play a crucial role in assessment of oxidative stress in OSMF patients and avoiding the possible consequences of malignant transformation.

Scope

In future there is scope for research on pre and post treatment evaluation of serum and salivary iron and vitamin C micronutrient levels in OSMF and its correlation with the molecular markers. Recognition of biochemical modifications in the sera of precancer patients may support in earlier diagnosis and prognosis of these lesions.

Recommendations

Further studies are required on large sample size which will include the uniform distribution of OSMF patient according to the grading. Moreover, serum iron and vitamin C are required to be correlated with molecular marker such as MMPs and TIMPs.

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