IMMUNOHISTOCHEMICAL EXPRESSION OF NUCLEAR FACTOR – KAPPA B IN ORAL SQUAMOUS CELL CARCINOMA, ORAL SUBMUCOUS FIBROSIS AND NORMAL MUCOSA

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CERTIFICATE

This is to certify that this dissertation titled **"IMMUNOHISTOCHEMICAL EXPRESSION OF NUCLEAR FACTOR - KAPPA B IN ORAL SQUAMOUS CELL CARCINOMA, ORAL SUBMUCOUS FIBROSIS AND NORMAL MUCOSA"** is a bonafide dissertation performed by **LAVANYA.C** under our guidance during her post graduate period between 2007 – 2010.

This dissertation is submitted to **THE TAMILNADU DR. M. G. R. MEDICAL UNIVERSITY,** in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY IN ORAL PATHOLOGY AND MICROBIOLOGY, BRANCH VI.** It has not been submitted (partial or full) for the award of any degree or diploma.

RAGAS DENTAL COLLEGE Chennai

Dr. K. Ranganathan, MDS, MS (Ohio)
Professor and Head
Department of Oral & Maxillofacial Pathology
Ragas Dental College & Hospitals
Chennai

Dr. K. RANGANATHAN, MDS, MS (OHIO)
Professor and Head of Department
Oral and Maxillofacial Pathology
RAGAS DENTAL COLLEGE & HOSPITAL
Chennai - 600 119.

Dr. Elizabeth Joshua, MDS
Professor
Department of Oral & Maxillofacial Pathology
Ragas Dental College & Hospitals
Chennai

DEPT. OF ORAL PATHOLOGY
RAGAS DENTAL COLLEGE & HOSPITAL
MADRAS

Dr. S. Ramachandran, MDS
Principal
Ragas Dental College & Hospitals
Chennai

PRINCIPAL
RAGAS DENTAL COLLEGE & HOSPITAL
CHENNAI

Oral submucous fibrosis (OSF) is a chronic progressive, scarring, high risk pre-cancerous condition of the oral mucosa seen primarily on the Indian sub continent and in South east Asia[1]. The disease was first reported in 1952 by Schwartz and in 1953, Joshi redesignated the condition as Oral Submucous Fibrosis, implying predominantly its histological nature[2]. The condition is characterized by mucosal rigidity of varying intensity due to fibroelastic changes in juxta epithelial layer, reduction in vasculature and epithelial atrophy resulting in a progressive inability to open the mouth.[3]

The precancerous nature of OSF was first postulated by Paymaster, (1955) who described the development of a slow growing squamous cell carcinoma in one third of OSMF cases.[4]The malignant transformation rate of 7.6% have been reported from Indian sub-continent over a period of 17 years.[5]

According to WHO, oral squamous cell carcinoma (OSCC) in males in developing countries, is the sixth most common cancer after lung, prostate, colorectal, stomach and bladder cancer. While in females it is the tenth most common site of cancer after breast, colorectal, lung, stomach, uterus, bladder and liver. (2006)[6]

The pathogenesis of OSF is multi factorial. The predisposing factors include areca nut chewing, genetic and immunologic process and nutritional deficiencies. Areca nut is the main etiologic agent for OSF.[7]

Nuclear factor kappa B (NF-κB) is a collective name for inducible, dimeric, ubiquitous transcription factors. They are made up of members of the Rel family and upon cell stimulation they are transferred from cytoplasm to nucleus. NF- κB was first identified as a regulator of expression of the kappa light chain gene in murine B-

lymphocytes, but has subsequently been found in many different cells. They play a crucial role in immune and inflammatory responses by regulating the genes encoding pro-inflammatory cytokines, chemokines and growth factors.[8]

NF-κB plays a role in the pathogenesis of many inflammatory conditions such as rheumatoid arthritis, asthma, and inflammatory bowel disease. NF-κB can also act as an amplifying mechanism that exaggerates disease specific inflammatory processes through coordinated activation of several genes involved in inflammation[9]. Persistent tissue inflammation plays an important role in the pathogenesis of tissue fibrosis and increases the risk of cancer development[10]. Many carcinogens including areca nut and various oncogenic events contribute to progressive increase in NF kappa B activation which leads to tumor cell proliferation, migration and angiogenesis.[11] The expression of NF kappa B proteins in pre malignant conditions such as OSF has the potential to be used as a marker for malignant transformation.

The present study was done to evaluate the expression of NF-κB in formalin fixed paraffin embedded tissues of normal buccal mucosa, oral submucous fibrosis and oral squamous cell carcinoma.

Aims and Objectives

- To evaluate the expression of NF - κB in formalin fixed paraffin embedded tissues of oral squamous cell carcinoma by immunohistochemistry.

- To evaluate the expression of NF- κB in formalin fixed paraffin embedded tissue of OSF by immunohistochemistry

- To evaluate the expression of NF- κB in formalin fixed paraffin embedded tissue of normal buccal mucosa by immunohistochemistry

- To compare the expression of NF- κB in oral squamous cell carcinoma, oral submucous fibrosis and normal buccal mucosa,

Hypothesis

There is altered expression of NF- κB in oral submucous fibrosis and oral squamous cell carcinoma when compared with normal buccal mucosa.

Study setting

- The study was conducted in Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, Chennai, using paraffin embedded tissues.

A cross-sectional study was done to evaluate the expression of NF-κB in oral squamous cell carcinoma, oral submucous fibrosis and normal buccal mucosa using immunohistochemistry in formalin fixed, paraffin embedded tissue specimens.

Study sample size

The study material comprised of 40 formalin-fixed, paraffin embedded tissue specimens (archival blocks).

- 20 histopathologically confirmed oral submucous fibrosis tissue specimens.
- 10 histopathologically confirmed oral squamous cell carcinoma tissue specimens.
- 10 normal buccal mucosa tissues specimens.

Study subject

The study comprised of 3 groups:

Group I – (CASES) (Fig. 3)

- 10 patients of oral squamous cell carcinoma, diagnosed clinically and confirmed histopathologically, were selected.

- **Inclusion Criteria-**

Clinical:

- A non-healing ulcerated lesion with indurated base or exophytic, fungating or papillary growth was considered as OSCC.

Histopathological

- Characterized by invasive islands and cords of malignant squamous epithelial cells in the connective tissue.

Group II- (CASES) (Fig. 4)

- 20 patients of OSF, diagnosed clinically and confirmed histopathologically, were selected.

Inclusion Criteria

Clinical:

- Habit of chewing areca nut in some form.
- The presence of fibrous bands in the labial and/ or buccal mucosa.
- Loss of elasticity of the buccal/ labial mucosa.
- Restriction of mouth opening.

Histopathological:

- Juxtaepithelial hyalinization.

- Submucosal dense and vascular collagenous connective tissue.
- Epithelial atrophy
- Variable number of chronic inflammatory cells

Group III- (CONTROLS)

- 10 patients who had clinically normal buccal mucosa, reporting to the outpatient department of oral and maxillofacial surgery for removal of impacted third molar constituted the control group.

Inclusion Criteria

- They had no habit of smoking, alcohol consumption or chewing areca nut.
- They were apparently healthy with no systemic disorders.
- They were not on any medications for systemic diseases like hypertension, diabetes.

Methodology

1. A detailed case history including age, sex, and occupation, past medical & dental history, history of habits, drugs and trauma were recorded.

2. General examination and intra oral examination was done.

3. Biopsy was done in both cases and controls.

4. The tissue taken was immediately transferred to 10 % buffered formalin.

5. After adequate fixation, tissues were embedded in paraffin.

6. From the paraffin embedded blocks 4 micron thick, sections were cut and used for routine hematoxylin and eosin (H & E) staining and immunohistochemical (IHC) staining.

7. This project was approved by Institutional Review Board (IRB) of Ragas Dental College and Hospital, Chennai and patient consent was taken.

HEMATOXYLIN & EOSIN STAINING

Reagents

- Harri's hematoxylin
- 1% acid alcohol
- Eosin

Procedure

- The slides were dewaxed in xylene and hydrated through graded alcohol to water.
- The sections on the slides were flooded with Harri's hematoxylin for 5 minutes.
- The slides were washed in running tap water for 5 minutes.
- The slides were differentiated in 1% acid alcohol for 5 minutes.
- The slides were washed well in running tap water for 5 minutes.
- The tissue sections on the slides were then stained in eosin for 30 seconds.
- The slides were washed in running tap water for 1 minute.

- The slides were then dehydrated through alcohol, cleared, mounted and viewed under light microscope (LM).

IMMUNOHISTOCHEMISTRY (IHC)

Armamentarium (Fig. 1)

- Microtome
- Autoclave
- Hot air oven
- Slide warmer
- Couplin jars
- Measuring jar
- Weighing machine
- APES coated slides
- Slide carrier
- Aluminium foil
- Micro-pipettes
- Toothed forceps
- Electronic timer
- Beakers
- Rectangular steel tray with glass rods
- Sterile gauze
- Cover-slips

- Light microscope

Reagents used

1. Conc. HCl

2. Laxbro soln

3. APES (3 amino propyl tri ethoxy silane)

4. Acetone

5. Citrate buffer

6. Phospho Buffer Saline (PBS)

7. 3% H2O2

8. Deionized distilled water

9. Haematoxylin

10. Absolute alcohol

11. Xylene

Antibodies used (Fig. 2)

1. Primary antibody – Rabbit polyclonal antibody (SantaCruz) (Clone: sc-114)

2. Secondary antibody – Rabbit Avidin Biotin Complex staining system (SantaCruz).

IHC Procedure

Pretreatment of the slides

- The slides were first washed in tap water for few minutes
- The slides were then soaked in detergent solution for 1 hour
- After 1 hour, each slide was brushed individually using the detergent solution and were transferred to distilled water.
- The slides were washed in two changes of distilled water.
- The slides were washed in autoclaved distilled water.
- The slides were immersed in 1 N HCL (100 ml HCl in 900 ml distilled water) overnight.
- The following day slides were taken out of acid and washed in two changes of autoclaved distilled water.
- All the slides were then transferred to slide trays, wrapped in aluminium foil and baked in hot air oven for 4 hours at 180 degrees centigrade.

APES (3 Amino propyl tri ethoxy silane) coating

Slides first dipped in couplin jar containing acetone for 2 minutes

↓

Dipped in APES for 5 minutes

↓

Dipped in two changes of distilled water for 2 minutes each

↓

Slides left to dry

Preparation of paraffin sections

After the slides were dry, tissue section of 4 micron thickness were made in a rotary manual microtome. The ribbons of tissue section were transferred onto the APES coated slide from the tissue float bath such that two tissue bits come on to the slide with a gap in between. One of the tissue sections was labeled positive (P) and the other negative (N).

Procedure

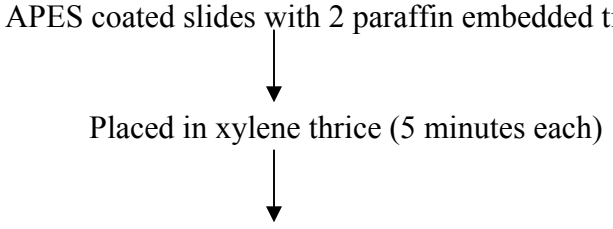
The slides with tissue sections were treated with three changes of xylene to remove paraffin wax. They were put in descending grades of alcohol and then rehydrated with water. Slides were then treated with 3% hydrogen peroxide for 30 minutes to quench endogenous peroxidase activity of cells that would otherwise result in non – specific staining. Then the slides were transferred to citrate buffer and autoclaved for antigen retrieval at 15 lbs pressure for 30 minutes. Then the slides were dipped in 3 changes of distilled water for 5 minutes each. Circles were drawn around the tissues, so that the antibodies added later on do not spread and are restricted to the circle. The tissues were incubated in protein blocking serum for one hour in an enclosed hydrated container. Then the slides were wiped carefully without touching the tissue section to remove excess of blocking serum. The primary antibody, rabbit polyclonal antibody, 1:50 dilution was added to P tissue on the slide and then to the N, PBS was added. The slides were incubated for one hour. Then the slides were wiped carefully without touching the tissue section to remove excess of antibody and washed with three changes of cold PBS for 5 minutes. Then a drop of biotin conjugated secondary antibody was added on both the sections and the slides were incubated for 30 minutes. Later slides were washed in three

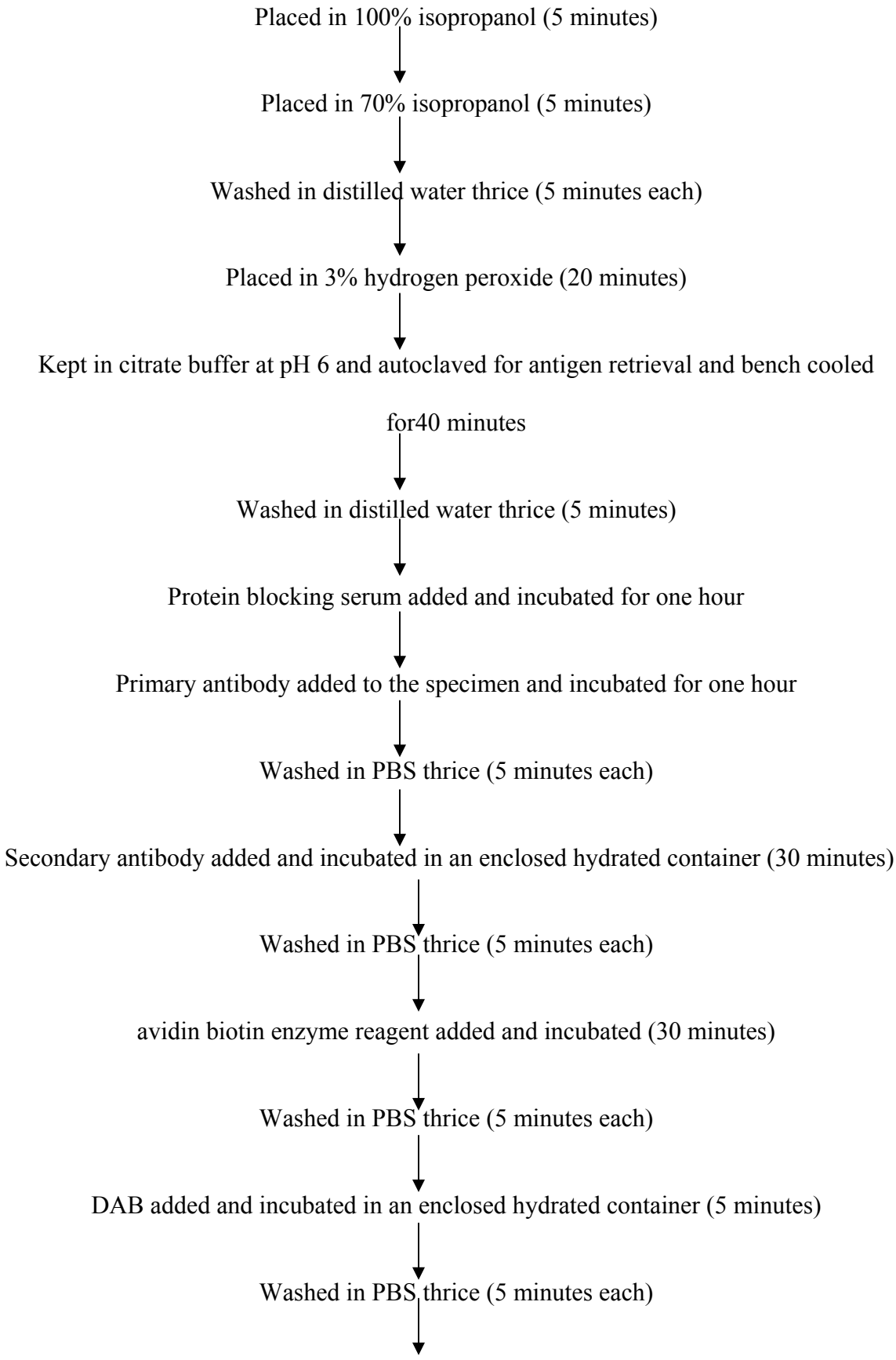
changes of cold PBS for 5 minutes in each. The slides were wiped carefully without touching the tissue section to remove excess PBS. Then a drop of avidin biotin enzyme reagent was added on both the sections and the slides were incubated for 30 minutes. The sections were washed in 3 changes of cold PBS for 5 minutes in each. Then the slides were wiped carefully without touching the tissue section to remove excess PBS. Then a drop of freshly prepared DAB (3' – Diaminobenzidine Tetra Hydrochloride – a substrate chromogen) was added on both sections. Slides were then washed in distilled water to remove excess DAB and counter stained with hematoxylin. The slides were placed in a tray with tap water for bluing. Then the slides were transferred to 70% alcohol, 100% alcohol and one change of xylene. The tissue sections were mounted with DPX. The slides were then observed under the microscope. Throughout the procedure care was taken not to dry the tissues.

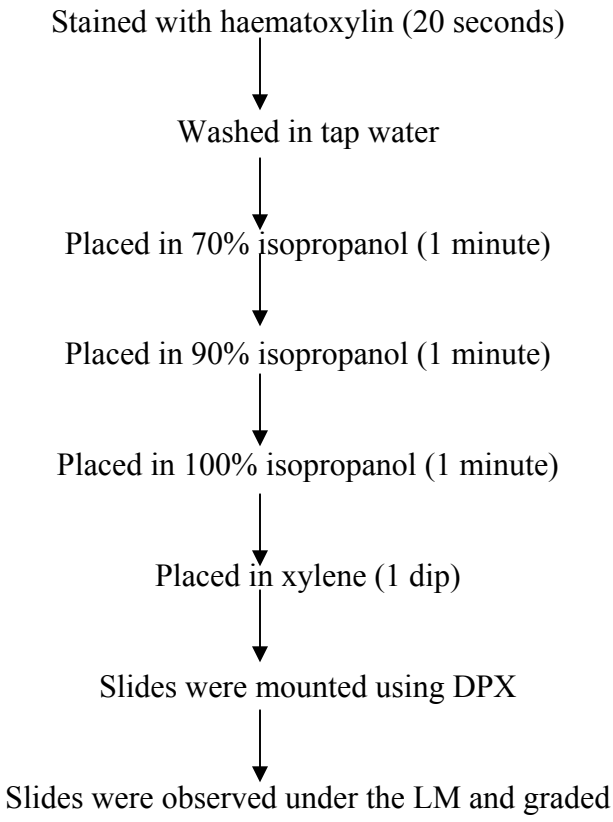
Positive Control

A known case of oral squamous cell carcinoma specimen tissue were fixed, processed, embedded, sectioned and stained in same manner and used as positive control. One positive control tissue slide was included for each batch of staining.

IHC PROCEDURE FLOW CHART




Placed in 100% isopropanol (5 minutes)
Placed in 70% isopropanol (5 minutes)
Washed in distilled water thrice (5 minutes each)
Placed in 3% hydrogen peroxide (20 minutes)
Kept in citrate buffer at pH 6 and autoclaved for antigen retrieval and bench cooled for40 minutes
Washed in distilled water thrice (5 minutes)
Protein blocking serum added and incubated for one hour
Primary antibody added to the specimen and incubated for one hour
Washed in PBS thrice (5 minutes each)
Secondary antibody added and incubated in an enclosed hydrated container (30 minutes)
Washed in PBS thrice (5 minutes each)
avidin biotin enzyme reagent added and incubated (30 minutes)
Washed in PBS thrice (5 minutes each)
DAB added and incubated in an enclosed hydrated container (5 minutes)
Washed in PBS thrice (5 minutes each)


Stained with haematoxylin (20 seconds)
Washed in tap water
Placed in 70% isopropanol (1 minute)
Placed in 90% isopropanol (1 minute)
Placed in 100% isopropanol (1 minute)
Placed in xylene (1 dip)
Slides were mounted using DPX
Slides were observed under the LM and graded

Criteria for evaluation of NF-κ B staining

The following parameters were used to evaluate NF-κ B staining

1. *Tissue localization of stain* – NF-κ B staining was present either present in the epithelium/connective tissue or was present in both. Localization in basal layer or suprabasal layer of the epithelium was assessed.

2. *Cellular localization of stain* – Nucleus and /or cytoplasm
3. *Intensity of the stain* – Each positive case was graded as no stain (0), mild (+), moderate (++), and intense (+++).

Statistical analysis was done using SPSS ^{TM} software (version 11.5). p value less than 0.05 was considered to be statistically significant.

- Pearson's Chi-square test was done to compare mean age, the distribution of gender and habits, tissue localization of stain, cellular location, nature of stain, intensity of stain and the percentage of cells stained among the three study groups.

- The inter-observer variability for the intensity of stain and percentage of cells stained was assessed using kappa statistics.

- To compare the mean labeling index between the groups, Mann Whitney U test was used.

NF – κB – STRUCTURE AND FUNCTIONS

The transcription factor NF - κB was first discovered in 1986 by David Baltimore and Ranjan Sen as a factor in nucleus that binds the promoter of the kappa chain of immunoglobulin in B cells[12]. NF- κB transcription factor include a collection of proteins with functions conserved from the fruit fly, Drosophila melanogaster to humans. Among the commonly used model organisms, these transcription factors are absent in yeast and Caenorhabditis elegans[13]. NF - κB is a collective name for ubiquitous inducible dimeric transcription factors that are made up of members of the Rel family of DNA binding proteins that recognize a common sequence motif [8].

Mammals express five NF- κB proteins that belong to two sub – families [8]:

- NF – κB proteins.
- Rel proteins.

All these proteins share a highly conserved DNA- binding / dimerization domain called the Rel Homology Domain (RHD) [14]. Members of NF- κB sub family include p105, p100 and Drosophilia Relish. They are encoded by NF- κB 1 and NF – κB2 genes, whose products are first synthesized as large precursors p105 and p100 respectively that require proteolysis processing to produce the mature p50 and p52 NF – κB proteins [8]. They contain long C- terminal domains that contain multiple copies of ankyrin repeats which act to inhibit these proteins. These members are not activators of transcription except when they form dimers with members of the Rel subfamily [13].

The Rel sub family includes c-Rel, RelB, Rel A (p^{65}), Drosophilia dorsal and Dif. They are synthesized as mature products and do not require proteolytic processing. They contain C- terminal transcription activation domains, which are not conserved at the sequence level across species [13].

NF- κB family proteins can form homodimers or heterodimers *in vivo* except for Rel B which can only form heterodimers *in vivo*[14].

PATHWAYS OF NF- ΚB

There are two pathways that lead to the activation of NF- ΚB.

Canonical / Classical pathway.
Non – canonical / alternate pathway[15].

In canonical pathway, NF- ΚB dimers such as p^{50}/ Rel A are maintained in the cytoplasm by inhibitor of NF- ΚB (IΚB). Activation of IΚB kinase (IKK) complex i.e. (IΚBα, IΚB β and IΚBγ) leads to the phosphorylation of IΚB α, which leads to ubiquitination and degradation by proteosome. NF-ΚB then enters the nucleus to turn on target genes [16]. In this pathway, one of the target gene activated by NF- ΚB is that which encodes IΚBα. The newly synthesized IκBα can enter the nucleus, remove NF-ΚB from DNA, and the complex can come back to the cytoplasm [13]. The activation of classical pathway is essential for innate immunity and also for inhibition of apoptosis [17].

In the non canonical pathway, p^{100}/ Rel B Complex are activated. Here the IKK complex does not contain IκBγ or NEMO. Activation of NF – κB inducing Kinase (NIK) cause phosphorylation of IκBα; which in turn phosphorylates p^{100} / Rel B. This cause proteolysis of p^{100} to P^{52} and liberation of p^{52} / Rel B complex occurs into the nucleus. The NF – κB enters the nucleus and activate target genes [15]. This pathway is important for the development of secondary lymphoid organs and adaptive immune response [17].

ACTIVATORS AND TARGET GENES FOR NF – κB

Many stimuli can rapidly activate NF – κB, such as stress induced stimuli, e.g.: Reactive oxygen intermediates (ROI) and ultraviolet light. Other stimuli include bacterial and viral infections and their products such as lipopolysaccharide and double-stranded RNA [18].

The various target genes for NF – κB include cytokines which activate the immune cells. The cell adhesion molecules such as V- CAM, E - CAM are needed for cell migration and repair. NF – κB also activates the acute phase response proteins during inflammation. NF- κB regulates transcription of genes involved in immunoregulation, inflammation, growth regulation, carcinogenesis and apoptosis[19, 20].

INHIBITORS OF NF – ΚB

NF – ΚB binds to 9 - 10 base pair DNA sites (ΚB sites) as dimers[18]. The activity of NF - ΚB is primarily regulated by interaction with inhibitory IΚB proteins. The IΚB are characterized by the presence of multiple ankyrin repeats and interact with NF- ΚB via RHD[8]. The RHD serves several functions; this domain can act as the dimerization and DNA binding domain of this class of proteins as it contains nuclear localization sequence and it is the site for binding of IΚB[21, 22].

IKK complex is composed of three subunits IΚBα IκBβ and IΚBγ (also known as NF- ΚB essential modulator (NEMO) [23]. In most cells, NF-ΚB is present as a latent, inactive IΚB – bound complex in the cytoplasm[18]. The stimulation by different pathogens and other inducers include viruses. Cytokines lead to the activation of signaling cascades that activates the IΚB complex and causes phosphorylation of IΚB. The NF – ΚB is then released and translocated to the nucleus where they bind with target genes and regulate their transcription[24].

FUNCTIONS OF NF – ΚB

NF – ΚB in Immune system

NF – ΚB is required for the normal functioning of the immune system. Cytokines and their corresponding receptors are key mediators of the immune system that are crucial for immune cell communication and effector functions during an active immune

response[25]. Studies on *c-rel*-deficient mice have demonstrated that *c-rel* is essential for IL-2, IL-3, expression in T lymphocytes, and IL-6 expression in B cells and TNF-α expression in macrophages and IL-12 expression in dendritic cells[26, 27, 28].

Mice deficient in the NF- ΚB2 gene mainly have defects in lymph node and splenic architecture, although development is normal. This leads to antigen presentation impairment from accessory cells such as dendritic cells and macrophages but does not affect B or T cells directly. All these studies emphasize the significance of NF – ΚB in normal development and functioning of the immune system[29, 30].

NF- ΚB activation is needed for haematopoiesis.

NF- ΚB is required for the development and function of B cells, T cells, thymocytes, dendritic cells, macrophages and fibroblasts[31]. Deletion of both p^{50} and p^{52} affects further maturation of committed progenitors of the lymphoid and osteoclast lineages[32, 33]. NF- ΚB/Rel plays a role in myeloid differentiation, as deletion of both p^{65} and c-Rel affects early common myeloid progenitors, leading to reduced colony forming unit progenitors, impaired erythropoiesis, aberrant expansion of granulocytes, and macrophage apoptosis[34].

In innate immunity, NF – ΚB is activated by various pathogens, which leads to microbicidal mechanisms of macrophages; production of inflammatory cytokines such as IL – 12 and activation of NK cells to produce IFN – γ. In adaptive immunity, IL – 1 family of proteins activate NF – ΚB, which results in T – helper cell responses[8].

NF – κB in Apoptosis

There are different activation pathways of NF- κB that cause expression of proteins which promotes apoptosis or inhibits apoptosis. This depends on the cell type or the type stimulation that determines which signaling pathways are activated. In most cells, NF- κB activation protects the cell from apoptosis through induction of genes such as TNF receptor associated factor 1 (TRAF 1), Bcl – X_L[35]. NF-κB activation more commonly counteracts p53 induced apoptosis by destabilizing p53 via enhanced Mdm2 expression[36]. But recent studies show that NF – κB has a pro-apoptotic role, by inducing FasL expression[37]. Another possible pro-apoptotic mechanism involves p^{100} mediated transcription independent cell death through activation of caspase -8[36]. It plays an essential role in activation of wild type p53 to initiate pre-apoptotic signaling in response to ROS accumulation[38].

Role of NF – κB in inflammatory disease

In chronic inflammatory disease, NF – κB increases the expression of genes for many cytokines and adhesion molecules. One such gene is that for inducible nitric oxide synthase which is increased in airway epithelial cells and macrophages in patients with asthma and in synovial cells in inflamed joints [8, 39, 40]. Cytokines including IL – 1β and TNF α can also activate the NF – κB pathway, which can result in amplification of inflammatory response and the persistence of chronic inflammation at local sites[41].

These metabolites contribute to the pathogenesis of inflammatory process. Although NF – κB acts as an initiator of inflammation, recent studies suggest that it is also involved in the resolution of inflammation[42]. In certain conditions during the resolution phase, NF – κB prolong rather than inhibit the inflammation, delaying tissue repair[43].

During onset of inflammation, NF – κB stimulation leads to p^{65} – p^{50} induced pro-inflammatory gene expression whereas during resolution, translocation of p^{50} – p^{50} homodimers is induced. This p^{50} homodimers can down regulate anti - apoptotic gene expression, but they cannot activate gene expression on its own. This p^{50} homodimers along with other co-factors can activate pro-apoptotic genes. The pro – inflammatory cells will receive apoptotic stimuli, and will be phagocytosed by macrophages which have a role in resolution of inflammation[10].

Role of NF – κB in cancer

NF – κB plays an important role in regulation of apoptosis. It suppresses apoptosis by regulating anti-apoptotic proteins such as inhibitor of apoptosis proteins (IAP), TNF receptor associated factor 1 (TRAF 1) and Bcl - X_L. NF – κB also blocks apoptosis by inhibiting Jun N-terminal kinase (JNK) pathway and accumulation of reactive oxygen species[17].

NF – κB plays an important role in invasion and angiogenesis of tumor cells by regulating matrix metalloproteinase 2 and 9, serine protease urokinase – type plasminogen activator (uPA).The production of angiogenic factors such as IL- 8, TNF,

VEGF produced by macrophages, neutrophils are regulated by NF – κB activation[44]. NF – κB promotes cell cycle progression by regulating the expression of gene involved in cell cycle such as cyclin D1, D2, D3 E and c- myc.

The earliest evidence for the role of NF- κB in oncogenic transformation was derived from the fact that v - Rel, a highly oncogenic retroviral homologue of c – Rel leads to carcinogenesis of avian lymphoid cells[41,45]. Later studies suggested that v – Rel has the capacity of transforming mammalian cells *in vivo*. Constitutive activation of NF – κB contributes to the malignant progression in major forms of human cancer such as breast cancer, esophageal cancer, Barrett's esophagus, head and neck squamous cell carcinoma, hepatocellular carcinoma, multiple myeloma, oral squamous cells carcinoma and prostate cancer[20].

Different abnormalities in the regulation of NF – κB pathway are seen in human malignancies. These changes are due to alterations in regulatory proteins that activate signaling pathways of NF – κB and in constitutive presence in the nucleus. In some tumors, mutations that inactivate the IκB proteins and amplification, rearrangements of genes encoding NF – κB family members or abnormalities that are seen in NF – κB pathway are seen. Constitutive activation of NF – κB pathway can lead to resistance to

tumor cell killing by chemotherapy and radiation therapy by enhanced expression of anti – apoptotic genes[41].

NF – κB regulates many genes that are involved in carcinogenesis such as in inflammation it is through upregulation of TNF, IL -1, Chemokines NF – κB activation promotes cell survival through increased expression of COX2, iNOS, MMP-9, uPA. The anti – apoptotic genes regulated by NF – κB are bcl – xl, TRAF. NF – κB regulates the genes involved in tumor cell invasion and metastasis through upregulation of VEGF, TNF, IL-1, IL-8, ICAM -1, VCAM -1, ECAM -1.[46]

Links between NF - κB, inflammation and Carcinogens in the development of cancer

Many chemical and physical carcinogens implicated in the promotion of human cancer activate NF – κB. Nicotine and carcinogens in tobacco and betel nut are associated in the pathogenesis of head and neck malignancies[11]. Chemotherapy and radiation induced DNA damages activates NF – κB through signaling mechanism of IKK complex.

In inflammation associated cancer, non-genetic stimuli encourage the survival and proliferation of cells. NF – κB has dual actions in tumor promotion; first by preventing the death of cells with malignant potential and second by stimulating the production of pro-inflammatory cytokines in inflammatory cells in tumor mass. These cytokines then signals the cell to promote their survival and proliferation[47].

Classical NF – κB pathway i.e. the IKK – β dependent NF – κB activation pathway might show the molecular link between inflammation and tumor promotion. Mouse studies of inflammation associated liver and colon cancer supports this hypothesis[48, 49].

So, in chronic inflammatory diseases, pro-inflammatory factors cause accumulating of DNA damage in dormant pre-malignant cells in tumor microenvironment to become malignant. NF – κB activation in these pre-malignant cells prevents apoptosis of these cells and enables tumor growth[10].

NF – κB IN PRE-CANCER AND ORAL CANCER

Frank G Ondrey, Gang Dong, John Sunwoo, Zhong Chen (1999)[50]

In this study, they examined the activation, composition and function of transcription factors in HNSCC cell lines that express pro-inflammatory cytokines by using electrophoretic mobility shift and reporter gene assay. Constitutive activation of NF – κB, Activated protein – 1 (AP – 1) and NF – IL6 DNA binding proteins was detected. Supershift analysis with antibodies specific for NF – κB showed that the NF – κB binding protein included p^{65}– p^{50}. Mutational analysis of NF – κB in the IL – 8 promoter region showed that constitutive IL – 8 reporter activity in HNSCC. So they concluded that NF – κB, AP – 1 and NF – IL6 promote the expression of pro-inflammatory & pro-angiogenic cytokines IL – 8 in HNSCC.

Nakayama H, Ikebe T, Beppu M et al (2001)[51]

The authors used immunohistochemistry to examine the expression of NF – κB and the signaling molecules leading to NF – κB activation in 36 untreated biopsy specimens from patients with squamous cells carcinoma (SCC) and in 15 specimens from patients with epithelial dysplasia of oral cavity. NF – κB (p65) and IκB α wtre expressed highly in all SCC specimens. The normal squamous epithelia and epithelial dysplasia were negative. The results suggest that high expression levels of p65 and IkB α contribute to malignant behavior and antiapoptotic activity in SCC of oral squamous epithelium.

Tamatani T, Azuma M, Bando T et al (2001)[52]

The authors examined the expression levels of proteins related to NF – κB activation and IκK activity in head and neck cancer cells and also in normal oral epithelium and salivary gland cells. They had shown that enhanced NF – κB activity is caused by the phosphorylation and degradation of IκB α protein. There was no significant difference in the expression levels of IκK β or IκK γ in cancer lines, compared to those in normal cells, but increased expression of IκK α protein was observed in cancer cells. These results suggest that enhanced NF – κB activity in head and neck cancer cells is due to IκK activity.

Nelson L. Rhodus, Vu Ho, Craig S Miller, Sandra Myers, Frank Ondrey (2005)[53]

The aim of this pilot study was to determine the level of NF – ΚB dependent cytokines in whole unstimulated saliva in subjects with oral premalignant lesions (OPML) as compared to those with squamous cell carcinoma (SCC). A sample size of 13 was taken in each group. The cytokines analysed were TNF – α, interleukin – 1, 6 and 8. The saliva samples were centrifuged and cytokine analysis was performed on the supernatants by ELISA. This study showed that these cytokines were elevated in saliva of OSCC and OPML when compared with normal. The cytokines with pro-inflammatory and pro-angiogenic activity are produced by SCC and they contribute to the progression of the disease. NF – ΚB is elevated in subjects with tobacco use and chronic inflammatory condition and it is associated with the expression of pro-inflammatory and pro-angiogenic cytokines such as IL – 1, IL – 6 and IL – 8. This study was done to test the hypothesis that in presence of chronic inflammation, irritation NF – ΚB will be elevated leading to the activation of pro-inflammatory, pro-angiogenic cytokines which is associated with progression of pro-malignant lesions to SCC.

Ni W F, Tsai C H, Yang S F, Chang Y C et al (2006)[54]

In this study, normal buccal mucosa and oral submucous fibrosis (OSF) were used to compare NF – ΚB expression. Seventeen OSF and six normal buccal mucosa specimens were examined by IHC. Primary human buccal mucosa fibroblasts were

established and challenged with safrole; a compound seen in Piper betel. NF – ĸB expression was significantly higher in OSF specimens and expressed mainly by fibroblasts, endothelial cells and inflammatory cells. Chewing areca quid may activate NF – ĸB expression that may be involved in pathogenesis of OSF.

Alok Mishra, Alok Bharti, Bhuder C Das (2006)[55]

In this study, they have analyzed the activation of NF – ĸB and its alterations, in the expression of different NF – ĸB proteins during oral carcinogenesis in vivo using tissue biopsies from precancerous and cancerous oral lesions. And also the role of high risk human papilloma virus (HPV – 16) which activates the p 50 / p65 NF – ĸB complex formation, which promotes differentiation of oral neoplastic cells led to better prognosis of the oral cancer. In this study, biopsy specimens were 110, n = 10 (normal) oral precancer (n = 34) and cancer n= 66.

Expression of p50 protein was 1000 in all the normal tissues and 40% of precancerous lesion & 70% of oral cancers showed moderate to high expression of NF – ĸB.

Bindhu O S, Ramadas K, Pillai M R et al (2006)[56]

The study investigated the pattern of expression and activity of gelatinases (MMP 2 and MMP 9) among the histologic groups of oral tissues such as normal oral tissues, leukoplakia and invasive oral carcinoma. The study also aimed at the role of NF – kB

and their inhibitor protein, IκB in all these group of tissues. 137 subjects were examined for the gelatinolytic activity assay for NF – κB (p50, p65) and IκB. High expression levels of p65 and IκB have shown to contribute to malignant behavior and anti-apoptotic activity in SCC of oral squamous epithelium. These observations suggest the regulatory role of NF – κB on the expression of gelatinases, and thereby in malignant transformation.

Chung H C, Parker S J, Carter J et al (2006)[57]

This study determined the feasibility of micro array gene expression analysis of formalin fixed tissue, and compared with gene expression of independent frozen tumor. This gene list which is highly predictive of recurrence free survival is a prognostic biomarker. These gene sets are involved in epithelial – mesenchymal transition, NF – κB activation and cell – adhesion, which are enriched in high risk tumors. This study provides the gene expression analysis to distinguish patients who are at high risk of recurrence.

Santhi W S, Sebastian P, Pillai M R et al (2006)[58]

This study investigated the role of NF – κB and COX – 2 in oral cancer progression and assessment of minimal residual disease (MRD). Expression of NF – κB and IκB was evaluated using IHC, while RT – PCR was used to detect COX – 2

expression.The present study demonstrated that nuclear localization of NF – ΚB and IΚB protein was increased with histological progression from normal to cancer tissue. This study also suggests an association between NF – ΚB activation and over expression of COX – 2 in oral cancer. NF – ΚB expression had a negative correlation in the evaluation of surgical margins, both p50 and p65 had a decreasing cytoplasmic expression from tumor to surgical margin to extra marginal tissue when evaluated by IHC. These markers are useful in predicting oral tumorigenesis and also for detection of MRD.

Sharma Chhavi, Kaur Jatinder, Aggarwal B B et al (2006)[59]

This study was designed to test the hypothesis that curcumin may inhibit the activation of NF – ΚB in smokeless tobacco exposed oral premalignant and cancer cells. Smokeless tobacco extract induced NF – ΚB activity was inhibited by curcumin in oral premalignant and cancer cells in vivo. Thus, curcumin can be used as chemo preventive agents in Leukoplakia & OSCC.

Meenakshi Sawhney, Nidhi Rohatgi, Aggarwal etal (2007)[60]

In this study, they analyzed the expression of NF – ΚB and COX -2 in oral tissues in hyperplastic, dysplastic lesions and also in OSCC by IHC and correlated with tobacco habits. Immunohistochemical analysis of p65 NF-ΚB protein was done in 15 normal tissues, 78 OPLs and 107 OSCC. In normal, 73% showed no cytoplasmic immune

reactivity while 26% showed mild NF – ΚB immune re activity. In precancerous lesions, 68% showed immune reactivity 58% of tumors showed p65 expression. The significant increase in expression of NF – ΚB & COX -2 from normal to OPL to OSCC suggest their association with development and progression of oral cancer. Among the ST products khaini consumption is association with NF – ΚB over expression in both OPL's and OSCC.

NF -kB in other Cancers

Cogswell C P, Guttridge D C, Baldwin Jr. S et al (2000)[61]

In this study, human breast cancer and adjacent normal tissue were analyzed by gel mobility shift assay and IHC for activation of NF-ΚB. The NF-ΚB genes were found to be elevated in breast tumors indicating functional NF – ΚB activity.

NairA, Venkatraman M, Maliekal T et al (2003)[62]

In this study immunohistochemical analysis was done using 106 paraffin embedded cervical tissue specimen of different histological grade. In normal cervical tissue & low grade squamous intra epithelial lesions, p50-Rel A were localized in cytosol, whereas in high grade squamous cell carcinoma of cervix, p50 – Rel A was seen in nucleus. Immunohistochemical analysis revealed that phosphorylation of IΚB- α occurs in squamous intra epithelial lesions, showing that it is degraded as tumor progress.

This study concluded that NF – κB activity is increased during cervical cancer progression.

Ismail H A, Lessard L, Saad F et al (2004)[63]

The aim of this study was to assess NF – κB expression in lymph node metastases of prostate cancer. 54 prostate cancer patients were analyzed of which 32 had lymph node metastases and 22 had no metastases. Nuclear localization of NF–κB was greater in metastatic lymph node when compared to controls, suggesting that NF – κB is upregulated in prostate cancer lymph node metastasis.

Ma J, Zhang Z, Xu S et al (2008)[64]

In this study, Tunel method was used to investigate the clinical significance of NF – κB on apoptosis in non-small cell lung cancers (NSCLC). The expression of NF – κB was correlated with the differentiation of NSCLC. NF – κB inhibits apoptosis in NSCLC.

PATIENT CHARACTERISTICS:

Ten cases of OSCC (Group I), 20 cases of OSF (Group II) and 10 cases of clinically appearing normal mucosa (Group III) were analyzed for immunoreactivity of NF –ĸB protein. All the samples in group I, II and III were taken from the buccal mucosa.

The patients' age ranged from 36 to 82 years (mean 52.5 ± 14) in group I, from 23 to 49 years (mean 34.3 ± 6.3) in group II and from 22 to 42 years (mean 28.7 ± 6.5) in group III **(Table 1, Graph 1).**

Of the 10 patients in group I, 80% (n=8) were men and 20% (n=2) were women. In group II all the cases were men, whereas in group III, 70% (n=7) were men and 30% (n=3) were women **(Table 2, Graph 2).**

Depending upon the habits, the patients were segregated into smokers, chewers and the third group included smokers and chewers. In group I, 60% (n=6) of patients were tobacco chewers, 10% (n=1) of patients were smokers and 30% (n=3) had no habits. In group II, 85% (n=17) of patients were areca nut and smokeless tobacco chewers, 15% (n=3) of patients were chewers and smokers. None of the patients in group III had any smoking or chewing habits. **(Table 3, Graph 3).**

According to the histopathological grading in OSCC, 70% (n=7) of patients had well differentiated OSCC, 30% (n=3) of patients had moderately differentiated OSCC.

DISTRIBUTION OF NF – ΚB STAINING AMONG 3 GROUPS:

NF – ΚB revealed positivity in group I (Fig 7 & 8), II (Fig 11 & 12) and III (Fig 15 & 16). In Group I all the cases showed 100% staining for NF – ΚB, whereas in Group II and Group III, positive staining was observed in 85% and 60% respectively.

Tissue localization of stain (Table 4, Graph4)

NF – ΚB staining was seen both in the epithelium as well as in the connective tissue. Of the 10 cases examined in group I, 100% (n=10) cases showed positive staining in both epithelium as well as connective tissue. 70% (n=7) of the cases showed cytoplasmic staining whereas 30% (n=3) showed nuclear staining. The labeling index of these cases which expressed nuclear positivity was 22.8%, 26%, 31.8% respectively. In connective tissue the staining was localized to muscle fibers and endothelial cells.

Of the 20 cases examined in group II, 85% (n=17) cases showed positive staining in epithelium and connective tissue, whereas 15% (n=3) cases showed negative staining. Of the positive staining cases in this group 30% (n=6) showed nuclear staining while 55% (n=11) showed cytoplasmic staining. The labeling index of the 6 cases which expressed nuclear positivity were 11%, 15%, 18%, 18.9%, 19.5%, 21.3% respectively. In connective tissue, staining was predominantly seen in lymphocytes, muscle fibers and endothelial cells.

In group III, of the 10 cases examined 60% (n=6) showed positive staining in epithelium whereas 40% (n=4) showed absence of staining. Of the positive stained cases, 50% (n=5) showed cytoplasmic staining and 10% (n=1) showed nuclear staining.

INTENSITY OF STAIN:

Distribution of staining intensity in the basal layer among the groups (Table 5, Graph 5).

When the staining intensity of NF – ĸB in the basal layer was compared between the study groups 10% (n=1) of OSCC showed negative staining whereas, 15% (n=3) of OSF and 40% (n=4) of normal showed negative staining. Mild intensity of staining was seen in 40% (n=4) of OSCC while mild staining was seen in 30% (n=3) of normal cases, 40% of OSCC showed moderate staining in comparison with 30% in OSF and normal. 10% (n=1) of OSCC showed intense staining while there was no intense staining in normal cases. But the results were not statistically significant (p = 0.058)

Distribution of the staining intensity in the suprabasal layer among the groups (Table 6, Graph 6)

In OSCC, none of the cases showed negative staining, whereas 20% (n=4) in OSF and 40% (n=4) in normal showed negative staining. 50% of OSCC (n=5) showed moderate staining when comparison with 45% (n=9) in OSF and 10% (n=1) in normal. . But the results were not statistically significant (p = 0.28)

Comparison of staining intensity in the basal layer between group II and group III (Table 7, Graph 7)

In OSF, 15% of the cases showed no staining in the basal layer while 40% had mild staining, 30% moderate staining, and 15% of the cases had intense staining in the basal layer. In normal mucosa, 40% exhibited no staining in the basal layer whereas mild and moderate staining was seen in 30% of the cases. None of the cases in the normal showed intense staining in the basal layer. But the results were not statistically significant ($p = 0.325$)

Comparison of staining intensity in the suprabasal layer between group II and group III (Table 8, Graph 8)

In OSF samples, 20% did not show any staining, while 30% had mild stain, 45% had moderate staining, and 5 % intense staining. In the normal mucosa, 40% had no staining whereas mild staining was seen in 40% cases, 10% showed moderate and intense staining. The difference in the staining intensity in the suprabasal layer between OSF and normal did not show statistical significance ($p=0.27$)

Comparison of staining intensity in suprabasal layer between group I and group III (Table 9, Graph 9)

In OSCC, mild, moderate and intense staining was seen in the suprabasal layers in 40%, 50% and 10% of the cases respectively. In normal mucosa, 40% showed no staining in the suprabasal layer, while 40%, 10% and 10% of the cases exhibited mild, moderate

and intense staining respectively. The difference in the staining intensity in the suprabasal layer between OSCC and normal did not show statistical significance (p=0.08)

Comparison of staining intensity between basal and suprabasal layer within the epithelium in Group II (Table 10, Graph 10).

Here 100% of cases showed negative staining in both basal and suprabasal layer. 75% of cases showed mild staining in both basal and suprabasal layer. 25% of cases showed mild staining in suprabasal and moderate staining in basal layer. 100% of cases showed moderate and intense staining in both basal and suprabasal layer. These results were statistically significant (p=0.000).

Comparison of staining intensity between basal and suprabasal layer within the epithelium in Group III (Table 11, Graph 11).

Comparison of staining intensity in normal oral mucosa showed that 75% of cases did not show staining in both basal and suprabasal layers. 25% of the cases showed mild staining in the basal layer and absence of staining in the suprabasal layer. 11.1% of the cases exhibited mild staining in the basal layer and moderate staining in the suprabasal layer. 66.7% of the cases had moderate staining in both basal and suprabasal layer. 22.2% of the normal cases showed intense staining in the basal and moderate staining in the suprabasal layer. These results were statistically significant (p=0.02).

Comparison of mean labeling index among the study groups

In OSCC, 3/10 cases showed nuclear staining and their labeling index were 22.8%, 26%, 31.8%. In OSF, 6/20 cases showed nuclear staining which had the mean

labeling index as follows, 11%, 15%, 18%, 18.9%, 19.5%, 21.3%. In normal, 1/10 case showed nuclear staining and its labeling index is 8%. The mean labeling index for OSCC 22.5, OSF 21.2, and normal 17.05, but this difference was not statistically significant.

Comparison of mean labeling index between Group I and Group III & Group II and Group III

Comparison of mean labeling index between OSCC (11.65) and normal (9.35) was done. Similarly the mean labeling index of OSF (16.65) was compared to normal (13.2), but these differences were not statistically significant.

The inter-observer agreement for the intensity of stain for all the 3 groups was arrived at kappa values: 0.648 and 0.5.

NF – κB is a transcription factor that is known to play an important role in controlling immune and inflammatory responses by regulating the genes encoding pro-inflammatory cytokines, chemokines and growth factors[61]. Constitutive activation of NF – κB contributes to the malignant progression in most of human cancers. It suppresses apoptosis by regulating anti-apoptotic proteins such as inhibitor of apoptosis proteins (IAP), TRAF – 1, Bcl – X_L and also by inhibiting JNK pathway and accumulation of reactive oxygen species. It also has a promoting role in invasion and angiogenesis by regulating MMP 2 and MMP 9[44]. It regulates many genes that are involved in carcinogenesis such as TNF, IL – 1, COX-2, iNOS, VEGF, ICAM – 1, VCAM – 1 and ECAM – 1[46].

OSF is a well recognized pre-cancerous condition with a multifactorial pathogenesis that include consumption of chillies, nutritional deficiency, chewing of areca nut, genetic susceptibility, altered salivary constituents, autoimmunity and collagen disorders[7]. Epithelial dysplasia in OSF is considered to be one of the indicators for predicting the potential malignant transformation of this condition. The malignant transformation of OSF is in the range of 7 – 13% and recently the carcinogenecity of areca nut without tobacco has also been identified. Though the induction, maintenance and progression of fibrosis in OSF have been addressed, the mechanism inducing carcinogenesis in OSF is not fully understood[65]. Therefore in order to predict the malignant potential in OSF, biomarkers could have a predictable role. Previous studies indicate that alterations in the expression of biomarkers can occur in the epithelium of OSF. These alterations may predispose to carcinogenesis and thus they could be used as surrogate markers of malignant transformation[66].

Since NF – ΚB is a transcription factor that is known to involve in growth, invasion and anti-apoptotic activity of cancer cells. In this study we wanted to ascertain its expression in OSCC and OSF and compare its activity with that of normal oral mucosa.

PATIENT CHARACTERISTICS

In our study the mean age in OSCC cases was 52.5 ± 14 years. **Mishra *et al*[55],** studied 66 cases of OSCC and reported the mean age in their study group as 52.9 ± 10.8 years. In the study done by **Nakayama *et al*[51],** the mean age was 70.2 years in 36 cases and **Sawhney *et al*[60]** reported a mean age of 55 years in 107 cases of OSCC.

In our study, the age presentation for OSF ranged from 23 to 49 years with a mean of 34.3 ± 6 years which is consistent with the study done earlier at our centre by **Ranganathan *et al*[67]**. In their study the youngest and oldest ages of occurrence of OSF was16 and 57 years respectively with the mean age of 32.4 ± 10.4 years. In the study by **Kiran kumar *et al*[68]** out of the 75 cases in OSF, 50% belonged to the age group of 20 – 29 years.

In our study, subjects who had OSCC were predominantly males and they constituted to about 80%. The male to female ratio was 4:1 and this finding was consistent with that of **Sawhney *et al*[60]** where the male: female ratio was 3.9:1and that of **Mishra *et al*[55]** who reported a male: female ratio of 6.3:1in patients who had OSCC.

In our study all the patients who had OSF were males and this finding is similar to a male: female ratio of 9.9: 1 reported by **Ranganathan *et al*[67]** in their study of 185 OSF

patients at our centre earlier. They also stated that number of males was around five times more than the females.

In this study, those patients who presented with OSCC, had the habit of chewing tobacco (60%) and 10% of the patient had the habit of only smoking tobacco. This finding is consistent with that of **Sawhney *et al***[60] who reported that the majority of patients in their study (28%) had the habit of chewing tobacco whereas 20.5% patients had the habit of only smoking

In this study of 20 patients who presented with OSF, 70% had the habit of chewing areca nut and 30% had the habit of both areca nut chewing and smoking. These findings are consistent with that of **Kiran kumar *et al***[68] who reported that 81% of the patients had the habit of chewing areca nut.

STAINING CHARACTERISTICS OF NF – κB

It has been shown that recurrent and persistent inflammation in the oral mucosa caused by continuous chewing of tobacco may induce, promote or influence susceptibility to carcinogenesis by causing DNA damage, inciting tissue reparative proliferation and creating a stromal environment enriched in cytokines and growth factors. NF – κB has an important role in stress response since physical, chemical oxidative and oncogenic stress can activate it[60]. NF – κB integrates multiple signals and regulates expression of multiple genes involved in different phenotypic alterations found in OSCC suggesting the hypothesis that NF – κB is an important molecular pathway for the development of cancer[56]. NF- is an inducible transcription factor that

mediates signal transduction between cytoplasm and nucleus. Normally, NF – ΚB presents in a latent inactive state in the cytoplasm. Upon stimulation it is translocated in the nucleus[58].

In our study, cytoplasmic expression of NF – ΚB was seen in 100% cases of OSCC. When we analysed the pattern of cytoplasmic staining in these OSCC cases, we observed that 40% of the cases showed mild intensity of staining, while 50% showed moderate and 10% showed intense staining. Nuclear expression of NF – ΚB was seen in 30% 0f cases. The nuclear percentage positivity increased with increasing grade of OSCC.

Dysregulation of NF – ΚB expression and its activation is frequently observed in many human cancers. Activated NF – ΚB shows nuclear expression and it indicates an early event in carcinogenesis. Once activated, it controls the expression of several genes that regulate cell cycle (cyclin D1), differentiation (p^{21}), cell survival (Bcl – 2), growth factors (VEGF) cell adhesion (VCAM, ECAM) and angiogenesis (MMPs).[55]

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Our results were consistent with **Nakayama *et al*[51]** who also reported 100% cytoplasmic expression in 36 cases of OSCC. **Mishra *et al*[55]** observed a 100% cytoplasmic expression out of which 70% showed a moderate expression and 30% showed weak immunoreactivity of NF – ΚB . **Sawhney *et al*[60]** in their study of 107 cases, reported cytoplasmic expression in only 68% of cases, unlike other studies where there was expression in 100% of the cases, but the reasoning of this finding was not

stated. Their study showed nuclear expression in 58% of those cases which showed cytoplasmic expression.

In the above studies there was an association between tumor progression and extent of nuclear translocation of NF – ĸB. In our study also the moderately differentiated SCC exhibited increased nuclear expression which is consistent with tumor progression. **Nakayama *et al*** [51] stated that high expression level of NF – ĸB contributed to the malignant behavior and anti apoptotic activity of OSCC. **Bindhu *et al*** [56] postulated that the increase in the nuclear Immunoreactivity could be attributed to the involvement of the p^{50}-p^{65} hetero dimmer formation. **Mishra *et al*** [55] reported that p^{50} homodynes transcriptionally regulate anti – apoptotic Bcl -2 which are over expressed in oral cancer cells and thus inhibits terminal differentiation of oral keratinocyte. The exact mechanism by which NF – ĸB is constitutively activated in OSCC is not fully understood, but has been suggested that the autocrine expression of IL and EGFR could play an important role in the activation of NF – ĸB

In inflammation associated cancer, non-genetic stimuli promote the survival and proliferation of cells. NF – ĸB has dual actions in tumor promotion; first by preventing the death of the cells with malignant potential and second by stimulating the production of pro-inflammatory cytokines in inflammatory cells in tumor mass. These cytokines then signals the cell to promote their survival and proliferation. Classical NF – ĸB pathway i.e., the IKK – β dependent NF – ĸB activation pathway might show the molecular link between inflammation and tumor promotion[48, 49]. So, in chronic

inflammatory diseases, pro-inflammatory factors cause accumulation of DNA damage in dormant pre-malignant cells in tumor microenvironment to become malignant[10].

In our study in OSF cases, cytoplasmic expression of NF – ΚB was seen in 85% of the cases. When we analyzed the pattern of staining, mild intensity of staining was observed in 40% of cases, 45% showed moderate intensity, and 5% showed intense cytoplasmic expression. Our study also showed nuclear expression of NF – ΚB in 30% of cases. When we further analyzed in order to understand the intensity of staining we observed that proliferating basal cells showed more expression than differentiated suprabasal cells. In those cases of OSF, which showed nuclear expression, when examined histologically they exhibited an atrophic epithelium, with an associated inflammatory response in the connective tissue. Alternatively those cases of OSF, which did not show NF – ΚB expression on histological examination, exhibited minimal inflammation in the connective tissue. We also observed that these cases showed minimal fibrosis in the associated connective tissue. Recent studies suggest that NF – ΚB is also involved in the resolution of inflammation[42]. During resolution, translocation of the p^{50}-p^{50} homodimers is induced. This p^{50} homodimer can down-regulate anti-apoptotic gene expression. This along with other co-factors can activate pro-apoptotic genes. The pro-inflammatory cells will receive apoptotic stimuli and will be phagocytosed by macrophages which have a role in resolution of inflammation[10].

Ni W F *et al*[53] observed intense staining of NF – ΚB expression in the epithelium of all OSF samples in their study. They also reported that intense staining was seen in basal and spinuous epithelial cells.

Ni W F *et al*[53] suggested that NF – ΚB is an important mediator in chronic inflammatory process. Aberrant and persistent tissue inflammation is believed to play an important role on the occurrence of tissue fibrosis and cancer. One of the etiological factors of OSF is area nut chewing habit. They proposed that areca nut extract was found to activate NF – ΚB in human oral keratinocytes and one of the pathogenic mechanisms of OSF may be due to increased expression of NF – ΚB in response to areca nut. NF – ΚB activates cytokines such as IL – 1 and TN F – α which can result in amplification of inflammatory, response and persistence of chronic inflammation at local site.

In OSF we observed nuclear staining in 30% of the cases. Thus the nuclear staining in our study in OSF could be attributed to the associated inflammation. In those cases, where nuclear expression was associated with moderate intensity of cytoplasmic staining, it could be suggestive of genotypic alterations due to NF – ΚB activity[60]. This can in turn lead to the expression of genes that are mediated in carcinogenesis. Based on the above concept, we state that expression of nuclear staining in OSF could be used as an indicator for carcinogenesis.

In our study NF – ΚB staining was seen in 60% of normal cases with 10% of staining showing in supra basal layer. Nuclear expression was seen in one case. This finding is consistent with **Mishra *et al*[55]** who reported that 20% of the normal cases showed positive expression. **Sawhney *et al*[60]** reported that 26% of normal cases showed mild cytoplasmic staining. The nuclear expression in this study was observed in those normal cases which histopathologically exhibited inflammatory response.

When we compared the cytoplasmic intensity and nuclear expression of NF – κB in OSCC and OSF we observed an increase in both the intensity and the nuclear Immunoreactivity but we did not observe any significant association between the two patterns of staining.

In conclusion, NF – κB expression is altered in OSCC and OSF when compared to normal with respect to localization and intensity, particularly in the basal layer, and has the potential to be used as a marker of malignant transformation.

- A total of 40 patients were included in the study, comprising of 10 cases of OSCC (group I), 20 cases of OSF (group II) and 10 patients with normal oral mucosa (group III).
- In OSCC, there was 100% positivity of NF – κB staining; with 70% of cytoplasmic expression and 30% of nuclear expression. Moderate to intense cytoplasmic expression was seen in suprabasal layer.
- In OSF; there was 85% positivity of NF – κB expression; with 55% of cytoplasmic expression and 30% of nuclear staining.
- Comparison of intensity of staining between the basal and suprabasal layer within the epithelium in OSF was statistically significant.
- In normal mucosa, there was 60% of positivity of NF – κB expression within 50% of cytoplasmic expression and 10% of nuclear expression. Comparison of intensity of staining between basal and suprabasal layer within epithelium of normal mucosa was statistically significant
- There was no statistically significant difference in the epithelial staining intensity between OSF & OSCC, OSF and normal, and also between OSCC and normal.
- Nuclear expression of NF – κB when compared between the groups was not statistically significant.

In conclusion our result showed that there is increased expression of NF – κB in OSCC and OSF when compared to normal. Although staining intensity and mean labeling index did not show any significant difference between OSCC and OSF, further

studies on a larger sample will help in ascertaining the significance of NF – ĸB expression in OSF samples.

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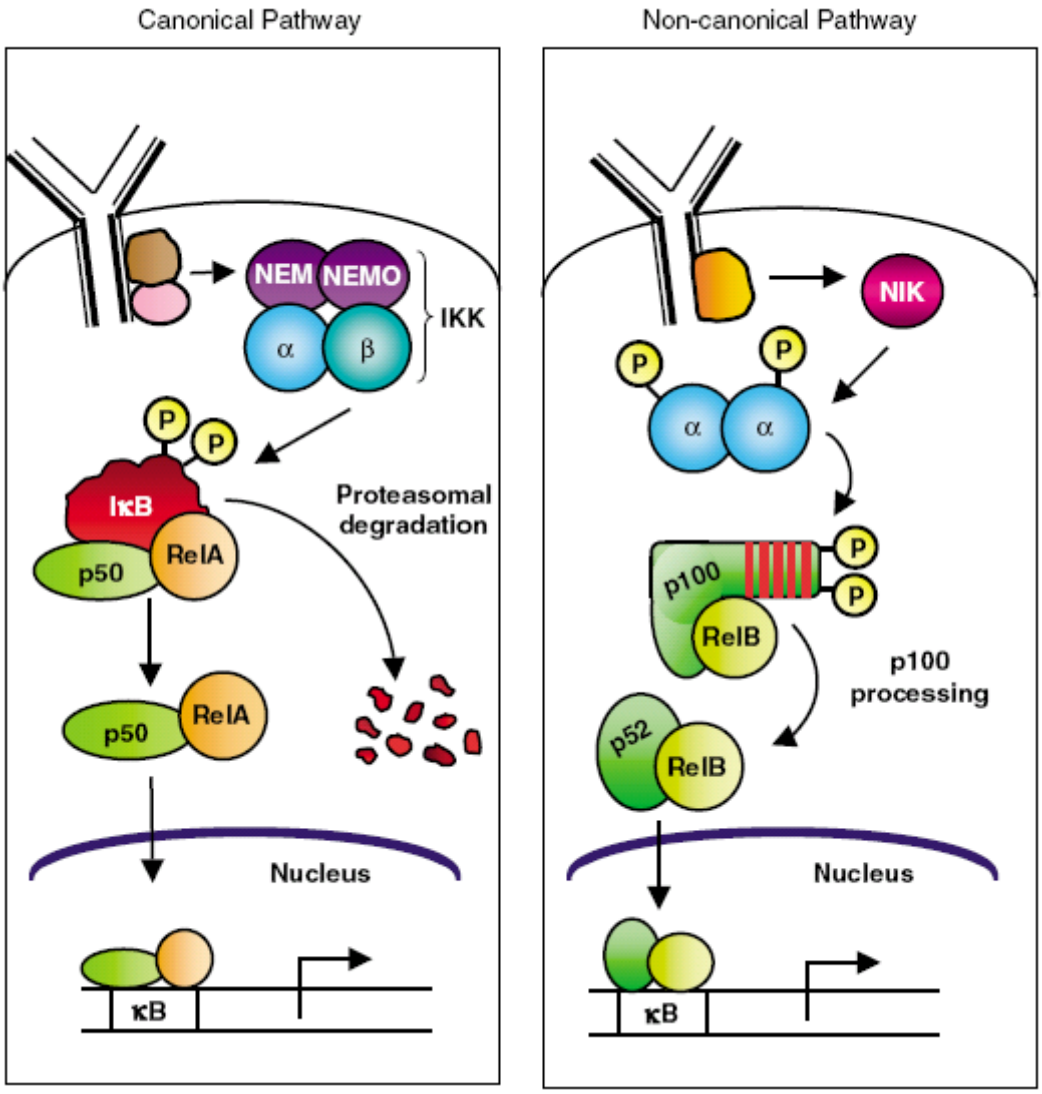
PATHWAYS OF NF- KB



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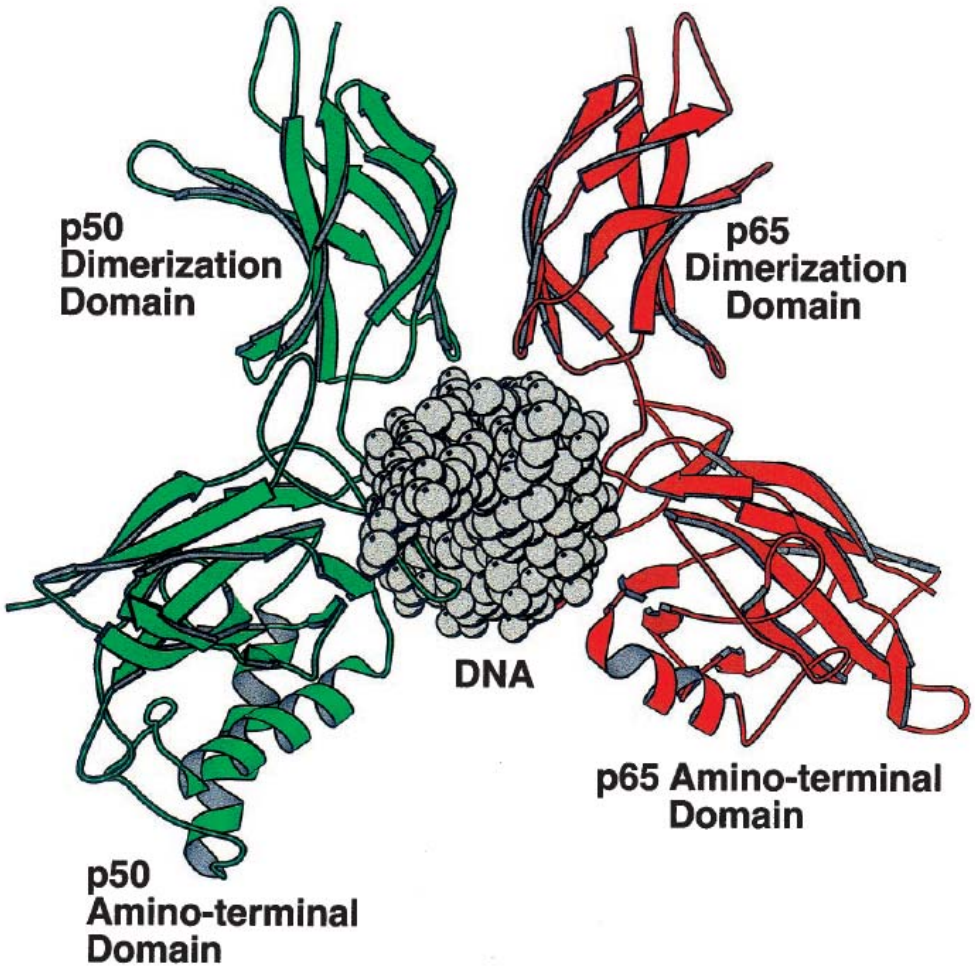
STRUCTURE OF NF-κB



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ABBREVIATIONS USED

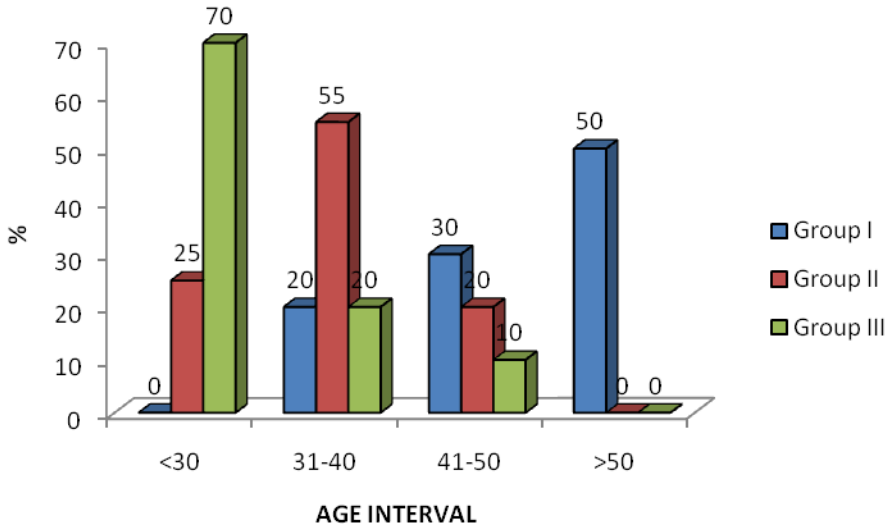
1. **RHD – Rel Homology Domain**
2. **IκB – Inhibitor of NF-κB**
3. **IKK – IκB Kinase**
4. **NEMO - NF-Kb Essential Modulator**
5. **NIK - NF-Kb Inducing Kinase**
6. **VCAM – Vascular Cell Adhesion Molecule**
7. **ICAM – Intercellular Cell Adhesion Molecule**
8. **TRAF – TNF – Receptor Associated Factor**
9. **IAP – Inhibitor of Apoptosis Protein**
10. **JNK – Jun N-terminal Kinase**
11. **TNF – Tumor Necrosis Factor**
12. **IL – Interleukins**
13. **COX – Cyclo oxygenase**
14. **iNOS – Inducible Nitric Oxide Synthase**
15. **MMP – Matrix Metallo Proteinase**
16. **uPA – Serine protease urokinas –type plasminogen activator**

TABLE 1: AGE OF THE SUBJECTS IN THE STUDY GROUPS

AGE	Group I		Group II		Group III		p value
	n	%	N	%	n	%	
<30	0	0	5	25	7	70	
31-40	2	20	11	55	2	20	0.000
41-50	3	30	4	20	1	10	
>50	5	50	0	0	0	0	

p value < 0.05 was considered to be statistically significant

GRAPH 1: AGE OF THE SUBJECTS IN THE STUDY GROUPS



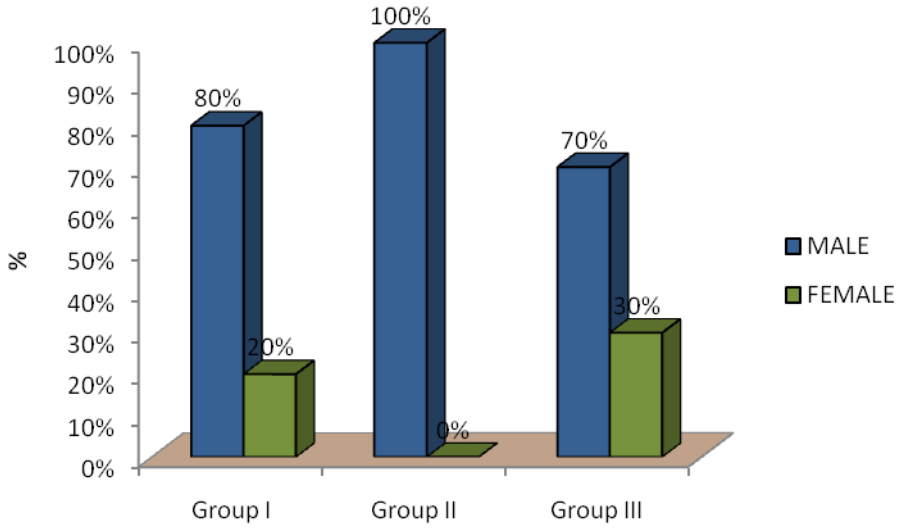
GROUP I – OSCC
GROUP II- OSF
GROUP III – NORMAL

TABLE 2: DISTRIBUTION OF GENDER IN THE STUDY GROUPS

CASES	MALE		FEMALE		p value
	n	%	n	%	
Group I	8	80	2	20	0.046
Group II	20	100	0	0	
Group III	7	70	3	30	

p value < 0.05 was considered to be statistically significant

GRAPH 2: DISTRIBUTION OF GENDER IN THE STUDY GROUPS



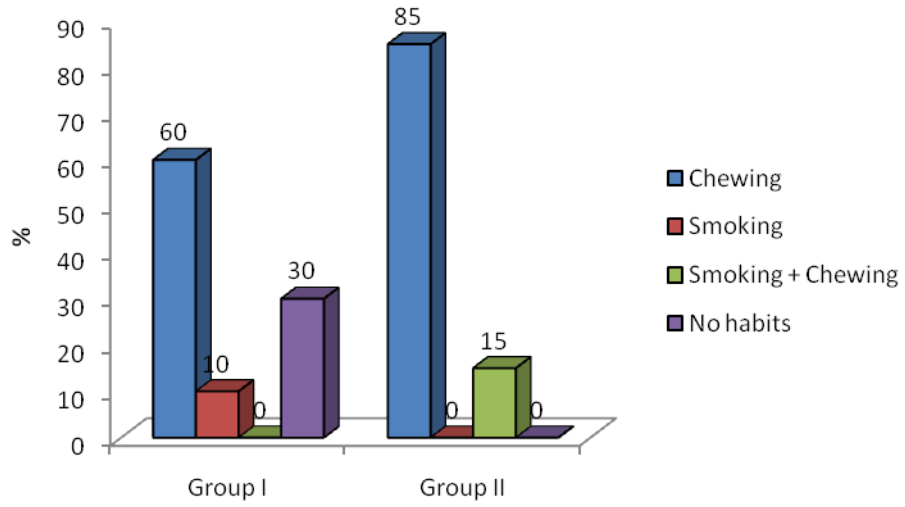
GROUP I – OSCC
GROUP II- OSF
GROUP III – NORMAL

TABLE 3: DISTRIBUTION OF HABITS IN THE STUDY GROUPS

Habits	Group I		Group II		p value
	n	%	n	%	
Chewing	6	60	17	85	0.000
Smoking	1	10	0	0	
Smoking + chewing	0	0	3	15	
no habits	3	30	0	0	

p value < 0.05 was considered to be statistically significant

GRAPH 3: DISTRIBUTION OF HABITS IN THE STUDY GROUPS

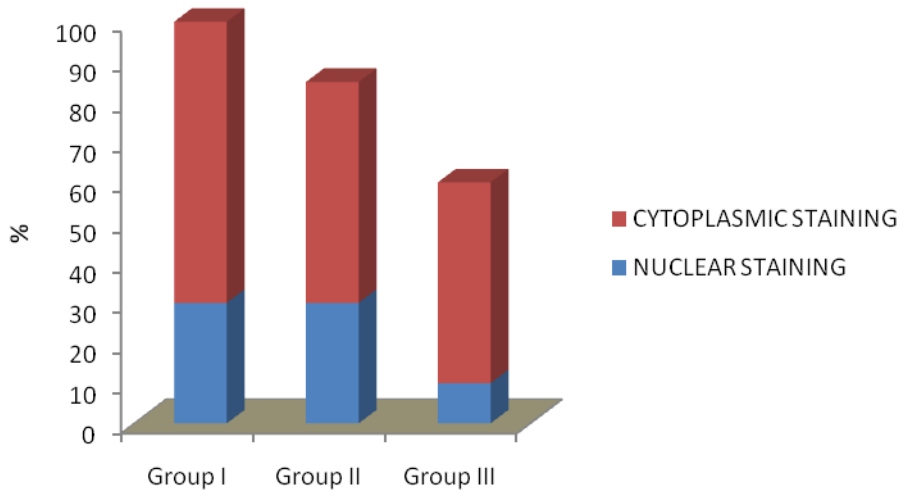


GROUP I – OSCC
GROUP II- OSF

TABLE 4: LOCALISATION OF NF – ĸB STAINING AMONG THE GROUPS

	GROUP I (%)	GROUP II (%)	GROUP III (%)
NUCLEAR	30	30	10
CYTOPLASMIC	70	55	50

GRAPH 4: LOCALISATION OF NF – ĸB STAINING AMONG THE GROUPS



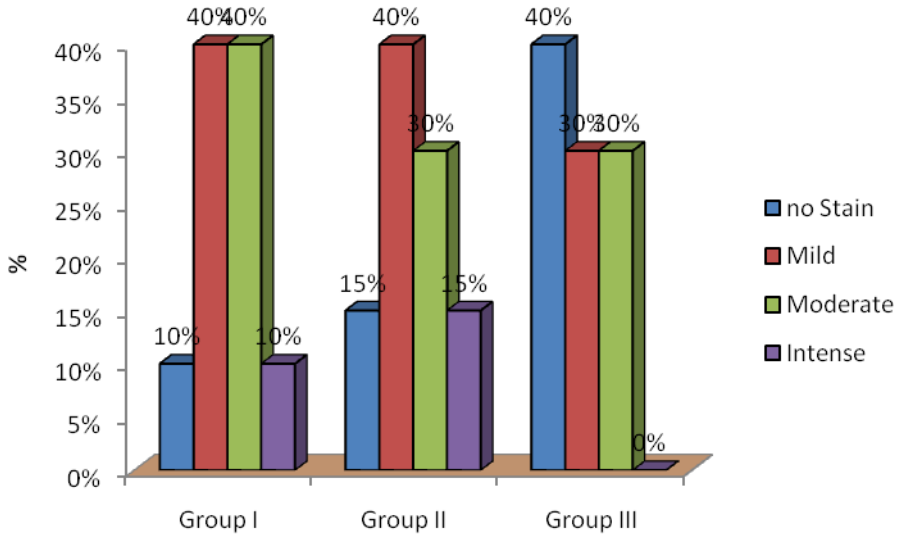
GROUP I – OSCC
GROUP II- OSF
GROUP III – NORMAL

TABLE 5: DISTRIBUTION OF STAINING INTENSITY IN THE BASAL LAYER AMONG THE GROUPS

Intensity	Group I		Group II		Group III		p value
	N	%	n	%	n	%	
no Stain	1	10	3	15	4	40	0.058
Mild	4	40	8	40	3	30	
Moderate	4	40	6	30	3	30	
Intense	1	10	3	15	0	0	

p value < 0.05 was considered to be statistically significant

GRAPH 5: DISTRIBUTION OF STAINING INTENSITY IN THE BASAL LAYER AMONG THE GROUPS



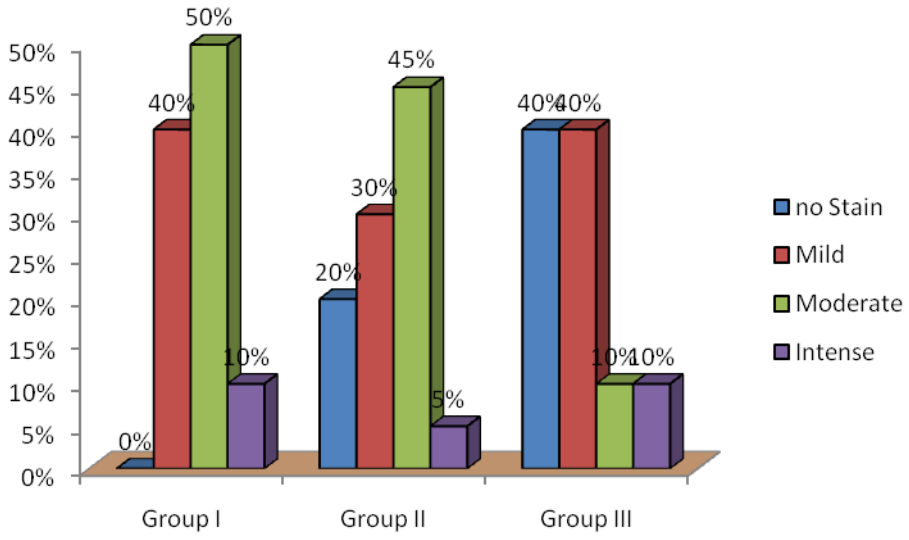
GROUP I – OSCC
GROUP II- OSF
GROUP III– NORMAL

TABLE 6: DISTRIBUTION OF STAINING INTENSITY IN THE SUPRABASAL LAYER AMONG THE GROUPS

Intensity	Group I		Group II		Group III		p value
	N	%	N	%	n	%	
no Stain	0	0	4	20	4	40	
Mild	4	40	6	30	4	40	0.28
Moderate	5	50	9	45	1	10	
Intense	1	10	1	5	1	10	

p value < 0.05 was considered to be statistically significant

GRAPH 6: DISTRIBUTION OF STAINING INTENSITY IN THE SUPRABASAL LAYER AMONG THE GROUPS



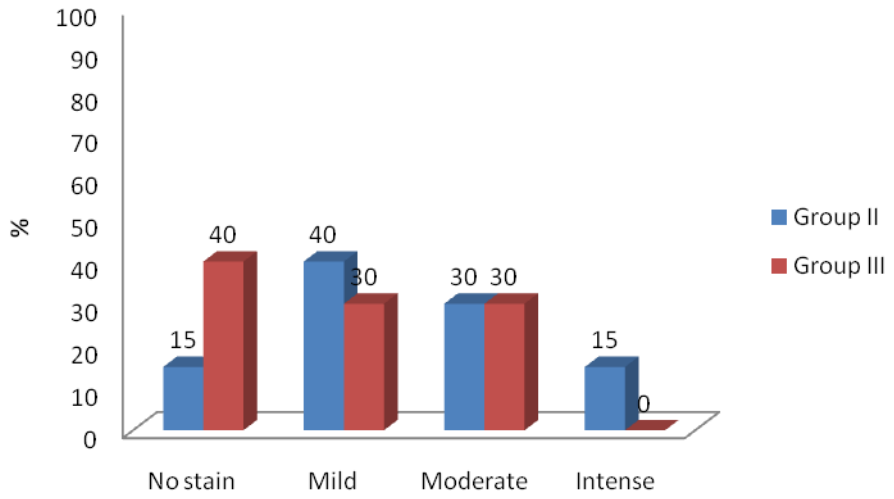
GROUP I – OSCC
GROUP II- OSF
GROUP III – NORMAL

TABLE 7: COMPARISON OF STAINING INTENSITY IN THE BASAL LAYER BETWEEN GROUP II AND GROUP III

	No stain (%)	Mild (%)	Moderate (%)	Intense (%)	p value
Group II	15	40	30	15	0.325
Group III	40	30	30	0	

p value < 0.05 was considered to be statistically significant

GRAPH 7: COMPARISON OF STAINING INTENSITY BETWEEN GROUP II AND GROUP III



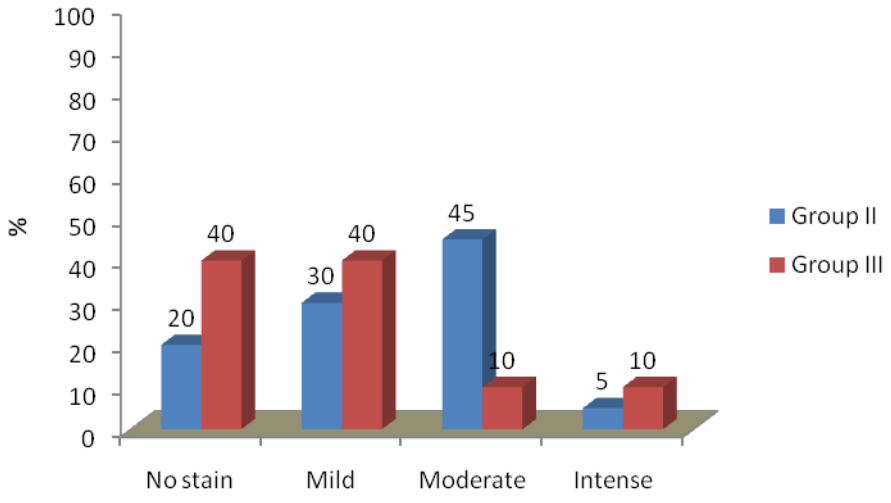
GROUP II- OSF
GROUP III – NORMAL

TABLE 8: COMPARISON OF STAINING INTENSITY IN THE SUPRABASAL LAYER BETWEEN GROUP II AND GROUP III

	No stain (%)	Mild (%)	Moderate (%)	Intense (%)	P value
Group II	20	30	45	5	0.272
Group III	40	40	10	10	

p value < 0.05 was considered to be statistically significant

GRAPH 8: COMPARISON OF STAINING INTENSITY IN THE SUPRABASAL LAYER BETWEEN GROUP II AND GROUP III



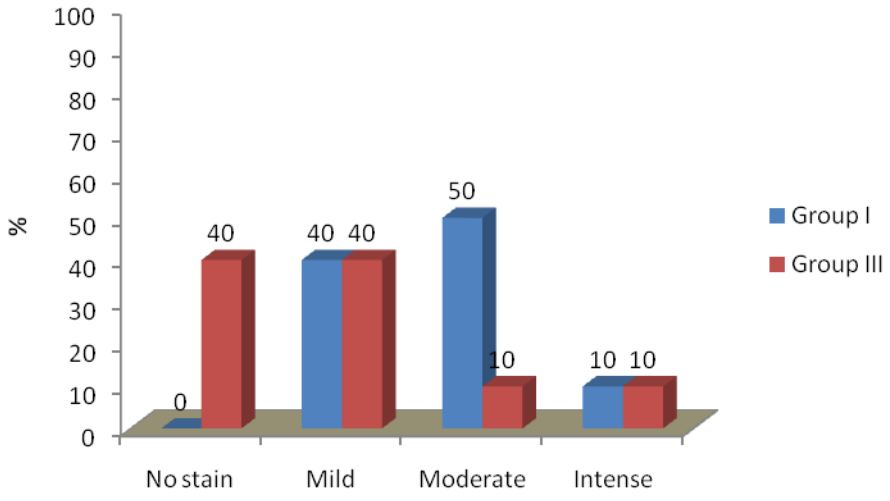
GROUP II- OSF
GROUP III – NORMAL

TABLE 9: COMPARISON OF STAINING INTENSITY IN SUPRABASAL LAYER BETWEEN GROUP I AND GROUP III

	No stain (%)	Mild (%)	Moderate (%)	Intense (%)	p value
Group I	0	40	50	10	0.083
Group III	40	40	10	10	

p value < 0.05 was considered to be statistically significant

GRAPH 9: COMPARISON OF STAINING INTENSITY IN SUPRABASAL LAYER BETWEEN GROUP I AND GROUP III



GROUP I- OSCC
GROUP III – NORMAL

TABLE 10: COMPARISON OF STAINING INTENSITY WITHIN EPITHELIUM IN GROUP II

	No stain in Supra basal layer (%)	Mild staining in suprabasal layer (%)	Moderate stain in suprabasal layer (%)	Intense stain in suprabasal Layer (%)	Pvalue
No stain in Basal layer	100	0	0	0	0.00
Mild staining in Basal layer	0	75	0	0	
Moderate stain in basal layer	0	25	100	100	

p value < 0.05 was considered to be statistically significant

GRAPH 10: COMPARISON OF STAINING INTENSITY WITHIN EPITHELIUM IN GROUP II

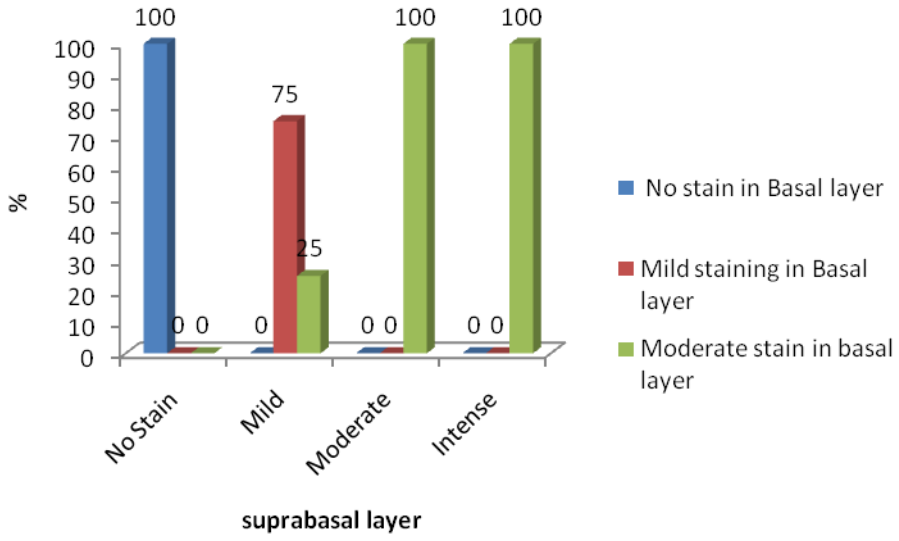


TABLE 11: COMPARISON OF STAINING INTENSITY WITHIN EPITHELIUM IN GROUP III

	No stain in Supra basal layer (%)	Mild staining in suprabasal layer (%)	Moderate stain in suprabasal layer (%)	Intense stain in suprabasal Layer (%)	p value
No stain in Basal layer	75	0	0	0	0.02
Mild staining in Basal layer	25	100	11.1	0	
Moderate stain in basal layer	0	0	66.7	0	
Intense stain in Basal Layer	0	0	22.2	100	

p value < 0.05 was considered to be statistically significant

GRAPH 11: COMPARISON OF STAINING INTENSITY WITHIN EPITHELIUM IN GROUP III

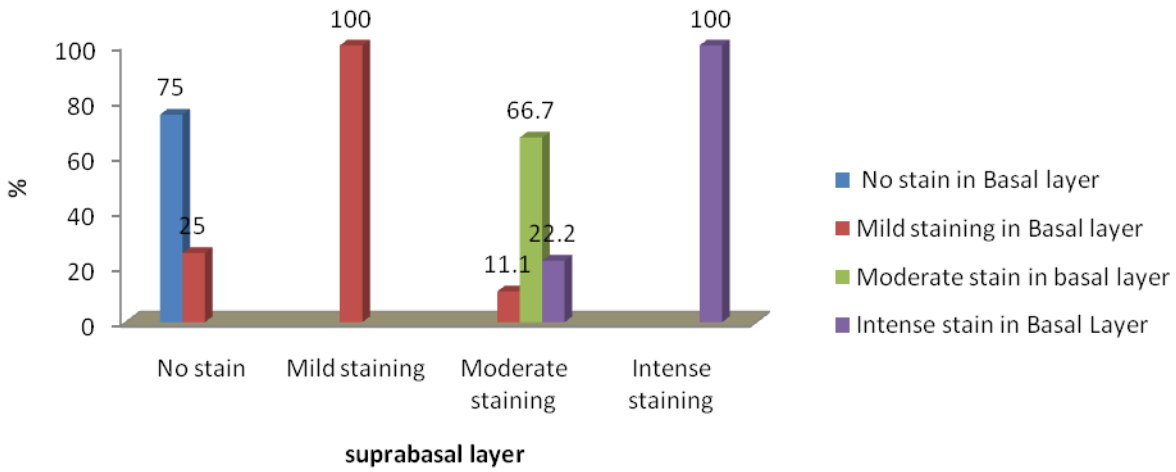


Figure 1: Armamentarium

Figure 2: Antibodies

Figure 3: Oral Squamous Cell Carcinoma

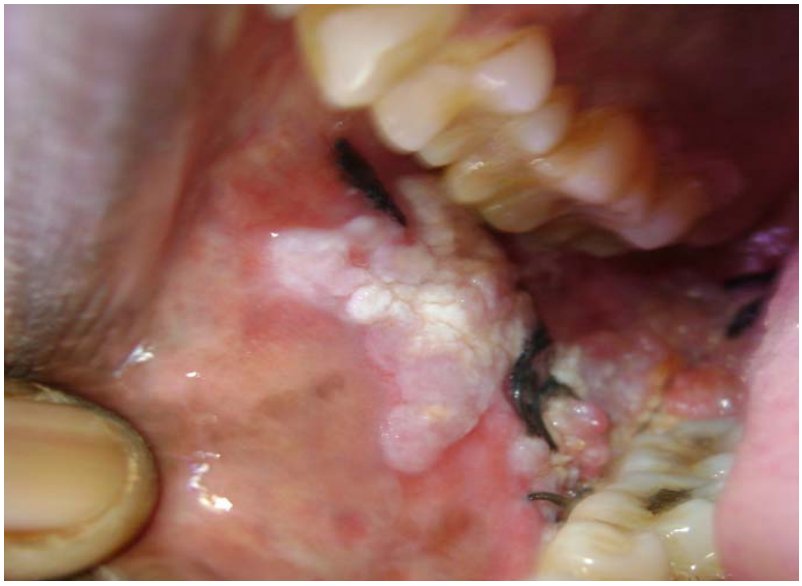
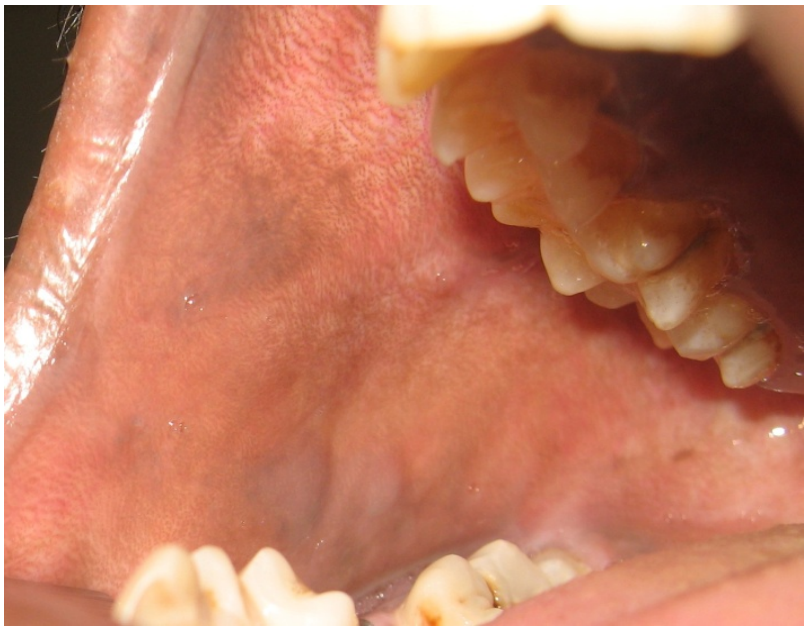
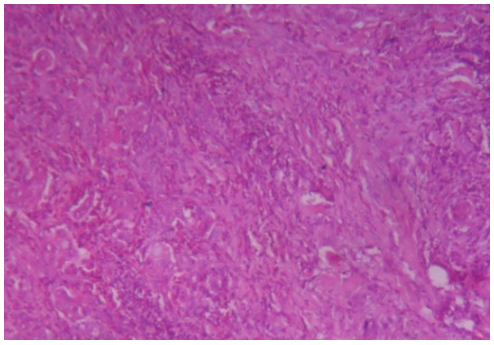


Figure 4: Oral Submucous Fibrosis



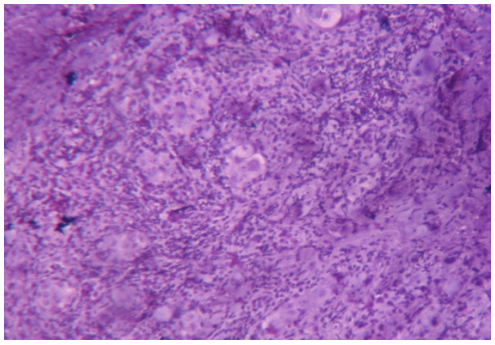
Oral Squamous Cell Carcinoma

Figure: 5



H & E stain; 10x

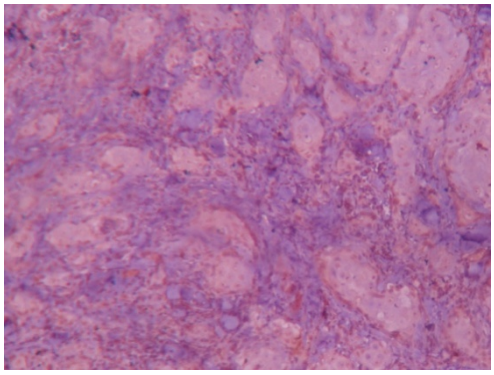
Figure: 6



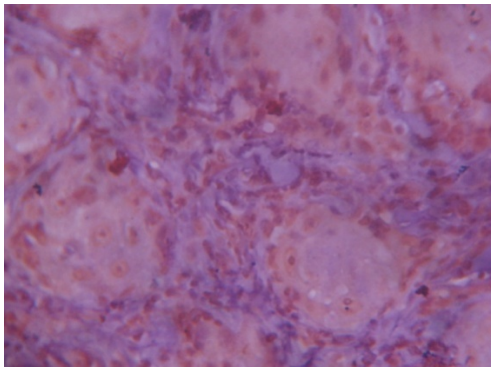
Negative; NF – κB stain; 10x

Figure: 7

Figure: 8



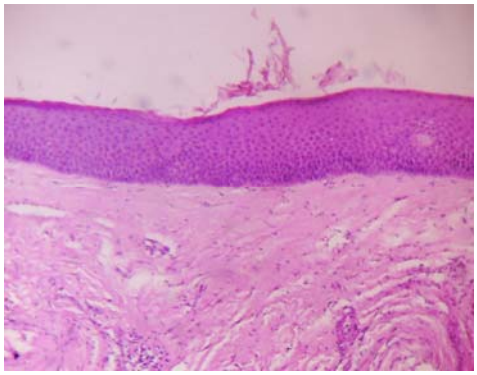
Positive; NF – κB stain; 10x



Positive; NF – κB stain; 40x

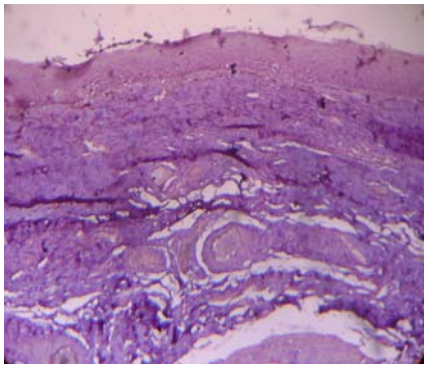
Oral Submucous Fibrosis

Figure: 9



H & E stain; 10x

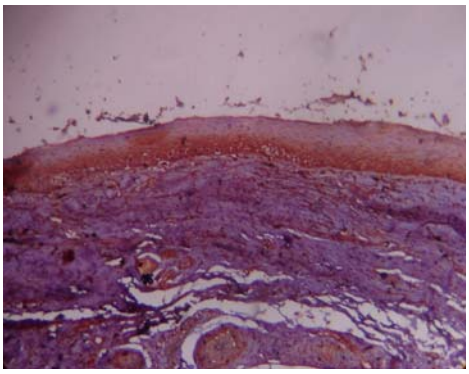
Figure: 10



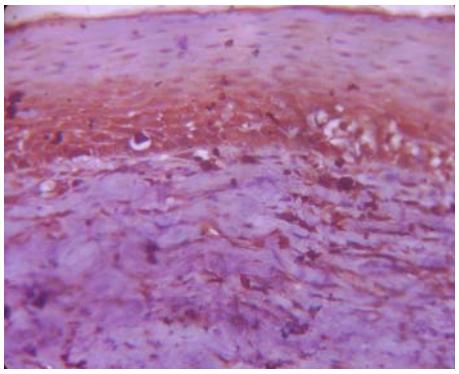
Negative; NF – κB stain; 10x

Figure: 11

Figure: 12



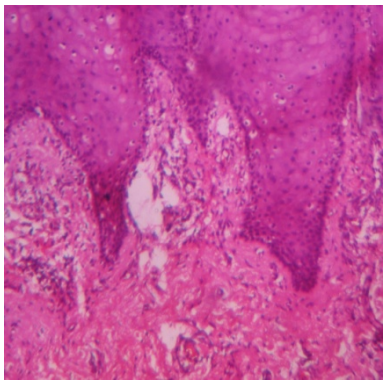
Positive; NF – κB stain; 10x



Positive; NF – κB stain; 40x

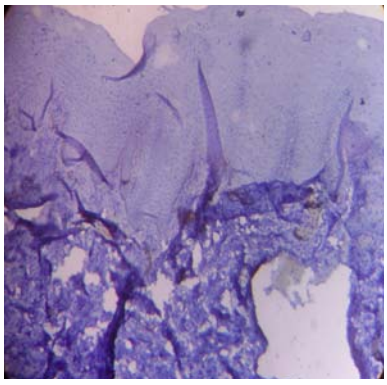
Normal Oral Mucosa

Figure: 13



H & E stain; 10x

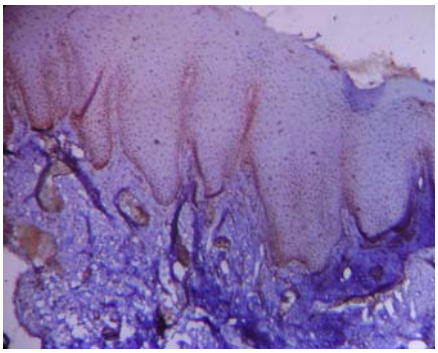
Figure: 14



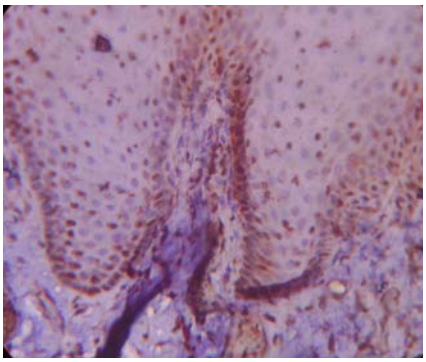
Negative; NF – κB stain; 10x

Figure: 15

Figure: 16



Positive; NF – κB stain; 10x



Positive; NF – κB stain; 40x