STUDY ON ANTIOXIDANT MICRONUTRIENT INTERVENTION OF ORAL PRE-CANCEROUS LESION IN SELECTED POPULATION OF BANGLADESH.

SUBMITTED BY

DR. MOHAMMOD BORHAN UDDIN HOWLADER

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SUBMITTED TO

THIS THESIS IS SUBMITTED TO THE INSTITUTE OF NUTRITION AND FOOD SCIENCE, UNIVERSITY OF DHAKA, BANGLADESH, AS A REQUIREMENT FOR THE PARTIAL FULFILLMENT OF THE DEGREE OF DOCTOR OF PHILOSOPHY.

DECLARATION

I do hereby declare that this thesis entitled “Study on antioxidant micronutrient intervention of oral pre-cancerous lesion in selected population of Bangladesh” is based on the research work carried out by me. No part of it was presented previously for any higher degree. The research work was carried out under the institute of Nutrition and Food Science, University of Dhaka under the supervision of Professor (Dr.) Khursheed Jahan, Professor and Former Director of INFS, Professor (Dr.) Khaleda Islam, Professor of INFS, Professor (Dr.) Motiur Rahman Molla, Professor of Oral and Maxillofacial Surgery, Anwar Khan Modern Medical College, Dhaka, Bangladesh.

_____

Dr. Mohammod Borhan Uddin Howlader

CERTIFICATE

This thesis entitled “Study on antioxidant micronutrient intervention of oral pre-cancerous lesion in selected population of Bangladesh” submitted by Dr. Mohammod Borhan Uddin Howlader for the award of PhD in Nutrition and Food Science, is an independent research work done under the University of Dhaka, Bangladesh, with our supervisions. This thesis was not used as the basis for the award of any degree or fellowship.

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ABBREVIATIONS

AKMMC	: Anwar Khan Modern Medical College
BIRDEM	: Bangladesh Institute of Research & Rehabilitation Diabetics, Endocrine and Metabolic Disorders
BMI	: Body Mass Index
CBC	: Complete Blood Count
DDCH	: Dhaka Dental College Hospital
DNA	: Deoxiribo Nuclic Acid
GDP	: Gross Domestic Product
HPLC	: High Performance Liquid Chromatography
ICDDRB	: International Center for Diarrheal Diseases and Rehabilitation of Bangladesh
N_2	: Nitrogen
NDI	: National Death Index
OPD	: Out Patient Department
ROS	: Reactive Oxygen Species
β	: β-Carotene
µL	: Micro Liter

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ABSTRACT

Oral precancerous lesion mainly oral leukoplakia is one of the common potentially premalignant disorders which may turn in to malignancy. It is commonly associated with bad oral hygiene, deficiency of antioxidant rich foods and sharp teeth. The use of tobacco either in the form of smoking or smokeless. Clinical studies suggest that antioxidant vitamins such as vitamin C, vitamin E and β-carotene prevent cancer development from oral leukoplakia.

This study investigated the effectiveness of antioxidants supplementations - vitamin C, vitamin E and β-carotene for the remission of oral leukoplakia. A total of 140 biopsy proven oral leukoplakia patient's were selected purposively with inclusion and exclusion criteria. These total 140 cases were divided into two groups: study group and placebo group. The study group was again divided 3 (three) groups and both the groups consisted of 35 subjects. A single blind clinical trial was carried out with antioxidant vitamin C, vitamin E and β-carotene for the study groups, while placebo for the placebo group. The dose of antioxidant vitamins were: vitamin C 500mg/day, vitamin E 400mg/day and β-carotene 30mg/day. The intervention was carried out for a period of 06 (six) months.

Anthropometric measurement and biochemical tests were performed at the base line and at the end of 6 (six) months intervention. The histopathological examination of the lesion was also conducted at the base line and at the end of 06 (six) months intervention. Dietary intake was assessed using 24 hours recall method for consecutive 3 (three) days at the base line and after 3 (three) months of intervention. Clinical examination was done at the base line, 01 (one) month, 03 (three) months and 06 (six) months interval of the intervention. Nutritional status was assessed using

Body Mass Index (BMI). Biochemical analysis such as complete blood count (CBC), serum vitamin C, vitamin E and β carotene were measured by standard method of analysis. Clinical evaluation was performed by naked eye observation and photograph of the lesion. Histopathological examination was conducted in the standard laboratory by hematoxilin and eosin stain method.

It was found that most of the patient's were between 50-60 years who were suffering from oral leukoplakia. Males were more affected than females. At the base line the mean serum level of vitamin C was 0.5125mg/dl, vitamin E 499.71µg/dl, β carotene 42.85µg/dl; while after supplementation, mean vitamin C was 1.29mg/dl, vitamin E 854.914µg/dl and β carotene 115.91µg/dl among the 3 (three) study groups. The mean serum level of vitamin C, vitamin E and β-carotene increased after supplementation in each study group. Increased serum level of antioxidants may have helped to the precancerous cell to normal cellular architecture and this was supported in histological examination. Abnormal oral cellular morphology and characters were changed to normal cellular architecture in smokers (38%), non-smokers (62%), tobacco receiving patients (40%) and non-tobacco receiving patients (60%) after supplementation. At the base line, 43% of the total population (intervention + placebo) had good nutritional status, 34% were malnourished, while 23% were overweight. After the intervention, it was found that subjects who had good nutritional status showed positive oral cellular change of the lesion compared to those who were malnourished. It is to be noted that nutritional status was measured anthropometrically. The mean BMI of the patients were not changed during the study period.

The remission rate of oral leukoplakia in case of vitamin C was 56%, vitamin E 60% and β carotene 64% based on both clinical and histological investigation. These results were statistically significant ($p < 0.05$). However, low rate of remission was observed for placebo patients (15%) which was not statistically significant.

Supplementation of antioxidant vitamin C, vitamin E, and β carotene offers overall benefits in the prevention oral cancer from oral leukoplakia. Antioxidants could play a positive role in the treatment of oral leukoplakia, especially β carotene which was found more effective than vitamin C and vitamin E.

10.1 Annexure-I

I. Informed Consent Form :

I hereby consent to participant in the study entitled vitamin C, E and β-carotene supplementation in oral leukoplakia patients to prevent pre-cancerous lesion to cancer A single blind randomized controlled trial study:

My collaboration will include:

a) Allowing blood collection for estimation of serum, Vitamin C, E and β-carotene level.
b) Responding to the interviewer regarding by socio-demographic, personal, dietary habits and other related queries.

I understand that all information will be kept strictly confidential, that I can contact study personnel if I have any questions. I further understand that I can withdraw from the study at any time. I am willing to participate in the study on antioxidant micronutrient intervention of oral precancerous lesion in selected population of Bangladesh. A single blind randomized controlled trial study.

Name.....

.....

Signature

Assigned by another person

I have read the information leaflet for the study and have no more questions. I agree that.....(Name of patient) should take part in the study.

Signature of person giving assigned

Relationship to patient

.....

10.2 Annexure-II

mvaZ cÎ

(CONSENT FROM)

আমি .....জানতে পারলাম যে, ডাঃ মোঃ বোরহান উদ্দিন হাওলাদার, পিএইচডি-এর জন্য একটি গবেষণা কাজ করছেন। যাতে তিনি মুখের ক্যান্সার রোগের উপর গবেষণা করছেন। তিনি আমাকে গবেষণা কাজটির উদ্দেশ্যে, পদ্ধতি ও সময়কাল, অংশগ্রহণের ফলে আমার দেয়া তথ্যাদির প্রয়োজনীয়তা সম্পর্কে অবহিত করেছেন। তিনি আমাকে আশ্বস্ত করেছেন যে, এই গবেষণায় অংশগ্রহণ সম্পূর্ণভাবে আমার ইচ্ছাধীন। আমি অবগত যে, গবেষণার প্রয়োজনে আমার দেয়া তথ্যাদির গোপনীয়তা রক্ষা করা হবে এবং গবেষণায় অংশগ্রহণ না করলে আমার চিকিৎসা সেবা ব্যাহত হবে না। আমাকে আরও জানানো হয়েছে যে, এই গবেষণালব্ধ জ্ঞান ভবিষ্যতে মুখের ক্যান্সার রোগ প্রতিরোধের ক্ষেত্রে গুরুত্বপূর্ণ ভূমিকা রাখতে পারে।

উপরে উল্লেখিত বিষয়াদি জেনে আমি স্ব-জ্ঞানে ও স্বেচ্ছায় এই গবেষণায় অংশগ্রহণের সম্মতি প্রদান করলাম।

রোগীর নাম ঃ.....বয়স ঃ.....রেজিঃ নং.....

প্রধান গবেষক

স্বাক্ষর.....

তারিখ.....

রোগী/অভিভাবক

স্বাক্ষর/টিপসহি.....

সম্পর্ক.....

তারিখ.....

10.3 Annexure-III

Questionnaire :

Study on antioxidant micronutrient intervention of oral precancerous lesion in selected population of Bangladesh.

Serial No.

01. Name :
02. Age (Year) :
03. Sex :
04. Residence :

    Rural=1, Urban=2, :

05. Occupation :

    1. Retirement, 2. Service holder, 3. Day laborer

06. Education (Year of schooling) :
07. Socioeconomic condition :

    Lower class=1, Middle class=2, Upper class=3

08. Intake of cigarette/day :
09. Onset of cigarette (Duration) :
10. History tobacco smoking : Yes=1, No=2
11. History of betel quid chewing : Yes=1, No=2
12. History ill fitting denture : Yes=1, No=2
13. History of taking pan :
14. History of taking zorda :
15. Site of lesion :

16. Size of the lesion :

17. Texture of the lesion :

18. Serum vitamin C, E, β-carotene, level at the entry of study (meq/L)

19. Histopathology : Yes=1, No=2

Annexure-III (A)

Questionnaire :

Study on antioxidant micronutrient intervention of oral precancerous lesion in selected population of Bangladesh.

Subsequent visit (After 1 month)

Serial No.

01. Name :
02. Age (Year) :
03. Sex :
04. Residence :

    Rural=1, Urban=2

05. Intake of cigarette/day :
06. Onset of cigarette (Duration) :
07. Intake of pan :
08. Intake of zorda :
09. Site of lesion :
10. Size of the lesion :
11. Texture of the lesion :
12. Serum Vitamin C, E and β-carotene, level after 1 month of intervention (meq/L)

Annexure-III (B)

Questionnaire :

Study on antioxidant micronutrient intervention of oral precancerous lesion in selected population of Bangladesh.

Subsequent visit (After 3 months)

Serial No.

01. Name :
02. Age (Year) :
03. Sex :
04. Residence :

    Rural=1, Urban=2

05. Intake of cigarette/day :
06. Onset of cigarette (Duration) :
07. Intake of pan :
08. Intake of zorda :
09. Site of lesion :
10. Size of the lesion :
11. Texture of the lesion :
12. Serum β-carotene, vitamin C, E level after 3 month of intervention (meq/L)

Annexure-III (C)

Questionnaire :

Study on antioxidant micronutrient intervention of oral precancerous lesion in selected population of Bangladesh.

Subsequent visit (After 6 months)

Serial No.

01. Name :
02. Age (Year) :
03. Sex :
04. Residence :

    Rural=1, Urban=2

05. Intake of cigarette/day :
06. Onset of cigarette (Duration) :
07. Intake of pan :
08. Intake of zorda :
09. Site of lesion :
10. Size of the lesion :
11. Texture of the lesion :
12. Serum β-carotene, vitamin C, E level after 6 month of intervention (meq/L)
13. Histopathology :

10.4 Annexure-IV

Ethical Clearance

BMRC

বাংলাদেশ চিকিৎসা গবেষণা পরিষদ
Bangladesh Medical Research Council

Ref: BMRC/NREC/2010-2013/597 Date: 03/05/2011

National Research Ethics Committee

Dr. Mohammod Borhan Uddin Howlader
Assistant Professor
Dhaka Dental College
Mirpur-14, Dhaka

Subject: Ethical Clearance

With reference to your application on the above subject, this is to inform you that your Proposal entitled **"Effect of Antioxidant Vitamins intervention on oral Leukoplakia in selected population of Bangladesh"** has been reviewed and approved by the National Research Ethics Committee (NREC).

You are requested to please note the following ethical guidelines as mentioned at page 2 (overleaf) of this memo.

(Professor Dr. Md. Habibe Millat)
Director

CHAPTER 1

INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1 Introduction:

Pre-cancerous lesions such as oral leukoplakia, erythoplakia, oral sub-mucous fibrosis are the common lesions may turn to malignancy if it is untreated[1].

Oral leukoplakia is defined as a white patch which can not be wiped off the mucosa and usually consists of elevated or flat which lesions of the oral mucosa that may be fissured; but some lesions are represented by an ulcer or an area of erythema. Males are affected more frequently than females (3:2), with the average duration of the lesion being 30 months. The vast majority of lesion occurs in fifth and sixth decades of life. Oral leukoplakia with dyskeratosis can be seen in the following area like- cheek, lip, floor of the mouth, tongue, palate and alveolar mucosa. The aetology of oral leukoplakia are- nutritional deficiencies, tobacco (smoking or smokeless), betel quid usage, high alcohol intake, sharp or rough teeth, genetic disorders and. The diagnosis of oral leukoplakia was done by clinical, bio-chemical and histological examination[2-5].

An antioxidants are inhibitors which are effective in preventive oxidation by molecular oxygen and have a great biological significance in the protection of cells by eliminating pro oxidants and scavenging free radicals[6]. Therefore health hazards by oxygen species can some extent be prevented by the bodies multi-level defense system against free radicals, which comprises a number of enzymatic antioxidants and some micronutrients that have to be regarded as antioxidant nutrients[7].

These antioxidants are categorized into two groups according to their nature of defense system against lipid per oxidation. First group acts through preventing initiation and this primarily accomplished by maintaining the structure of tissues and architecture with in the cell. The other group, the radical quenching antioxidants acts through preventing radical propagation and chain elongation. Among them alpha-tocopherol and β-carotene were the main ones obtained from the diet[8].

When the free radicals are first generated in the aqueous phase of the whole blood, the water soluble antioxidants especially vitamin C acts as a first defense[9]. Vitamin C scavenges aqueous radical but cannot scavenge lipophilic radicals within the membranes. Vitamin C also as an enhancer of the effectiveness of vitamin E, because vitamin C can effectively reduce the vitamin E radicals and there by regenerate vitamin E to maintain it in a functional form to act as lipid antioxidant[10]. Thus nutritional adequacy of vitamin C is essential in the control of membrane damage initiated by the free radicals and which may be expected to be at considerable importance in prevention of a number of diseases. Mega doses of vitamin C supplementation was found to play a protective role in neoplasm[11]. Vitamin C also prevent the formation of nitrosamines. Vitamin C is needed a large dose amount to protect the body from cancer by walling the cancer off from the rest of the body. It inhibits cancer from spreading by neutralizing hyaluronidase made by cancer[12].

Antioxidant including vitamin C, E and β-carotene are inhibitors which are effective in preventive oxidation by molecular oxygen. These antioxidants have a great biological significance in protection of cells by eliminating pro-oxidants and

scavenging free radicals[6.7]. The anticancer effects of antioxidants are thought to be due to inhibition of cell proliferation and promotion of cell differentiation. Recently, progress has been made in establishing a possible mechanism of action at the cellular level, says that squamous cell carcinomas of the oral cavity can be preceded by clinically obvious pre-malignant changes and they have a high rate of incidence of development of secondary tumors[12].

There are several studies regarding prevention of oral pre-cancerous lesion and oral squamous cell carcinoma in association with different antioxidant such as β carotene, vitamin C, E are the micronutrients with the strongest evidence of having a link to cancer prevention and control[13-15].

Vitamin C is known as ascorbic acid and is a derivative of the nexose-a-glucose. The daily requirements vitamin C is 45mg in adult. Vitamin E is known as tochopherol. It is fat soluble vitamin and daily requirement is 15 to 20 IU for adult[16].

Vitamin C has anti-oxidant properties and reacts with super-oxidant produced as a result of the cells normal metabolic process; this inactivation of super-oxidant inhibits the formation of nitrosamines during protein digestion and helps to avoid damage to DNA and cellular protein.

Ascorbic acid has anti oxidizing properties and reacts with super-oxidant produced as a result of cell normal metabolic processes, this inactivation of super-oxidant inhibits the formation of nitrosamines during protein digestion and helps avoid damage to DNA and cellular proteins.

Vitamin C is essential for collegen, elastic fibers and neurotransmitter, anti-carcinogenic.

Vitamin E is the commonest and most active form of vitamin E. It is an effective antioxidant at high levels of oxygen, protecting cellular membranes from lipidic per-oxidation. Main actions of alpha tocoferol includes;

- Free radical scavenging
- Maintenance of membrance integrity, immune function
- Inhibition of cancer cell growth/ differentiation
- Cytotxicity
- Inhibition of DNA and RNA, protein synthesis in cancer cells

Free radicals, such as super-oxide, hydroxyl ions and nitric oxide all contain an unpaired electron. Theses radicals can have a negative effect on cell causing oxidative damage that leadas to cell death. Antioxidants, such as vitamin E, prevent cell damage by binding to the free radical and neutralizing its unpaired electron.

β carotene is a vitamin A precursor. Main action of β carotene includes;

- Anti-oxidant and free radical scavenging
- Immunomodulation, stimulation of increase in the number of T- helper and NK cells as well as cells with IL-2 receptors
- Inhibition of mutagenesis
- Inhibition of cancer cell growth

The use of β carotene has been recommended in order to prevent OL and possibly oral cancer. The potential benefits and protective effects against cancer are possibly6 related to its anti-oxidizing action. This fuvction is accomplished through a ligation between β carotene and oxygen, which is an unstable reactive molecule, thus diminishing the damaging effects of free radicals.

The use of β carotene has been recommended in order to prevent oral leukoplakia and possibly oral cancer. The potential benefits and protective effects agaionts cancer are possibly related to its anti-oxidizing action. This function is accomplished trough a ligation between β carotene and oxygen, which is an unstable reactive molecule, thus diminishing t6he damaging effects of free radicals. A diet supplemented with β carotene can prevent changes in the oral mucosa, especially in smoker patients, who present low serum levels of vitamin C and β carotene when compared to nonsmokers. The β carotene has a better therapeutic clinic respose in the prevention of OL lesions in smoker patients than in the nonsmoker ones.

Vitamins such as A, β-carotene C, E, Fe and micronutrients with the strongest evidence of having a link to cancer prevention and control. Deficiency of these vitamins at the dietary, systemic or mucosal level will interact with tobacco use and increase the risk oral pre-cancerous lesions[17].

Tobacco chewing and or smoking are strongly related to several cancers. Several studies on diet and cancer links suggest that micronutrients particularly antioxidant vitamins and minerals are risk modifier of cancers of epithelial origin[18].

Epidemiological and experimental data have suggested that some micronutrients including various carotenoids, tochopherol may have chemo preventive activity against certain types of cancers. Oral cancer and preneoplastic oral leukoplakia occur in buccal mucosal cells. It has been shown that intake of β-carotene, 2-tochopherol and retinol (vitamin A) on its analogues causes regression of oral leukoplakia, thus preventing its progression to cancer. In India, tobacco and or betel use is accepted as the most important risk factors for oral pre-cancers and oral cancer. Smokeless tobacco users are at high risk of pre-cancerous lesions which transform to neoplasm at a rate of 3-5% per year[12].

Our objective is to find out the baseline levels of these micronutrients in oral leukoplakia patients with or without tobacco smoking and to compare these level after clinical intervention and also to investigate the effect on oral leukoplakia.

1.2 Justification of the Study:

Micronutrients have a significant role to play in the prevention of cancers from pre-cancer. Several pre-cancerous lesions are encountered both due to chewing and smoking tobacco. If the pre-cancerous lesions is untreated that may turned into malignancy. It can also create social burden and affect the national economy and ultimately national GDP. So, by intervention of micronutrient supplement to prevent the pre-cancer to cancer and possibly remission of oral leukoplakia. As a results to improve the personal, social and economical life that will develop our national economy. In Bangladesh, so far only few study was done about pre-cancerous lesion and no satisfactory statistical data available about pre-cancerous lesion in relation to micronutrient.

This data will give the appropriate guide line to the management of pre-cancerous lesion. The expected outcome of the study-hypothesis is to demonstrate the effectiveness of the micro-nutrient intervention to the prevention and remission of oral leukoplakia[19].

1.3 Operational definitions:

Pre-cancerous lesion: Pre-cancerous lesions are those lesions in which carcinoma may develop. Oral mucosal lesions, particularly red lesions (erythroplakia) and some white lesions (oral leukoplakia) have a potential for malignant change.

Anti-oxidant: Antioxidants are inhibitors which are effective in preventive oxidation by molecular oxygen. These antioxidants have a great biological significance in the protection of cells by eliminating pro oxidants and scavenging free radicals[6]. Therefore health hazards by oxygen species can to some extent be prevented by the bodies multi-level defense system against free radicals, which comprises a number of enzymatic antioxidants and some micronutrients that have to be regarded as antioxidant nutrients. They includes vitamin A, E, C and β-carotene

Oral Leukoplakia: Oral leukoplakia is defined as a white patch which can not be wiped off the mucosa and usually consists of elevated or flat which lesions of the oral mucosa that may be fissured; but some lesions are represented by an ulcer or an area of erythema.

Free Radical : Free radical can be defined as any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital. The presence of an unpaired electron results in certain common properties that are shared by most radicals.

Placebo : "A Placebo is a control" that mocks the real treatment, but lacks any active ingredients.

Oxidative Stress : Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage.

Biopsy : A biopsy is a medical test commonly performed by a surgeon, interventional radiologist, or an interventional cardiologist involving extraction of sample cells or tissues for examination to determine the presence or extent of a disease. The tissue is generally examined under a microscope by a pathologist.

Chemoprevention : Chemoprevention is the use of substances to stop cancer from developing.

Micronutrients : These are essential organic nutrients, most of which are not made in the body, or only in insufficient amounts, and are mainly obtained through food. When their intake is inadequate, vitamin deficiency disorders are the consequence.

Hyperkeratosis : Hyperkeratosis is thickening of the stratum corneum often associated with the presence of an abnormal quantity of keratin, and also usually accompanied by an increase in the granular layer.

Acanthosis : Acanthosis is diffuse epidermal hyperplasia (thickening of the skin). It implies increased thickness of the Malpighian layer.

Epithelial dysplasia : Epithelial dysplasia, a term becoming increasingly referred to as intraepithelial neoplasia, is the sum of various disturbances of epithelial proliferation and differentiation as seen microscopically. Individual cellular features of dysplasia are called epithelial atypia.

Serum : In blood, the serum is the component that is neither a blood cell (serum does not contain white or red blood cells) nor a clotting factor; it is the blood plasma not including the fibrinogens.

Plasma : Blood plasma a yellowish coloured liquid component of blood that normally holds the blood cells in whole blood in suspension; this makes plasma the extracellular matrix of blood cells.

Pro-oxidants : Pro-oxidants are chemicals that induce oxidative stress, either by generating reactive oxygen species or by inhibiting antioxidant systems.

Ulcer : A mouth ulcer is an ulcer that occurs on the mucous membrane of the oral cavity. Mouth ulcers are very common, occurring in association with many diseases and by many different mechanisms, but usually there is no serious underlying cause.

Sample : A small part or quantity intended to show what the whole is like.

Study Population : A population study is a study of a group of individuals taken from the general population who share a common characteristic, such as age, sex, or health condition. This group may be studied for different reasons, such as their response to a drug or risk of getting a disease.

Sample Population : Population sampling is the process of taking a subset of subjects that is representative of the entire population.

Dose : A quantity of a medicine or drug taken or recommended to be taken at a particular time.

Intervention : Intervention is the act of inserting one thing between others, like a person trying to help.

Nutritional Status : The condition of the body in those respects influenced by the diet; the levels of nutrients in the body and the ability of those levels to maintain normal metabolic integrity.

1.4 Hypothesis:

Vitamin C, vitamin E and β carotene interventions will increase the remission rate of the patients suffering from Oral Leukoplakia.

1.5 Objectives:

1.5. a. General objective:

To examine the effectiveness of antioxidant micronutrient interventions of vitamin C, vitamin E and β Carotene on remission of oral leukoplakia.

1.5.b. Specific objectives:

- To asses and compare the serum level of vitamin C, vitamin E and β Carotene of oral leukoplakia patients before and after intervention
- To examine the clinical and histological responses of oral leukoplakia patients to vitamin C, vitamin E and β Carotene
- To determine the association of blood level of vitamin C, vitamin E and β Carotene with normalization of oral leukoplakia cell morphology

CHAPTER 2

LITERATURE REVIEW

LITERATURE REVIEW

Pre-cancerous lesions are those lesions in which carcinoma may develop. Oral mucosal lesions, particularly white lesion (oral leukoplakia) and some red lesion (erythroplakia) have a potential for malignant change.

Oral leukoplakia is a white patch which can not be wiped off the mucosa and usually consists of elevated or flat white lesions of the oral mucosa that may be fissured; but some lesions are represented by an ulcer or an area of erythema. Males are afflicted more frequently than female (3:2), with the average duration of the lesion being 30 months. The vast majority of lesion occurs in fifth and sixth decades of life. Oral leukoplakia with dyskeratosis can be seen in the following area like-cheek, lip, floor of the mouth, tongue, palate and alveolar mucosa. The etiology of oral leukoplakia are-tobacco (smoking or smokeless), betel quid usage, high alcohol intake, sharp or rough teeth, genetic disorders and nutritional deficiencies. The diagnosis of this lesion by clinical photograph, biopsy and treatment of oral leukoplakia involves avoidance of predisposing factors-tobacco cessation, smoking, quitting betel chewing, abstinence from alcohol and avoidance of chronic irritants such as sharp edges or teeth. A biopsy should be done and the lesion surgically excised if precancerous changes or cancer is detected[20].

Oral leukoplakia is the best known precursor lesion. The evidence that oral leukoplakia are pre-malignant is mainly derived from follow-up studies showing that between 1% to 18% of oral pre-malignant lesion will develop into oral cancer; it has

been shown that certain clinical sub-types of leukoplakia are a higher risk for malignant transformation than others. The presence of epithelial dysplasia may be even more important in predicting malignant development than the clinicla characteristics. There major problems, however are attached to the importance of epithelial dysplasia in predicting malignant development: (1) The diagnosis is essentially subjective (2) it seem that not all lesions exhibiting dysplasia will eventually become malignant and some may even regress and (3) carcinoma can develop from lesion in which epithelial was not diagnosed in previous biopsies. There is, therefore a substantial need to improve the histological assessment of epithelial dysplasia or since epithelial dysplasia does not seem to be invariably associated with or even a necessary prerequisite for malignant development, it may be necessary to develop other method for predicting malignant potential of premalignant lesions. As a consequence of these problems, numerous attempts have been made to relate biological characteristics to the malignant potential of leukoplakias. Molecular biological markers have been suggested to be of value in the diagnosis and prognosis evaluation of leukoplakias. Markers of epithelial differentiation and more recently genomic markers could potentially be good candidates for improving the prognostic evaluation of precursor of oral cancer. As yet, one or a panel molecular markers has not been determine that allows for a prognostic prediction of oral pre-cancer which is any more reliable than dysplasia recording. However this new markers could be consider complementary to conventional prognostic evaluation[21].

Oral cancer research in India stated that the prevalence of leukoplakia in India varies from 0.2% to 5.2%. leukoplakia may persistent regress spontaneously, recur or progress to cancer. Regression occurs more significantly in lesions associated with the chewing tobacco of betel quid than in those associated with smoking. Worldwide, 3.6% of leukoplakia shows malignant transformation[22].

The study has been made of the survival times of 100 terminal cancer patients who were given supplemental associate, usually 10 g/day, as part of their routine management and 1000 matched controls, similar patients who had received the same treatment except for the ascorbate. The two sets of patients were in part the same as those used in our earlier study. Tests confirm that the ascorbate-treated patients and the matched controls are representative subpopulations of the same population of untreatable patients. Survival times were measured not only from the date of untreatability but also from the precisely known date of first hospital attendance for the cancer that eventually reached the terminal stage. The ascorbate-treated patients were found to have a mean survival time about 300 days greater than that of the controls. Survival times greater than 1 yr after the date of untreatability were observed for 22% of the ascorbate-treated patients and for 0.4% of the controls. The mean survival time of these 22 ascorbate-treated patients is 2.4 yr after reaching the apparently terminal stage; 8 of the ascorbate-treated patients are still alive, with a mean survival time after untreatability of 3.5 yrs[23].

It was stated that vitamin C scavenges aqueous radicals but cannot scavenge lipophilic radicals within the membranes. Vitamin C also as an enhancer of the effectiveness of vitamin E, because vitamin C can effectively reduce the vitamin E radicals and there by regenerate vitamin E to maintain it in a functional form to act as lipid antioxidant[24].

It was indicated that thus nutritional adequacy of vitamin C is essential in the control of membrane damage initiated by the free radicals and which may be expected to be at considerable importance in prevention of a number of diseases. Mega doses of vitamin C supplementation was found to play a protective role in neoplasm. Vitamin C also prevent the formation of nitrosamines. Vitamin C needed a large dose amount to protect the body from cancer by walling the cancer off form the rest of body. It inhibit cancer from spreading by neutralizing hyaluronidase made by cancer[23].

It was noticed that these antioxidants are categorized into two groups according to their nature of defense system against lipid per oxidation. First group acts through preventing initiation and this primarily accomplished by maintaining the structure of tissues and cell and architecture with in the cell[25, 26].

The study found that in this respect vitamin A, C can be considered preventive antioxidants. The other group, the radical quenching antioxidants acts through preventing radical propagation and chain elongation. Among them alpha-tocopherol are the ones obtained from the diet[27,28].

The literature found that reactive oxygen species in tissues and can damage DNA, proteins, carbohydrates and lipids. These potentially deleterious reactions are controlled by a system of enzymatic and nonenzymatic antioxidant which eliminate prooxidants and scavenge free radicals. The ability of the lipid soluble carotenoids to quench single molecular oxygen may explain some anticancer properties of the carotenoids, independent of their provitamin A activity. Tocopherols are the most abundant and efficient scavengers of hydroperoxyl radicals in blological membranes. Water-soluble antioxidants include ascorbate and cellular thiols. Glutathione is an important substance for enzymatic antioxidant functions and is capable of nonenzymatic radical scavenging. Thiols associated with membrance proteins may be also important to the antioxidant systems. Interaction between the thiols, tocopherols and other compounds enhance the effetiveness of cellular antioxidant defense[29].

The study suggested that interest in free radical events has stimulated speculation thal their disorder may be involve in a number of diseases. The reduction of dioxygen to water involve several active intermediates. The control of this depends on the integrity of an enzymatic system that requires adequate intake of selenium, cooper, zinc and manganese, if their level of intake is low, proliferation of active oxygen metabolites may occur. Targets for attack are DNA, proteins and polynsatured phospholipida. Perixidation polyunsaturated phospholipids will result in disruption of membrane architecture. Vitamin E, perhaps with ascorbic acid can prevent this and vitamin A and β-carotene also intervene. The implication of this in the etiology of a number of diseases depends on theory and on evidence of linking low intake of

antioxidant nutrients with a high disease incidence. Improvement in epidemiology have resulted in glimpses into what prove to be links between diet and disease[7].

The study reported that the influence of free radical-mediated oxidations is amplified because it proceeds by a chain mechanism, i.e. only one radial can initiate chain reaction which may propagate over and over again. It was found that the in vitro oxidations of erythrocyte membranes proceed by a chain mechanism with a ling kinetic chain length. Thus, the role of chain breaking antioxidants is quite important, since they scavenge chain-carrying radicals to break a chain reaction. In fact, it has been found experimentally that vitamin E, a lipophilic chain breaking antioxidant present within the membranes, suppresses the oxidative damage of the membranes more efficiently than water-soluble chain-breaking antioxidants such as vitamin C, which scavenges aqueous radicals but can not scavenge chain carrying radicals within the membranes[9, 30-37].

The study described that therefore health hazards by oxygen species can to some extent be prevented by the bodies multi level defense system against free radicals, which comprises a number of enzymatic antioxidants and some micronutrients that have come to be regarded as antioxidant nutrients. They include vitamins vitamin A, E, C and β-carotene[7].

The study showed the free radicals first generated in the aqueous phase of the whole blood, the water soluble antioxidants especially vitamin C acts as a first defense[38].

In a study at University of Melbourne, Australia showed the mean serum levels of β-carotene and retinol were statistically significantly lower when the cases were compared with another set of 88 male controls of a similar age of head and neck squamous cell carcinoma .

The study showed the ultimate proof that a curative chemo preventive agent does prevent cancer is a demonstration of reduced cancer incidence in a targeted population. However, because of practical and logistical consideration, such trials are virtually impossible to conduct for the majority of cancers. Therefore a conclusion regarding the efficacy of chemo preventive activity is based on consideration of a variety of indirect lines of evidence, including laboratory studies, animal model systems, epidemiologic surveys, intervention trials involving reversal of premalignant change, and the prevention of malignancies in particularly high risk subjects. Furthermore, the only agents worth testing are those with limited, or preferably, no toxicity, since the final use will be prevention in a generally health population. β-carotene and vitamin E both fulfill all the criteria for suitable chemo preventive agents; several lines of evidence point toward preventive roles for them in oral cancer. In numerous epidemiologic studies, low intake of β-carotene has been associated with higher cancer risk. Both intake and supplemental use of vitamin E have been associated with a lowered risk of cancer. Smokers, whose habit is a major risk factor, have lower β-carotene levels in oral mucosal cells when compared with non-smokers in several laboratory and animal model systems, including the very relevant hamster cheek pouch model, these agents strongly inhibit oral cavity carcinogenesis. B-carotene and vitamin E produce regression of oral leukoplakia, a premalignant lesion

for oral cancer. This has now been shown in seven clinical trials: five with β-carotene alone, one with vitamin E, and one with a combination of both. Actual cancer incidence reduction trials in high risk groups have targeted the prevention of second malignancies in patients cured of an oral cancer. Such trials are now in progress. These data, together with the lack of any significant side effects, and an emerging role for these agents in the prevention of other life-shortening chronic diseases such as atherosclerosis, are strongly supportive of a very significant disease-preventive role for these nutrients, including chemopreventive role in oral cancer .

It was stated that an antioxidants are inhibitors which are effective in preventive oxidation by molecular oxygen. These antioxidants have great biological significance in the protection of cells by eliminating pro oxidants and scavenging free radicals[39].

The study looks at the impact of micronutrients such as vitamin A, riboflavin, zinc and selenium as intervention agents in subjects with and without precancerous lesions in a high risk group (reverse smokers of chutta-rolled tobacco leaf). Reverse smokers from four villages were enrolled in the study. 150 subjects were supplemented with four nutrients, namely vitamin A, riboflavin, zinc and selenium in the form of a capsule twice a week for 1 year. 148 controls received a placebo capsule containing lactose for the same period. Clinical history and anthropometric data were collected from all the subjects and a clinical photograph of the palate was taken. Micronutrients were estimated in random blood collected from a sub-sample before and after the study. Micronutrients improved the vitamin A, riboflavin and selenium nutriture in the supplemented group with a concomitant regression of precancerous lesion present on the palate. Clinically complete remission of white, red and combination lesions

was seen in 57% of subjects on supplements, whereas 8% on placebo showed a positive response. Further progression of these lesions was seen in 10% of the supplemented group compared with 47% in the placebo group ($p<0.001$ in the non-lesion group, new lesions appeared in 12% on the placebo developed new lesions ($p<0.02$)[40].

The study shows currently there is little evidence that vitamins of any type are able to greatly modify the progression of established malignancy with the exception of promyelocytic leukoplakia. In contrast, there is considerable laboratory evidence from chemical, cell culture and animal studies that antioxidant vitamins and related micronutrients are able to slow, or possible prevent the carcinogenic process. There is a good theoretical basis for these findings. Current theories for the mechanism of tumourigensis suggest that reactive species and prooxidants promote and encourage the process whilst antioxidants are inhibitory and protective. Retinoids and folate with limited or no antioxidant activity may protect DNA in other ways. In man there is support for this role the extraordinarily high concentrations of ascorbate and possible alpha-tocopherol at sites where oxidant stress is likely to be most intense, with loss of such antioxidant protection in some conditions which predispose to malignancy. There is also impressive epidemiological agreement, particularly from observation studies, where the lowest fruit and vegetable intake has been consistently associated with increased risk of cancer, especially of the lung and gastrointestinal tract, but much less evidence that such low intakes can encourage the development of cancers which are under hormonal control. Where individual micronutrients have been considered, β carotene appears to have the strongest protective effect followed by

vitamin C and vitamin E. Whilst the experimental studies have suggested a role for retinoids, this has not been confirmed by the observational studies. Unfortunately, with the exception of oral leukoplakia, studies investigating reversal of premalignant conditions have been disappointing and two intervention studies aimed at prevention in large populations have produced conflicting results. All this begs the question as to what dietary advice or intervention, if any, should be provided prior to the publication of the many randomized intervention studies that are presently investigating the role of micronutrients in cancer prevention. Gey [73] has produced recommendations for minimum blood concentrations and intake of antioxidant micronutrients[41-48].

It was indicated that the vitamins, such as A, β carotene, C, E, B_{12} and folate are the micronutrients with the strongest evidence of having a link to cancer prevention and control. Deficiency of these vitamins at the dietary, systemic or mucosal level will interact with tobacco use and increase the risk of oral precancerous lesions. The objective of this study was to (1) establish the baseline circulating levels of these vitamins in our normal population with and without tobacco use and (2) compare these levels with the values obtained in cases of oral leucoplakias. 50 normal controls with 25 each in chewers and non-chewers, matched for age and sex, were selected. 50 cases of oral leucoplakias (clinically detectable white patches) from the field constituted the study group. Simultaneous measurement of serum vitamin B_{12} and folate were carried out by radio assay. The other serum vitamins were estimated spectrophotometrically. Except for serum vitamin E, all the other serum vitamin levels were significantly decreased in oral leucoplakia compared to the controls.

Cancer chemo preventive agents acting as inhibitors of both initiation and promotion, as analyzed in our population, is promising for further intervention trials[49].

The study evaluate the duration of response and the need for maintenance therapy in subjects who respond to β-carotene. In this multicenter, double-blind, placebo-controlled trial, subjects were given β-carotene, 60 mg/d, for 6 months. At 6 months, responders were randomized to continue β-carotens or placebo therapy for 12 additional months. Fifty-four subjects were enrolled in the trial, with 50 being evaluable, At 6 months, 26 subjets (52%) had a clinical response. Twenty-three of the 26 responders completed the second, randomized phase. Only 2 (18%) of 11 in the β carotene arm and 2 (17%) of 12 in the placebo arm relapsed. Baseline biopsies were performed in all patients. A second biopsy was obtained at 6 months in 23 subjects who consented to this procedure. There was improvement of at least grade 1 of dysplasia in 9 (39%), with no change in 14 (61%) Nutritional intake was assessed using food frequency questionnaires. There was no change in carotenoid intake during the trial. Responders had a lower intake of dietary fiber, fruits, folate and vitamin E supplements than did no responders. B-carotene levels were measured in plasma and oral cavity cells. Marked increases occurred during the 6 month induction. However, baseline levels were not restored in subjects taking placebo for 6 to 9 months after discontinuation with oral leukoplakia was confirmed. The responses produced were durable for 1 year. Khanna R (2005) concluded that 20 new cases of histological proven oral squamous cell carcinoma, 20 of leukoplakia and 20 age and sex matched healthy controls were included. Intra oral pH of patients and controlled were measured by quantitative litmus paper test and serum was analyzed for

malonialdehyde (MDA), super oxide bismuths (SOD) cataloes and glutathione peroxides (GP). Patients with leukoplokia were treated with exogenous antioxidants for 3 months and the same were reassessed. Oral pH of oral cancer patients were neutral (PH-7) but that of leukoplakia and controls were mildly acidic (6.64 and 6.58 respectively). Serum malonialdehyde levels were highest in oral cancer group. With antioxidant enzymes super oxide bismuths, cataloes and glutathione peroxides different pattern was noticed. Antioxidant enzymes remained almost the same ($p<0.005$ each) in patients with leukoplakia after 3 months of Vitamin A, C and E. But there was marginal increase in cataloes level ($p<0.05$). The study shows the positive benefit of Vitamin (A,C,E) and nutrition supplementation on the antioxidant enzyme defense system hence prevention of oral carcinogenesis in patients with leukoplakia[50].

The results showed an overall inverse association of antioxidants, including retinol, β-carotene, and Vitamin C and E and risk of head and neck squamous cell carcinoma. The protective effect of these antioxidants was seen in both men and women. These effects are thought to result from changes in the expression of genes that regulate cell growth and differentiation[51].

A case-control study conducted of 385 histologically confirmed cases of HNSCC (193 oral, 132 pharyngeal and 60 laryngeal), excluding nasal and par nasal cancer and 1925 age matched and sex-matched cancer-free outpatient controls using data from the hospital based epidemiologic research program at aichi cancer center, Japan. The intake of nutrients and food groups was assessed with a food frequency questionnaire

and multivariate-adjusted odds ratios for cancer were estimated for smoking and drinking habits using logistic models. The results showed an overall inverse association between the intake of dietary antioxidants, including carotene and vitamin C and E and risk of HNSCC. The protective effect of these antioxidants was seen in both men and women. High consumption of antioxidants was associated with a decreased risk of HNSCC among smokers, drinkers and those with both smoking and drinking habits. These findings suggest that dietary antioxidant intake prevents HNSCC in smokers and drinkers[51].

A study from 8171 women who were randomly assigned in the Women's Antioxidant Cardiovascular Study, a double-blind placebo-controlled 2×2×2 factorial trial of vitamin C (500mg of ascorbic acid daily), natural-source vitamin E (600 IU of α-tocopherol every other day) and β carotene (50mg every other day), 7627 women who were free of cancer before random assignment were selected for this study. Diagnosis and deaths from cancer at a specific site were confirmed by use of hospital reports and the National Death Index. Cox proportional hazards regression models were used to assess hazard ratios (represented as relative risks [RRs) of common cancers associated with use of antioxidants, either individually or in combination. Subgroup analyses were conducted to determine if duration of use modified the association of supplement use with cancer risk. All statistical tests were two-sided. During an average 9.4 years of treatment, 624 women developed incident invasive cancer and 176 women died from cancer. There were no statistically significant effects of use of and antioxidant on total cancer incidence. Compared with the placebo group, the RRs were 1.11 (95% confidence interval [Cl]= 0.95 to 1.30 in the Vitamin C group, 0.93

(95% Cl=0.85 to 1.17) in the β carotene group. Similarly, no effects of these antioxidants were observer on cancer mortality. Compared with the placebo group, the RRs were 1.28 (95% Cl=0.95 to 1.73) in the Vitamin C group, 0.87 (95% Cl=0.65 to 1.17) in the Vitamin E group and 0.84 (95% Cl=0.62 to 1.13) in the β carotene group. Duration and combined use of the three antioxidants also had no effect on cancer incidence and cancer death. Supplementation with Vitamin C Vitamin E or β carotene offers no overall benefits in the primary prevention of oral incidence or cancer mortality52.

CHAPTER 3

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 Study Design

Single blind Clinical trial of selected patients

3.2 Place of study:

DDCH

AKMMCH

ICDDRB

BIRDEM

3.3 Durational Study:

3 Years

3.4 Study Population:

Biopsy proven oral leukoplakia patient's having attending in the outpatient department

3.5 Inclusion Criteria:

- Patients suffering from homogenous, non plaque type oral leukoplakia
- Age range: 21 to 60 years
- Sex-Both males and females
- Family income - all income group population
- Geographic location (Leaving place)- both rural and urban
- Level of education – both illiterate and literate population

3.6 Exclusion Criteria:

- Any white lesion in the oral cavity other than oral leukoplakia
- Plaque type oral leukoplakia
- Severely medically compromised patients
- Pregnant and lactating women
- Patient with oral leukoplakia receiving treatment

3.7 Sample Size:

3.7a. Sample size Determination[53]:

The conventional remission rate of 10% cases (approximate) and intervention remission rate of 50% cases (approximate) with antioxidant micronutrient on oral leukoplakia. The sample size was determined in this study by the following formula –

$$n = 2pi^2 \times \frac{\bar{P}(1-\bar{P})}{(P_1-P_2)}$$

n= number of sample

Pi= power index

P_1= conventional remission rate (10%)

P_2= intervention remission rate (50%)

P= average remission rate ($\frac{.10+.50}{2}$) =0.30

α level= 0.05(1.96)

β level = 0.20 (1.28)

Power index (Pi) is-

1.96(0.05 α, one tailed)+ 1.28(.20 β, one tailed)=3.24

So, n =

$$\frac{2 \times (3.24)^2 \times .30 \times .70}{(.1-.5)^2}$$

= 27.50

The required sample size is 28. To compensate the drop out of the patients from the study, 25% sample will be added and total sample size was (28+7) 35. So the total sample size for 4 groups were (35×4) = 140.

3.7 b. Sampling technique :

Purposive sampling – The final sample size 140 was achieved by non randomized purposive sampling technique

3.7.c. Study groups :

Four groups. The 140 subjects were equally distributed among the four groups in such a way, each groups contain equal cell volume (35) and supplement was done by random sampling with

lottery System

Group I : Vitamin C Supplementation

Group II : Vitamin E Supplementation

Group III : β Carotene Supplementation

Group IV : Placebo Supplementation

3.8 Study Variables:

3.8a.

i) Anthropometric variable: Body Mass Index (BMI)

ii) Biochemical variables: Analysis of Vitamin C, Vitamin E and β carotene.

iii) Clinical variables :

Site of the lesion

Size of the lesion- 0-1 cm^2, >1-2cm^2, >2- 4cm^2

Colour of the lesion

iv) Histopathological variable

v) Dietary variables[54]:

Consumption of vitamin C, vitamin E and β carotene were found from diet by repeated 24 hours recall method for consecutive 3 days. These were calculated from the self reported consumption history of the patients. It was done at the base line and 3 months of intervention.

3.8 b. Measurement of Study Variables

- Anthropometry: Anthropometric measurement was done by Body Mass Index (BMI). It was recorded at the base line and at the end of 6 months interval of intervention.
- Bio-Chemical analysis: Bio-Chemical analysis of vitamin C, vitamin E and β-carotene was done by standard colorimetric and HPLC method. These were recognized by association of American Chemistry (AOAC). There were done at the base line and at the end of 6 months intervention.

3.9 Bio-Chemical Analysis of vitamin C, vitamin E and β carotene

3.9.a. Biochemical analysis of vitamin C[55]:

- Vitamin C was analyzed by colorometric method
- Took 2.0 ml of freshly prepared meta-phosphoric acid in test tubes add 0.5 ml of sample or standard
- Mixed and centrifuged at 2500 rpm for 15 minutes
- Filtered and took supernatant for blank, sample and standard
- Added 0.4 ml of DTCS in each and waited for 3 hrs at 37^0C
- Added 2 ml of 12 molar sulphuric acid in each
- Read the absorbance at 520 nm against the reagent blank

3.9.b Biochemical analysis of vitamin E and β carotene [56]:

- Vitamin E and β carotene was analyzed by HPLC method
- 100µl plasma was de-protinized with 100µl ethanol
- Added hexane
- Shaken for 30 seconds
- Centrifuged at 3500 rpm for 4 minutes
- The hexane layer was separated and transferred in a clear glass vial
- It was evaporated to dry under nitrogen (N_2)
- It was re-dissolved into mobile phase (100µl methanol) and injected into HPLC

3.10 Clinical Examination:

- Clinical examination was taken at the base line, 1 month, 3 months and 6months of intervention.
- Site of the lesion - Floor of the mouth, buccal mucosa, lateral surface of the tongue, inner surface of the lip
- Size of the lesion - 0-1 cm^2, >1-2cm^2, >2- 4cm^2
- Colour of the lesion - Elevated or flat white lesion in the affected area

3.11 Histological Examination:

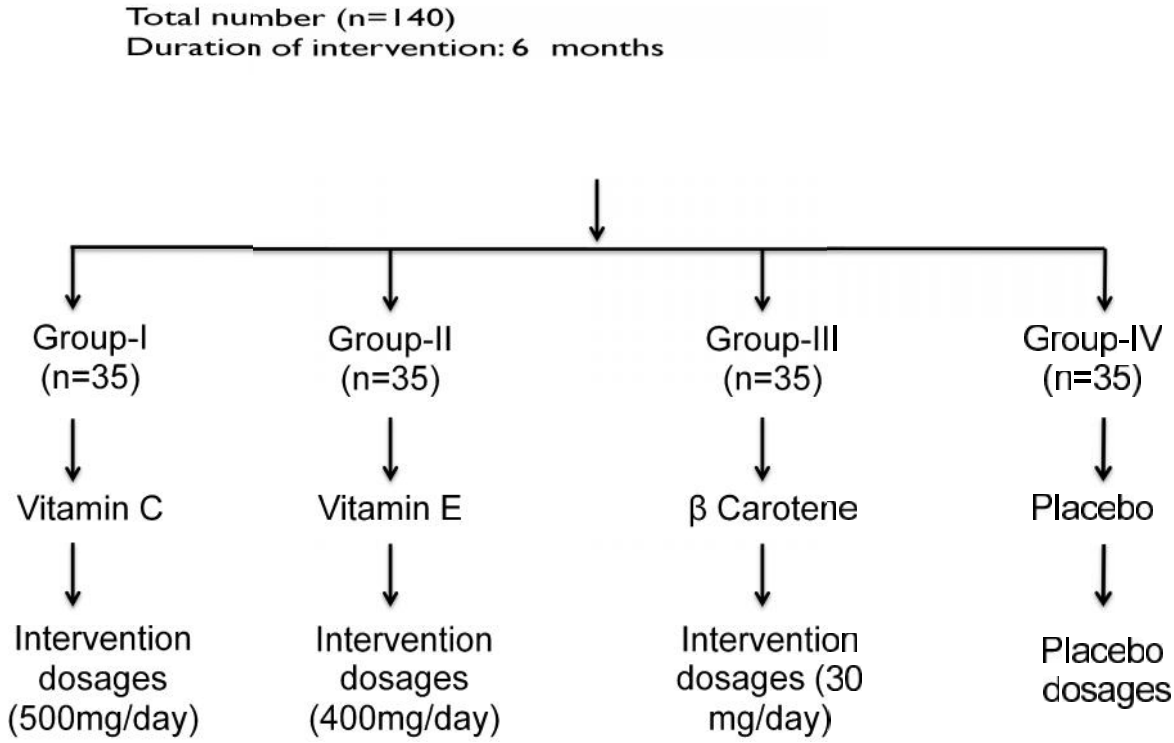
3.11.a. Histopathological examination:

Histopathological examination was investigated by hematoxyline and eosin stain method. It was done at the base line and at the end of 6 months of intervention.

3.11.b. Histopathological Features :

- Hyper keratosis of the epithelium
- Acanthosis of the epithelium
- Chronic inflammotory cell
- Epithelial dysplasia –
    - Loss of polarity of basal cell
    - Increased nuclear cytoplasmic ratio
    - Abnormal mitotic figures
    - Nuclear pleomorphism
    - Keratinazation

3.12 Duration of Intervention and dosage of Anti-oxidant [57]:



Intervention was done in random sampling technique by lottery system. Coated antioxidants- vitamin C, Vitamin E and β carotene was administered for both study and placebo groups. The Same outer coating was done of all antioxidant vitamins – vitamin C, vitamin E, β carotene and also for placebo

The coating was prepared by Pharmaceuticals Company. All the preparation was prepared in such a way their color, shape, size were similar.

3.13 Statistical data analysis:

After coding and editing data will be analyzed by using statistical packaging for social science (SPSS) version 16.0 software programs and co-efficient of correlation. According to the variable and appropriateness mean, standard deviation will be calculated and presented by tables, figures. According to data appropriateness significance test (T test, Chi-square test, Correlation test and coefficient of variation) will be done.

3.14 Ethical Clearance:

The Ethical Clearance was administrated from the Ethical review committee of Bangladesh Medical and Research Council.

CHAPTER 4

RESULTS

RESULTS

Table 1 : Socio demographic characteristics of the patient's before supplementation of vitamin C, vitamin E, β Carotene and placebo (n=140)

Supplement / Treatment	Number / Count	Percentage
Vitamin C	35	25
Vitamin E	35	25
β Carotene	35	25
Placebo	35	25
Total	**140**	**100**
Gender	**Number / Count**	**Percentage**
Male	84	60
Female	56	40
Total	**140**	**100**

Above table shows that each groups contain 35 number of respondents. Out of them male was 60% and female was 40%.

Table 2 : Socio demographic characteristics of the patient's before supplementation of vitamin C, vitamin E, β Carotene and placebo (n=140)

Age range	Number / Count	Percentage
21 – 30	11	7.5
31 – 40	21	14.5
41 – 50	40	29
51 – 60	68	49
Total	**140**	**100**
Leaving area	**Number / Count**	**Percentage**
Rural	78	56
Urban	62	44
Total	**140**	**100**

Table 2 shows that 49% of the patients were 51-60 years of age followed by 29%, 14.5% and 7.5% were 41-50 years, 31-40 years and 20-30 years respectively.

The rural respondents were 56% and urban were 44%.

Table 3 : Socio demographic characteristics of the patient's before supplementation of vitamin C, vitamin E, β Carotene and placebo (n=140)

Income status	**Number / Count**	**Percentage**
< 10000/Month	45	32
10000-30000/Month	60	43
>30000/Month	35	25
Total	**140**	**100**
Educational status	**Number / Count**	**Percentage**
Illiterate	12	9
Primary and high school	68	49
High school and above	60	42
Total	**140**	**100**

Table 3 shows that 43% had income of BDT 10,000-30,000/ Month and followed by 32% and 25% had income were BDT <10,000/Month and >30,000/ Month respectively.

The 49% of the respondents were primary and high school, followed by 42% and 9% were high school and above and Illiterate respectively.

Table 4: Distribution of the patient's according to personal habits (n=140)

Smoking status	**Number / Count**	**Percentage**
Smoker	88	63
Non Smoker	52	37
Total	**140**	**100**
Tobacco status	**Number / Count**	**Percentage**
Tobacco	80	57
Non tobacco	60	43
Total	**140**	**100**

Above table shows that 63% of the respondents were smoker and 37% were non-smoker.

Among the respondents 57% were tobacco chewer and 43% were non-tobacco chewer.

Table 5: Distribution of the patient's according to personal habits (n=140)

Food intake pattern	**Number / Count**	**Percentage**
Anti-oxidant rich foods	16	12
protein rich foods	48	34
Process foods	56	40
Fats and oils	20	14
Total	**140**	**100**

Above table shows that among the respondents 40% were intake Process foods, 34% were protein rich foods, 14% were fats and oils and 12% were anti oxidant rich foods.

Table 6: Distribution of the patient's according to clinical features (n=140)

Oral hygiene status	**Number / Count**	**Percentage**
Good oral hygiene	48	34
Bad oral hygiene	92	66
Total	**140**	**100**
Nutritional status	**Number / Count**	**Percentage**
Malnourished (BMI <18.5)	26	19
Good (BMI 18.5-24.99)	74	53
Over weight (BMI >25)	40	28
Total	**140**	**100**

Above table shows that among the respondents 66% were bad oral hygiene and 34% were good oral hygiene.

The out of 140 respondents 53% were good nutritional status, 28% were overweight and 19% were malnourished.

Table 7: Distribution of the patient's according to clinical features (n=140)

Area/Size of the lesion (Size in cm^2)	**Number / Count**	**Percentage**
Up to 1 cm^2	24	17
>1-2 cm^2	96	69
>2-4 cm^2	20	14
Total	**140**	**100**
Location / Site of the lesion	**Number / Count**	**Percentage**
Inside the check	97	69
Lateral surface of the tongue	25	18
Floor of the mouth	3	2
Inside the lip	5	4
Alveolar Mucosa	10	7
Total	**140**	**100**

Table 7 shows that among the respondents 69% were >1-2 cm^2, 17% were up to 1 cm^2 and 14% were >2-4 cm^2.

The out of 140 respondents 69% of the lesion were inside the check, 18% were lateral surface of the tongue, 7% were alveolar mucosa, 4% were inside the lip and 2% were floor of the mouth.

Table 8: Association of blood level before supplement and after supplement of vitamin C, vitamin E, β Carotene and Placebo (n=140)

Anti-oxidants	Before intervention (Mean±SD)	After 6 months of intervention (Mean±SD)	Mean Change	*p-value*
Study group				
Vitamin C (mg/dl)	0.51±0.29	1.30±0.68	0.78	0.04
Vitamin E (µg/dl)	499.8±179.48	854.91±255.70	352.20	0.01
β carotene(µg/dl)	42.28±18.70	115.91±69.21	73.63	0.001
Placebo group				
Vitamin C (mg/dl)	0.53±0.307	0.50±0.25	-0.017	0.38
Vitamin E(µg/dl)	498.62±169.38	494.77±156.32	-4.94	0.45
β carotene(µg/dl)	41.68±17.80	39.37±20.80	-2.91	0.56

The above table shows that the study results were statistically significant in all study groups ($P<0.05$). Among them β carotene was highly significant.

Table 9: Association of blood level and histological change in study group (n=105)

Group	Mean Change of serum level	Adjusted odds ratio	95% Confidence interval
Vitamin C(mg/dl)	0.78	2.01	1.01-3.04
Vitamin E (µg/dl)	352.20	2.08	1.07-4.06
β carotene(µg/dl)	73.63	2.45	2.04-5.69

Above table shows that-

- Positive histological change due to increasing blood level were found by Odds ratio.
- Incase of vitamin C, 2.01 times positive histological change was found for per unit blood change.
- Incase of vitamin E, 2.08 times positive histological change was found for per unit blood change.
- Incase of β carotene, 2.45 times positive histological change was found for per unit blood change.

Table 10: Association of the supplement of vitamin C, vitamin E, β carotene and placebo with the change of color, size and histopathology (n=140)

Supplement Received	Total Number	Positive Change by color and size	Positive Change by histopathology
		Percentage / Number	Percentage / Number
Vitamin C	35	63(22)	56(20)*
Vitamin E	35	65(23)	60(21)*
β carotene	35	69(24)	64(22)*
Placebo	35	15(5)	15(5)

*$p<0.05$

Above table shows that anti-oxidant was highly significant in supplement group compare to placebo by color, size and histopathology (P<0.05). But supplement group was not significant compare to each other, but higher proportion of the supplement of β-Carotene was found (64%).

Table 11: Measurement of selected anti-oxidant consumption level from diet by repeated 24 hours dietary recall method for consecutive 3 days before and after intervention (n=105)

Name of the nutrient	Amount of 3 days mean consumption	
	At the base line	After 3 months of intervention
Vitamin C (mg)	35±6.51	36±7.90
Vitamin E (mg)	5±1.632	5.50±1.82
β carotene (µg)	2203.25±144.20	2210.6±152.36

Above table state that dietary intake level was not satisfactory change at the base line and after 3 months of intervention.

Table 12: Association of gender with the change of histopathological test after the supplementation of vitamin C, vitamin E, β carotene and placebo (n= 140)

Sex	**Total Number**	**Histological Changes**		***P-value***
		Yes	No	
Male	84(100)	52(44)	48(40)	0.107
Female	56(100)	43(24)	57(32)	

Above table shows that the remission rate was not significant in male and female group

(p-value=0.107).

Figure 1: Association of gender with the change of histopathological test after the supplementation of vitamin C, vitamin E, β Carotene and placebo (n=140)

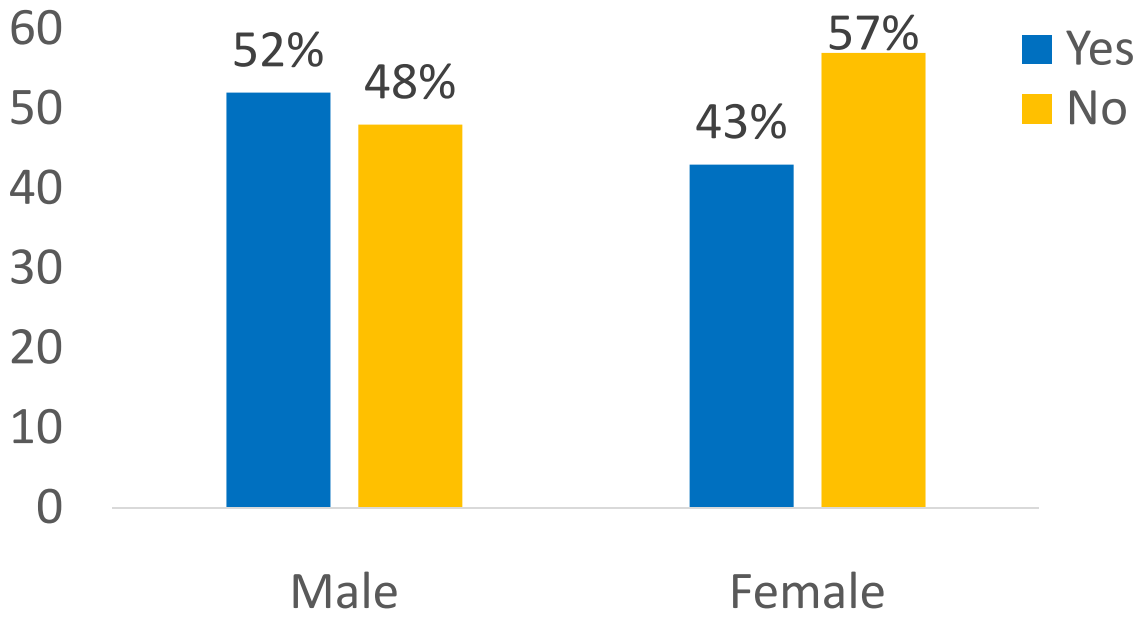


Figure 1 shows that positive oral histological changes within the male was 52% and female was 43%.

Table 13: Association of smoking status and histopathological test after the supplementation of vitamin C, vitamin E, β carotene and placebo (n=140)

Smoking status	**Total Number**	**Histological Changes**		***P-value***
		Yes	No	
Smoker	88(100)	38(33)	62(55)	0.005
Non Smoker	52(100)	65(34)	35(18)	

Above table shows that histological changes were significant in non smoker (65%) than compare to smoker (38%) (p=0.005).

Figure 2: Association of Smoking status and histopathological test after the supplementation of vitamin C, vitamin E, β Carotene and placebo (n=140)

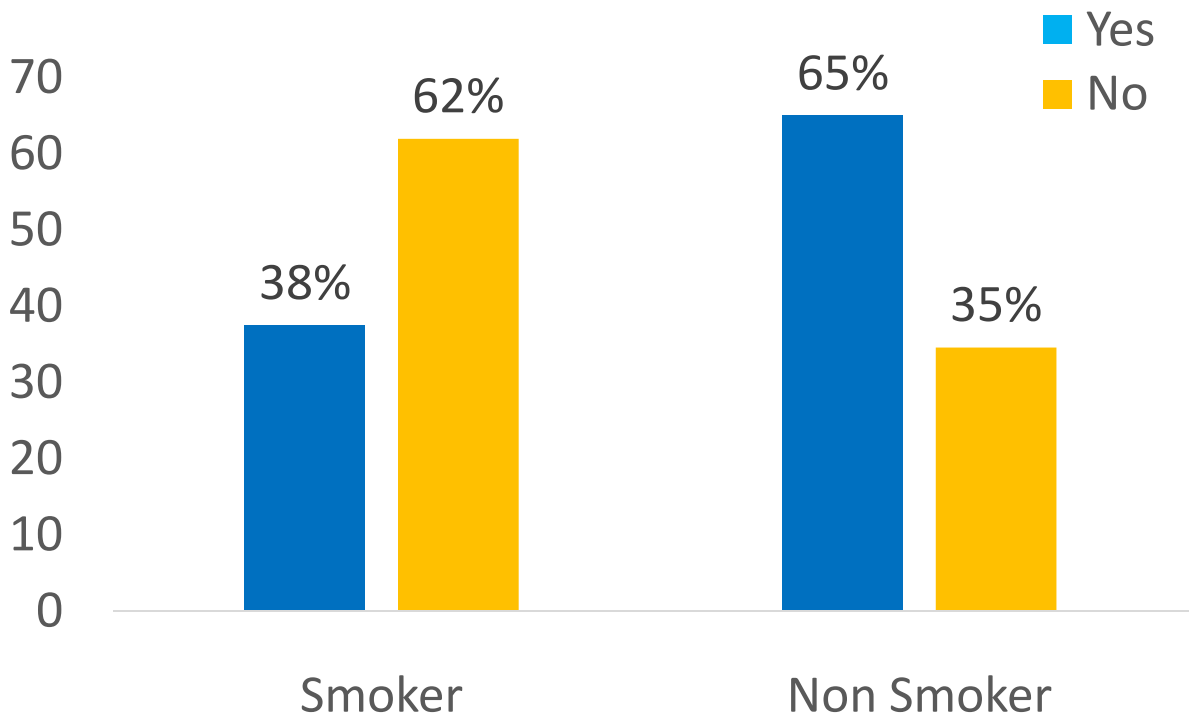


Figure 2 shows that positive oral histological changes within the smoker was 38% and non smoker was 65%.

Table 14: Association of smokeless tobacco and histopathological test after the supplementation of vitamin C, vitamin E, β Carotene and Placebo (n=140)

Smokeless Tobacco	**Total Number**	**Histological Changes**		***P-value***
		Yes	No	
Tobacco	80(100)	40(32)	60(48)	0.019
Non Tobacco	60(100)	60(36)	40(24)	

Above table shows that histological changes were statistically significant in non tobacco than compare to tobacco (p=0.019).

Figure 3: Association of smokeless tobacco and histopathological test after the supplementation of vitamin C, vitamin E, β Carotene and placebo (n=140)

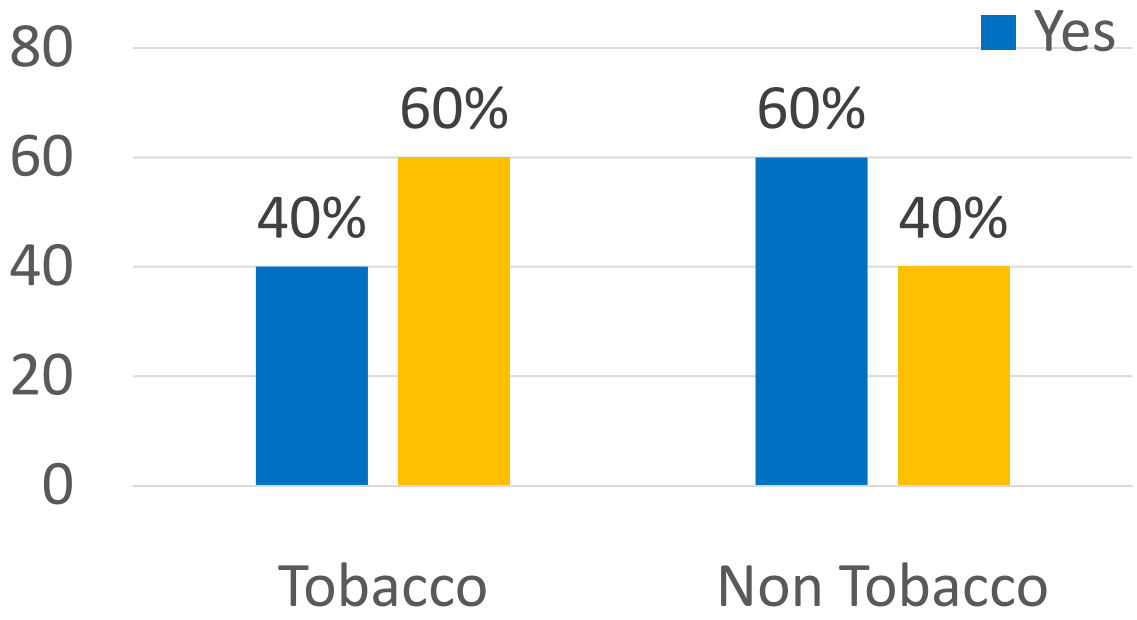


Figure 3 shows that positive oral histological changes within the tobacco was 40% and non tobacco was 60%.

Table 15: Association of nutrition status and histopathological test after the supplementation of vitamin C, vitamin E, β Carotene and placebo (n=140)

Nutrition status	**Total Number**	**Histological Changes**		***P-value***
		Yes	No	
Good	74(100)	61(45)	39(29)	0.043
Malnourished	26(100)	35(9)	65(17)	
Overweight	40(100)	43(17)	57(23)	

Above table shows that histological changes were significant in good nutritional status than Malnourished and overweight person (p=0.043).

Figure 4: Association of nutrition status and histopathological test after the supplementation of vitamin C, vitamin E, β Carotene and placebo (n=140)

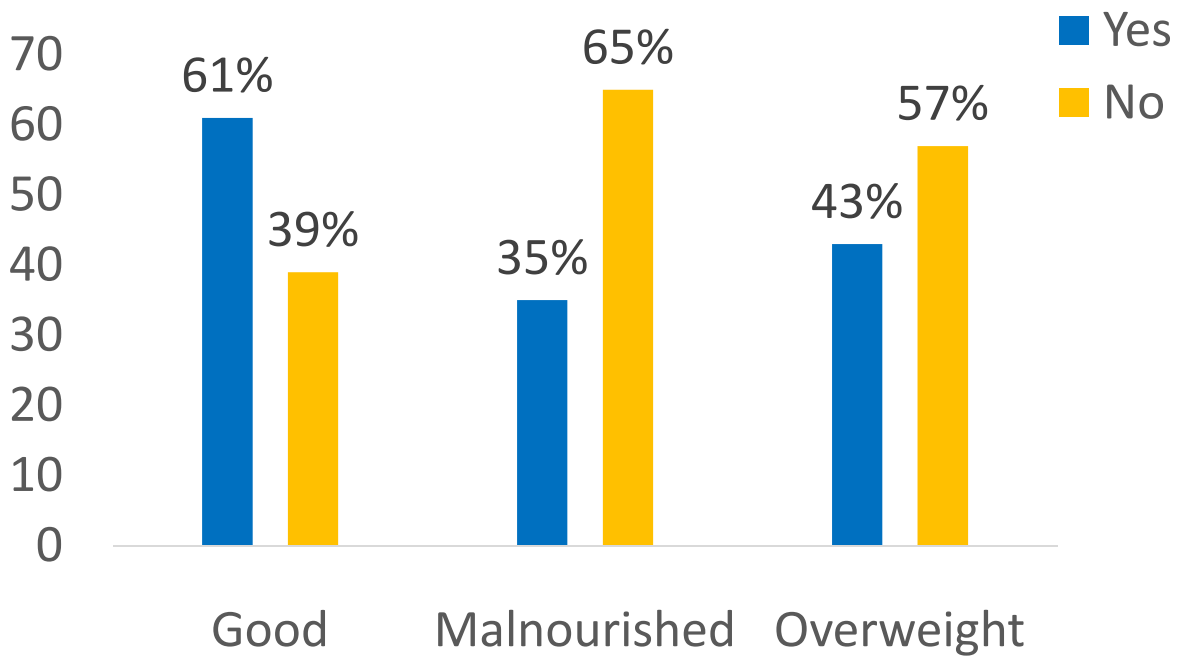


Figure 4 shows that positive oral histological changes within the good nutritional was 61%, malnourished was 35% and overweight was 43%.

Table 16: Logistic regression analysis to estimate the effect of Supplement of vitamin C, vitamin E, β Carotene and Placebo on histological change controlling the other risk factors (n=140)

Supplement Received	OR	*P*	95% Confidence Interval
Placebo	1		
Vitamin C	15.70	0.004	3.93- 20.66
Vitamin E	21.11	0.003	5.06-31.07
β Carotene	24.23	0.001	5.77- 32.83

- Multi – Variable stepwise logistic method (backward elimination)
- Controlling factors/risk factors : Age, sex, smoking, tobacco and nutritional status
- OR means Odds Ratio.

Above table shows that supplement of vitamin C, vitamin E and β carotene was highly significant for the remission of oral leukoplakia ($P<0.05$).

Table 17: Logistic regression analysis to estimate the effect of supplement of vitamin C, vitamin E, β Carotene and Placebo on histological change controlling the other risk factors (n=140)

Supplement Received	OR	*P*	95% Confidence Interval
Smoking status			
Yes	1		
No	11.88	0.001	3.20-18.11
Tobacco status			
Yes	1		
No	2.25	0.019	1.20 -4.90
Nutritional Status			
Malnourished	1		
Overweight	1.41	0.609	0.78-4.20
Good	7.99	0.001	2.26-14.42

Table 17 shows that – Positive oral histological changes were 11.88 times more in non smoker compare to smoker Positive oral histological changes were 7.99 times more in good nutritional status compare to overweight and malnourished respondents.

Table 18: Logistic regression analysis to estimate the effect of supplement of vitamin C, vitamin E, β Carotene, and Placebo on histological change controlling the other risk factors (n=140)

Food Intake pattern	**OR**	***P***	**95% Confidence Interval**
Fats and Oils	1		
Process Food	1.25	0.12	0.43-3.20
Protein rich food	2.20	0.08	0.74-4.90
Anti Oxidant rich food	2.86	0.04	1.25-6.40

Table 18 shows that – Positive oral histological changes were 2.86 times more in anti oxidant rich food compare to fats and oils, process food and protein rich food.

CHAAPTER 5

DISCUSSIONS

DISCUSSIONS

The present study was conducted among 140 patients with oral leukoplakia to established the effect of vitamin C, vitamin E and β Carotene on oral leukoplakia. The total 140 cases were equally divided into four groups such as group I, group II, group III and group IV. They were supplemented them by Vitamin C, Vitamin E, β carotene and placebo. Regarding selection of subjects strict guide line was followed to pick only the newly diagnose histopathologically documented oral leukoplakia in the Oral cavity. Among the oral leukoplakia patients 49% were aged 51-60 years, 29% were aged 41-50 years, 14.5 were aged 31-40 years and 7.5% were aged 21-30 years. It is similarities with Adriana Spinola Ribeiro (2010). The oral leukoplakia is more found among older and elderly men and its prevalence increases with age advancement. The male female ratio in study was 3:2. The study also showed that recurrence rate of male was 44 (57%) and female was 24 (43%). It is correlates with Adriana Spinola Ribeiro (2010). In this study males were more affected than female.

After interventions of vitamin c, vitamin E and β carotene on oral leukoplakia patients, the percentage of normal Oral cellular change within the male was 52% and female was 43%. It correlates with the study findings of Braz, Oral, res (2012). In this study oral leukoplakia lesions were more frequent in males compared to females. The percentage of normal Oral cellular change within the smoker 38% and within the non-smoker 65%. These findings are in agreement with the findings of Braz, Oral, res (2012). Although the frequency of oral leukoplakia lesions were higher among the smokers but the oral cellular change within the smokers were less. The percentage of normal oral cellular change of the lesion within the tobacco chewer was 40% and within the non tobacco chewer was 60%. (P-Value=0.019) . The similar results were

observed by Kumar S., Muniyandi M. (2015). In that study found, however the percentage of oral leukoplakia was specially high among chewers who also smoked tobacco (21.9%), but the normal Oral cellular change within the smoker was less after the intervention of vitamin C, vitamin E and β carotene. Increased serum level of vitamin C, vitamin E and β carotene were significantly and positively associated with histological changes ($p<0.05$). The remission rate of vitamin C was 56%. It is similar with the study conducted by Nago T. (2005), the remission rate of oral leukoplakia were found 17.4% cases after intervention of vitamin C at the duration of 1 year. It is dissimilar with the study conducted by Elitsa G. Deliverska (2017). In this study- regarding the efficacy of the use of vitamin C alone for treatment of oral leukoplakia was less. The remission rate of vitamin E was 60%. It was statistically significant ($p<0.05$). It correlates with this study Benner S.E (1993). In this study administration of vitamin E for 24 weeks had 46% clinical and 21% histological response were found. The remission rate of β carotene was 64%. It is similar with this study conducted by H.S.Gareweal (1990). In this study the remission rate of oral leukoplakia was found in 52% cases after intervention of β carotene at the duration of 1 year. The remission rate of oral leukoplakia was found in 15% cases in case of placebo. The rest 36-40% of oral leukoplakia lesions were not satisfactory change after intervention. The other literature suggest that it may be a case of individual physiology. It may be occur due to individual genetic factor, immunological factor, other micro-nutrient deficiency phenomena, energy requirement, work load and duration. The individual variation that hampers the effectiveness or utilization of antioxidant intervention and ultimately affect the remission rate of oral leukoplakia.

CHAPTER 6

CONCLUSIONS

CONCLUSIONS

Oral leukoplakia is a common potentially pre-malignant disorder in the oral cavity. The aged patients, specially 51-60 years were more affected by oral leukoplakia. The males were more sufferer than females. The anti-oxidant vitamins- vitamin C, vitamin E and β carotene were important nutrients of clinical and histological change of oral leukoplakia. The remission rate of oral leukoplakia in case of- vitamin C was 56%, vitamin E was 60% and β carotene was 64%. Oral histological changes were 11.88 times more in non smokers than smokers patients. Oral histological changes were 2.25 times more in non-tobacco chewer than tobacco chewer patients. After supplementation of anti-oxidant causes increasing the serum blood level and positive oral histological changes were found. So, an anti-oxidant could be used as a nutritional regimen/supplement for the remission of oral leukoplakia.

CHAPTER 7

STRENGTHS AND LIMITATIONS

7.1 STRENGTHS

- Clinical study
- It is a new study in Bangladesh

7.2 LIMITATIONS

- Short duration
- Small amount of study subjects

CHAPTER 8

RECOMMENDATIONS

RECOMMENDATIONS

The following recommendations are put forward-

- An anti-oxidants could be used as a nutritional regimen / supplement in addition with topical steroid for the remission of oral leukoplakia
- Oral hygiene should be maintained
- Correction of sharp teeth, ill fitting denture
- Tobacco products should be prohibited
- Future study with larger sample size, longer follow up and additional biomarkers of oxidative stress could be investigated to ascertain the results

CHAPTER 9

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CHAPTER 10

ANNEXURE