Serum aflatoxin B1 antibody titer, percent hemolysis and transaminases in oral submucous fibrosis

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Abstract

Introduction: Areca nut is deeply rooted sociocultural habit in India. Areca nut reported to be infested by fungi during the field and storage conditions. Areca nut alkaloids, nitrosamines, tobacco and aflatoxin are cytotoxic, immunotoxic to red blood cell and epithelial cell. Hence, the present study was conducted to assess the serum aflatoxin B_1 (AFB_1) antibody titer, percent hemolysis and transaminases in oral submucous fibrosis (OSMF) patients.

Materials and Methods: In this study, 128 participants of which 88 were suffering from OSMF. Twenty participants were areca nut habitual without OSMF (habitual control) and 20 participants without any habit (healthy control). For the detection of AFB_1 antibody titer, AFB_1 antigen (Sigma) A6636 from *Aspergillus* flavus was used. Percent hemolysis was estimated as per the procedure described by Mathuria and Verma. Serum Glutamic oxalo acetic transaminase (SGOT) and Serum Glutamic pyruvic transaminase (SGPT) were estimated by the optimized ultraviolet method using the enzyme-linked immunosorbent assay kit.

Results: Mean SGOT, SGPT, percent hemolysis and AFB_1 antibody titer were significantly higher in participants with OSMF than the habitual and healthy controls. AFB_1 antibody titer and % hemolysis in both OSMF and habitual without OSMF were showed significant correlation, i.e., increased AFB_1 antibody titer with increased % hemolysis.

Conclusions: A study result demonstrates that aflatoxin causes increase in serum transaminases which is indicative of liver damage in OSMF. The combined toxic effects of areca nut alkaloids, tobacco and AFB_1 on red blood cell (RBC) cell wall might be responsible for increased percent hemolysis in OSMF and habitual control.

Keywords: Aflatoxin B1, hemolysis, transaminases and oral submucous fibrosis

INTRODUCTION

Oral submucous fibrosis (OSMF) is the morbid condition of areca nut habitual primarily affecting the nonkeratinized oral mucosa form superficial epithelium to the basal layer. It involves any part of the oral cavity from the anterior to posterior oropharyngeal region and sometimes upper aerodigestive tract. Slowly progressive chronic inflammatory change from epithelium to underlying connective tissue causes the deposition of abnormal

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fibroblast which is responsible for rigidity of oral mucosa and atrophic epithelium. Masticatory microtrauma and irritation from the areca nut may be responsible for recurrent vesicle formation. Rigid oral mucosa leads to difficult opening of mouth, inability to blow/whistle, and eat.[1-5] The prevalence of OSMF in India is in the range of 0.2%–1.2%.[4,5] Malignant transformation of OSMF varied from 2.3% to 7.6% in different studies conducted in India.[4-6] Gadbail *et al.* reported early malignant potential with OSMF at the younger age of 25 years and detected the higher expression of marker used for proliferation, neoangiogenesis, and myofibroblast expression in low-risk epithelial dysplasia of OSMF patients which may show higher malignant potential.[7,8] Sarode *et al.* suggested that basal cell proliferation more than 26%–50% in OSMF should be strictly followed for malignant changes.[9]

A multi-factorial pathogenesis is responsible for OSMF but leaching out compounds from areca nut remains the principal causative factor.[10,11] Various studies conducted in India suggested that areca nut (areca catechu) product with or without tobacco, slaked lime is responsible for OSMF.[5,6,12,13]

Areca nut proved to be contaminated by various toxin yielding fungi, including *Aspergillus flavus* in the course of farming and storage.[14] Kolhe and Patil studied the cytotoxicity of aqueous extract of areca nut and tobacco on human erythrocytes red blood cells (RBCs).[15] Therefore, this study was carried out to assess serum aflatoxin B$_1$ (AFB$_1$) antibody titer, percent hemolysis, and transaminases in OSMF patients.

MATERIALS AND METHODS

The present study was conducted in the Department of Oral Medicine and Radiology, SPDC, after approval from the Institutional Ethics Committee of DMIMS (DU), Sawangi (M), Wardha, and Maharashtra. The participants were well informed regarding the study procedures, and their consent was obtained.

Study population: A total 88 clinically diagnosed OSMF individuals, aged 18 years and above were included in the present study by the purposive sampling method. Areca nut habit with or without tobacco for more than 12 months was included in the present study, without any medicinal or surgical intervention in the last 12 months for OSMF.

- Habitual control: Twenty individuals with areca nut chewing habit for more than 12 months with or without tobacco and without OSMF were included in the present study
- Healthy controls: Twenty individuals without areca nut/tobacco/habit were included in the present study.

An exclusion criterion for all three groups was individual suffering from inflammatory disorder, autoimmune and hematological disorders, treated for any malignancy, alcoholic, systemic diseases, and pregnant women. Habitual and healthy controls were selected from the individual accompanying the OSMF individuals.

Habit history related to areca nut chewing was documented in structured format. 5 ml blood was used for the determination of percent hemolysis and AFB$_1$ antibody titer. Obtained serum was transferred to cryotubes with protease inhibitor cocktail (10 µl/ml) for long storage purpose and kept at −20°C. 100 µl aliquots for single use were prepared for each sample to prevent repeated fluctuations in the temperature of cryotubes and further degradation. 0.5 mL of blood was transferred to EDTA containing (40 µL) bulb, mixed well by stirring for 15 s and then kept undisturbed for 15 min.

The percent hemolysis was estimated for each patient as per the procedure described by Mathuria and Verma.[16] The serum SGOT and SGPT were estimated by the optimized UV method using AST kit by Euro Diagnostic Systems and Liquipath (Pathozyme Diagnostics)-SGPT estimation Kit, respectively. Indirect enzyme-linked immunosorbent assay for the detection and quantification of AFB$_1$ antibody was done for all 128 participants as per the protocol and procedure given by Engvall and Perlman[17] and Avrameas.[18] Aflatoxin antigen used for the procedure was AFB$_1$ Antigen (Sigma A6636) from *Aspergillus* flavus.

One-way ANOVA (F-test), Dunnett D test, Z-test, and Pearson's correlation coefficient test were used for the statistical analysis.

RESULTS AND OBSERVATIONS

The mean age of OSMF patients was found to be 29.47 ± 8.52 years. Maximum patients with OSMF were in the range of 21–30 years (*n* = 44) and 31–40 years (*n* = 23). 94.32% (83 out of 88) areca nut chewers with OSMF were males and 5.68% (5 out of 88) were females (M:F ratio of 16.6:1). The mean age of areca nut chewers without OSMF (habitual control) was 34.05 ± 11.37 years with male:female ratio of 9:1, while that of the individuals in healthy control was 23.5 ± 1.40 years.

In OSMF group, 4 (4.54%) of the 88 patients were only areca nut habitual. 63 (71.59%) of the 88 individuals were kharrha

chewers (manually prepared mixture of fully ripened areca nut pieces, tobacco and lime). Three (3.40%) individuals were gutkha chewers. 14 (15.90%) individuals chewed both gutkha and kharrha. 4 (4.54%) chewed paan-containing areca nut. All twenty individuals were habitual of kharrha in habit without OSMF group. In OSMF group, the mean duration of habit was 7.95 ± 6.61 years and in areca nut chewers without OSMF (habitual control) 11.7 ± 7.35 years. Mean inter-incisal opening in OSMF group was 24.68 mm. Mean daily frequency in OSMF was 6.44 ± 1.29 and in habit without OSMF 6.45 ± 2.15. Amount of areca/tobacco consumed in OSMF was 24.47 ± 3.31 grams per day and in habit without OSMF 24.51 ± 4.23 per day [Table 1].

Mean SGOT level in participants of OSMF was 33.90 ± 13.23 IU/L, with areca nut habit without OSMF (habitual control) was 27 ± 8.30 IU/L, and in the healthy control, it was 25.62 ± 9.07 IU/L. One-way ANOVA showed a significant variation in serum SGOT levels amongst the groups (F = 5.32, $P = 0.006$) and Dunnet D test revealed remarkable contrast between OSMF and healthy control groups ($P = 0.012$) [Table 2]. Mean SGPT level in the participants of OSMF was 28.32 ± 12.82 IU/L, in areca nut chewers without OSMF was 21.82 ± 6.02

IU/L, and in healthy control, it was 20.96 ± 8.01 IU/L. One-way ANOVA revealed notable variation in the SGPT levels in the three groups (F = 5.12, $P = 0.07$). Dunnett D test revealed significant difference between OSMF and healthy control group ($P = 0.018$) [Table 3].

Mean percent hemolysis in OSMF group was 6.09 ± 8.38; in areca nut habitual without OSMF 3.01 ± 2.27, and in healthy control, it was 1.06 ± 1.02. One-way ANOVA showed significant variation in % hemolysis in three groups (F = 4.15, $P = 0.018$). Dunnett D test for comparisons between the three groups showed significant difference in percent hemolysis between the OSMF group and healthy control group ($P = 0.021$) [Table 4].

Mean serum AFB$_1$ antibody titer in participants of OSMF was 0.67 ± 0.24; in areca nut habit without OSMF was 0.47 ± 0.15, and in the healthy control, it was found to be 0.34 ± 0.07. One-way ANOVA suggested remarkable variation in the serum AFB$_1$ antibody titer among the three groups (F = 22.29, $P = 0.00$). Dunnett D test for multiple comparisons revealed a significant difference between the AFB$_1$ antibody titer of OSMF and healthy controls [Table 5].

Table 1: Characteristics of study and control group: descriptive statistics

Characteristics	OSMF (*n*=88)	Habit without OSMF (habitual control) (*n*=20)	Healthy control (*n*=20)
Age (years), mean±SD	29.47 ±8.52	34.05±11.37	23.5±1.40
Gender (%)			
Male	83(94.32)	18 (90)	20 (100)
Female	5 (5.68)	02 (10)	00
Male:female ratio	16:1	9:1	
Type of habit (%)			
Only areca nut	4 (4.54)		
Only Kharrha	63 (71.59)	20 (100)	
Only Gutkha	3 (3.40)		
Kharaa + Gutkha	14 (15.90)		
Pan quid with areca nut	4 (4.54)		
Frequency per day, mean±SD	6.44±1.29	6.45±2.15	
Amount of betel nut/tobacco consumed per day (g), mean±SD	24.47±3.31	24.51±4.23	
Duration of habit (years), mean±SD	7.95±6.61	11.7±7.35	
Mean inter-incisal distance (mm)	24.68±6.02		

SD: Standard deviation, OSMF: Oral submucous fibrosis

Table 2: Comparison of serum glutamic oxaloacetic transaminase in three groups

Descriptive statistics								
Group	*n*	Mean (IU/L)	SD	SE	95% CI for mean		Minimum	Maximum
					Lower bound	Upper bound		
OSMF	88	33.90	13.23	1.41	31.10	36.71	13.90	77.80
Habit without OSMF (habitual control)	20	27.35	8.30	1.85	23.46	31.24	14.20	41.70
Healthy control	20	25.62	9.07	2.02	21.37	29.86	14.60	49.10
One-way ANOVA								
Source of variation		Sum of squares	df	Mean square	F		P	
Between groups		1543.64	2	771.82	5.325		0.006 (significant), *P*<0.05	
Within groups		18117.97	125	144.94				
Total		19661.61	127					

SD: Standard deviation, SE: Standard error, CI: Confidence interval, OSMF: Oral submucous fibrosis

Table 3: Comparison of serum glutamic pyruvic transaminase in three groups

		Descriptive statistics						
Group	*n*	Mean (IU/L)	SD	SE	95% CI for mean		Minimum	Maximum
					Lower bound	Upper bound		
OSMF	88	28.32	12.82	1.36	25.61	31.04	11.70	78.10
Habit without OSMF (habitual control)	20	21.82	6.02	1.34	19.00	24.63	12.70	30.40
healthy control	20	20.96	8.01	1.79	17.21	24.71	11.30	35.90
		One-way ANOVA						
Source of variation		Sum of squares	df	Mean square		F		P
Between groups		1329.85	2	664.92		5.12		0.007 (significant), P<0.05
Within groups		16215.33	125	129.72				
Total		17545.19	127					

SD: Standard deviation, SE: Standard error, CI: Confidence interval, OSMF: Oral submucous fibrosis

Table 4: Comparison of percentage hemolysis in three groups

		Descriptive statistics						
Group	*n*	Mean	SD	SE	95% CI for mean		Minimum	Maximum
					Lower bound	Upper bound		
OSMF	88	6.09	8.38	0.89	4.32	7.87	0.20	68.50
Habit without OSMF (habitual control)	20	3.01	2.27	0.50	1.95	4.08	0.90	10.50
Healthy control	20	1.60	1.02	0.22	1.12	2.08	0.30	4.10
		One-way ANOVA						
Source of variation		Sum of squares	df	Mean square		F		P
Between groups		414.39	2	207.19		4.15		0.018 (significant), P<0.05
Within groups		6235.29	125	49.88				
Total		6649.69	127					

SD: Standard deviation, SE: Standard error, CI: Confidence interval, OSMF: Oral submucous fibrosis

Pearson's correlation coefficient for percent hemolysis and AFB$_1$ antibody revealed significant positive correlation ($r = 0.560$, $P = 0.000$). It indicates that increased titer of AFB$_1$ antibody in OSMF patient results in increased RBC destruction [Table 6]. Similarly, positive connection between the percent hemolysis and levels of AFB$_1$ antibodies was found in areca nut habit without OSMF ($r = 0.593$, $P = 0.006$) [Table 7].

DISCUSSION

OSMF is predominantly seen in user of areca nut.[10,12,13,19] It is a serious morbid and gradually developing disease.[3,5,6] Marked rigidity of oral mucosa is due to the abnormal deposition of fibroblasts in underlying connective tissue causing, a gradually developing reduction in opening of the mouth. Areca nut chewer encountered with burning sensation, intermittent oral mucosal ulceration of soft palate, and lips.[3] There is a strong association of chewing areca nut and occurrence of OSMF, leukoplakia, and oral cancer.[2,6,20,21] The peculiarity of the disease is that it is confined to a particular geographic region because of prevalent dietary or cultural habits. In the Indian population, areca nut is mostly chewed in combination with tobacco. The frequency of chewing was directly correlated

with OSMF.[22] Comprehensive experimental studies have proved the mutagenicity and carcinogenicity of areca nut.[19,23,24] Mainly, arecoline and arecadine from the areca nut are responsible for abnormal fibroblast growth and collagen synthesis which leads to sequential alteration in the connective tissue in OSMF.[25-28] Cross linking of collagen occurs through lysyl oxidase and further get stabilized by areca nut flavonoids, chatechin, and tanin.[29,30]

The present study revealed that it is the disease of younger age group and predominantly affects male compared to female which is comparable to previous studies.[31-35] All OSMF individuals and habitual controls were areca nut users, either alone or with combination of tobacco, lime, or piper betel leaf. Majority of the study population (71.59%) was habitual of kharrha. Kharrha is a manual preparation which contains the pieces of matured white half cut areca nut mixed with tobacco and lime. Only three (3.41%) patients consumed Gutkha. The mean duration of chewing areca nut in any form was 7.95 ± 6.61 years. Mean frequency of consumption was 6.44 ± 1.29 per day, and amount of daily consumption in any form was 24.47 ± 3.31 g. Shah and Sharma reported that daily consumption was more significant than total duration of habit.[22] A similar finding was also reported by Maher *et al*. who stated that

Table 5: Comparison of Aflatoxin B$_1$ antibody titer in three groups

Group	n	Mean	SD	SE	95% CI for mean		Minimum	Maximum
					Lower bound	Upper bound		
OSMF	88	0.67	0.24	0.02	0.62	0.72	0.23	1.58
Habit without OSMF (habitual control)	20	0.47	0.15	0.03	0.40	0.54	0.23	0.80
Healthy control	20	0.34	0.07	0.01	0.31	0.37	0.21	0.49

One-way ANOVA					
Source of variation	Sum of squares	df	Mean square	F	P
Between groups	2.08	2	1.04	22.29	0.000 (significant), $P<0.05$
Within groups	5.83	125	0.04		
Total	7.91	127			

SD: Standard deviation, SE: Standard error, CI: Confidence interval, OSMF: Oral submucous fibrosis, Df: Degrees of freedom

Table 6: Correlation of serum Aflatoxin B$_1$ antibody titer and percentage hemolysis in oral submucous fibrosis

Pearson's correlation coefficient			
	Mean±SD	n	Correlation "r"	P
Percentage hemolysis	6.09±8.38	88	0.560	0.000 (significant), $P<0.05$
Aflatoxin B$_1$ antibody titer	0.67±0.24	88		

SD: Standard deviation

Table 7: Correlation of serum Aflatoxin B$_1$ antibody titer and percent hemolysis in betal nut habitual without oral submucous fibrosis (habitual control)

Pearson's correlation coefficient			
	Mean±SD	n	Correlation "r"	P
Percentage hemolysis	3.01±2.27	20	0.593	0.006 (significant), $P<0.05$
Aflatoxin B$_1$ antibody titer	0.47±0.15	20		

SD: Standard deviation

daily consumption is a high risk factor than the life-long duration of the habit.[36] However, the present study in agreement with Ahmed *et al.*, who reported that continuous user of areca nut for more than 5 years have greater chance to suffer from OSMF.[37]

Mean serum SGOT and SGPT were increased in OSMF, and a positive correlation was found when compared with habitual and healthy controls. CD Anuradha and C. S. Shyamala Devi revealed a significant increase in the transaminase levels in OSMF patients.[38] Choube and Bhowate reported increased transaminase levels, alkaline phosphatase bilirubin, and HCV RNA in OSMF and OSMF with oral lichenoid lesion.[39] A significant increase in liver enzymes in areca nut users considered a high risk for developing liver cancers and liver cirrhosis as evidenced by studies conducted in Thailand.[40-42] Hepatotoxicity and hepatocarcinogenicity of areca nut and its metabolites are attributed to high content of safrole, alkaloids, and nitrosamines and its interaction with bacterial nitrite

compounds while chewing and swallowing.[42,43] Areca nut specific nitrosamines may methylate and cyanoethylate liver DNA which is genotoxic to hepatocytes.[42] Lysyl oxidase has also been implicated in other fibrotic diseases, liver fibrosis, and scleroderma.[40,41,44] Nonessential suppression of matrix metalloproteinase enzymes leads to extensive deposition of collagen similar to OSMF and arecoline and safrole synergistically increase expression of MMP-9 (TIMP-1).[45] Increased TIMP-1 in circulation leads to not only fibrosis of the oral cavity but could be implicated for the fibrotic changes in the liver.[42]

AFB$_1$ antibody titer and % hemolysis in both OSMF and habitual control OSMF were showed significant correlation, i.e., increased AFB$_1$ antibody titer with increased % hemolysis [Tables 6 and 7]. AFB$_1$ is a well-documented immunotoxic,[14,46] hepatotoxic,[42,47,48] mutagenic,[49] and emrbryotoxic.[50] Misara and Raisuddin reported that aflatoxin and other mycotoxins infested in areca nuts may regulate the toxic potential when consumed with tobacco.[14] Raisuddin and Misara reported that 37.5% of Indian areca nut are contaminated by Aflatoxin, particularly AFB$_1$ favored by its nutritive constituents.[51] Lipid and moisture in areca nut are abundant to encourage the growth of fungi.[52] A serum level of Aflatoxin is directly proportional to the amount of aflatoxin consumed and individual host response. It has been reported that both metabolized (aflatoxicol, M$_1$ and M$_2$) and unmetabolized (B$_1$, B$_2$, G$_1$, and G$_2$) form of Aflatoxin can get excreted in the saliva, stool, urine, and milk. Swallowed salivary Aflatoxin might be absorbed from the gastrointestinal tract and again comes into blood circulation responsible for recycling of aflatoxin in the body.[49] Circulating aflatoxin binds with albumin and to forms aflatoxin albumin-adduct responsible for liver mutagenesis.[53] These aflatoxin albumin adduct represent cumulative amount which is responsible for prolonged toxicity of aflatoxin even after cessation of exposure.[46,49] Lin *et al.* reported that the use of AFB$_1$ invaded areca nut

suppress cell-mediated immune response of the body leading to the increased risk of HBV/HCV infection and perturbed copper metabolism in the liver.[41]

Kolhe and Patil studied the dose-dependent cytotoxic effects of areca nut and tobacco extract on human erythrocytes RBCs and reported cytotoxic effect on cell wall causing RBC lysis.[15] Verma and Raval reported cytotoxic effect of aflatoxin on RBCs and showed that AFB$_1$ causes morphological alterations and hemolysis of human RBCs.[54] Various studies have indicated that greater concentration of aflatoxin in circulation may be responsible for self-destruction of RBCs. On the other hand, lower concentration results in morphological changes and elimination of RBCs in reticuloendothelial tissues.[16,53,54] Liberated iron after RBCs hemolysis may not be available for body utilization. It may deposit in the reticuloendothelial system or form the complexes with haptoglobulin leading to anemia.[55,56]

Fully ripened white half cut areca nut favored the infestation of *Aspergillus* flavus due to its composition and poor storage condition.[51,57] Mathew *et al.* conducted a study on the effect of copper-based fungicide and Bordeaux mixture on copper content of areca nut. He reported that increased concentration of copper in ripened areca nut is due to its less moisture content compared with unripe areca nut.[58] The above facts demonstrate that the toxic component of areca nut, tobacco and Aflatoxin which target the hepatocytes as indicated by raised serum transaminases indicative of liver damage in OSMF. The combined effects of arecoline and AFB$_1$ on RBC might be responsible for increased percent hemolysis in OSMF and habitual control group [Figure 1]. This might be due to the daily and prolonged consumption of areca nut contaminated with *Aspergillus* Flavus species. Hemolysis in disease condition may aggravate its cytotoxic and immunotoxic effect on oral mucosa due to liberated free iron. This increases the cellular damage by increasing oxidative stress.[59] Copper content of areca nut and free liberated iron during hemolysis perturbed Iron and copper metabolism in areca nut habitual increases the potential of malignant transformation in OSMF.

CONCLUSION

The present study showed the significant correlation between increased AFB$_1$ antibody titer and percent of hemolysis of RBCs in OSMF, habitual control and healthy control group. This indicates that area nut alkaloid and aflatoxin might be responsible for altered metabolism of iron and copper, increases oxidative stress, hypoxia and toxicity to oral epithelium causing increased
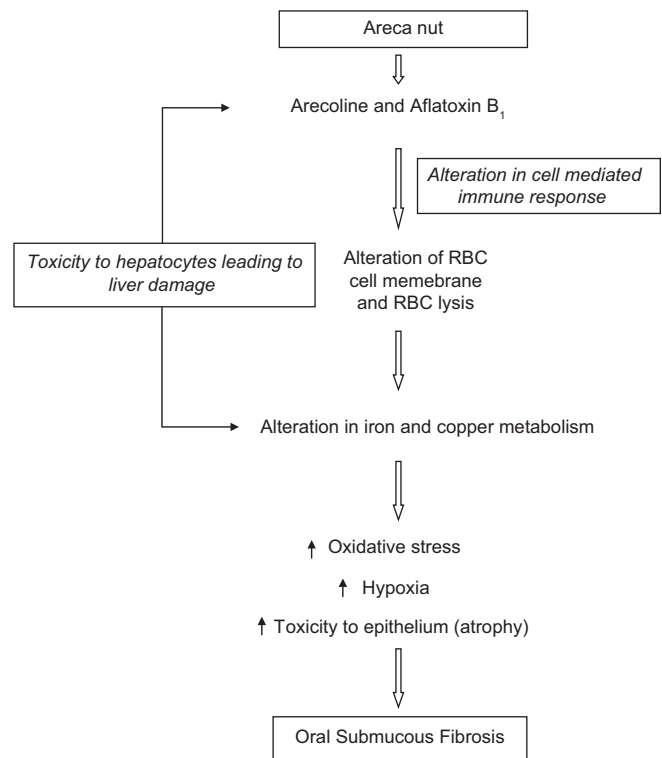

Figure 1: Role of area nut alkaloids and aflatoxin B1 in the progression of oral submucous fibrosis

chance of malignant potential in preexisting OSMF condition. Clinician treating the cases of OSMF must consider the alteration of iron and copper metabolism in planning the treatment protocol and should consider the hepatoprotective therapeutic agent considering ensuing hepatotoxicity due to arecholine and aflatoxin.

Conflicts of interest

There are no conflicts of interest.

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