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Evaluation of Serum Proteins in OSMF Separated by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Chinar Fating[1*] Rolly Gupta[2] Mekhala Lanjewar[3] Anjan Basak[4]

[1]Reader, Department of Oral Medicine and Radiology, Chhattisgarh Dental College and Research Institute, Rajnanagaon, Chhattisgarh, India.
[2]Reader, Department of Oral and Maxillofacial Pathology and Microbiology, Chhattisgarh Dental College and Research Institute, Rajnanagaon, Chhattisgarh, India.
[3]Private Consultant, Department of Oral and Maxillofacial Pathology and Microbiology, Bilaspur, Chhattisgarh, India.
[4]Professor and Head, Department of Biochemistry, Jawaharlal Nehru Medical College, Wardha, Maharashtra, India.

ABSTRACT

Aim: Evaluation of serum proteins was done in oral submucous fibrosis (OSMF) patients of different clinical stages and healthy controls.

Materials and Method: Serum samples of 40 diagnosed cases of OSMF without any other systemic disease and age and sex matched 40 normal controls were subjected to SDS-PAGE on a 10% resolving gel, followed by staining with Coomassie Brilliant Blue R-250. Protein fractions were analyzed using computer software “Alpha Digi Doc 1201”.

Results: The OSMF group showed increased mean total protein i.e. 7.69 g/dl when compared with control group i.e. 7.52g/dl. The serum sample of all patients showed altered protein profile for protein fractions ranging in molecular weight 136kDa, 120kDa and 93kDa, when they were subjected to SDS-PAGE and gels were analyzed. The milder grades of OSMF showed overexpression for the above mentioned protein fractions and severe grade which is accompanied by increased duration, frequency and amount of betel nut consumption, these protein fractions were observed to be consistently absent or under expressed.

Conclusion: The above result establishes the fact of alteration in serum protein profile in OSMF. The preliminary analysis of serum protein profile may act as diagnostic marker in oral Submucous fibrosis, a premalignant condition with a marked malignant potential. As our results specify that in severe grades of OSMF there is decreased or loss of particular protein fractions so this could also predict the prognosis and progression of the condition to malignancy with greater accuracy.

Keywords: Oral submucous fibrosis, serum protein profile, SDS-PAGE.

INTRODUCTION

OSMF is a well established precancerous condition affecting the oral mucosa. Its literature dates back to as far as 2500 to 3000 BC when a renowned Indian physician Sushruta mentioned about a condition – “Vidari” which simulates OSMF.1 The incidence of OSMF, chiefly in the younger generation is on the upsurge and occurs with a high prevalance in India and South East Asian countries. Etiological factors hypothesized to trigger the disease process include areca nut chewing, nutritional deficiencies, immunologic processes and genetic predisposition. 2,3. However leaching out compounds from areca nut remains the main etiology for OSMF. It has been estimated that worldwide 6, 00,000,000 people chew areca nut.4

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***Correspondence Dr. Chinar Fating.**
Department of Oral Medicine and Radiology, Chhattisgarh Dental College and Research Institute, Rajnanagaon, Chhattisgarh, India.
Email: chinarfating@rediffmail.com

Chewing areca nut in its various form is widely prevalent in Indian subcontinent giving rise to increased prevalence of

OSMF.[1,5]

The precancerous nature of OSMF was first reported by J.C Paymaster (1956)[6] who described the development of slow growing squamous cell carcinoma in one third of the cases of OSMF seen among 650 patients in Bombay. leukoplakia in 26.9% of 101 OSMF. J.J Pindborg (1965)[7] demonstrated higher incidence of patients from North India. Malignant transformation of OSMF varied from 2.3%-7.6% in different studies conducted in India. [8,9,10] Discrimination between OSMF and OSMF transforming into malignancy is difficult purely on clinical grounds. Additional improvement in early detection, diagnosis and prognosis will likely require the analysis of a panel of protein biomarkers.

Proteins initiate all cell functions and pathways, identifying differentially expressed proteins between normal and pathological state, leading to a better understanding of the cellular mechanisms involved in disease. Some proteins are down-regulated and others are up-regulated with the onset of disease, depending on a protein's specific function, undergoing disease-specific

posttranslational modifications[11]. This identification of changes in protein profile that occur during pathological condition could lead to the discovery of protein biomarkers and novel strategies for the improvement of early detection, diagnosis and treatment of OSMF. Early detection and intervention will minimize incidences of malignant transformation thereby reducing mortality rate. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a versatile and powerful technique widely used for protein separation based on their molecular weights[11]. In this study protein fractions were studied by SDS-PAGE due to its importance in the resolution of serum proteins. In this study the aim of performing SDS-PAGE was to make a comparison between protein profiles found in healthy subjects and OSMF cases and to identify some novel proteins that are lost or changed (raised or lowered) in concentrations in diseased subjects.

MATERIALS AND METHODS

This study was undertaken in the Department of Oral Medicine and Radiology, Sharad Pawar Dental College & Hospital, Sawangi (Meghe), Wardha, Maharashtra in association with Department of Biochemistry, Jawaharlal Nehru Medical College, Sawangi (Meghe) Wardha, Maharashtra. The study protocol was approved by institutional ethical committee . After obtaining written informed consent, clinical profile of the pateints was worked out by taking thorough case histoy and clinical examinaton. Interincisal opening, duration and frequency of betelnut chewing were noted for all the pateints. This study consists of clinicopathologically diagnosed 40 cases of OSMF. Depending on the interincisal opening and degree of fibrosis these 40 cases were divided into grade I, grade II and grade III. The mouth opening (interincisal distance of maxillary and mandibular incisors at maximum possible mouth opening) was measured and graded as follows : grade I (>40mm) , grade ii (20 -39 mm) and grade iii(<19mm)[3]. In 40 patients 95% were having mixed habit (Kharra, Gutka, tobacco/lime and betelnut/fennel). Only 5% were arecanut chewers.

Inclusion criteria

The patients with signs and symptoms of OSMF, aged 18 years and above, with betel nut chewing habit more than 6 months with or without tobacco were included in the present study. The control group included 40 subjects (free from betel nut, tobacco habit and systemic disease) with age and sex matched healthy volunteers.

Exclusion criteria

Patients with infectious & inflammatory disease affecting the oral cavity or TMJ may also lead to trismus eg. Myofacial pain dysfunction syndrome, TMJ arthritis & arthralgia, Trauma to maxillofacial skeleton, patient with systemic diseases like CVS, RS, Endocrine, GIT, Liver, Kidney, autoimmune and hematological disorders and patient suffering/treated case of any malignancy. 5ml Blood samples of 40 diagnosed cases of OSMF were collected. Age and sex matched 40 healthy subjects were included from general community as control group. Sera were separated and stored at -20 C till analysis.

Table 1: Total Serum Proteins in OSMF Patients.

Patient number	Total protein (g/dl)	Duration of habit (years)	Amount of betel nut consumed (g)
1	6.0	3	9
2	7.13	20	18
3	7.12	4	48
4	9.34	4	36
5	7.04	25	75
6	7.33	27	12
7	8.43	4	12
8	8.48	11	36
9	6.71	10	21
10	8.63	12	12
11	7.90	3	18
12	8.04	9	9
13	7.43	7	15
14	7.63	20	18
15	7.18	7	-
16	5.37	3	12
17	11.98	2	60
18	8.21	16	12
19	9.45	20	18
20	9.44	14	54
21	7.47	4	45
22	7.88	6	18
23	8.14	14	36
24	7.66	16	24
25	7.24	4	30
26	7.55	4	18
27	7.80	10	30
28	7.59	4	21
29	7.28	3	15
30	7.60	2	51
31	6.71	15	12
32	7.63	32	
33	6.77	10	60
34	7.66	1	30
35	7.24	6	30
36	7.55	5	27
37	7.22	4	66
38	6.98	1	45
39	7.46	6	18
40	7.52	3	30
Normal	7.52		

Table 2: Serum Proteins Expression in OSMF Patients by SDS-PAGE.

						10% gels						
		136 kDa protein			120 KDa protein			97 kDa protein				
Patientsnumber	over	expressed	Underexpressed	Absent	overexpressed		Underexpressed	Absent	overexpressed	Underexpressed		absent
1	–		–	–	–		–	–	–	–		–
2	–		–	√	–		–	–	–	–		–
3	–		–	–	–		–	√	–	–		–
4	–		–	–	–		–	√	–	–		–
5	–		–	–	–		–	√	–	–		–
6	–		–	–	–		–	–	–	–		–
7	–		–	–	–		–	–	–	–		–
8	–		–	–	–		–	–	–	–		–
9	–		–	–	–		√	–	–	√		–
10	–		–	–	–		–	–	–	√		–
11	–		–	–	√		–	–	–	–		–
12	–		–	–	–		–	√	–	√		–
13	–		–	–	√		–	–	–	–		–
14	–		–	–	–		–	√	–	–		–
15	–		–	–	√		–	–	–	–		–
16	–		–	–	–		–	√	–	√		–
17	–		–	–	–		–	–	–	–		–
18	–		–	–	–		–	–	–	–		–
19	–		–	–	–		√	–	–	√		–
20	–		–	–	–		–	–	–	–		–
21	–		–	–	–		–	–	–	–		–
22	–		–	–	–		–	√	–	–		–
23	–		–	–	–		–	–	–	–		–
24	–		–	–	–		–	–	–	–		–
25	–		–	–	–		–	–	–	√		–
26	–		–	–	√		–	–	√	–		–
27	–		–	–	√		–	–	√	–		–
28	–		–	–	–		–	√	–	–		–
29	–		–	–	–		√	–	–	–		–
30	–		–	–	√		–	–	–	–		–
31	–		–	–	–		–	√	–	–		–
32	–		–	–	√		–	–	–	–		–
33	–		–	–	–		√	–	–	–		–
34	–		–	–	–		–	–	–	–		–
35	–		–	–	–		–	–	–	–		–
36	–		–	–	–		–	–	–	–		–
37	–		–	√	–		–	√	–	–		–
38	–		–	–	–		–	–	–	–		–

39	–		–	–	–		–	–	–	–		–
40												

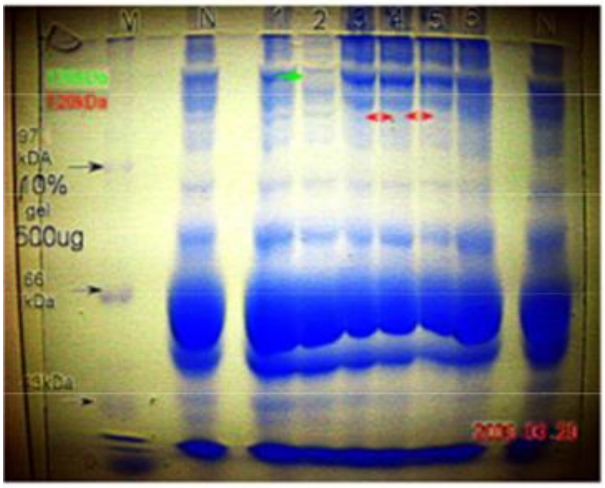

Fig 1: Serum protein profile of control (N) and cases (1-6) resolved on 10% resolving Gel by SDS-PAGE. M: Protein size markers (from top to bottom): 136, 120, 97, 66, 43 kDa. Serum protein 136 kDa is found missing in patient 2, serum protein 120kDa is found missing in patient 4 and 5.

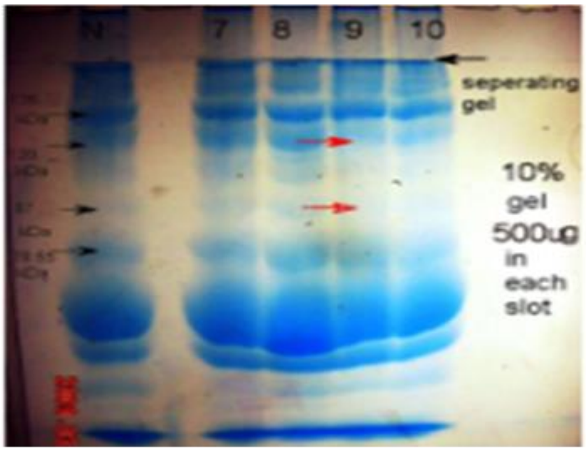

Fig 2: Serum protein profile of control (N) and cases (7-10) resolved on 10% resolving Gel by SDS-PAGE. M: Protein size markers (from top to bottom): 136, 120, 97, 66, 43 kDa. Serum protein 120kDa and 94kDa is underexpressed in patient 9.

REAGENT PREPARATION

12% resolving gel

8mls were prepared by dissolving 30% acrylamide-bisacrylamide, 3.35ml of 3M Tris-HCl (pH-8.8),

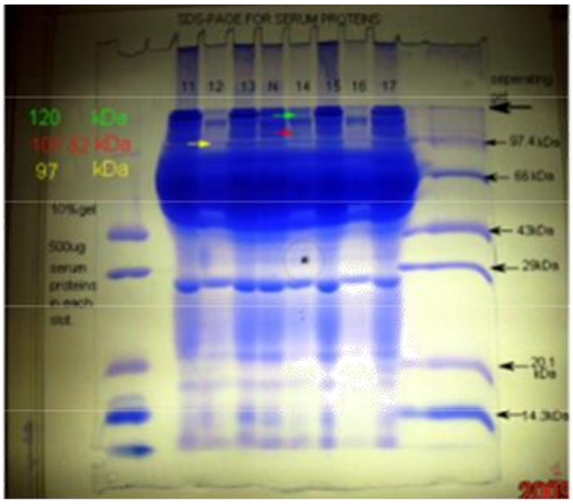

Fig 3: Serum protein profile of control (N) and cases (11-17) resolved on 10% resolving Gel by SDS-PAGE. M: Protein size markers (from top to bottom): 120, 101.32, 97, 66, 43 kDa. Serum protein 120kDa is overexpressed in pateint 11,13and 14and is absent in patient 12,14 and 16. Serum protein 97kda is underexpressed in patient 12 and 16.

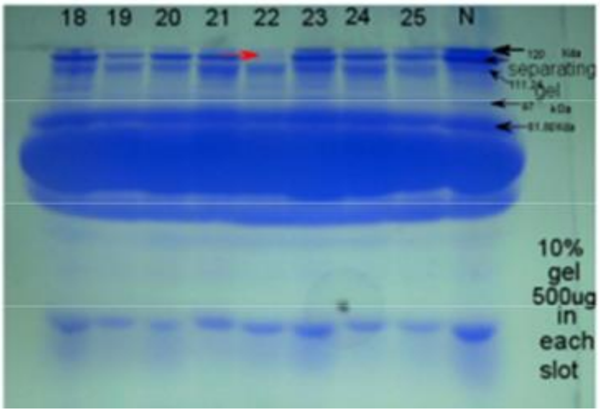

Fig 4: Serum protein profile of control (N) and cases (18-25) resolved on 10% resolving Gel by SDS-PAGE. M: Protein size markers (from top to bottom): 120, 111, 97, 66 kDa. Serum protein 120kDa is absent in pateint 22 and underexpressed in patient 19. Serum protein 97kda is underexpressed in patient 19 and 25.

0.2ml of 10% SDS in 8.45ml of distilled water, followed by 4ul TEMED and 65ul of 10% ammonium persulfate.

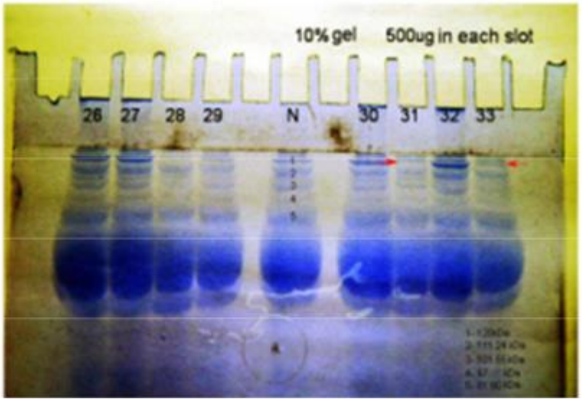

Fig 5: Serum protein profile of control (N) and cases (26-33) resolved on 10% resolving Gel by SDS-PAGE. M: Protein size markers (from top to bottom): 120, 111, 97, 66 kDa. Serum protein 120kDa is overexpressed in pateint 26 ,27,30and 32 and absent in patient 28and 31. Serum protein 97kda is overexpressed in patient 26 and 27.

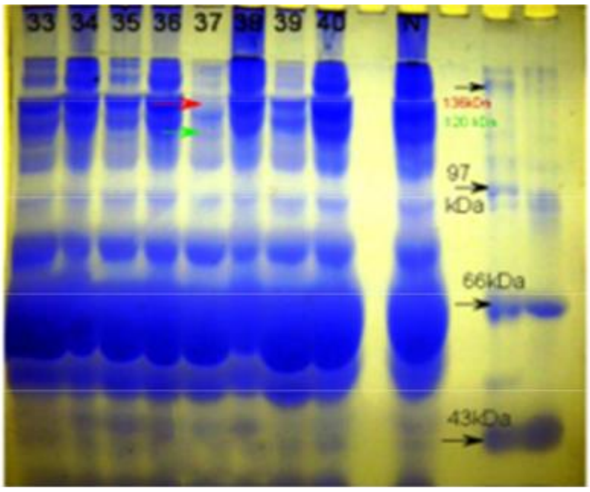

Fig 6: Serum protein profile of control (N) and cases (33-40) resolved on 10% resolving Gel by SDS-PAGE. M: Protein size markers (from top to bottom): 136, 120, 97, 66 and 43kDa. Serum protein 136kDa is absent in patient 37. Serum protein 120kDa is underexpressed in pateint 33and absent in patient 37,

Stacking gel

It was prepared by dissolving 0.9ml of 30% acrylamide-bisacrylamide, 55µl 10% SDS, 0.35ml of 1M Tris-HCl (pH 6.8) in 4.9ml distilled water followed by addition of 38µl bromophenol blue, 5µl TEMED and 55µl 10% ammonium persulfate.

Tris-glycine buffer

15g of trizma base, 72g of glycine and 5g SDS were dissolved in distilled water and final volume was made up to 1000ml. The solution was 5x diluted before electrophoresis.

Staining solution

0.5g Coomassie Brilliant Blue R-250 were dissolved in 450ml methanol and then mixed with 90ml glacial acetic acid and 450ml distilled water.

Destaining solution

It was prepared by mixing 300ml (30%) methanol, 100ml (10%) acetic acid and 600ml water. Since equal quantities of protein from each sample under comparison must be loaded on to gels for electrophoretic analysis, so the total protein content of each serum sample (cases and controls) was estimated by colorimetric analysis and 500µl protein was added in each slot. In each group serum samples of control and OSMF cases were separated by loading in their respective wells. The gel was then electrophoresed at constant supply of 12mA and voltage of

150V in a minicold lab maintained at 4 C. The gel was stained using Coomassie Brilliant Blue R-250 and then destained. Protein fractions appeared as dark bands on a light background. Gels were photographed using digital camera and their images were formatted and analysed by using computer software program “Alpha Digi Doc 1201”. The molecular weight of each protein fraction was determined by using molecular weight markers as standard. The raw volume was calculated by the software using band height and intensity, which was a measure of relative quantity of protein in each sample.

RESULTS

In the present study mean total proteins were 7.69 g/dl in OSMF and 7.52g/dl in control group. The maximum amount of protein in a patient sample was 11.98 g/dl and minimum was found to be 5.37 g/dl. The present study reported increased total proteins level in 25% of OSMF patients (Table 1). In the present study, all patient samples showed altered protein profile i.e. under expression, over expression or no expression for protein fractions ranging in molecular weight 136kDa, 120kDa and 97kDa, when they were subjected to SDS-

PAGE.(Table 2). 5% pateint samples (sample 2, sample 37) [figure1 and 5] showed no protein expression in 136kDa range and rest 95% showed normal expression. In 120kDa range, 17.5% patient samples (sample 11, 13 ,15, 26 ,30 and 32) showed overexpression, 10% patient samples (sample 9, 19, 29 and 33) showed under expression whereas 25% of patient samples (sample 3, 4, 5, 12, 14, 16, 22, 28, 31 and 37) showed no expression respectively. In 93kDa range only 5% samples (sample 26 and 27) showed over expression, 15% samples (sample 9, 10, 12, 16, 19 and 25) showed under expression and rest 80% showed normal expression. (figure 1-6).

DISCUSSION

Patients with OSMF have an assertive habit history of areca nut chewing, and a clear dose dependent relation is observed for both frequency and duration of chewing areca nut[12]. OSMF does not regress and there is no known cure .1 In India most of the habitual chewers of betel nut use tobacco. A malignant transformation rate of 7.6% in an Indian cohort over a period of 17 years has been reported.[10] The direct contact and continuous irritation from the various components of the areca nut products to the oral tissues produces a hypersensitivity reaction in the oral mucosa, thus initiating the disease. The period between initiation of the chewing habit and the development of clinical symptoms of OSMF varies tremendously ranging from a few months to several decades, depending on the type and duration of the areca nut chewing habit. Soluble irritants, such as alkaloids present in areca nut act as initiating factor causing a juxta-epithelial inflammatory reaction thus leading to burning sensation, vesiculation and ulceration of the oral mucosa, which renders a phase for difficulty Protein biomarkers are very useful for diagnosis and prognosis of various forms of cancers and other diseases. Certain proteins can be up or down regulated during disease progression. Detection of various protein profiles, as a function of disease progression is important for the prognosis of any disease. To distinguish between normal, OSMF and OSMF cases transforming to malignancy, a qualitative analysis of Coomassie Brilliant Blue-stained proteins separated by SDS-PAGE was undertaken in this study.

In the present study protein expression pattern in gels and their relation with type of habit, duration, frequency and amount of betelnut consumed by patient was correlated. (table 1) In range of 136kDa protein fraction, the expression was absent in 5% of patients ,who were habitual chewer's of kharra with mean duration of 12 years, for 12-15 times /day and consumed betel nut approximately 27 g/day. In 120 kDa protein range 17.5% showed over expression with mean duration of their habit since 8.71 years. All were habitual chewer's of kharra and ghutka 9-10 times /day and consumed approximately 21 g/day of betel nut. Under expression of proteins was found in 10% patient with mean duration of their habit[10]. 75 years, with habit of chewing kharra in mean frequency of 8 times/day in mean quantity of 28.5 g/day. No expression of protein was found in 25% patients in range of 120 kDa, of which all patients were habitual of chewing kharra/gutka or both with mean duration of 9.5 years, mean frequency was 10.5 times/day in the mean quantity of 31.5 g/day.

In a range of 97kDa protein 5% showed over expression of bands, with habit of eating kharra and gutka in quantity of 24 g/day, mean frequency was 8 times/day and mean duration was 7 years. Under expression of bands was found in 15% patients with mean quantity of betelnut intake 17 g/day for mean duration of 9.6 years and with frequency of 5.6 times/day. In the present study, altered protein profile was observed for protein fractions ranging in molecular weight 136kDa, 120kDa and 97kDa, which are belonging to higher molecular weight proteins. In previous studies conducted, protein fraction with molecular weight ranging 107 to 331kDa is considered as glycoprotein so we may also consider these protein fractions as glycoprotein.[13,14,15]

In present study it was observed that in initial stages of OSMF higher molecular weight proteins showed overexpression and in later stages of OSMF, which is accompanied by increased duration, frequency and amount of betel nut consumption, serum protein was observed to be consistently absent or under expressed. Overexpression of protein fractions in serum profile could be attributed to the increased production of acute phase reactants from liver in response to inflammation present in initialstages of OSMF 16.

Disease progression is not only governed by the increased frequency of habit, duration and amount of consumption of betel nut but is also equally effected by our body's immune mechanism.[17,18] Various studies have reported that the betel nut, main culprit for OSMF, is contaminated with aflatoxin in high proportion (up to 37.5%), which is a well documented hepatotoxin.17,19,20 . Severe grades of OSMF directly reflects increase in hepatotoxicity as there is cumulative increase in amount of aflatoxin consumption. Hepatotoxicity produces alteration in serum proteins where some protein fractions may also be lost.

In the present study, samples of 30% patients showed complete loss of all protein fractions. Almost similar findings were reported in other studies where the protein maps of normal and malignant prostate tissue when compared showed loss of 20 different proteins in malignant transformation.11 It could be hypothesized that under expression or no expression of protein fractions from serum profile reflect the advanced stage and malignant transformation of OSMF respectively and need to be reassessed by other methods.

CONCLUSION

Other than the stagnation of leachants from betel nuts, gutkha/kharra, lime etc and generation of reactive oxygen species (ROS) which leads to oxidative damage to oral mucosa, the depression of immune mechanism plays important role for transforming OSMF into malignancy. This study emphasizes on the hampering of the immune mechanism because of the hepatotoxicity caused by aflatoxin. We recommend that the liver profiling be added in the investigation parameters of OSMF patients.

Future scope:

We recommend extensive studies with increased number of cases be conducted with aim to discover and characterize the altered proteins in OSMF. Identification of those proteins will provide the rationale for the development of drugs or protein supplements with ability to modulate the diseased condition. Future researches could focus upon other premalignant conditions and specific biomarkers be identified for discrimination between premalignant and the malignant stages of various diseases. This unfolding fact may decrease the morbidity and mortality rate of the society.

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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