REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 25, No. 2 (2024)
http://www.veterinaria.org
Article Received: Revised: Accepted:

"Evaluation Of Langerhans Cells Using Cd1a In Oral Submucous Fibrosis: An Immunohistochemical Study"

Dr.Sourab Kumar[1*], Dr.Abhishek Jadhav[2], Dr. Sejal Vijay Palve[3], Dr. Nisha Kumari Jaiswal[4], Dr. Saniya Subir Puthanpurakkal[5], Dr. Priyanka Anil Patil[6]

[1*]Professor, Department of Oral Pathology & Microbiology, D.Y.Patil School of Dentistry, Nerul, Navi Mumbai – 400706, Email - sourab.kumar@dypatil.edu, Contact - 9819614239
[2]Associate Professor, Department of Oral Pathology & Microbiology, D.Y.Patil School of Dentistry, Nerul, Navi Mumbai – 400706, Email - abhishek.jadhav@dypatil.edu, Contact - 9892007738
[3]Junior Resident, D.Y.Patil School of Dentistry, Nerul, Navi Mumbai – 400706, Email - palvesejal01@gmail.com, Contact - 8850238900/9594953871
[4]Junior Resident, D.Y.Patil School of Dentistry, Nerul, Navi Mumbai – 400706Emial - jaiswalnisha1609@gmail.com, Contact - 7070755504
[5]Junior Resident, D.Y.Patil School of Dentistry, Nerul, Navi Mumbai – 400706Email - saniyasubir2@gmail.com, Contact - 9136776223
[6]Junior Resident, D.Y.Patil School of Dentistry, Nerul, Navi Mumbai – 400706, Email - patilpriyanka3146@gmail.com, Contact - 8600339202

ABSTRACT
The burden of cancer is still increasing worldwide despite advances for diagnosis and treatment. Epidemiological studies have shown that many cancers may be avoidable. It is widely held that 80–90% of human cancers may be attributable to environmental and lifestyle factors such as tobacco, alcohol and dietary habits. It was estimated that in the year 2000, worldwide over 10 million new cases of cancer occurred (approximately 5.3 million men and 4.7 million women) and over 6 million people died from cancers.
This study aims to evaluate the presence of langerhans cells in various histological stages of OSMF using CD1a antibody, which has been suggested as the most reliable marker of langerhans cells differentiation & to determine whether alterations in the number of LCs impairs oral mucosal immunologic protection against mutagens such as areca nut (betel quid), tobacco constituents, which might play a role in oral epithelial dysplasia& oral squamous cell carcinoma.

Keywords : OSMF, CD1a,Langerhans cells, Immunohistochemical study

INTRODUCTION

Fibrous collagen is synthesized as a protein (procollagen) in a manner similar to protein of other cells. As suggested by various researchers pathogenesis of OSMF shows a definite increase of collagen which is a protein, in the areas affected by this condition locally. Exuberant collagen formed being the primary tissue component in OSMF has been compared with an excessive scar tissue formation in healing wounds because of constant irritation. Fibroblasts are widely recognized as a critical cell type involved in wound healing and tissue repair. Fibrogenic cytokines are secreted by activated macrophages or T lymphocytes are very important in the development of fibrotic disorders. Less generally appreciated is the notion that the transformation of fibroblast to myofibroblasts is a key, perhaps essential, event for the cells to perform these functions.

The oral mucosa that is continuously exposed to a variety of injurious agents with antigenic properties, such as tobacco & tobacco smoke. **Langerhans cells (LCs)** may well represent a "**first-line**" of sensitization of the immune system, leading to clearance of antigen or to pathological phenomena as their absence can lead to disease initiation or progression. Recent studies have highlighted mucosal LCs as important arbiters of mucosal immune responses. According to Daniels et al., alterations in the number of Langerhans cells in tobacco-associated lesions is a result of changes caused by tobacco & is one factor in the long term pathogenesis of oral carcinoma.

LCs are bone marrow-derived epidermal dendritic cells & are present in the epithelium of the skin & mucosa, including the oral cavity. They are readily identified in the epithelium by special stains or with the help of immunohistochemical markers (CD1a) in addition to the presence of dendritic processes & suprabasal position.

LCs perform important immunologic functions by participating in cutaneous & mucosal immune reactions that can have both local & systemic effects. LCs have a specialized role in the presentation of non-peptide antigens to T cells, & this function is mediated by the antigen presenting molecule CD1a, which is highly expressed by these cells.

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 25, No. 2 (2024)
http://www.veterinaria.org
Article Received: Revised: Accepted:

AIMS AND OBJECTIVES

Aims:

1) To assess the severity of the disease by clinical and functional staging.
2) To correlate clinical and functional staging with histopathological staging.
3)To study the expression of CD1a in Oral Submucous Fibrosis patients &to assess the number and distribution of Langerhans cells in OSF tissue & compare with normal control.
4) To evaluate possible correlation of Langerhans cells using CD1a marker in Oral Submucous fibrosis and its other histological grades.

Objectives:

1) To help assess the prognosis of the disease.
2) In future, to aid in planning the treatment of OSMF.

Through the present study, it was intended to establish a possible correlation between habits, clinical features, histopathological grading of oral submucous fibrosis & its correlation with langerhans cells expression using CD1a marker.

MATERIALS AND METHODS

This study was carried out in the Department of Oral Pathology and microbiology in Dr. D. Y. Patil Dental College and Hospital, Nerul, Navi Mumbai.

MATERIAL USED IN THE STUDY:

1. Mouth Mirror, probe and tweezer.
2. Gloves.
3. BP Blade 15 no.
4. Tissue holding Forceps
5. Sterile Cotton
6. Sterile bulb for sample collection
7. 10% Formalin.
8. Grossing Table
9. Measuring Scale
10. Leukhardts 'L' Block
11. Paraffin Wax
12. Coplin Jars
13. 60%, 75%, 90%,100% Graded Ethanol
14. Acetone (Pure)
15. Xylene
16. Harris's Haematoxylin
17. Acid Alcohol.
18. Eosin
19. Blotting Paper
20. Glass slide.
21. Cover slip
22. Mounting Media. (DPX – Disterenedibutylphthalate xylol)
23. Semi-automatic Microtome (Leica, Germany)
24. Hot Plate.
25. Water Bath
26. Light Binocular Microscope.
27. Poly L lysine coated slides (Biogenex, USA).
28. Di sodium hydrogen phosphate – anhydrous (Purified)
29. Sodium Di hydrogen phosphate
30. Distilled Water.
31. Conical Flask.
32. Glass Stirrer
33. Glass Beaker.
34. pH meter.
35. Weighing Scale
36. Incubator – Wooden Make
37. Antigen-retrival Machine (EZRetriver- Biogenex, CA, USA).
38. Anti CD1a Anitbody (Biogenex, CA, USA) – Prediluted.
39. Secondary Anitbody kit (Biogenex, CA, USA)

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 25, No. 2 (2024)
http://www.veterinaria.org
Article Received: Revised: Accepted:

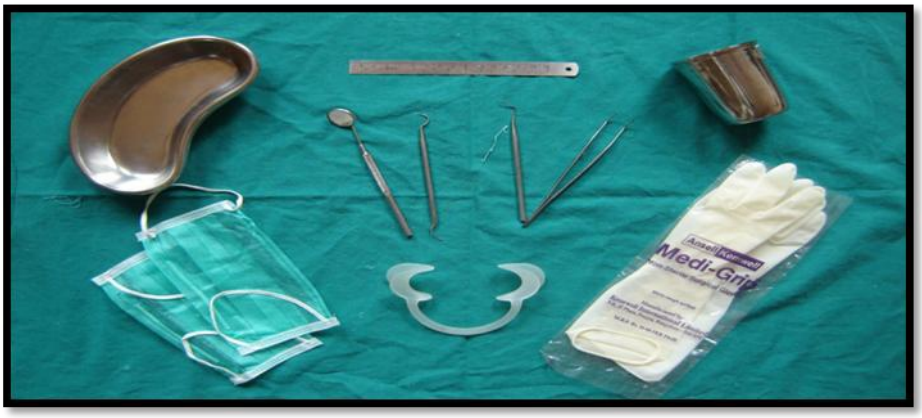

Fig 1: Clinical Examination Tools

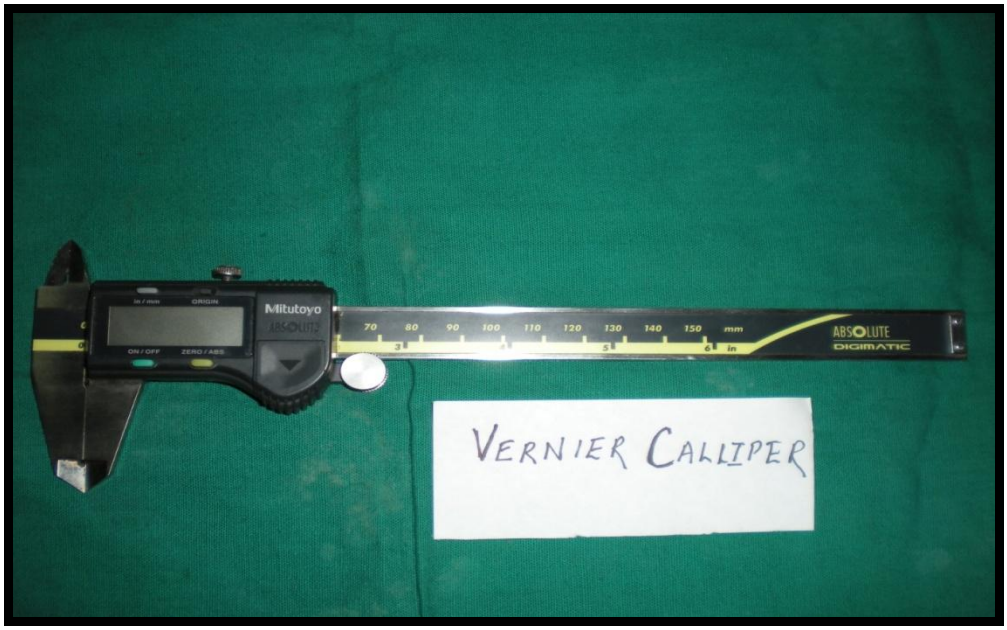

Fig 2: Vernier Calliper

METHODOLOGY:

Steps involved in carrying out the study:

I. Sample selection.
II. Sample Collection.
A. Blood Sample collection.
B. Tissue sample collection.
III. Haematoxylin & eosin staining of biopsy tissue sample.
IV. Staining of biopsy tissue sample using CD1a via immunohistochemistry.
V. Paraffin embedded sections of biopsies of the study groups will be stained by H&E.
VI. The serial sections of the same will be stained by immunohistochemical marker using CD1a and observed under microscope.
VII. Qualitative and quantitative analysis of langerhans cells will be done.
VIII. Two hot spots will be viewed under low magnification (4x).
IX. Images of these hot spots will be captured under higher magnification (40x).
X. The number of LCs will be counted manually from the images captured.
XI. The average figures which were obtained in the counted hot spot fields were considered as LC density.
XII. All counts were performed by a single investigator to eliminate an inter-observer variation.
XIII. Correlation between habits, clinical features, histopathological grading of oral submucous fibrosis & its correlation with langerhans cells expression.

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 25, No. 2 (2024)
http://www.veterinaria.org
Article Received: Revised: Accepted:

I.SAMPLE SELECTION.

Sample size:

The total number of the samples in the present study comprised of 40 patients.

The participants were categorized into the following subdivision,

1) 30 cases of OSMF
2) 10 cases were selected as control group

Methods and criteria used for sample selection

Inclusion criteria – The control patients were selected according to criteria such as, clinically healthy persons without the history of any oral habits like tobacco, pan, and alcohol, consumption, smoking and normal appearing oral mucosa.

Exclusion criteria – Antibiotics in the course of the previous month , bleeding risk (anticoagulant therapy) chemotherapy and / or immunosuppressant therapy, endocarditis risk, patients radiated in the head and neck region, infection risk (e.g. ,HIV , HBV ,HCV ,TB) .

Patients with clinically evident palpable bands of OSMF comprised the cases. Specially prepared case sheets (Annexure 1) were used to record data of patients. At first examination each patient was interviewed to obtain case history with special reference to possible etiological factors and detail clinical examination. Routine haematological examination was performed. Clinical photographs were taken. Each patient was subjected to biopsy procedure. Proper medication and postoperative instructions were given to the patients.

All the samples were collected from the Out Patient Department of Dr.D.Y.Patil Dental College and Hospital. Nerul, Navi Mumbai, Maharashtra. All the study participants were given clear explanations about the objective of the study and a written informed consent was taken after a standard questionnaire interview was performed to obtain the history. It was ensured that adequately sterilized instruments were used for oral examination and sample collection to prevent cross-infection.

For clinical grading of OSMF criteria of Lai DR (1995) was taken in to consideration in present study. Mouth opening of each patient was measured using a Vernier Calliper

Group A: Mouth opening greater than 35mm
Group B: Mouth opening between 30- 35 mm
Group C: Mouth opening between 20- 30 mm
Group D: mouth opening less than 20 mm.

Fig 3: Group A

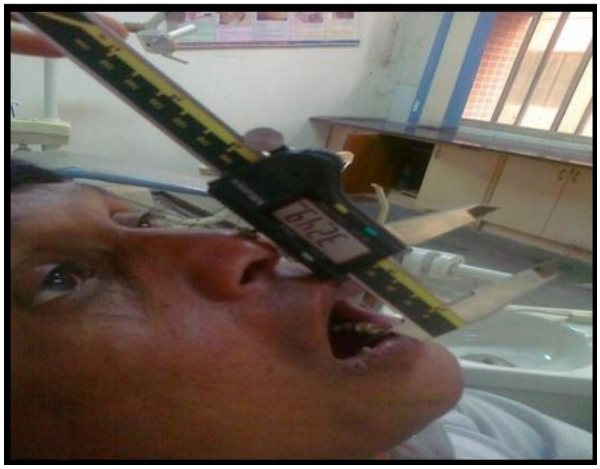

Fig 4: Group B

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 25, No. 2 (2024)
http://www.veterinaria.org
Article Received: Revised: Accepted:

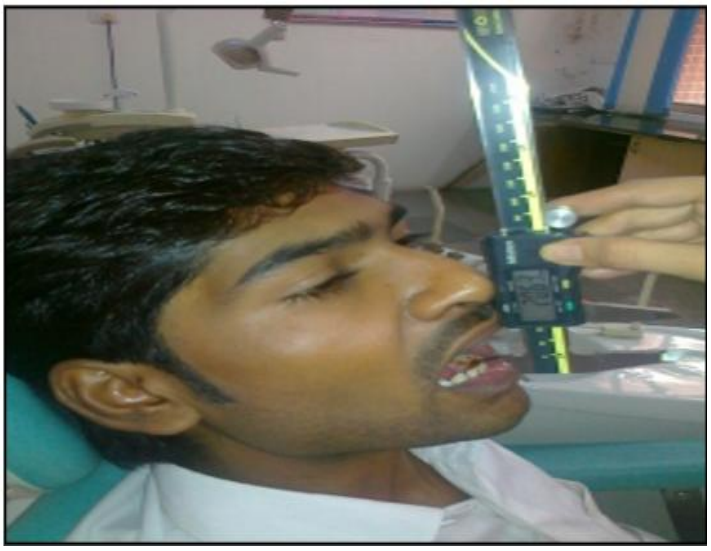

Fig 5: Group C

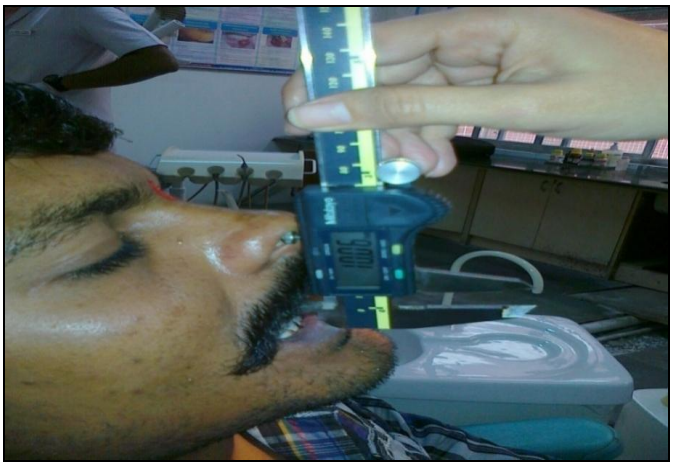

Fig 6: Group D

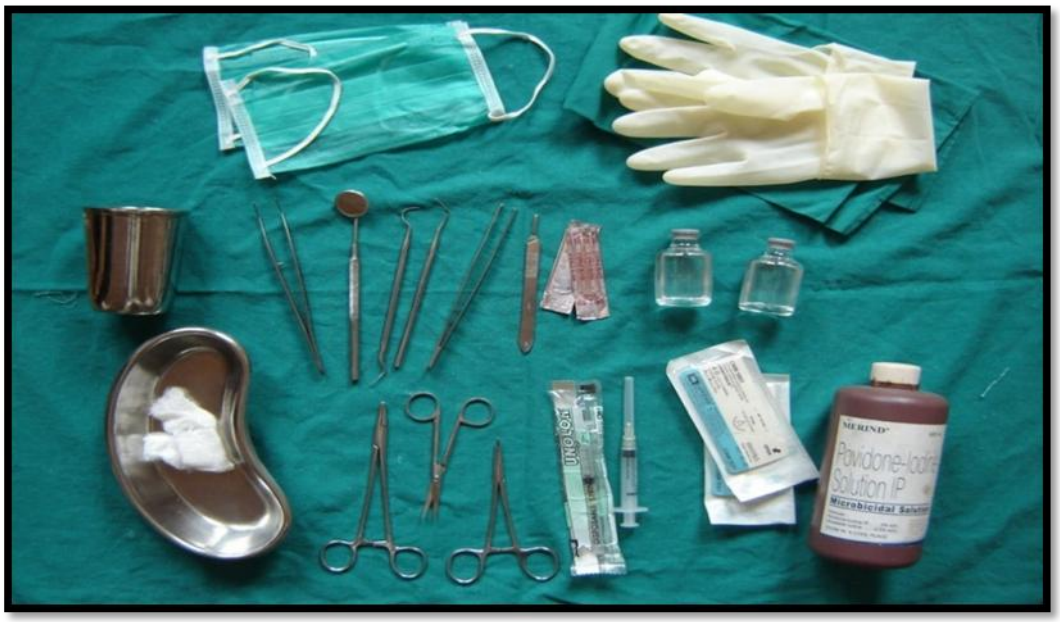

Fig 7: Biopsy Armamentarium

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 25, No. 2 (2024)
http://www.veterinaria.org
Article Received: Revised: Accepted:

Fig 8: H and E Tray

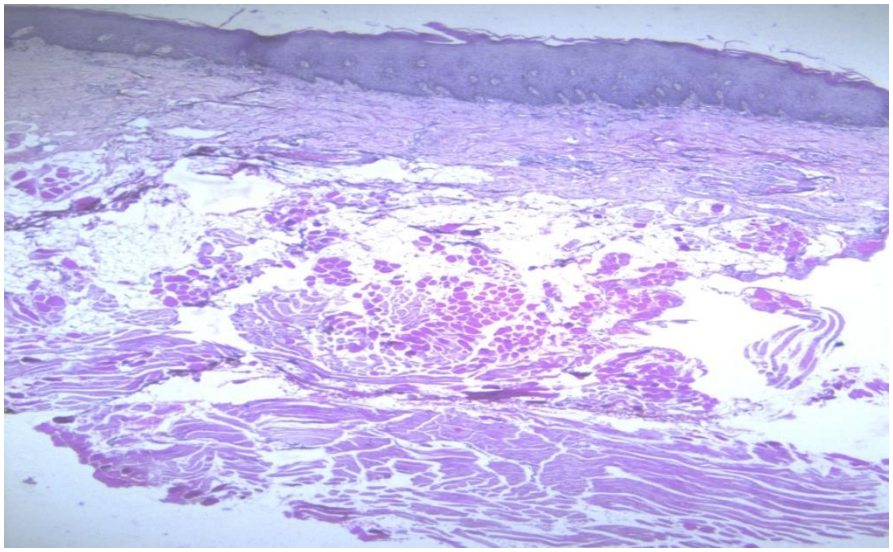

Fig 9: Very Early Stage OSMF (10X)- H & E

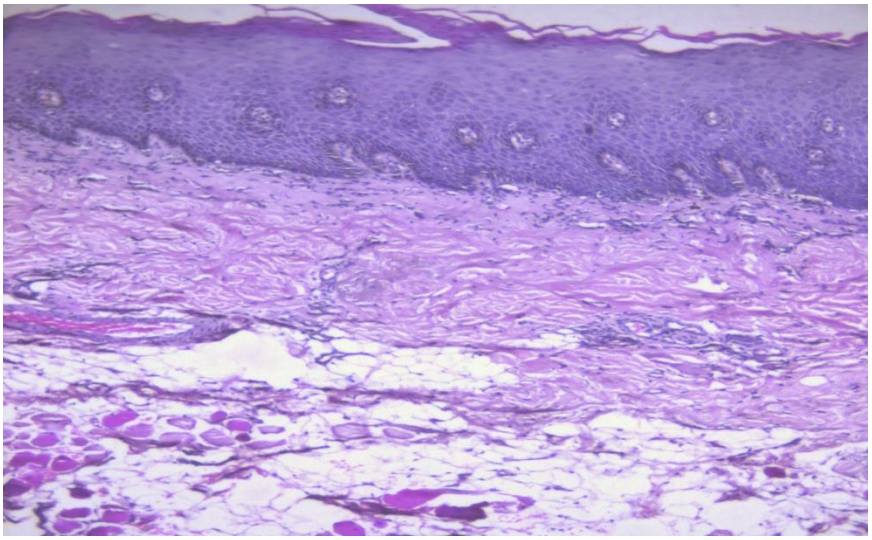

Fig 10: Very Early Stage OSMF (40X)- H & E

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 25, No. 2 (2024)
http://www.veterinaria.org
Article Received: Revised: Accepted:

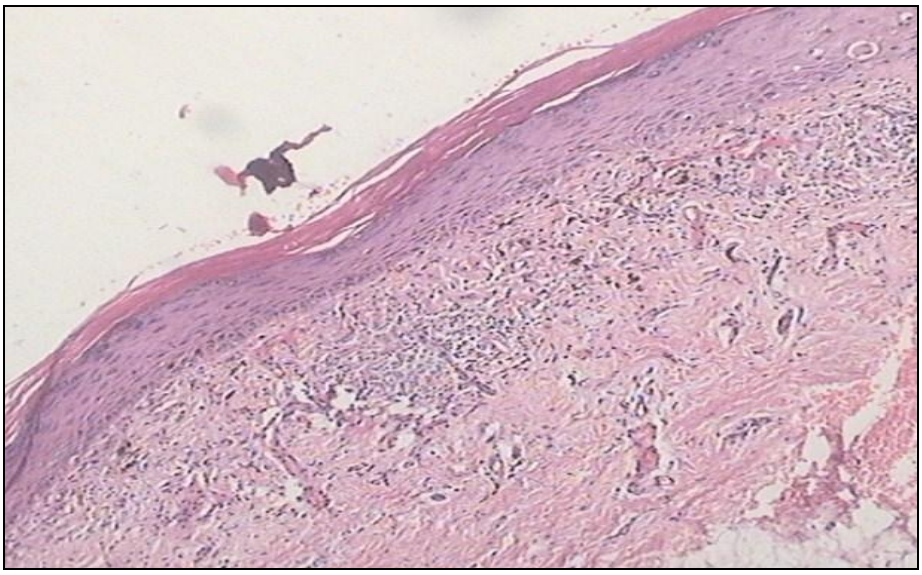

Fig 11: Early Stage OSMF (10X)- H & E

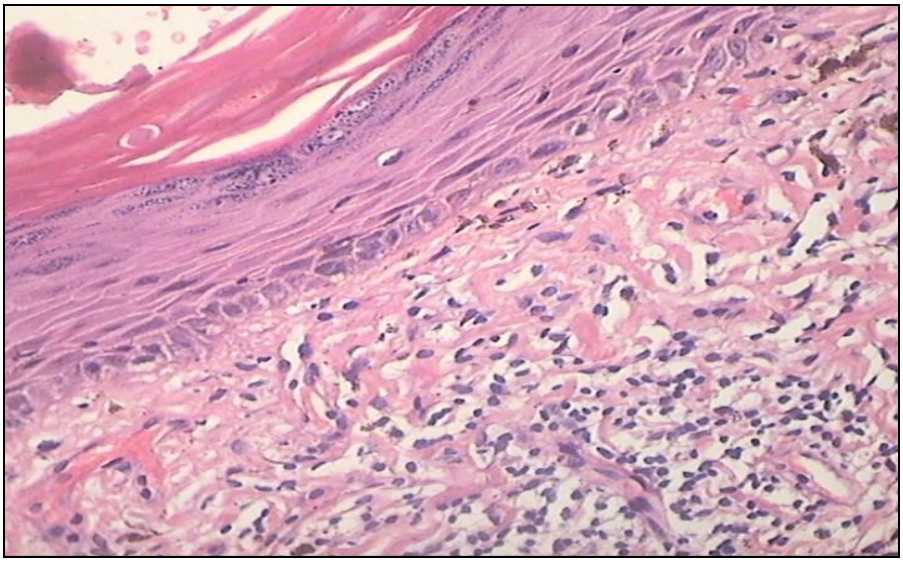

Fig 12: Early Stage OSMF (40X)- H & E

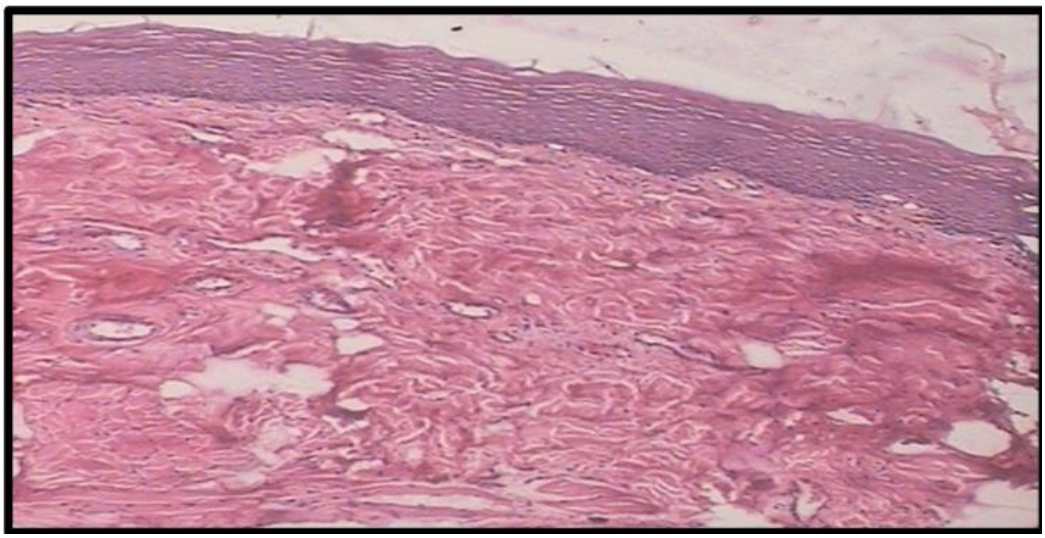

Fig 13: Moderately Advanced Stage OSMF (10X)- H & E

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 25, No. 2 (2024)
http://www.veterinaria.org
Article Received: Revised: Accepted:

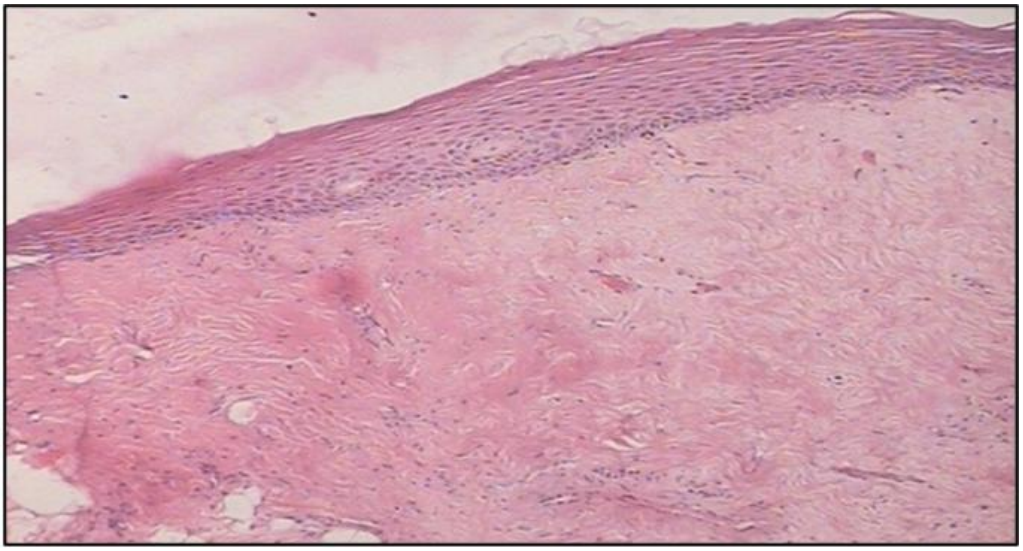

Fig 14: Moderately Advanced Stage OSMF (40X)- H & E

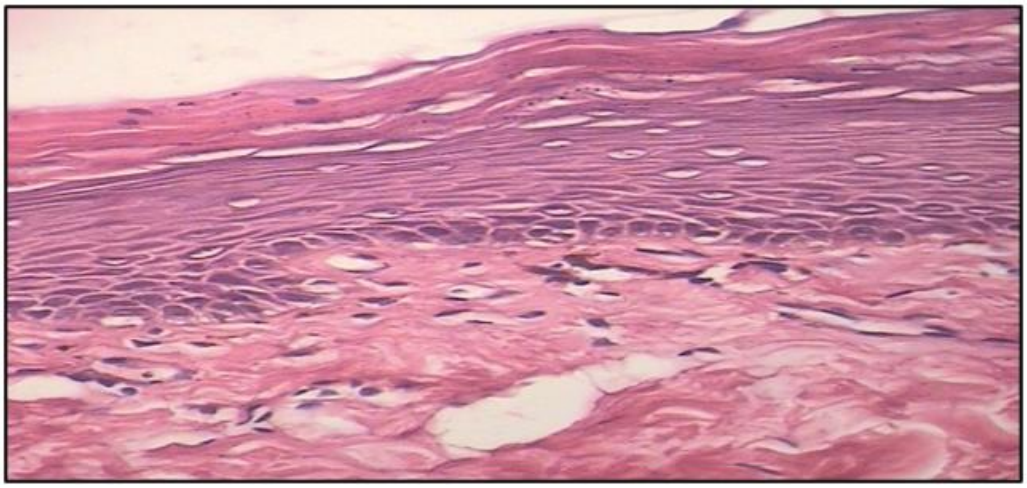

Fig 15: Advanced Stage OSMF (10X)- H & E

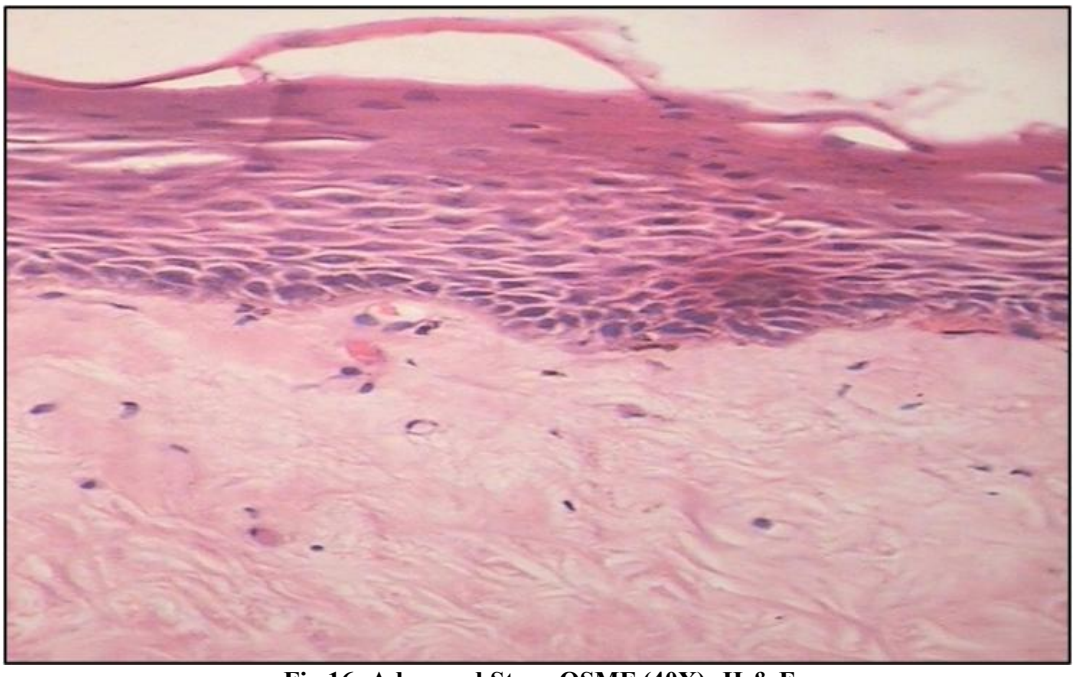

Fig 16: Advanced Stage OSMF (40X)- H & E

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 25, No. 2 (2024)
http://www.veterinaria.org
Article Received: Revised: Accepted:

II. SAMPLE COLLECTION

A. Tissue sample collection.

Tissue biopsy samples were obtained from the affected site in case of OSMF and from normal buccal mucosa from control subject who were undergoing surgical procedure.

Processing of the tissue biopsy

Tissue biopsy samples were obtained. The tissues were processed and later stained by the routine Haematoxylin and eosin procedure and slides was interpreted for the confirmation of the diagnosis.

Specimen Accessioning

Tissue specimens after the surgical pathology laboratory had a request form that listed the patient information and history along with a description of the site of origin. The specimens were accessioned by giving them a number to identify each specimen for each patient.

Gross specimen examination

Gross examination consisted of describing the specimen and placing all or parts of it into a small cassette which holds the tissue (fig 14). Than all the tissues were processed in automated tissue processing machine (YORCO) for paraffin embedding technique as follows:

Fixation

The purpose of fixation was to preserve tissues permanently in as life-like as state as possible. Fixation was carried out immediately after removal of the tissues. 10% formalin was used for fixation.

Tissue Processing

Once the tissue was fixed, it was processed into a form in which it can be made into thin microscopic sections.

1. **Dehydration**: Wet fixed tissues (in aqueous solutions) cannot be directly infiltrated with paraffin. First, the water from the tissues was removed by dehydration. This was done with a series of alcohols, 70% to 95% to 100%.
2. **Clearing**: It consists of removal of the de-hydrant with a substance that is miscible with the embedding medium (paraffin). The clearing agent we used was xylene which is:

- highly volatile flammable
- tissues are rendered transparent by xylene
- prolonged treatment makes tissue brittle
- Tissue block of less than 5 mm thickness will be cleared in an hour.

3. **Infiltration**: The tissue was infiltrated with the embedding agent paraffin.
4. **Embedding**: Tissues that came off the tissue processor were manually put into the blocks by picking the tissues out of the cassette and then tissues was aligned, or oriented, properly in the block of paraffin.
5. **Paraffin section cutting**: Once the tissues were embedded, they were cut into sections that can be placed on a slide.

a.Semi automated microtome was used to cut 5µ thick sections.

b. Water bath was used to unfold the tissue section.

- The thermostatically controlled type was used.
- The temperature of water was maintained 10degree c below the melting point of paraffin wax.
- Allowing the section tooflatten out with greater ease.

6. **Slides**: once the tissue sections were unfolded they were taken on slide.

- 76 by 25 mm slides in dimension and thickness 1 to 1.2 mm were used.
- "Ground & polished edge" slides were used to reduce dangers of finger cut.

7. **Section adhesives**: Protein adhesives egg albumin was used as adhesives.
8. Slides were placed in slide stand to dry.

III.HAEMATOXYLIN & EOSIN STAINING OF THE TISSUE SAMPLE.

The cut sections were then subjected to Haematoxylin & Eosin staining.

The stained slides were interpreted for the conformation of the diagnosis.

Staining Protocol: For histopathological grading of fibrosis criteria given by **Ranganathan K** in 2007 was taken into consideration in present study.The histological diagnosis of OSMF was established if the following findings were observed:

- Epithelium: Type of keratinization–para / orthokeratosis
- Thickness–atrophic / hyperplastic
- Dysplasia–mild / moderate / severe
- Connective tissue: Type of fibrous tissue–loose / dense
- Hyalinization–partial / complete
- Inflammatory cells: Acute / Chronic; focal / diffuse;

• juxtaepithelial / perivascular

Staining protocol

1. Deparaffinize the slide:

a.Slides are placed on hotplate.

b. Dip in xylene: I, II, III.

c.Rinse in decreasing grades of alcohol: 100% > 90%>80%.

d. Keep under running water 15min and rehydrate.

2. Stain the slide with haematoxylin - 10 minutes.
3. Wash the slide in running tap water - 15minute.
4. Differentiate in acid alcohol.
5. Wash the slide in running tap water - 15minute.
6. Stain with eosin - 1 minutes.
7. Dehydrate, clear and mount.
8. The slides were than observed under microscope.

IV. IMMUNOHISTOCHEMICAL STAINING FOR TISSUE SAMPLE

The technique used was based on the labelled streptavidin biotin (LSAB) method. Endogenous peroxidase was blocked by first activating the section in 0.6 % H_2O_2. The specimen was then incubated with primary antibody followed by the sequential incubations with biotinylated link antibody and peroxidase labelled streptavidin staining was completed after incubation with substrate chromogen solution.

PROCEDURE

A. PARAFFIN EMBEDDED TISSUE

The tissues were fixed in 10 % formal saline for 24 hours. The tissues were then processed and embedded in paraffin.

B. ADHERENCE OF SECTIONS OF SLIDES

Polylysine – coated (Biogenex CA, USA) slides were used for the proper adherence of tissue section (other adhering agents which can be used are gelatin chrome, APES, Ainenopropyltriethoxy silane). Unstained section of 5 um thickness was cut using microtome and was transferred on polylysine coated slides from the paraffin blocks.

C. DEPARAFFINIZATION AND REHYDRATION

Sections were de-waxed thoroughly on slide warming table at 65 degree for half an hour.
Then sections were given two changes in xylene I, xylene II for 7 minutes each and then slides were allowed to dry.
Rinsed in alcohol for 3 minutes – two changes
The embedding medium must be completely removed. Any residual medium can cause increase in background staining and obscure specific staining.
Sections were kept in distilled water .

D. ANTIGEN RETRIEVAL

Antigen retrieval is the process by which antigenic epitopes, made unavailable because of fixation associated protein cross linking, are rendered accessible to antibodies for binding.
For this procedure buffer solution Tris- buffer was freshly prepared in which sodium citrate and citric acid was added with distilled water.

Preparation of Sodium Citrate Buffer: for 300ml

Buffer A: Citric Acid 2.10gm in 100ml distilled water
Buffer B: Sodium Citrate 2.950gm in 100ml distilled water
Buffer A + Buffer B
(9ml) + (41ml) = 50ml + 450ml distilled water = 500ml.
Slides were then placed in plastic coplin jars containing Tris buffer solution (PH-6) and irradiated in enzyme reteriver (microwave oven) (Biogenex CA, USA) at 96 degree for 10 minutes (two cycle)After heating the jar were removed from the oven and then slides were allowed to cool.Slides were then placed in the phosphate buffer saline (PBS) which is freshly prepared.

Phosphate buffer saline contains

Sodium chloride: 8.5gm
Di sodium hydrogen phosphate anhydrous (purified): 1.84g (Merck, India)
Sodium di- hydrogen phosphate (purified): 8.6gm in 1000ml of distilled water.
Here the slides are dipped in PBS solution for 5 minutes.

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 25, No. 2 (2024)
http://www.veterinaria.org
Article Received: Revised: Accepted:

E. STAINING PROTOCOL

STEP 1: Excess buffer was wiped off.
Specimen is incubated with **PEROXIDASE BLOCK(Biogenex CA, USA)** for 15 minutes .
Then slides were again washed with PBS solution. As the PBS acts as aqueous medium.

STEP 2: POWER BLOCK (BIOGENEX CA, USA) is used almost covering the specimen on the slide for 10 minutes in wooden box .

STEP 3: PRIMARY ANTIBODY (BIOGENEX CA, USA)
Slides are blot dried and then sections were covered with optimally diluted primary antibody of CD1a (Biogenex CA, USA) incubated for 1 hour.
Here precautions must be taken to avoid dryness of the tissue.

STEP 4: SUPER ENHANCER (Biogenex CA, USA)
Incubated on the specimen for 20 minutes followed by rinse with PBS

STEP 5: **POLY HRP REAGENT (HORSERADISH PEROXIDASE) (BIOGENEX CA, USA)**
Excess buffer was wiped off.
Enough drop of Poly HRP reagent were applied to cover the specimen and incubated for 30 minutes at room temperature. Again wash in PBS solution.

STEP 6: SUBSTRATE CHROMOGEN SOLUTION
(BIOGENEX CA, USA)
Slides were wiped as before.
Enough of freshly prepared substrate chromogen solution was applied to cover the section and incubated at room temperature for 10 minutes.

STEP 7: COUNTERSTAIN
SECTIONS WERE COUNTERSTAINED WITH HEMATOXYLIN
DAB chromogen gives alcohol insoluble end product. Therefore alcohol based haematoxylin eg Harris Haematoxylin used.

STEP 8: MOUNTING
Stained sections were dehydrated, cleared and mounted in DPX (DistreneDebutyl – Pthalate Xylene)

INTERPRETATION OF STAINING
The positive control was examined for the presence of a coloredend product at the site of the target antigen (Dab chromogen brown end product). The presence of these colors was interpreted as a positive staining result indicating proper performance of kit reagents.The negative control was examined. The absence of specific staining in the negative control confirmed the specificity of primary antibody.LCs were identified in the epithelium by their intensely staining dendritic processes & generally suprabasal location. Criteria for LC identification :- only cells with a definable cell body & nucleus & at least one dendritic process were considered LCs.The average number of LCs were calculated & compared with the normal oral epithelium.

Fig 17: Armamentarium for solution preparation

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 25, No. 2 (2024)
http://www.veterinaria.org
Article Received: Revised: Accepted:

Fig 18: Weighing Machine

Fig 19: Antigen Retrieval Reagents

Fig 20: Reagents For Phosphate Buffer Saline

Fig 21: Hot Plate

Fig 22: Antigen Retrieval Machine

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 25, No. 2 (2024)
http://www.veterinaria.org
Article Received: Revised: Accepted:

Fig 23: Immunohistochemistry Kit

Fig 24: Humid Chamber

Statistical Methods:
Discrete statistical data was analysed by Chi square test & Independent Student t- test & LCs were counted by manual tagging on Leica Suite Image Analysis Software.
Grid Microscope was used to measure the depth of connective tissue fibrosis.
Number of positive cells are expressed as mean +/- standard deviation.

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 25, No. 2 (2024)
http://www.veterinaria.org
Article Received: Revised: Accepted:

OBSERVATIONS AND RESULTS

30 patients with OSMF and 10 controls were selected for our study and were analysed for correlation of LC density in various grades of OSF epithelium with normal epithelium and depth of connective tissue fibrosis& degree of chronic inflammation in subepithelial connective tissue with help of detailed clinical examination, routine histopathological evaluation (H & E), and immunohistochemistry. All the findings were compiled and analysed for various comparative analyses using all the parameters with the help of Chi – square test and Student t- test.

All the observations were categorized in 5 groups as per aims and objectives

I.To study the clinical profile of oral Submucous fibrosis Patients.

II. Correlation of clinical groups according to Lai D.R *et al*. criteria with histopathological grade according to Rooban T. *et al*.

III. Correlationof no. of LCs in the epithelium with Histopathological stages/grades of OSMF.

IV. Correlation of LC density in OSF epithelium with depth of connective tissue fibrosis.

V.Correlation of LC density in OSF epithelium & degree of chronic inflammation in subepithelial connective tissue.

Correlation of H/P grade by H &E and clinical groups:

		CLINICAL GRADING					P-Value
		Group A	Group B	Group C	Group D	Total	
H/P grades	Very early	6	0	0	0	6	0.181
	Early	2	5	0	0	9	
	Moderately advanced	0	1	6	2	10	
	Advanced	0	1	3	1	5	
Total		8	7	8	7	30	

Profile distribution of subject group according to areca nut with tobacco& lime habit

	Areca Nut + Tobacco+Lime	
Frequency per day	**No of Patients**	**Percentage**
4	5	16.66
5	4	13.33
6	2	6.7
7	2	6.7
8	1	3.3
13	1	3.3

Correlation of No. of LCs in the epithelium with Histopathological stages/grades of OSMF.

1. The study group had 6(20%) subject's in group A with the LC density(37.00+/-7.69)with mean range (23-48),9(30%) subjects in group B with LC density(29.22+/-3.32) and mean range(28-38),10(33.33%) subjects in group C with LC density (23.2+/-8.30) and mean range(15-33) & 5(16.66%) subjects in group D with LC density (20.2+/-5.10) and mean range(9-26).Normal subjects10(33.3%) had LC density (54.90+/-11.91) with mean range (37-68)
2. Correlation Coefficient was estimated which gave value for H/P grade and number of LCs is0.000.This shows that there is strong positive relationship between H/P grade and no of LCs as compared to the normal mucosa.

Correlation of No. of LCs in the epithelium with histopathological stages/grades of OSMF

Oral epithelium	No.of subjects	LC density (cells/mm2 area)Mean +/-SD	Mean Range	P – value
Normal	10	54.50+/-11.91	37-68	0.000
OSF(very early) group A	6	37.00+/-7.69	33-48	
OSF(early) group B	9	29.22+/-3.32	28-38	
OSF(moderately dvanced) group C	10	23.2+/-8.30	15-33	
OSF(advanced) group D	5	19.2+/-5.10	9-26	

III.Correlation of LC density in OSF epithelium with depth of connective tissue fibrosis

1. Correlation Coefficient was estimated which gave value for LC density with depth of connective tissue fibrosis in OSF as 0.001 , which shows that there is strong positive relation between these two.

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 25, No. 2 (2024)
http://www.veterinaria.org
Article Received: Revised: Accepted:

2. The study group had had 6(20%) subject's in group A with the LC density (34.90+/-5.69), 9(30%) subjects in group B with LC density(31.0+/-4.32),10(33.33%) subjects in group C with LC density (23.4+/-8.30) & 5(16.66%) subjects in group D with LC density (19.4+/-5.10)

Correlation of LC density in OSF epithelium with depth of connective tissue fibrosis

Depth of connective tissue fibrosis.	No.of subjects	LC density (cells/mm2 area) (mean)	P-value
OSF(very early) group A	6	34.90+/-5.69	0.001
OSF(early)group B	9	31.0.+/- 4.32	
OSF(moderately advanced) group C	10	23.4+/-8.30	
OSF(advanced) group D	5	19.4+/-5.10	

Correlation of LC density in OSF epithelium & degree of chronic inflammation in subepithelial connective tissue.

1. Correlation Coefficient was estimated which gave value for LC density with degree of chronic inflammation in subepithelial connective tissue in OSF as 0.005, which shows that there is strong positive relation between these two.
2. The study group had had 6(20%) subject's in group A with the LC density(34.63+/-4.69), 9(30%)subjects in group B with LC density(30.44+/-3.32), 10(33.33%) subjects in group C with LC density (24.0+/-8.30) & 5(16.66%) subjects in group D with LC density (19.2+/-5.10) All the correlations were done using independent student-t test ($p<0.001$)

Correlation of LC density in OSF epithelium & degree of chronic inflammation in subepithelial connective tissue

Degree of chronic inflammation in subepithelial connective tissue.	No .of subjects	LC density (cells/mm2 area)(mean)	P-value
OSF(very early) group A	6	34.63+/- 4.69	0.005
OSF(early) group B	9	30.44+/-3.32	
OSF(moderately advanced) group C	10	24.0+/-8.30	
OSF(advanced) group D	5	19.2+/-5.10	

Fig25 Early OSMF CD1a positive 40x

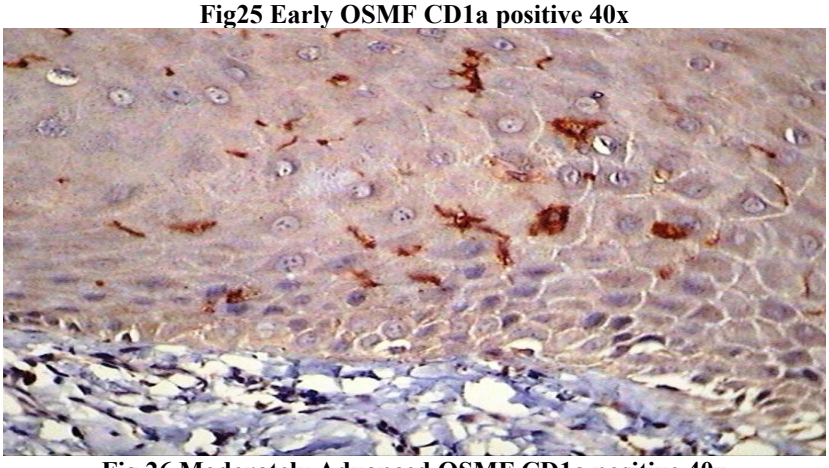

Fig 26 Moderately Advanced OSMF CD1a positive 40x

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 25, No. 2 (2024)
http://www.veterinaria.org
Article Received: Revised: Accepted:

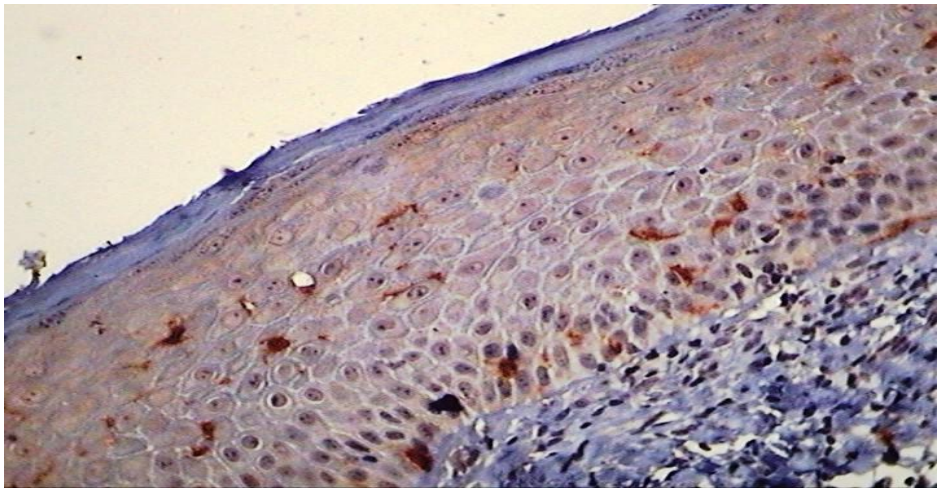

Fig.27 Advanced OSMF CD1a positive 40x

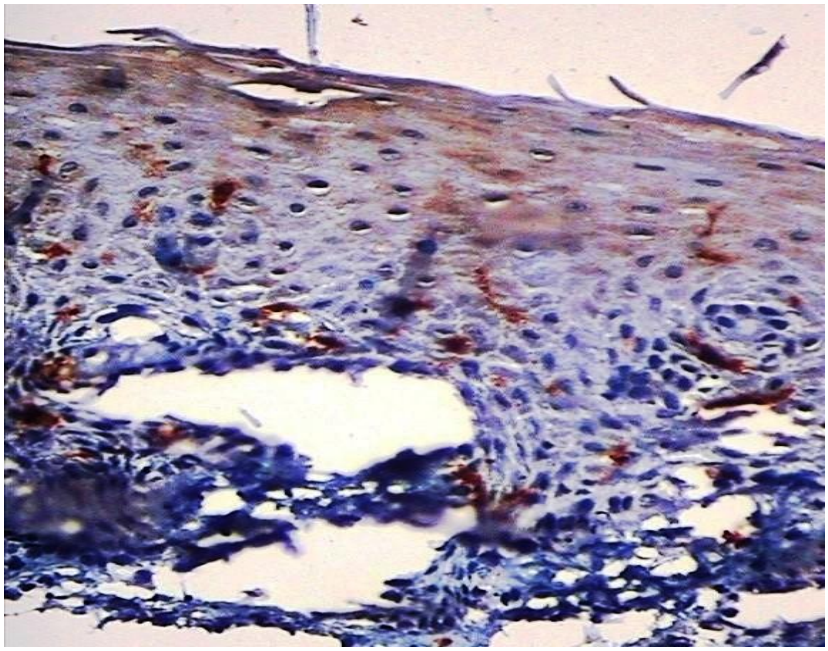

Fig 28: Normal Mucosa (10X)- CD1a positive.

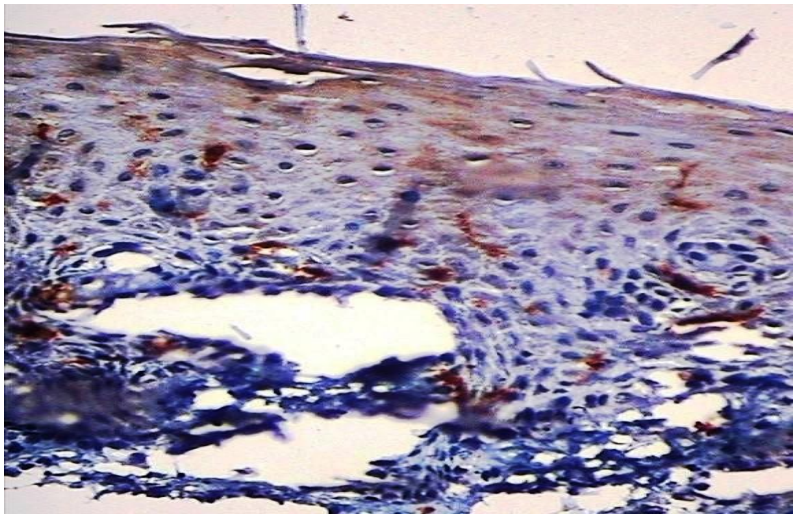

Fig 29: Normal Mucosa (40X)- CD1a positive.Cell counting –Leica Image Analysis

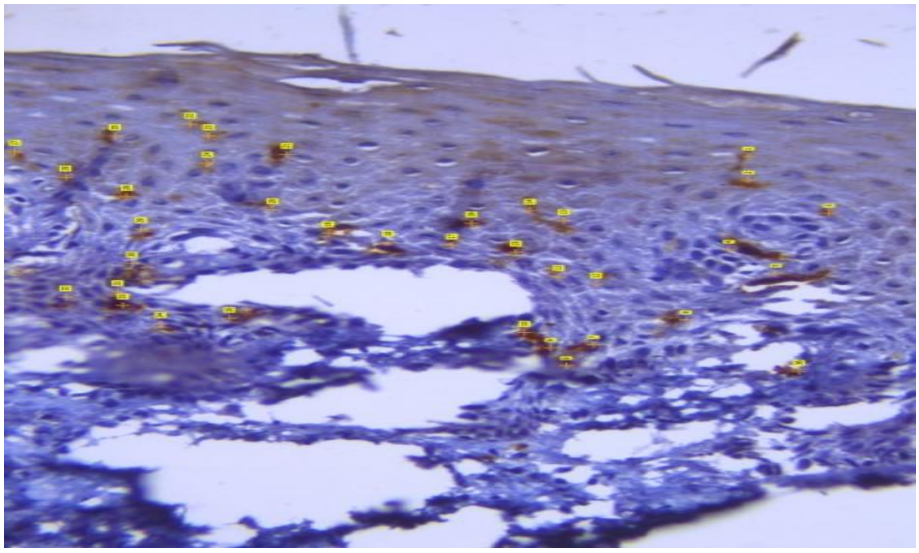

Fig 30: Very Early OSMF (10X)- CD1a positive

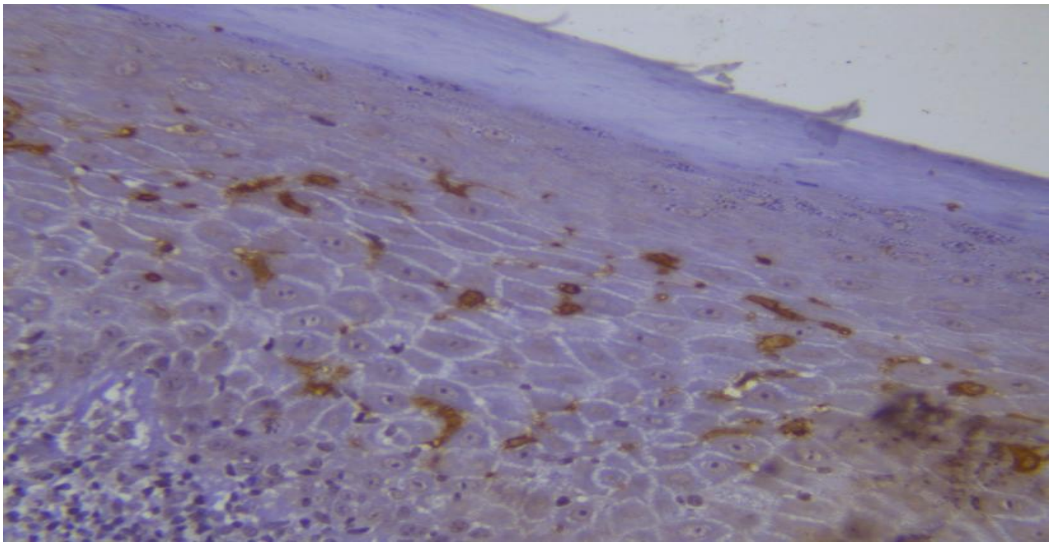

Fig 31: Very Early OSMF (40X)- CD1a positiveCell counting- Leica Image Analysis

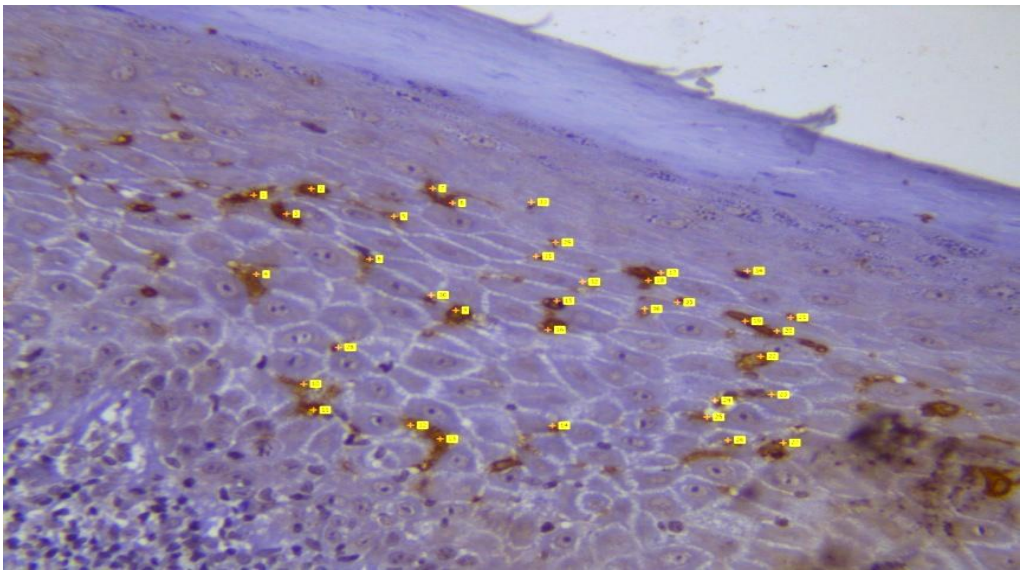

Fig 32: Early OSMF (10X)- CD1a positive

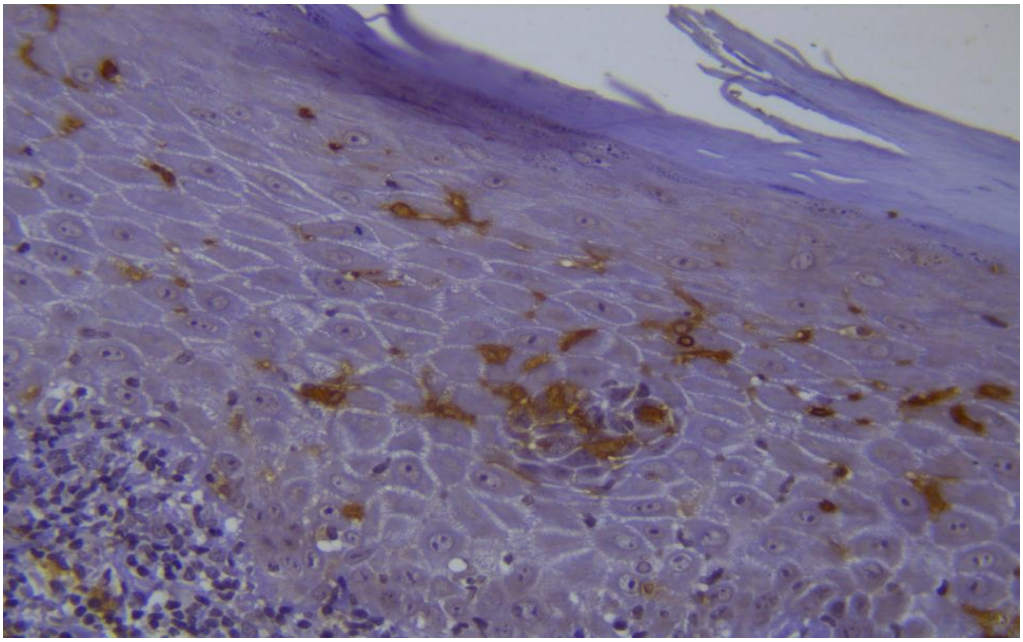

Fig 33: Early OSMF (40X)- CD1a positiveCell counting- Leica Image Analysis

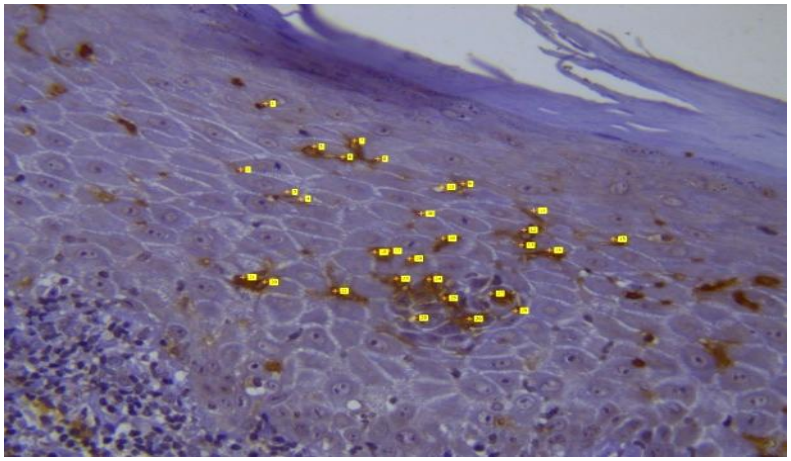

Fig 34: Moderately Advanced OSMF (10X)- CD1a positive

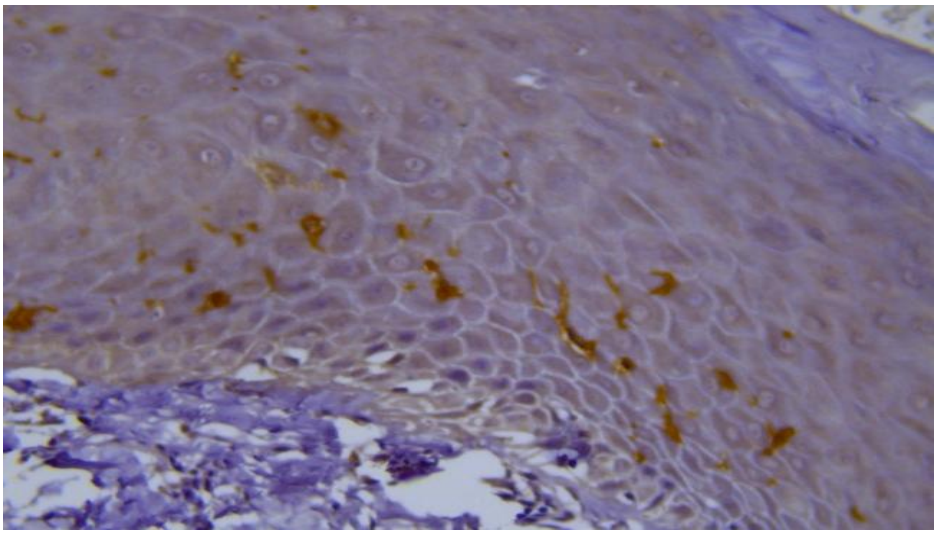

Fig 35: Moderately Advanced OSMF (40X)-CD1a positiveCell counting- Leica Image Analysis

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 25, No. 2 (2024)
http://www.veterinaria.org
Article Received: Revised: Accepted:

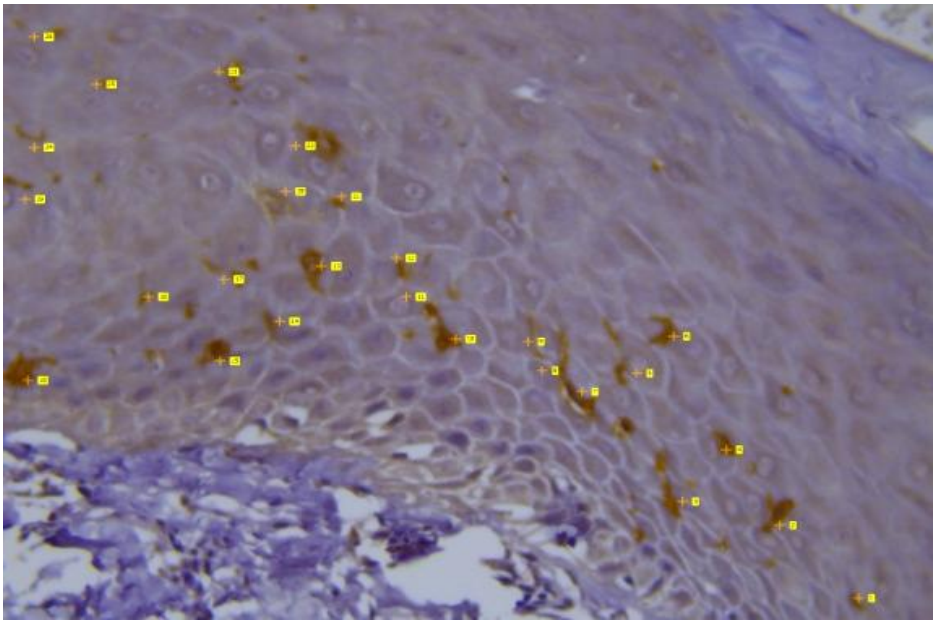

Fig 36: Advanced OSMF (10X)-CD1a positive

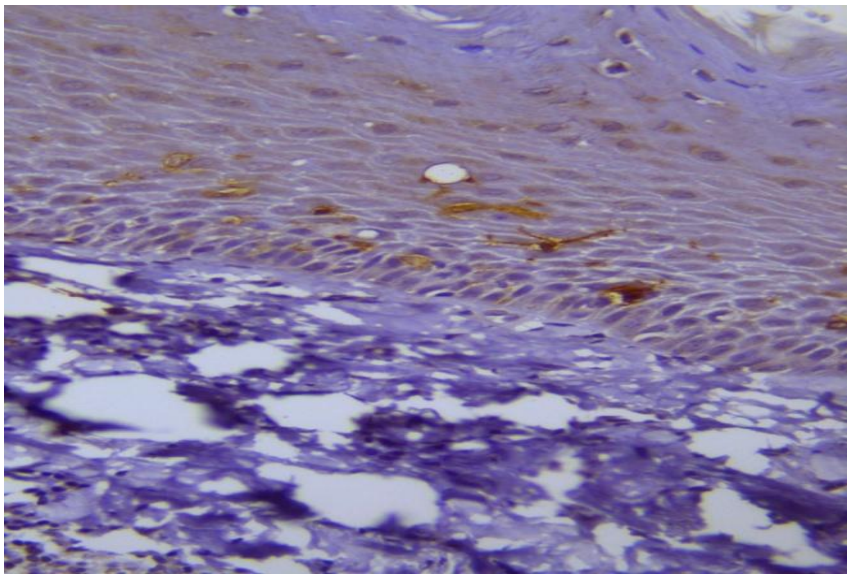

Fig 37: Advanced OSMF (40X)- CD1a positiveCell counting- Leica Image Analysis

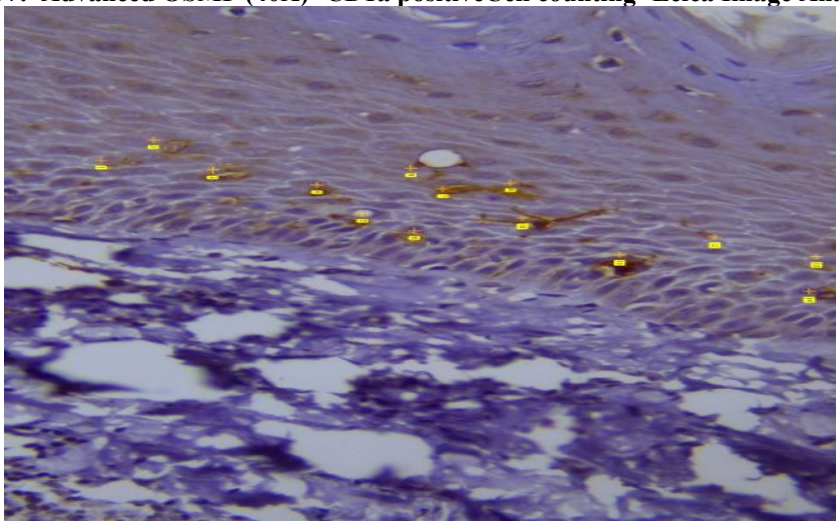

Fig 38: Advanced OSMF (40X)- CD1a positiveCell counting- Leica Image Analysis

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 25, No. 2 (2024)
http://www.veterinaria.org
Article Received: Revised: Accepted:

DISCUSSION

Oral submucous fibrosis (OSMF) predominantly affects Asiatic people & is characterized by inflammation & a progressive fibrosis of the juxta-epithelial & deeper connective tissue of oral mucosa. The major presenting complaint is a progressive inability to open the mouth because of accumulation of inelastic fibrous tissue in the subepithelial region of the oral mucosa, along with concomitant muscle degeneration. Recent epidemiological studies suggest the habit of betel nut chewing as a major etiologic factor. Histologically, OSMF is characterized by atrophy of the overlying epithelium with variable degrees of dysplastic change, & subepithelial fibrosis and hyalinization with a progressive loss of vascularity. Often there is variable chronic inflammatory cell infiltrate in the lamina propria & submucosa. OSMF is also regarded as a precancerous condition. It is regarded as possessing a high degree of malignant potential. Its precancerous nature was first mentioned by Paymaster in 1956, who observed the development of slowly growing squamous cell carcinoma in one-third of his patients. Since then more data have been presented by Pindborg to substantiate the hypothesis that submucous fibrosis is precancerous condition.

In the present study, LC density in OSMF epithelium was decreased compared with normal oral epithelium. The study group had normal subjects,10(33.3%) with LC value 54.50+/-11.91, 6(20%) subjects with the LC density values 37.00+/-7.69 in H/P grade 1, 9(30%) had LC density value 29.22+/-3.32 in H/P grade 2, 10(33.33%) had the value 23.2+/-8.30 in H/P grade 3and 5(16.66%) had a value 19.2+/-5.10 in H/P grade 4. Correlation Coefficient was estimated which gave value for H/P grade and LC density when compared with normal as 0.000 This shows that there is significant co-relationship between H/P grade and LC density.

If OSF is considered a precancerous condition, this result was consistent with that obtained by Mc Ardle & Muller. They demonstrated that LC density is significantly decreased in cervical condyloma, which may demonstrate premalignancy, especially when infected with HPV-16&18. The reduction of LC density in OSMF could reflect a defective local immune surveillance & may promote the development of oral cancer. The reason for decrease in the LC density in OSMF epithelium is reasonable to hypothesize that the recruitment of bone-marrow derived LCs from the circulation is decreased because fibrosis & hyalinization of subepithelial connective tissue & subsequent loss of vascularity usually develop as the stage of OSMF advances. Consistent with this hypothesis is that; tissue fibrosis – induced decrease in LC number has also been reported in a group of tongue squamous cell carcinoma.

Although the LC number in OSMF was significantly reduced as the stage advances as compared to the normal oral epithelium, this study did not appreciate much of a difference between the moderately advanced & advanced histologic stages. This result may be due to the fact that though there are histologic differences, no significant loss of vascularity & recruitment of LCs from the circulation is detectable. LC density is not noticeably decreased in the early stage (as compared to the mean ranges) of OSMF, but is markedly decreased in the late stage.

Diminished nutritional supply resulting from subepithelial connective tissue fibrosis might shorten the life span of LCs & thus decrease the number of LCs in the OSMF epithelium. Hence with the severity of OSMF(as observed in the H&E stained sections), the depth of connective tissue fibrosis is increased as a result there is decrease in the LC density ie ; there is significant correlation of LC density with the depth of connective tissue fibrosis.

There was no significant difference observed between the various histopathological parameters & the LC density.
Similarly, as the grade/stage of OSMF advances the inflammatory cell infiltrate is hardly seen. Number of blood vessels are dramatically small in subepithlial zone. Marked fibrosis with hyaline changes seen extending from subepithelial to superficial muscle layers. This was observed by **Utsunomiya H, Tilakratne WM, Oshiro K et al in 2005.** Hence, decrease in local immune surveillance causes difficulty in the recruitment of LCs from circulation. Hence our results show decrease in LC density when compared to the degree of chronic inflammation in subepithelial connective tissue(as observed in H &E stained sections) as severity of OSMF progresses.

Our results on LC numbers in OSMF(precancerous lesion) were similar to those of **Rich & Reade** who found significantly fewer LCs in tobacco-associated leukoplakia; **Anjali Narwal &SusmitaSaxena.** A statistically significant difference was noted in the presence of LCs between normal, early and advanced OSMF (independent student's t-test; $p < 0.001$) Similar findings were reported by **Chun-Pin Chiang, Chi-Yuan Kao, Bu-Yuan Liu, Jeng-Tzung Wang,1998.**

CONCLUSION

There is great deal of interest in how Dendritic Cells (DCs) might be exploited as a form of immunotherapy. DCs are being studied as adjuvants for vaccines or as a direct therapy to induce immunity against cancer. DCs loaded with tumor lysates, tumor antigen-derived peptides, MHC class I restricted peptides, or whole protein have all been shown to generate anti-cancer immune responses & activity, including in some cases the ability to induce complete regression of existing tumor.

Thus, there is a great desire to test these strategies & use tumor-antigen bearing DCs as a vaccine in humans.
Thus, the reduction of LC density in OSF epithelium, which may indicate a decrease in local immune surveillance, not only provides histologic evidence for its premalignant character but also has an effect of fanning the flame-on the epithelial carcinogenesis.Also, more studies need to be carried out in the future in order to clarify the exact relationships among areca nut, tobacco, LCs, & premalignant & malignant oral lesions.

BIBLIOGRAPHY

1. N. S. Murthy and Aleyamma Mathew. Cancer epidemiology, prevention and control.Current Science 2004; 86: 4-25.
2. SamanWarnakulasuriya. Global epidemiology of oral and oropharyngeal cancer. Oral Oncology 2009; 45: 309–316.
3. *S. EL Mofty.* Early detection of oral cancer .*Egypt. J. Oral Maxillofac. Surg. 2010; 1: 25- 31.*
4. Crispian Scully, José V. Bagan, Colin Hopper, &Joel B. Epstein. Oral cancer: Current and future diagnostic techniques.Am J Dent 2008;21:199-209.
5. R.B. Zain, N. Ghazali. A review of epidemiologicalstudies of oral cancer andprecancer in Malaysia. Annal Dent Univ Malaya 2001; 8: 50-56.
6. Chris de Souza, UdayPawar, PankajChaturvedi. Precancerous lesions of oral cavity.Otolaryngeology Clinics: An International Journal May- Sept 2009; 1(1):7-14.
7. R. Rajendran. Oral submucous fibrosis: etiology,pathogenesis, and future research. WHO Bulletin OMS 1994; 72.
8. GanigaChannaiahShivakumar, ShivakumarSahana. Correlation between the Functional and Histological Staging of Oral Submucous Fibrosis. Journal of Indian Academy of Oral Medicine and Radiology, July-September 2010;22(3):133-135.
9. Ahmad M. S., Ali S. A., Ali A. S., Chaubey K. K. Epidemiological and etiological study of oral submucous fibrosis among Gutka chewers of Patna, Bihar, India. J Indian SocPedodPrev Dent - June 2006; 84-89.
10. R. Rajendran, G.K. Raju, S.M. Nair,& G. Balasubramanian. Prevalence of oral submucous fibrosis in thehigh natural radiation belt of Kerala, South India. Bulletin of the World Health Organization, 1992;70(6): 783-789.
11. Vanaja Reddy, P.V.Wanjari, Naveen Reddy Banda, Prashanti Reddy. Oral Submucous Fibrosis: Correlation of Clinical Grading to various habit factors. International Journal Of Dental Clinics 2011:3(1).
12. AjitAuluck, Miriam P. Rosin, Lewei Zhang, Sumanth KN. Oral Submucous Fibrosis, a Clinically Benignbut Potentially Malignant Disease:Report of 3 Cases and Review of the Literature. October 2008; 74(8) .
13. Saba Khan, LaxmikantChatra, Shenai KP, K M Veena, Prasanna Kumar Rao. Pathogenesis of oral submucous fibrosis. Journal of Cancer Research and Therapeutics- April- June 2012; 8(2): 199-203.
14. N. Afroz, S.A. Hasan, S. Naseem. Oral Submucous Fibrosis A Distressing Disease with Malignant Potential. Indian Journal of Community Medicine October-December 2006; 31(4): 270-271.
15. Chung-Jung Chiu, Min-Lee Chang, Chun-Pin Chiang,Liang-Jiunn Hahn, Ling-Ling Hsieh, and Chien-Jen Chen. Interaction of Collagen-related Genes and Susceptibility to BetelQuid-induced Oral Submucous Fibrosis. Cancer Epidemiology, Biomarkers & Prevention- July 2002;11: 646–653.
16. RamachandranSudarshan, Rajeshwari G Annigeri, SreeVijayabala G. Pathogenesis of Oral Submucous Fibrosis: The Past and Current Concepts. International Journal of Oral & Maxillofacial Pathology. 2012;3(2):27-36.
17. C.P. Chiang, H.Y. Wu, B.Y. Liu, J.T. Wang, M.Y.P. Kuo. Quantitative analysis of immunocompetent cells in oral submucousfibrosis in Taiwan. Oral Oncol 2002; 38: 56–63.
18. Anjali Narwal&SusmitaSaxena: A comparison of langerhans cell numbers in tobacco-associated leukoplakia& oral squamous cell carcinoma. Int J Oral –Med 2011; 10(3):125-131.
19. Kalpana A P, Rajkumar N P, Sangeeta PW. Correlation of salivary and serum IgG, IgA levels with total protein in oral submucous fibrosis. Jr of Oral Science 2011; 53(1): 97-102.
20. Pindborg JJ, Sirsat SM. Oral Submucous Fibrosis. Oral Surg, Oral Med, Oral Pathol 1966; 22(6): 764-779.
21. P. C. Gupta, p. N. Sinor, r. B. Bhonsle, v. S. Pawar, h. C. Mehta. Oral submucous fibrosis in India: A new epidemic? The national medical journal of india 1998; 11(3): 113-116.
22. Pindborg, J. J., Chawla, T. N., Shrivastava, A. N., Gupta, D. &MehrotraSubmucous Fibrosis of the Oral Cavity : 2. Studies on Epidemiology .Actaodont. scand.1964; 22: 793-799.
23. Peter A. Hayes. Oral submucous fibrosis in a 4-year-old girl. Oral Surg, Oral Med, Oral Pathol 1985; 59(5): 475-478.
24. . Fali S. Mehta, Gupta, J. J. Pindborg, R. B. Bhonsle,P. N. Jalnawalla, & P. N. Sinor. An intervention study of oral cancer and precancerin rural Indian populations: a preliminary report. Bulletin of the World Health Organiztion 1982; 60(3): 441-446.
25. Pindborg JJ. Oral Submucous Fibrosis: A Review. Annals of the Academy of Medicine, Singapore1989; 18(5):603-607].
26. Nektorios IL. OraSubmucous Fibrosis. http://www.emedicine,com/derm/topic 653. htm (1 of 19) 10/2/2007.
27. Prakash C Gupta. Areca nut use in India. Indian J Med Science 2007; 61: 317-319.

28. P. N. Sinor, P. C. Gupta, P. R. Murti, R. B. Bhonsle, D. K. Daftary, Mehta, J. J. Pindborg. A case-control study of oral submucous fibrosis with special reference to the etiologic role of areca nut. Journal of Oral Pathology & Medicine 1990; 19(2):94–98.
29. Binu J Jacob, Kurt Straif, Gigi Thomas, KunnambathuRamadas, Babu Mathew, Zuo-Feng Zhang, RengaswamySankaranarayanan, Mia Hashibe. Betel quid without tobacco as a risk factor for oral precancers. Oral Oncol2004; 40: 697–704.
30. A. Ariyawardana, A. D. S. Athukorala, A. Arulanandam. Effect of betel chewing, tobacco smoking and alcohol consumption on oral submucous fibrosis: a case–control study in Sri Lanka.Jr of Oral Pathol& Med April 2006; 35(4): 197–201.
31. W.M. Tilakaratne, Klinikowski, Takashi Saku, T.J. Peters, SamanWarnakulasuriya. Oral submucous fibrosis: Review on aetiology and pathogenesis. Oral Oncol 2006; 42: 561– 568.
32. Soma Babu, Ramesh VenkataramanaBhat, PutchaUday Kumar, BoindalaSesikaran, KakarapartiVisweswaraRao, PakerlaAruna and Puttam Reddy Ramachandra Reddy. A Comparative Clinico-Pathological Study of Oral Submucous Fibrosis in Habitual Chewers of Pan Masala and Betelquid. Clinical Toxicology 1996, Vol. 34, No. 3 , Pages 317-322.
33. N. Shah, P. P. Sharma. Role of chewing and smoking habits in the etiology of oral submucous fibrosis (OSF): a case-control study. Jr of Oral Pathol& Med- November 1998; 27(10): 475–479.
34. Mohammad Sami Ahmad, SharíqueAther Ali, Ayesha Sharique Ali, Krishna Kumar Chaubey. **Comparative Severity of Oral Submucous Fibrosis among gutkha and other areca nut products chewers.** http://www.priory.com/den/areca.htm (2 **of 20) 10/2/2007.**
35. Saraf S. Textbook of Oral Pathology. 1st edition 2006; Jaypee Publishers.
36. Yu-Chao Changa, Shun-FaYangb, Kuo-Wei Taia, Ming-Yung Chouc, Yih-Shou Hsieh. Increased tissue inhibitor of metalloproteinase-1 expression and inhibition of gelatinaseA activity in buccal mucosal fibroblasts by arecoline as possible mechanisms for oral submucous fibrosis. Oral Oncol 2002; 38: 195–200.
37. Chung-Hung Tsai, Shun-Fa Yang, Yu-Chao Chang. The upregulation of cystatin C in oral submucous fibrosis. Oral Oncol 2007; 43: 680– 685.
38. Supriya S Koshti, Suresh Barpande. Quantification of plasma fibrinogen degradation products in oral submucous fibrosis: A clinicopathologic study J Oral MaxillofacPathol 2007;11: 48-50.
39. Harvey W, Scutt A, Meghji S, Canniff JP. Stimulation of human buccal mucosa fibroblasts in vitro by betel-nut alkaloids. Arch Oral Biol. 1986;31(1):45-9.
40. Van Wyk CW, Stander I, Padayachee A, Grobler-Rabie AF: The areca nut chewing habit and oral squamous cell carcinoma in South African Indians. A retrospective study. S Afr Med J 1993 Jun; 83(6): 425-9.
41. Haque MF, Meghji S, Khitab U, Harris M. Oral submucous fibrosis patients have altered levels of cytokine production. J Oral Pathol Med 2000: 29: 123–8.
42. Rajalalitha P, Vali S. Molecular pathogenesis of oral submucous fibrosis – a collagen metabolic disorder. J Oral Pathol Med 2005; 34: 321–328.
43. Tsai CC, Ma RH, Shieh TY. Deficiency in collagen and fibronectin phagocytosis by human buccal mucosa fibroblasts in vitro as a possible mechanism for oral submucous fibrosis. J Oral Pathol Med 1999; 28:59–63.
44. Shieh DH, Chiang LC, Lee CH, Yang YH, Shieh TY. Effects of arecoline, safrole and nicotine on collagen phagocytosis by human buccal mucosal fibroblasts as a possible mechanism for oral submucous fibrosis in Taiwan. J Oral Pathol Med 2004; 33: 581–587.
45. Shieh DH, Chiang LC, Shieh TY. Augmented mRNA expression of tissue inhibitor of metalloproteinase1 in buccal mucosal fibroblasts by arecoline and safrole as a possible pathogenesis for oral submucous fibrosis. Oral Oncol 2003; 39: 728–735.
46. Tsai CH, Chou MY, Chang YC. The up-regulation of cyclooxygenase-2 expression in human buccal mucosal fibroblasts by arecoline: a possible role of pathogenesis in oral submucous fibrosis. J Oral Pathol Med 2003; 32: 146–153.
47. Jeng JH, Ho HS, Chan CP, Wang YJ, Hahn LJ, Chang MC. Arecanut extract up-regulates prostagladin production, cyclooxygenase-2 mRNA and protein expression of human oral keratinocytes.Carcinogenesis 2000; 21: 1365–70.
48. Rajendran R, Shirley V. Inducible nitric oxide Synthase expression is upregulated in oral submucous fibrosis. Indian J Dent Res. 2007; 18 (3): 94-100.
49. Seedat HA, van Wyk CW: Betel-nut chewing and submucous fibrosis in Durban. S Afr Med J 1988 Dec 3; 74(11): 568-71.
50. Liao PH, Lee TL, Yang LC, et al: Adenomatous polyposis coli gene mutation and decreased wild-type p53 protein expression in oral submucous fibrosis: a preliminary investigation. Oral Surg Oral Med Oral Pathol Oral RadiolEndod 2001 Aug;92(2):202-7
51. Rajendran R, Shivpathasundaram S. Shefarstext book of oral pathology. Fifth edition; Elsevier publications. Pg. 138 – 139.
52. V. Jayanti, Probert CSJ, Sher KS, Mayberry JF. OSMF: A preventable disease. Gut 1992; 33: 4-5

53. Wahi PN, Kapur VL, Luthra UK, Srivastava MC. Submucous fibrosis of the oral cavity: 1.Clinical Features. Bull World Health Organ 1966; 35: 789-792
54. Rajendran R, Radhakrishnan NS, Kartha CC. Light and electron microscopic studies on oral submucous fibrosis. J Indian Dent Assoc 1993; 64: 157-161.
55. El-Labban NG, Canniff JP. Ultrastructural findings of muscle degeneration in oral submucous fibrosis. J Oral Pathol 1985; 14:709-717.
56. Shivakumar GC, Sahana S. Clinical Staging of Oral Submucous Fibrosis: A Review. Int J of Oral Med- Science 2011; 10(3): 216-219.
57. Ahuja SS, AgrawalGD :Submucous fibrosis of the oral mucosa. J Oral Med 1971; 26: 35-36.
58. Bhatt AP, DholakiaHM .: Mast cell density in oral submucous fibrosis. Journal of Indian Dental Association 1977; 49: 187-191.
59. 58.Gupta DS, Gupta MK, GolharBL : Oral submucous fibrosis -clinical study and management by physiofibrolysis (MWD). JIDA 1980; 52: 375-378, 1980.
60. 11.Mathur RM , Jha T : Normal oral flexibility a guideline for SMF cases. JIDA, 64: 139-143.
61. Khanna JN, Andrade NN : Oral submucous fibrosis: A new concept in surgical management.Report of 100 cases. Int J Oral MaxillofacSurg 1995; 24: 433-439.
62. Lai DR, Chen HR, Lin LM, Huang YL, Tsai CC: Clinical evaluation of different treatment methods for oral submucous fibrosis. A 10-year experience with 150 cases. J Oral Pathol Med 1995; 24: 402-406.
63. HaiderSM , Merchant AR, Fikree FF, Rahban MH. Clinical and functional staging of oral submucousfibrosis. Brit J Oral MaxilofacSurg 2000; 38: 12-15.
64. Baillor DN, Nagesh KS. Oral Precancer. Findamentals of oral medicine and radiology.2nd edition; 2001: 282-289.
65. Aziz SR : Oral submucous fibrosis; case report andreview of diagnosis and treatment.J Oral MaxillofacSurg 2008; 66: 2386-2389.
66. Kerr AR, Warnakulasuriya S, Mighell AJ,DietrichT,NasserM ,RimalJ,JalilA,BornsteinMM ,NagaoT,Fortune F, Hazarey VH, Reichart PA, Silverman S,JohnsonNW : A systematic review of medical interventions for oral submucous fibrosis and futureresearchopportunities.Oral Disease 2011; 17: 42-57.
67. Eric Wieder : Dendritic Cells: A Basic Review. International society of cellular therapy; 2003
68. Chun-Pin Chiang, Chi-Yuan Kao, Bu-Yuan Liu, Jeng-Tzung Wang: Reduction of Langerhans cell density in oral epithelium of patients with oral submucous fibrosis. Chin Dent J 1998;17(3):137-147
69. ShwetaJaitley, TR Saraswati: Pathophysiology of Langerhans Cells. J of oral Maxillofac pathol,2012; vol.16, issue: 2.
70. T.Lombardi, C.Hauser, & E. Budtz-Jorgensen: Langerhans Cells – structure, function & role in oral pathological conditions. J of Oral Pathol Med 1993:22;193-202.
71. Troy E. Daniels, Laisheng Chou, John S. Greenspan, Deborah G. Grady: Reduction of Langerhans cells in smokeless tobacco – associated oral mucosal lesions. J Oral Pathol Med 1992; 21:100-4.
72. Cruchley AT, Williams DM, Farthing PM, Speight PM, LeschCA : Langerhans cell density in normal human oral mucosa & skin: relationship to age, smoking & alcohol consumption. J Oral Pathol Med 1994; 23:55-59.
73. Khoo SP, Lee KW: The Oral Mucosa & Langerhans Cells in Smokers: Evidence for Carcinigenesis. Annals Dent, Univ.Malaya 1995; 2:1-4.
74. Robert E.Hunger, Peter A.Sieling, Maria Teresa Ochoa, MakotaSugaya: Langerhans cells utilize CD1a &Langerin to efficiently present nonpeptide antigens to T cells: The Journal of clinical Investigation 2004:113(5).
75. L.Perez, M.R.Shurin, B.Collins, D.Kogan, I.L.Tourkova&G.V.Shurin :Comparative analysis of CD1a, S-100, CD83 & CD11c human dendritic cells in normal, premalignant & malignant tissues: Histology & Histopathology 2005; 20:1165-1172.
76. Celia carrillo, Miguel Penarrocha, Maria Penarrocha, Francisco Vera, David Penarrocha : Med OralPathol Oral Cir Bucal, 2010: 15(2).
77. Troy E. Daniels, Louis S. Hansen, John S. Greenspan, Laisheng Chou, Deborah G. Grady, Walter W. Hauck, John C. Greene, and Virginia L. Ernster: Smokeless tobacco-associated Epithelial & Langerhans Cell changes. Smoking & Tobacco Control Monograph No.2; supported by National Institute of Dental Reseach grant no.DE-08547.
78. Pindborg JJ, Murthi P.R, R.B Bhonsle, P.C Gupta, D.K Daftary and FaliS.Mehta. Oral submucous fibrosis as a precancerous condition. Scand J Dent Res 1984; 92:224-229
79. J.J. Pindborg, R.B. Bhonsle , P.R. Murti , P.C. Gupta, D.K. Daftary and Fali S. Mehta.Incidences and early forms of oral submucous fibrosis. Oral surgery ,Oralmedicin and Oral pathology 1980; Vol. 50: 40-44.
80. Ranganathan K, Devi MU, Joshua E, KiranKumar K, Saraswathi TR. OSMF: a case control study in chennai, South India. Journal of Oral pathology medicine 2004; 33(5):274-277.
81. V. K. Hazarey, D.M.Erlewad, K. A. Mundhe, S. N. Ughade.Oral submucous fibrosis: study of 1000 cases from central India. Journal Of Oral Pathology and Medicin 2007; 36: 12-16.
82. Chandramani More, Sunanda Das, HetulPatel,ChhayaAdalja, VaishnaveeKamatchi :Proposed clinical classification for oral submucous fibrosis. Oral Oncology 48(2012)200-202.

83. P.NWahi ,V.LKapur, Usha K. Luthria and M.C Srivastava: Submucous Fibrosis of Oral Cavity-Clinical Features. Indian Journal of Medical Research 1966, 16:189
84. P.C Gupta, C S Ray: Epidemiology of Betel Quid Usage. Ann Acad Med Singapore 2004; 33(Suppl):31S-36S.
85. C.R Trivedi, G.Craig, S.Warnakulasuriya: the Oral Health Consequences of chewing areca nut. Addiction Biology (2002) 7; 115-125
86. Eric Oakle ,L.Demaine& S. Warnakulasuriya: Areca (betel) nut chewing habit among high school children in the commonwealth of the Northern Mariana Islands (Micronesia): Bulletin of World Health Organisation 2005;83:656-660.
87. Kiran Kumar, Saraswathi TR, K. Rangnathan, M. Uma Devi, Joshua Elizabeth: Oral SubmucousFibrosis : A Clinico –histopathological study in Chennai. Indian J Dent Res, 18(3),2007.
88. Ching-Hung-Chung, Yi-Hsin-Yang, Tung-Yiu Wang, Tein-Yu Shieh, S. Warnakulasuriya: Oral precancerous disorders associated with areca nut chewing, smoking & alcohol drinking in southern Taiwan. J Oral PatholMed(2005)34;460-6
89. Ranganathan K, Gauri Mishra: An overview of classification schemes for oral submucous fibrosis. Journal of Oral and Maxillofacial Pathology 2005; 10(2): 55-58.
90. Vineet Singh Cheema, V. Ramesh & P.D. Balamurali: The Relevance of Mast Cells in Oral Squamous Cell Carcinoma. Journal of Clinical & Diagnostic Research, 2012;6(10):1803-1807.