

# A Study on Mast Cell Number and Lipid Profile in Oral Submucous Fibrosis

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## Abstract

**Aims:** Prevention of oral precancer is most desirable and should take precedence over its diagnosis and therapy. As both, mast cell counts in connective tissue and serum lipids are altered in Oral Submucous Fibrosis (OSF), so study of both the factors is vitally important in OSF.

**Methods:** A total of 50 persons were included in the study of which 40 were OSF patients and 10 controls. Tissue sections and blood samples were collected for mast cell count and lipid profile estimation for both OSF patients and controls.

**Results:** Mean mast cells in all grades of OSF were higher as compared to apparently normal appearing oral mucosa but as severity of OSF increases (from Grade II to Grade IV), count of Mast cell decreases. Further, the mean mast cells in OSF Grade II, III and IV groups were found 95.5%, 93.8% and 92.2% higher respectively as compared to normal (highly significant for all the groups  $p < 0.001$ ). The mean serum lipid profile of OSF groups was comparatively lower than age and sex matched healthy controls but was highly significant for all the groups ( $p < 0.001$ ).

**Conclusions:** Thus, it can be said that in the present study serum lipid profile decreases in OSF patients and mast cell count is increased when compared with apparently normal appearing mucosa but with the advancement of the grades mast cell number decreases in tissue sections of OSF. It can be suggested that biochemical and histological assessment of OSF patients may help in earlier diagnosis and/or prognosis of this disease.

*Key words: Mast cells, Lipid profile, Oral submucous fibrosis, Early diagnosis, Prevention*

## Introduction

Oral Submucous Fibrosis (OSF) is a chronic, progressive, scarring, high-risk precancerous condition of oral mucosa [1], now globally accepted as an Indian disease, having one of the highest rates of malignant transformation (7.6%) amongst potentially malignant oral lesions and conditions [2,3]. In Southeast Asia, oral cancer is a major public health problem and over 90% of oral malignancies are known to arise from pre-existing potentially malignant lesions and conditions [4].

An interesting etiological factor of OSF is the mast cell. Mast cells are large granular cells that arise from a multipotent CD 34<sup>+</sup> precursor in the bone marrow and are local, permanent, connective tissue residents [5]. Studies on mast cells in normal and various pathologic conditions have shown them to be complex, well-engineered, multifunctional cells playing a central role in acquired and innate immunity. They release preformed secretory mediators like histamine, heparin, tryptase, lipid derived mediators, pro-inflammatory cytokines, mitogenic cytokines and immunomodulatory cytokines. Mast cells have been studied in various conditions like wound healing, chronic inflammation, keloid, pulmonary fibrosis, angiogenesis, and scleroderma [6]. OSF is always associated with juxta-epithelial inflammatory reaction, thus even in OSF average mast cell numbers/density might get altered in connective tissue stroma in comparison to normal oral mucosa [7-10].

Lipids are major cell membrane components essential for various biological functions including cell growth and division of normal and malignant tissues. They are homogeneous group of compounds related more by physical than chemical properties [11,12].

Areca nut is the major etiological factor for OSF [11,13-

16]. Fundamentally, development of malignancy requires the uncontrolled and excessive proliferation of cells [14]. Excessive use of areca nut may cause fibrosis due to increased synthesis of collagen and induce the generation of free radicals and reactive oxygen species. Due to lipid peroxidation, there is a greater utilization of lipids including total cholesterol, lipoproteins and triglycerides for new membrane biogenesis. An alteration in the circulatory cholesterol levels has been found to be associated with premalignant conditions [11,12,15]. Some investigators have also found relation of low serum cholesterol with increased risk of cancer occurrence and mortality. The question that whether hypolipidemia at the time of diagnosis, is a causative factor or is a result of cancer has remained unanswered [12]. However, only a few reports are available on plasma lipid profile in OSF [3,14,17].

As both, mast cell counts in connective tissue and serum lipids are altered in OSF, so study of both the factors is vitally important. Only few reports are available on mast cell count in tissue sections and lipid profile (serum) of OSF. Prevention of oral precancer is most desirable and should take precedence over its diagnosis and therapy [15]. Disease can be prevented easily only if it can be diagnosed earlier during its development.

Keeping all these curiosities in mind, the present study was aimed to assess the serum lipid profile and mast cell numbers in connective tissue of OSF cases, since detection of both these factors in the patient of OSF might be favorable in early diagnosis, early therapy and prevention of the disease.

## Materials and Methods

The present study was undertaken to evaluate the mast cell number and lipid profile in OSF. A total of 50 persons constituted the study of which 40 were biopsy proven OSF

patients and 10 were controls whose tissue was normal mucous membrane. Toluidine blue staining for histopathological evaluation of mast cells in OSF, and lipid profile of the blood was done. Tissue sections and blood samples were collected for mast cells and lipid profile for both OSF patients and controls. Controls were the apparently normal appearing mucosa and blood samples from the age and sex matched healthy individuals.

#### Inclusion criteria

Only biopsy proven cases of OSF, patients of all age groups, patients with all kind of habits (gutkha, betel quid, tobacco, pan masala, chewing, smoking, khaini, mawa) and all the biopsies of OSF included were taken from the buccal mucosa.

#### Exclusion criteria

Patients with a history of previous treatment of OSF, patients with any systemic ailment, OSF associated with squamous cell carcinoma, blood samples of hypertensive and diabetic patients and blood samples of Hypothyroidism and those receiving lipid-altering drugs.

#### Data collection

Detailed clinical history and blood examination was carried out as per proforma and the following criteria were used to histopathologically confirm the diagnosis of OSF. For grading of OSF, Khanna and Andradæ classification of OSF given in the year 1995 was followed which covers both clinical and histopathological aspect of OSF [16].

1. Presence of atrophic epithelium.
2. Loss of rete ridges.
3. Juxtaepithelial hyalinization.
4. Thickened collagen bundles.
5. Constricted blood vessels.
6. Young or mature fibroblast.

Written consent of patients diagnosed with OSF was taken. After explaining the procedure and outcome, the incisional biopsy was done under local anesthesia. Tissue biopsies and resected specimens were collected in 10% formalin, and paraffin sections were made for histological examination. Two sections of 4µm thickness were obtained. One was stained with H&E and other with 1% Toluidine blue for mast cells. The H&E stained tissue sections were analyzed by three different intra-department observers to remove inter-observer variability bias and were graded into different grades of OSF [16] (Figure 1).

#### Toluidine blue staining

Paraffin sections were deparaffinized and hydrated. Sections were flooded with 1% toluidine blue for 30 seconds. The sections were kept in running water for 2 minutes and then differentiated with 95% and absolute alcohol. The sections were warmed and cleared in xylene and mounted mounted with DPX. The mast cells were counted using an oculometer grid with 10 X 10 divisions. The mast cell counting was carried out in 10 grid fields at 40x. The oculometer grid was oriented parallel to the epithelial rete ridges and the mast cells were counted in 10 grid fields within the connective tissue stroma at 40x (Figure 1).

#### Blood Sample Collection

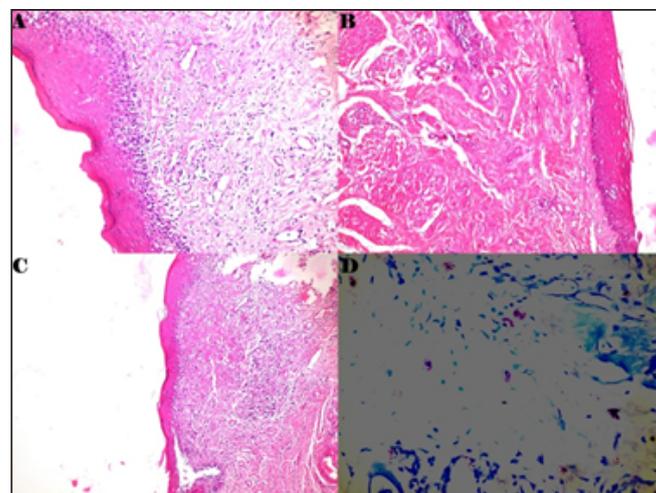
Fasting blood samples of histologically confirmed cases of OSF were collected in plain vials. Histopathologically

proven patients of OSF were studied. In these patients serum lipids including: (i) Total cholesterol (ii) LDL cholesterol (LDLC) (iii) HDL cholesterol (HDLC) (iv) VLDL cholesterol (VLDLC) (v) triglycerides were estimated by spectrophotometric method using liquid gold kits obtained from Autospan Reagents (Span Diagnostics Limited, India) in a Semiautomatic Biochemical Analyzer (Merck). 10 µl serum sample was mixed with 1000 µl of triglycerides mono reagent containing Pipes buffer, 4-chlorophenol, Magnesium ion, ATP, lipase, peroxidase, glycerol kinase, sodium azide, 4-amino antipyrine, glycerol-3-phosphate oxidase and detergents. The mixture was then incubated for 10 minutes at 37°C and absorbance was read at 505 nm using the analyzer. (Figure 2).

Statistical analysis was done by Newman-Keuls post hoc test. A two tailed ( $\alpha=2$ )  $p<0.05$  was considered statistically significant and all analyses were performed on Graph Pad Prism (Windows version 5.0).

### Results

There were total 10 subjects (M=8 and F=2) in Normal (Control) group and 40 in OSF cases (M=35 and F=5). The prevalence of OSF was higher in males (87.5%) than females (12.5%). The distribution of OSF according to age and gender showed that the OSF was most prevalent in middle age (20-40 yrs) group, followed by higher age (> 40 yrs) group, and the least in (<20 yrs) group. Further, the prevalence of OSF



**Figure 1.** A photomicrograph showing the various grades of OSF. A, B, C: show the early, advanced and very advanced cases of OSF stained by H/E, 10X. D: shows the mast cells in OSF by Toluidine staining, 40X.



**Figure 2.** A photograph of the lipid profile biochemical analysis of OSF patients. A: shows serum sample mixed with reagent in the various test tubes. B: shows serum sample of OSF patients collected after blood centrifugation.

in males (77.5%) at middle age was significantly ( $p < 0.05$ ) higher than females (5.0%) ( $\chi^2 = 7.58$ ,  $p = 0.023$ ). Comparing the sex proportion (M/F) between the two groups,  $\chi^2$  test revealed similar ( $p > 0.05$ ) proportion of sex in two groups (8/2 vs. 35/5,  $\chi^2 = 0.37$ ;  $p = 0.541$ ). Similarly, t test revealed that the mean age of two groups was similar ( $28.60 \pm 1.82$  vs.  $29.13 \pm 1.88$ ,  $t = 0.14$ ;  $p = 0.893$ ) (Tables 1 and 2).

The distribution of OSF according to site types showed that among all 40 OSF cases, the OSF was present in 30 different types of sites. The most prevalent site was bilateral buccal mucosa and retromolar region (17.5%) followed by bilateral buccal mucosa, retromolar region, shrunken uvula and floor of mouth (7.5%), bilateral buccal mucosa, retromolar region and shrunken uvula (5.0%), and bilateral molar and retromolar region (5.0%) while in rest of the site it remains similar with 2.5% prevalence. The distribution of OSF according to histopathological grading showed that OSF cases with Grade III (47.5%) were the highest followed by Grade IV (35.0%) and Grade II (17.5%), the least.

The distribution of OSF according to histopathological grading is summarized in Table 3. It showed that OSF cases with Grade III (47.5%) were the highest followed by Grade IV (35.0%) and Grade II (17.5%), the least.

The mast cells of normal group and OSF groups (Grade II, Grade III and Grade IV) showed that the mean mast cells in all grades of OSF were comparatively higher as compared to normal. It also revealed that as severity of OSF increases (from Grade II to Grade IV), mast cells decreases. Further, the mean Mast cells in OSF Grade II, III and IV groups were found 95.5%, 93.8% and 92.2% higher as compared to Normal group and were said to be significantly different and higher (93.8%) than normal subjects ( $1.40 \pm 0.45$  vs.  $22.50 \pm 0.91$ ,  $t = 11.47$ ;  $p < 0.001$ ). (Table 4, Bar Chart 1)

The lipid profiles (TC, TG, HDL, LDL and VLDL) of Normal group and OSF group showed that the mean lipid profile of OSF group was comparatively lower than Normal group. Further, the mean TC, TG, HDL, LDL and VLDL of OSF group lowered by 39.8%, 44.8%, 55.4%, 33.6% and 44.8% respectively as compared to Normal group and were said to be significantly lower ( $p < 0.001$ ) in cases of OSF than normal subjects. (Table 5, Bar Chart 2)

**Table 1.** Frequency distribution of gender and age summary of two groups.

Gender	Normal group (n=10)	OSF group (n=40)	$\chi^2$ / t value	p value
Sex: M	8 (80.0%)	35 (87.5%)	0.37	0.541
F	2 (20%)	5 (12.5%)		
Age (yrs)	$28.60 \pm 1.82$ (22-40)	$29.13 \pm 1.88$ (18-70)	0.14	0.893

The numbers in parenthesis represent the age (min-max)  
Inference: The subjects of two groups were sex and age matched

**Table 2.** Frequency distribution of OSF according to age and gender.

Age groups	Male (n=35)	Female (n=5)	$\chi^2$ value (DF=3)	p value
< 20 yrs	2 (5.0%)	1 (2.5%)	7.58	0.023
20-40 yrs	31 (77.5%)	2 (5.0%)		
> 40 yrs	2 (5.0%)	2 (5.0%)		

Inference: The subjects of two groups were sex and age matched

**Table 3.** Frequency distribution of OSF according to Histopathological grading.

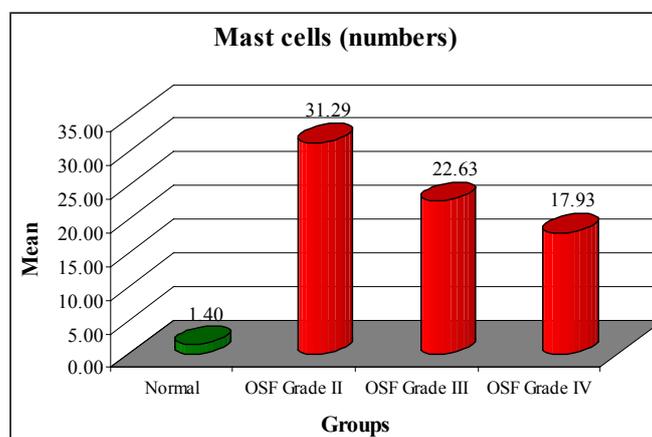
Histopathological grading	N	%
II	7	17.5
III	19	47.5
IV	14	35.0

Inference: The most of the cases were of Grade III (47.5%)

**Table 4.** Summary (Mean  $\pm$  SE) of Mast cells of Normal group and OSF groups.

Normal group (n=10)	OSF groups (n=40)			ANOVA F value (3,46 DF)	p value
	Grade II (n=7)	Grade III (n=19)	Grade IV (n=14)		
$1.40 \pm 0.45$ (0-4)	$31.29 \pm 1.63$ (25-36)	$22.63 \pm 0.72$ (15-31)	$17.93 \pm 0.93$ (13-25)	144.87	$p < 0.001$

The numbers in parenthesis represent the range (min-max).  
Inference: The mean Mast cells in OSF patients are significantly different and higher (93.8%) than normal subjects ( $1.40 \pm 0.45$  vs.  $22.50 \pm 0.91$ ,  $t = 11.47$ ;  $p < 0.001$ ).



**Bar Chart 1.** Mean Mast cells of Normal group and OSF groups.

## Discussion

Oral Submucous Fibrosis(OSF) is a chronic oral mucosal disease characterized by epithelial atrophy and progressive accumulation of collagen fibers in the lamina propria and submucosa of the oral mucous membrane. The abnormal fibrosis causes blanching and stiffness of the mucous membrane, with eventual immobility of lips, cheeks, tongue, soft palate and uvula [2-4,8-10,18].

Mast cells have been implicated in the pathogenesis of OSF [19]. Changes in Lipid profile have long been associated with malignancies, as lipids play a key role in maintenance of cell integrity [15]. Our study showed that OSF was most prevalent in middle age (20-40 yrs) group (82.5%) with a male predominance (87%). These observations were supported by the studies done earlier which state that the disease mainly occurs between 20 to 40 years of age [7,20] and male predominance [3,18,20] although some studies have shown female predominance as well [8,10].

The statistics showed that mean mast cells in all grades of OSF were comparatively higher as compared to normal and as severity of OSF increased (from Grade II to Grade IV), the mast cell count decreased. Ankle et al had also reported an increase in the number of mast cell count in OSF (48.25/sq.mm) as compared to normal oral mucosa (25.20/sq.mm)

**Table 5.** Summary (Mean  $\pm$  SE) of TC of Normal group and OSF groups.

Variables	Normal (n=10)	OSF (n=40)	% mean change	t value (DF=48)	p value
TC (mg/dl)	206.05 $\pm$ 2.99 (193.90-224.20)	123.96 $\pm$ 4.05 (75.40-180.30)	39.8%	9.89	p<0.001
TG (mg/dl)	142.80 $\pm$ 2.55 (131.40-154.20)	78.78 $\pm$ 4.34 (36.24-148.08)	44.8%	7.25	p<0.001
HDL (mg/dl)	44.29 $\pm$ 0.79 (40.00-46.60)	19.74 $\pm$ 1.08 (10.00-38.20)	55.4%	11.14	p<0.001
LDL (mg/dl)	133.20 $\pm$ 2.67 (119.88-148.36)	88.47 $\pm$ 3.70 (52.88-148.42)	33.6%	5.91	p<0.001
VLDL (mg/dl)	28.56 $\pm$ 0.51 (26.28-30.84)	15.76 $\pm$ 0.87 (7.25-29.62)	44.8%	7.25	p<0.001

The numbers in parenthesis represent the range (min-max).

Inference: The mean levels of TC, TG, HDL, LDL and VLDL are significantly different and lower (p<0.001) in cases of OSF than normal subjects.

TC: Total Cholesterol

TG: Tri Glycerides

HDL: High Density Lipoprotein Cholesterol

LDL: Low Density Lipoprotein Cholesterol

VLDL: Very Low Density Lipoprotein Cholesterol

[6]. The mast cell increase could be attributed to histamine release from mast cells [19]. Ankle et al. also suggested that histamine is responsible for the submucosal edema which is seen in early stages of OSF. Increased vasopermeability, ECF is released from the mast cells and this could lead to the presence of eosinophils that are sometimes a part of inflammatory cell infiltrate seen in the early stages of the disease [6]. It was also suggested that in early cases when the tissue reaction to the irritant is strongest, the mast cell counts are highest. As the tissue gets converted to less reactive hyaline, the mast cells become fewer than normal [21].

The mean mast cells of OSF in Grade II was found to be highly significant (p<0.001) and higher as compared to Grade III and Grade IV and we also found that mean mast cells in Grade III was significantly different (p<0.01) and higher as compared to Grade IV. These results were similar to those observed by Bhatt and Dholakia, who had hypothesized that the release of mast cell granules may initiate a change in the connective tissue ground substance by changing intracellular fluid or free tissue water into mucinous fluid. The mast cell histamine chain may be terminated by the fibroblastic production of hyaluronic acid or by depositing collagen, fibrosis or other processes [19].

The progressive depletion of mast cell number from early to advanced stage could be due to the degranulation of mast cells as the disease progressively advances. The toluidine blue only stains the mast cell granules. Thus, the lack of granules in mast cells in advanced stages may not have stained them with toluidine blue. This was substantiated by the observations of previous studies [21,22]. Thus, it could be inferred in the present study that with the advance in the disease process of OSF there was a significant reduction in the mast cell number. This could be because the degranulated mast cells are not visible under the light microscope when stained with toluidine blue.

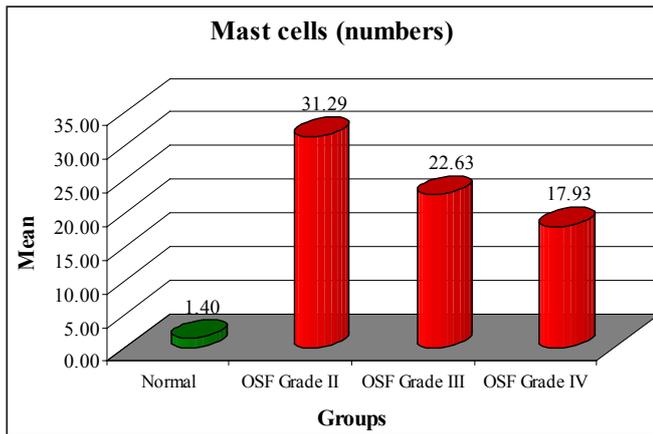
Regulation of cholesterol is mediated by lipoprotein receptors. Serum triglycerides and cholesterol are packed into lipoproteins for transport. Cholesterol is an essential constituent of lipoprotein fractions like LDL, HDL, and VLDL and 75% of the plasma cholesterol is transported in the form

of LDL. In some malignancies, serum cholesterol undergoes early and significant changes. Low levels of cholesterol in the proliferating tissues and in blood compartments could be due to the rapidly dividing cells in malignancies. Several prospective and retrospective studies have shown an inverse association between blood lipid profiles and different cancers. Some scientists have observed an inverse trend between lower serum cholesterol and premalignant conditions as well as head and neck cancers [11,12,14,15].

In our study, it was found that serum TC, HDL, LDL, VLDL and TG levels in case of OSF group were significantly less when compared with the healthy control (normal) group. This may be due to greater utilization of lipids including total cholesterol, lipoproteins and triglycerides for new membrane biogenesis as well as accumulation of esterified cholesterol in tumoral tissues. A significant decrease in levels of HDL was also observed in this study. This was in accordance with previous reports which reported that lower HDL is an additional predictor of oral premalignant condition and it might be a consequence of disease that is mediated by utilization of cholesterol by membrane biogenesis [12,15].

It was concluded that serum cholesterol level was inversely associated with incidence of colon cancer in one study but Feinlieb reported evidence relating hypercholesterolemia to an increased risk of cancer as controversial [23]. An inverse association was observed between blood cholesterol and overall cancer risk. Tulinius reported that serum retinol and serum cholesterol are both lowered in people who later develop cancer. Broitman reported an inverse relationship between serum or plasma cholesterol levels and risk for colon cancer. According to them the risk is greatest at serum cholesterol levels <180 mg/dl [24,25]. It was also found that in an alcoholic population compared to non-alcoholics, the cholesterol (187 mg/dl) and carotene (94 mg/dl) concentrations were markedly reduced [15]. The LDL cholesterol was highly correlated with carotene and they suggested that alterations due to alcohol intake may partially account for the relationship of alcohol to increased cancer risk.

It was found that there was significantly lower value of TC and LDL cholesterol in men with cancer (lung cancer



**Bar Chart 2.** Mean Mast cells of Normal group and OSF groups.

and hematological cancer) as compared to controls [26]. A decrease in TC and HDLC cancer patients as compared to controls was also observed [12]. A reduction in TC, HDL, LDL in the patients with oral precancerous condition was also seen and it was suggested that these changed levels might indicate the progression of the lesions towards malignancy. A significant decrease in plasma total cholesterol, HDLC, and triglycerides in oral precancerous conditions as compared to controls was proven and it was suggested that lower levels

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of plasma cholesterol and other lipid constituents in patients might be due to their increased utilization [15]. These results were similar to our study performed on OSF patients.

Variability of the values of serum lipid profile in precancerous condition and cancer patients may be due to multiple reasons, such as age, nutritional status, body mass index, exercise habits, tobacco and alcohol consumption. Thus, in the present study serum lipid profile decreases in OSF patients as compared to normal and the mast cell count in OSF is increased when compared with apparently normal appearing mucosa but with the advancement of the grades of OSF mast cell number decreases in tissue sections of OSF stained with 1% toluidine blue.

After going through the available literature thoroughly, we have not come across any study which has considered all these 5 parameters (TC, HDL, LDL, VLDL and TG) as done in our study. To the best of our knowledge very few studies has been conducted to assess Mast cell count and lipid profile in OSF patients, so the findings in the present study strongly warrant an in-depth study of alterations in serum Lipid profile and Mast cell count in tissue sections of the patients with OSF. Thus, this would be helpful in early treatment and better prognosis of the patient.

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