Changes in Blood Constituents Due to Smoking and Chewing of Betel-Nuts and Tobacco

BY

Shanthi G.

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Introduction

I - INTRODUCTION

Epidemics of bubonic plague and cholera those great waves of disease used to start in the orient and spread progressively to the West, killing millions in their path. Ignorance of their cause prevented effective action. Today a new epidemic starting in the west and potentially as dead as earlier plagues threatens the world, (Ball, 1980). Smoking as a habit has produced an epidemic of serious diseases. Equally potent is the habitual chewing of betel-nut and tobacco in spreading a plethora of diseases. (Potts, 1970).

In most European countries more than 50 per cent of adults smoke cigarettes. In Asian countries not less than 40 per cent of male smoker with the participation rate rising to 70 per cent more in Japan, some parts of India, (Fitch, 1973). India is the third biggest producer of tobacco in the world, with 80 per cent of the tobacco grown being consumed locally, mostly in traditional form (bidi, hookah, chutta etc.,). Tobacco chewing and the taking of snuff are also practised, (WHO Chronicle, 1982). For women smokers the figure rarely exceeds 30 per cent. Smoking surveys in Australia revealed a lower percentage of male smokers between 1969 and 1974 (from 45 per cent to 41 per cent) but a slightly higher percentage of female smokers (28 per cent to 29 per cent) (Willard, 1983). In Denmark 48 per cent of male population and 46 per cent of the female population are smokers, (Egsmose, 1983). In the period from the early twenties to 1970 the consumption of cigarettes rose

from 750 per adult to 3970 per adult per year in United States, (Fitch, 1973).

The human beings need fresh air to maintain a healthy body. Anything that cuts off its supply may bring on sickness. Cigarette smoking does it and the major health risk of this habit is lung cancer, (Potts, 1970). Two studies in India showed that the lung cancer risk for cigarette smokers is 8.6 times the risk for nonsmokers, (Stjensward, 1981).

Epidemiological evidence from many countries implies that smoking is a causative factor in lung cancer, leukoplakia, chronic bronchitis, ischaemic heart disease and obstructive peripheral vascular disease, (WHO Chronicle, 1975).

Smokers have a three times greater risk of getting heart attack than do nonsmokers and also that if they get a heart attack smokers have a 21 times greater risk of dying from that attack. The carbon monoxide in cigarette smoke can cause a rare disease of the blood itself which was given the name, "Smoker's Polycythemia" and the result is that the heart gets so thick with red cells, that the flow of blood is reduced, (Rodale, 1979).

Cigarette smoke plays a perpetuating role in hypertension and peptic ulcer, (Sanjivi, 1976). Studies have established a strong association between smoking and laryngeal cancer. Eighty four per cent of all laryngeal cancer among men could be attributed to smoking (Olsen, 1985).

The estimated annual excess mortality from cigarette smoking in the United States exceeds 350,000 more than the total number of American lives lost in World War I. It has been estimated that an average of 5½ minutes of life is lost for each cigarette smoked, (Fielding, 1985).

Smoking results in an increase in the percentage of blood carboxyhaemoglobin values. In heavy cigarette smokers who inhale while smoking it is common to note carboxyhaemoglobin values of upto 8 per cent as compared with values of less than 1.0 per cent in non smokers. The acute effects of tobacco smoking include increase in plasma catecholamine, ACTH as well as increase in serum cortisol. The changes in these hormones will also result in important effects on the peripheral leukocyte count, a decrease in eosinophils, an increase in neutrophils and a increase in monocytes. The changes in hormone values also lead to an increased value for plasma non-esterified fatty acids, (Henry, 1980).

Increased blood haemoglobin, increased mean corpuscular volume and increased white blood cell count are the chronic effects of smoking, (Conn, 1985). It has been reported that serum estradiol values are higher in male smokers than in male non-smokers, (Klaiber, 1984). The concentration of nicotine and thiocyanate generally found in urine and saliva of active smokers are clearly much higher than those found in passive smokers, (Robert, 1984).

Smokers showed lower HDL cholesterol and higher LDL cholesterol and total cholesterol than nonsmokers, (Stampford et al., 1984

In general smokers were lighter than nonsmokers and had a lower body mass index. There was no difference in energy intake but in general smokers had lower uptakes of vitamins, minerals and dietary fibre, (Grosslight, et al 1984).

It has been known for many years that certain countries in South-East Asia have a much higher frequency of oral cancer than other areas of the world. Indians as a whole seem to have much higher incidence rates of oral cancer than other ethnic groups and this difference is most probably the result of chewing tobacco together with betel-nut, (WHO, 1981).

Sanghivi et al., (1955) put forward evidence that extrinsic carcinoma of larynx is associated with bidi smoking and betel-nut chewing. Sarma (1960) reported that in Assam there is a close relationship between high frequency of extrinsic carcinoma of larynx and Assamese type of betel-nut chewing habit. Dimethyl sulphoxide extract of betel-nut is capable of inducing significant leukoplakia and tumours (Squamous cell carcinoma) in hamster cheek pouch mucosa, (Baruah, 1984).

In women between 18 to 45 years old using tobacco or oral contraceptives, triglycerides were significantly higher and HDL cholesterol were significantly lower than in controls. In women using both these was a cumulative effect on those values, (Ladd, et al., 1985). There was a significant increase in serum copper level in the oral cancer patients who had the habit of betel-nut chewing as compared to normals, (Sashidhar et al., 1983).

Betel-nut chewing causes gingivitis, irregularities of

teeth and sepsis resulting in chronic irritation and cancer, (Jussawalla et al., 1971).

Harmful effects of smoking and betel-nut and tobacco chewing have been well documented. The present study was designed to find out the biochemical profile of smokers and betel-nut and tobacco chewers. The biochemical parameters determined in the blood and serum samples were cholesterol, triglyceride, non-esterified free fatty acids, lipase activity, total protein, Creatinine, thiocyanate, carboxy haemoglobin and leukocytes count. It is hoped that the study would throw more light on the effects of smoking and betel-nut chewing.

Review of Literature

II - REVIEW OF LITERATURE

The literature pertaining to the study of 'Changes in blood constitutents due to smoking and chewing of betel-nuts and tobacco' are discussed under the following headings:

I. Epidemiology

II. Effects of smoking on
- a) Oral cavity
- b) Respiratory system
- c) Circulatory system
- d) Gastrointestinal tract

III. Effects of smoking on
- a) Lipids
  - (i) Total cholesterol
  - (ii) Triglyceride
  - (iii) Free fatty acids and lipase activity
- b) Serum proteins and creatinine
- c) Minerals

IV. Effect of smoking on Haemoglobin

V. Smoking and thiocyanate concentration in serum

VI. White blood corpuscles in smokers

VII. Betel-nut and tobacco chewing and health risks

I. EPIMEMIOLOGY

Approximately 60 million Americans smoke more than 200 types or brand, of cigarette and spend more than 23 million annually in the process. More than 35 per cent of American

men and approximately 30 per cent of American women smoke, (Kaplan, C. 1980). Maya and Stiaika, N. (1984) stated that 7.04 per cent of students from pune city and 10.69 per cent of students coming from other urban areas in our country were either smokers or ex-smokers. A survey in China shows that 56 per cent of the male population and 1.0 per cent of the female were smokers. In remote and very poor hilly areas of Nepal, as many as 85 per cent of men and 72 per cent of women smoke, (WHO Chronicle, 1982). In Asia, Africa, America and Oceania it is rare to find less than 40 per cent of regular smokers among males; in most European countries more than 50 per cent of adult males smokes cigarettes at an average of 15 per day, (WHO Chronicle, 1979).

II. EFFECTS OF SMOKING ON

a) ORAL CAVITY

The most hazardous of smoking is the oral cancer, Of the total of 9,484 primary malignant tumours, 2,450 arose in the hypopharynx and larynx. This constitutes 26.83 per cent of all carcinomas. Sanghvi et al., (1955) put forward evidence that extrinsic carcinoma of larynx is associated with both bidi smoking and betel-nut chewing. Mehta et al., (1969) studied oral habits VIS-a-VIS oral cancer and observed the pattern and prevalence of smoking, chewing pan, gudaku, misri in Bihar, Gujarat, Andhra and Kerala, over the rate of prevalence of oral cancer which ranges from 0.01 per cent in Bihar to 0.1 per cent in Andhra and Kerala.

b) RESPIRATORY SYSTEM

The major health risk related to cigarette smoking is lung cancer. The Kreyberg group I tumour are more common among cigarette smokers, (WHO Chronicle 1983). Smoking adversely effects total respiratory compliance in smokers. Smoking sets in obstructive changes which are reversible with bronchodilator to begin with but become irreversible with increasing duration of smoking and quantities of tobacco smoked, (Mahajan et al., 1984). Epidemiological studies have shown that bidi smoking is related to the development of cancer of larynx, pharynx, lung and oesophagus. Bidi smoke shows a greater level of total particulate matter and nicotine than that of Indian cigarette, (Menon, M.M, 1985). Children of smokers had a 20 per cent to 80 per cent greater risk of respiratory problems than children of non-smokers. (Marshall, 1982).

c) CIRCULATORY SYSTEM

A recent case control study in India has shown that cigarette smokers are more than twice likely to develop myocardial infarction than non smokers, (WHO Chronicle, 1983). Nicotine in cigarette increase the diurnal secretion of cortisol which has been associated with myocardial infarction and with more frequent and complex ventricular arrythmias. Smoking causes vasoconstruction probably by increasing catecholamine secretion. The rise in fatty acids probably due to catecholamine stimulation, may aggravate cardiac dysfunction and contribute to the fatty cellular lesions of atherosclerosis (Castelli et al., 1981).

Smoking results in an increase in the percentage of blood carboxyhaemoglobin values (Conn, B. 1985). Carboxyheamoglobin can severly reduce oxygen delivery to the myocardium. Carbonmonoxide also combines with myoglobin thus impairing the diffusion of oxygen to mitochondria in heart muscle, (Castelli, et al., 1981). At blood concentrations commonly found in smokers nicotine causes an increase in heart rate and blood pressure. Hence nicotine increases the demand the myocardium for oxygen while carbon monoxide decreases the supply, (Jones, R.M. 1985).

d) GASTROINTESTINAL TRACT

Smoking of cigarettes is associated with an increased, approximately doubled incidence of gastric and duodenal ulcers. It impairs ulcer healing and favours ulcer relapse. In a recent study it was demonstrated that cigarette smoking usually significantly reverses the inhibitory effect of antisecretory drugs on the inter digestive gastric secretion in patients with ulcers. Smoking increases the transpyloric reflux of bile acids and other duodenal contents, which may damage gastric mucosa, (Domschke and Sigurd, 1984). Nicotine slows the digestive process by as much as an hour and acidity in the stomach may increase causing heart burn (James, H. 1976).

III EFFECTS OF SMOKING ON SERUM

(a) LIPIDS

(i) Cholesterol

Smoking is belived to reduce HDL cholesterol and smoking is recognised as coronary risk factor, (Stampford, et al., 1984 The protective role of HDL in atherosclerosis is due to its ability to remove cholesterol deposited in arteries and transport it to the liver for catabolism and excretion, (Narang, N.K. et al., 1985).

Baldawa et al., (1983) showed a positive association between smoking and cholesterol and LDL - cholesterol values with an inverse relation between smoking and HDL level. A similar association was observed with the amount and duration of smoking.

In subjects who were alcoholic smokers, serum cholesterol, phospholipid and LDL - cholesterol were higher and HDL-cholesterol was lower than in the control group. It may be inferred that the favourable effect of chronic alcoholism on coronary heart disease in the form of low LDL and high HDL-cholesterol was not seen in subjects who were smokers too. On the contrary the HDL-cholesterol increased to an extent equal to or more than in pure smokers. Consequently the protective effect alcohol confers from coronary heart disease on the individual is completely nullified by the accompanied habit of smoking. (Garrison. et al., 1981).

Cigarette smoking is associated with low HDL - cholesterol values whereas exercise is associated with high HDL - cholesterol values. It was hypothesized that smoking may attenuate the effects of exercise to increase HDL - cholesterol, (Cresanta et al. 1985). Changes in serum HDL - cholesterol, after a fatty meal were studied in smokers and non smokers. In the nonsmokers there was an increase in the HDL_2 : HDL_3cholesterol ratio after a fatty meal but not in smokers. The findings suggest that smoking interferes with the conversion of $HDL_3$to HDL_2 and the lipolysis of triglyceride - rich lipoproteins. (Elkeles, R.S. 1984).

(ii) Triglyceride

Baldawa et al., (1983) showed a positive association between smoking and triglyceride values. In subjects who were alcoholic smokers serum triglyceride level was higher than in the control group.

Serum levels of triglyceride were significantly higher in smokers than those in the nonsmokers. The serum levels of triglyceride should be considered from aspects of both anabolism and catabolism of triglyceride since serum lipoprotein lipase level was increased in smokers. In that case the anabolism of triglyceride might be increased in the smokers compared with the nonsmokers. (Yoshida et al., 1986). The findings suggest that smoking interferes with the lipolysis of triglyceride - rich lipoproteins, (Elkeles, R.S. 1984).

(iii). Free fatty acids and Lipase activity

Brunzell et al., (1980), reported that the fasting levels of lipoprotein lipase activity were significantly higher in smokers than those in nonsmokers matched for age and sex. Adipose tissue lipoprotein lipase activity significantly higher than in controls of similar weight, 5.4 against 1.9 $m\mu/10^6$.

Smoking causes an increase in the circulating free fatty acid secondary to the secretion of noradrenalin which results in raised triglyceride, (Pozner et al, 1982). The acute effects of tobacco smoking include increases in plasma catcholamine and the change in this hormone lead to an increased value for plasma non-esterified free fatty acids. (Henry, J.B. 1980).

Linoleic acid was significantly lower in the adipose tissue of current cigarette smokers than in that of non smokers. Those smoking twenty-one or more cigarettes per day had the lowest proportion of adipose tissue linoleic acid. Platelet linoleic acid was significantly lower in current cigarette smokers than in nonsmokers. The lowest level of platelet linoleic acid was seen in heavy smokers (David Wood, 1984).

(b) Serum Total Protein and Creatinine

The serum protein levels of healthy controls is comparatively higher than that of healthy smokers (Vijayalakshmi and Lakshmi,C.V. 1985). Cigarette smoking decreases protein utilisation probably due to decreased metabolic availability of amino acid nitrogen.

(Anthony, et al., 1972). Smokers showed increases in serum total amylase, pancreatic isoamylase,, cationic trypsinogen and pancreatic trypsin inhibitor after stimulation with secretin, (Rogers et al., 1981). Increased N-nitrosation of proline was noticed in smokers compared to nonsmokers, (Ladd et al., 1985). Smoking was also associated with a lower serum creatinine as was drinking three or more drinks daily, (Savdie et al., 1984).

(c). Minerals

Smokers tend to have lower intakes of minerals, the mean intake of calcium was 6 per cent lower and iron 8 per cent lower. (Karin et al., 1984). Six ng of mercury are released in smoke per unit length of cigarette and 80 per cent of mercury in smoke are retained in the human body. (Suzuki et al., 1976). Cigarette smokers and those in smoke filled room divide between them from 1 to 2 µg of cadmium per package of cigarettes, (Holum, 1977).

Zinc

The zinc content and prostaglandin metabolism of leucocytes from control nonpregnant women and mothers 24-48 hours after delivery were estimated and related to foetal growth and maternal smokings. Monocytes from nonsmokers tended to produce more prostaglandins and zinc than those from smokers but the differences were not significant, (Simmer et al., 1985).

IV. EFFECTS OF SMOKING ON HAEMOGLOBIN

Mc Donough et al, (1965) reported elevated hematocrit values in cigarette smokers in contrast to nonsmokers. Moreover observed that smokers had a higher erythrocyte count, high haemoglobin content, an elevated erythrocyte volume, and an increased mean haemoglobin concentration than nonsmokers. (Thronton, 1978).

Cigarette smoke contains carbonmonoxide which is the villian of the drama. It enters the lungs and disrupts the mechanism of oxygen delivery to the heart. Haemoglobin, the blood substance that picks up oxygen in the lungs and delivers it to the heart, goes bersetk in a peculiar way. Haemoglobin perversely prefers carbonmonoxide to oxygen-infact prefers it 250 times as much. So levels above five per cent of carbon monoxide in the air drastically reduce the amount of oxygen that the heart is able to get.

Putting carbonmonoxide into the air breathed by the lungs not only cuts off oxygen from getting to the heart in sufficient amounts but signals the body to create more red cells and haemoglobin to feed that vital gas to the heart. As a result 'Polycythemia' results (Rodale, 1979).

In heavy cigarette smokers who inhale while smoking it is common to note carboxyhaemoglobin values of upto eight per cent as compared with values of less than one per cent in nonsmokers, (Conn, 1985). Smokers had a higher haemoglobin level

than nonsmokers. (Vijayalakshmi and Lakshmi,C.V., 1985).

Haemoglobin values were inversely correlated with birth weight quartiles, equally in mothers who smoke and those who did not smoke. When birth weights were grouped according to mothers haemoglobin value at term, the tobacco related effect on birth weight appeared only with haemoglobin values above 12g/dl, being particularly strong for values above 13g/dl. Haemoglobin values of 9.0 to 11.0 g/dl seemed to protect from growth retardation of the fetuses of anaemic mothers who smoked, (Nilsen et al., 1984).

V. SMOKING AND THIOCYANATE CONCENTRATION

It has long been known that one of the toxic substances of the tobacco aerosol is cyanide which after its entry into the blood steam is primarily detoxified as thiocyanate. A higher thiocyanate level in the blood of smokers therefore is likely to occur and many reports corroborate it. Smoking of bedies and cigarettes both affect the thiocyanate and alkaline phosphatase levels of blood (Bhown, 1972).

Salivary thiocyanate was significantly greater in habitual tobacco chewers than in nonchewers and was further increased after the chewing of betel quid with tobacco (Shivapurkar et al., 1980).

Dalferes used a procedure for determining the thiocyanate concentration in plasma so as to detect the presence or absence

of cigarette amoking in normal volunteers. The mean value of plasma thiocyanate concentrations in adult smoker was 161 μmol/litre as compared with mean plasma thiocyanate concentration in 24 non smokers of 62 μmol/litre (Henry, 1980).

VI. WHITE BLOOD CORPUSCLES IN SMOKERS

The acute effects of tobacco smoking include increasd in plasma catecholamines and cortisol. The changes in these hormones will also result in important effects on the peripheral leucocyte count; a decrease in eosinophils, an increase in neutrophils and an increase in monocytes.(Conn, 1985).

The total WBC count was strongly related cross sectionally to cigarette smoking and smoking status. Smokers on average had a WBC count of 7,750/cu.mm compared to 6,080/cu.mm for non smokers. A strong relationship was also noted between WBC count and cigarette smoking and the association between WBC count and myocardial infarction was diminshed when cigarette smoking was controlled. Prentice *et al.*, have reported the increase in coronary heart disorder risk to be primarily associated with neutrophils and to a lesser extent eosinophils and monocytes.

Previous studies have demonstrated increased production of oxygen radicals by the neutrophils of cigarette smokers with leukocytosis. Free radical damage has been widely postulated as pathogenetic of cancer and lung disease, (Neaton *et al.*, 1985).

VII. BETEL-NUT AND TOBACCO CHEWING AND HEALTH RISKS

Balendra (1949) from Ceylon stated that components of betelquid themselves are not cancer producing agents; betel-nut chewing causes gingivitis, irregularities of teeth and sepsis resulting in chronic irritation and cancer. Jussawalla (1971) studied the risk of the habit of the betel-nut chewing without tobacco compared to non-chewers and found that risk was three times per almost all sites and somewhat higher for cancer of the hypopharynx.

Chewing of tobacco especially in the form of pan, has been found to play an important role in the etiology of oral cancer in most part of central and South-East Asia. The risk of developing oral cancer increases with the frequency of chewing and is higher in prolonged chewers. (WHO Chronicle, 1983).

Chang (1966) working in Formosa showed that the continuous chewing of betel-nut with slaked lime even without chewing or smoking tobacco have a definite carcinogenic effect. Ranadive et al., (1976), confirmed the potent carcinogenecity of betel-nut particularly the tannin - containing polyphenolic fraction in Swiss mice and hamsters.

Leukoplakias were almost exclusively found among those who had tobacco habits. Tobacco chewing have been found to promote faster blood clotting, while the stillbirth rate for female tobacco chewers was found to be 50 per 1000 births as against 17.1 per 1000 in the case of nonchewers. (WHO Chronicle, 1985).

Sinha and Rao (1985) investigated the fetotoxic potential of betel-nut in Swiss albino rats. Total aqueous extracts of ripe betel-nuts of unprocessed and processed varieties were administered to pregnant animals. Hematomas, curved tails and a few incidence of rib anomalies were observed. The results indicated a delay in skeletal maturity particularly in those fetuses exposed prenatally to the betel-nut extract of the unprocessed variety, Rats given 15 per cent betel-nut meal showed enlargement of spleen and those given 20 per cent had catarrhal enteritis also. Besides enlargement of the spleen they had fatty changes in the liver.(Saikia and Vaidheki, 1983).

In the hypopharyngeal cancer cases studied by Baruah (1984). 51.1 per cent of females used to take betel-nut without tobacco and 48.8 per cent tobacco. In the laryngeal cancer cases all the female cases used betel-nut without tobacco.

In women between 18 and 45 years old using tobacco and oral contraceptives, triglycerides were significantly higher and high density lipoprotein cholesterol significantly lower than in controls. In women using both there was a cumulative effect on those values.

The salivary amylase was lower in tobacco chewers, this reduction was thought to be due to increased salivary flow with its dilutional effect, (Reddy, 1980). It was seen that

the polyphenolic fraction and tannic acid of betel-nut decreased protein content in almost all tissues, (Ranadive et al., 1976).

All the betel-nut constituteuts increased DNA synthesis and RNA ase activity in different tissues of male Swiss mice, (Shivapurkar et al., 1979). The Salivary potassium and sodium concentration were lower in betel-nut and tobacco chewers and these reduction were thought to be due to increased salivary flow with its dilutional effect, (Reddy et al., 1980).

Vasudevan et al., (1983) noticed a significant increase in the level of serum copper in the oral cancer patients who had the habit of tobacco chewing as compared to normals. It has been suggested that elevation in serum copper level in oral cancer is due to the destruction and necrosis of the tissues by chewing tobacco and leading to the release of copper present in the affected tissues into the circulation.

Experimental Procedure

III - EXPERIMENTAL PROCEDURE

The experimental procedure pertaining to the study of "Changes in blood constitutents due to smoking and chewing of betel-nuts and tobacco" are disscussed under the following headings:

I. Selection of subjects

II. Collection of blood samples
   a) Collection of blood by veinipuncture
   b) Separation of serum

III. Estimation of biochemical parameters in serum
   i) Total protein
   ii) Cholesterol
   iii) Triglycerides
   iv) Free fatty acids
   v) Lipase activity
   vi) Creatinine
   vii) Carboxyhaemoglobin (in blood)
   viii) Thiocyanate
   ix) Total and differential leukocyte count

I. SELECTION OF SUBJECTS

Twenty nine volunteers from rural areas were at random selected for the present study. Of them thirteen were smokers, eight betel-nut chewers and eight were betel-nut and tobacco chewers. They all belonged to the age group 20 to 60 years.

Seven nonsmokers and eight voluenteers who are not habitual chewers of either betel-nut and/or tobacco, matching in age and sex were chosen to serve as controls.

II. COLLECTION OF BLOOD

The blood was collected as follows (Oser, 1976). Tied a tourniquet (of soft rubber tubing or a strip of bandage) tightly around the arm of the patient, a couple of inches above the elbow. Made the subject clench his fist firmly, washed the skin surface about the prominant vein on the inner surface of the elbow to dry held the vein immobile by pressingon it with the thumb below the elbow and into the vein inserted a sharp, sterile hypodermic needle (No.20) an inch and a half long which was attached to a dry sterile syringe of suitable capacity. The needle should penetrate the vein from the side at an angle of 50° with the surface of the arm, the level or opening of the needle being kept upward or to the side. As soon as the blood was seen to enter the syringe, retracted the plunger slowly until the desired amount of blood had entered the syringe. Before removing the needle from the vein, loosened the tourniquet had the patient unclench his fist and on the skin, at the point of entrance of needle held in place a small pad of folded gauze moistened with 70 per cent alcohol. Withdrew the needle, detached it from the syringe (not too vigorously which might cause hemolysis) and then transferred the blood to a centrifuge tube.

About 8.0ml of blood was distributed into two tubes as follows:

1) 5.0ml of blood was used for serum separation in a clean non-oxalated tube.

2) The remaining blood was collected in another tube which contained Ammonium oxalate and Potassium oxalate in the ratio of 2:1 for the estimation of carboxyhaemoglobin.

SEPARATION OF SERUM (Tietz, 1976)

After the blood was drawn from patient by using a syringe, It was immediately transferred to a clean dry tube after the needle has been removed.. The blood was then allowed to clot for atleast 10-15 minutes at room temperature. The clot may adhere to the wall of the tube so that 'ringing'(making a gentle sweep around the inside walls of the tube with a wooden applicator stick) should be performed before centrifugation. Excessive ringing is unnecessary and can produce hemolysis. By allowing the clot to retract for a long period of time hemolysis is minimised and the yield of serum is greater. After the blood had clotted, the tube was centrifuged and the supernatant serum was removed.

III. ESTIMATION OF BIOCHEMICAL PARAMETERS

(i) ESTIMATION OF TOTAL PROTEINS

The serum total protein level was found to be decreased in smokers by Vijayalakshmi and Lakshmi (1985). Therefore serum total protein was estimated by Biuret method (Tietz, 1976). The details of which are presented in Appendix - I.

(ii) ESTIMATION OF SERUM CHOLESTEROL

Baldawa et al (1983) have reported an increase in serum cholesterol level in smokers. The serum cholesterol level was therefore estimated by Zak's method (Varley, 1975) in the selected subjects. The procedure for the same is given in Appendix - II.

(iii) ESTIMATION OF TRIGLYCERIDE

Ladd et al (1985) noticed an increase in serum triglyceride level in tobacco chewers. The serum triglyceride level was estimated by the of Chin et al (1971) method (Varley, 1980). The details of the method are given in Appendix - III.

(iv) ESTIMATION OF FREE FATTY ACIDS

Yoshida et al (1986) reported an increased level of free fatty acids in serum. The serum free fatty acids was estimated by the method using copper soap formation, (Falholt et al, 1973). The details of the method are given in Appendix - IV.

(v) ESTIMATION OF LIPASE ACTIVITY

Serum lipase activity was estimated by Tietz and Fiereck method, (Tietz, 1976).

(vi) ESTIMATION OF CREATININE

Decreased levels of serum creatinine was noticed in smokers by Savdie et al (1984). Serum total creatinine was estimated by alkaline picrate method, (Varley, 1975). The details of the method are given in Appendix - VI.

(vii) ESTIMATION OF BLOOD CARBOXYHAEMOGLOBIN

Blood carboxy haemoglobin was estimated by Whitehead and Worthington method (Varley, 1980). The details of the method are given in Appendix - VII.

(viii) ESTIMATION OF THIOCYANATE

Increased serum thiocyanate was found in smokers by Conn, (1985). Serum thiocyanate concentration was estimated Bowler's method (1957). The details of the method are given in Appendix - VIII.

(ix) ESTIMATION OF WBC COUNT

Conn, (1985) found an increase in white blood cell count of the smokers. The total count and differential count of the white blood cells were done by the method of Truck and Leishman's Stain method. The details of the methods are given in Appendix - IX.

Results and Discussion

RESULTS AND DISCUSSION

The study aimed at assessing the Bio-chemical changes in smoking, betel-nut chewing and betel-nut and tobacco chewing in 29 volunteers. All of them were from rural areas and their age range was 20 to 60 years. Age matched normal persons who were free from the habits of smoking, betel-nut chewing and betel-nut and tobacco chewing and any type of disease, from the same area served as the controls. All of them belonged to low socio-economic group. The results obtained in this study are discussed under the following headings.

I. Serum cholesterol, triglycerides, free fatty acid levels and lipase activity in smokers and the controls.

II. Serum creatinine and total protein levels in smokers and controls.

III. Blood carboxyhaemoglobin and serum thiocyanate levels in smokers and controls.

IV. Total and differential count of leukocytes in smokers and controls.

V. Serum cholesterol, triglycerides, free fatty acids and lipase activity in betel-nut chewers, betel-nut and tobacco chewers and controls.

VI. Serum creatinine and total protein levels in betel-nut chewers, betel-nut and tobacco chewers and controls.

VII. Blood carboxyhaemoglobin and serum thiocyanate levels in betel-nut chewers, betel-nut and tobacco chewers and controls.

VIII. Total and differential leukocyte count in betel-nut chewers betel-nut and tobacco chewers and controls.

IX. Serum cholesterol, triglycerides, free fatty acids, lipase activity, total protein and creatinine level in betel-nut chewers, betel-nut and tobacco chewers and smokers.

X. Blood carboxyhaemoglobin and thiocyanate level in serum of betel-nut chewers, betel-nut and tobacco chewers and smokers.

XI. Total and differential count of leukocytes in betel-nut chewers, betel-nut and tobacco chewers and smokers.

Table I presents the mean levels of serum cholesterol, triglycerides, free fatty acids and lipase activity in smokers and the controls and the individual values are given in Appendix - X.

TABLE -I

SERUM CHOLESTEROL, TRIGLYCERIDES, FREE FATTY ACIDS AND LIPASE ACTIVITY IN SMOKERS AND THE CONTROLS.

Parameters	Controls $n=7$ Mean $\pm$ S.D	Smokers N=13 Mean $\pm$ S.D	't' Value
Cholesterol (mg/100ml)	192.1 $\pm$ 15.5	240.5 $\pm$ 34.3	3.360**
Triglyceride (mg/100ml)	104.7 $\pm$ 15.0	146.3 $\pm$ 17.6	5.030**
Free fatty acids (m.mol/lit)	0.8 $\pm$ 0.2	1.1 $\pm$ 0.8	0.841 NS
Lipase activity (U/lit)	182.8 $\pm$ 22.9	261.5 $\pm$ 79.5	2.430**

NS - Non-significant.

** - $P < 0.01$.

As seen from table-I the mean serum cholesterol level of smokers and controls were 240.5 ± 34.3 and 192.1 ± 15.5mg per 100ml of serum respectively. The serum cholesterol level was found to be high in smokers compared to that of controls and this difference is statistically significant ($P < 0.01$). The normal level of cholesterol in serum is 140-250mg per 100ml (Varley et al., 1976). According to this standard the serum cholesterol level of the smokers was found to be closer to the upper limits of the normal range whereas that of the controls was found closer to the lower limit of the normal range. Increased level of cholesterol in smokers has also been reported by stampford, A (1984).

Mean triglyceride level in smokers was found to be 146.3 ± 17.6mg per 100ml and that of normals was 104.7 ± 15.00mg per 100ml. This difference in the serum triglyceride level of smokers and controls was found to be statistically significant ($P < 0.01$). This finding is in accordance with the work of Baldawa et al (1983) who showed a positive association between smoking and serum triglyceride values.

The serum free fatty acid level was found to be 1.1 ± 0.8 and 0.8 ± 0.2 m.mol per litre in smokers and controls respectively. The difference between the mean free fatty acid levels in serum of smokers and that of the controls was found to be not statistically significant. According to Varley et al (1980) the normal range of serum in free fatty acids is 0.1 to 0.9 m.mol/lit. Based on this criterion, the serum free fatty acids level of the smokers was found to be above the normal range while that of the controls selected for comparision was within the normal range.

Poznæret al (1982) have also reported an increased level of free fatty acids in smokers.

The mean lipase activity in serum of the smokers was found to be 261.5 ± 79.5 U/litre against the value of 182.8 ± 22.9 U/litre in the controls. Increased lipase activity was seen in the smokers compared to that of the controls and was found to be statistically significant. ($P < 0.01$). This is in accordance with the findings of Brunzell et al (1986) who also have reported significant rise in serum lipase activity in smokers.

Thus the serum cholesterol, triglycerides, free fatty acids and lipase activity were found to be elevated in smokers when compared to those of the controls.

Table-II presents the mean total protein and creatinine levels of smokers and controls and the individual values are given in Appendix -X.

TABLE - II

SERUM CREATININE AND TOTAL PROTEIN LEVELS OF CONTROLS AND SMOKERS

Parameters	Controls n=7 Mean ± S.D	Smokers n=13 Mean ± S.D	't' value
Creatinine (mg per 100ml)	1.0 ± 0.4	0.55 ± 0.2	3.198**
Total protein (g/100ml)	7.8 ± 0.3	7.00 ± 0.4	4.400**

** $P < 0.01$

The mean total protein level in serum of smokers was found to be 7.0 ± 0.4 g per 100 ml while it was 7.8 ± 0.3 g per 100ml in controls. Smoking was found to decrease the serum total protein level and the difference is statistically significant ($P<0.01$). Vijayalakshmi and lakshmi (1985) also have reported a low level of total protein in smokers.

Mean level of serum creatinine in smokers and controls was found to be 1.0 ± 0.4 mg/100ml and 0.55 ± 0.2 mg/100ml and the difference is statistically significant ($P<0.01$). Both creatinine and total protein values were found to be low in smokers compared to those found in controls.

Carboxyhaemoglobin and serum thiocyanate levels in smokers and controls are given in Table III and the individual values are given in Appendix - X.

TABLE - III

CARBOXY HAEMOGLOBIN AND THIOCYANATE LEVEL IN CONTROLS AND SMOKERS.

Parameter	Controls n=7 Mean ± S.D	Smokers n=13 Mean ± S.D	't' values
Carboxyhaemoglobin (%)	1.2 ± 0.3	2.3 ± 0.5	4.739**
Thiocyanate mg/100ml	2.0 ± 0.2	3.5 ± 0.6	5.830**

** - $P<0.01$.

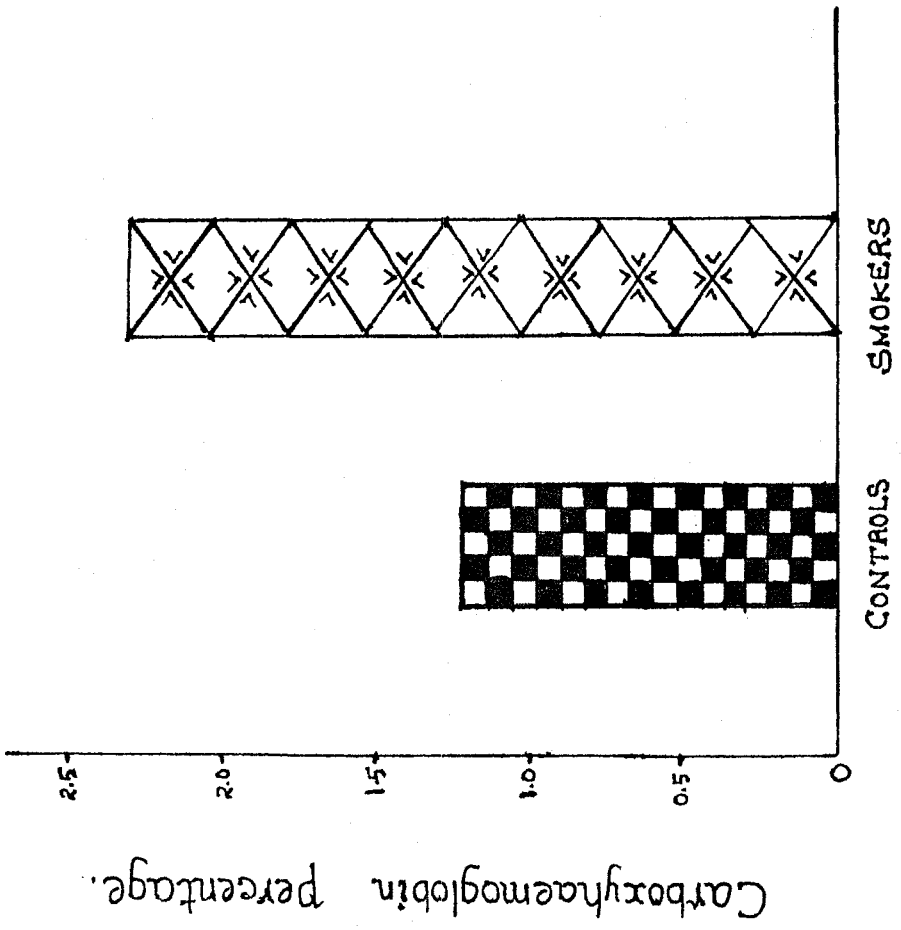


FIG-1. BLOOD CARBOXYHAEMOGLOBIN LEVEL IN CONTROLS AND SMOKERS

As revealed in table-III, the mean carboxyhaemoglobin level in smokers was 2.3 ± 0.5 percent while that of the controls was found to be 1.2 ± 0.3 per cent. The results showed that in smokers a singificantly ($P<0.01$) high level of carboxyhaemoglobin was found compared to that of the controls. Conn (1985) has also reported an increase in blood carboxyhaemoglobin level in smokers. The normal carboxyhaemoglobin level in blood as per Varley et al (1980) is less than 1.5 per cent.

The serum thiocyanate level in smokers and controls were found to be 3.5 ± 0.6mg per 100 ml and 2.0 ± 0.2,g per 100ml respectively. The difference in the level of serum thiocyanate is statistically significant, ($P<0.01$). Elevated serum thiocyanate in smokers was also noticed by shivapurkar et al ,(1980).

Table IV shows the total and differential count of leukocytes in controls and smokers and the individual values are given in Appendix - XI.

TABLE - IV

TOTAL AND DIFFERENTIAL COUNT OF LEUKOCYTES IN CONTROLS AND SMOKERS

Parameter	Controls n=7 Mean ± S.D	Smokers n=13 Mean ± S.D	't'value
Total count per cu.mm	7040 ± 478.7	7768 ± 942.0	2.260*
Eosinophils per cent	2.5 ± 0.9	2.4 ± 1.7	0.254NS
Lymphocytes per cent	26.3 ± 4.4	22.7 ± 4.5	0.250NS
Monocytes per cent	1.4 ± 0.6	1.0 ± 0.9	0.091NS
Polymorphs per cent	69.7 ± 4.8	74.9 ± 4.7	2.213*

* - $P<0.05$.

As seen from Table IV the mean total leukocyte counts in smokers and controls were found to be 7768 ± 942.0/cu.mm and 7040 ± 478.7/cu.mm respectively. Total leukocyte count is elevated in smokers compared to controls and is statistically significant, ($P<0.05$).

The eosinophil count in smokers was found to be 2.4 ± 1.7 per cent and that of controls was 2.5 ± 0.9 per cent. The normal eosinophil count is 0.4 per cent. The eosinophil count in smokers was also within the normal range indicating that smoking has not affected the eosinophil count of the blood in the smokers chosen for the study.

The mean value of lymphocytes in smokers and controls was found to be 22.7 ± 4.5 per cent and 26.3 ± 4.4 per cent respectively and the difference is not statistically significant.

The monocyte count was found to be 1.4 ± o.6 per cent and 1.0 ± 0.9 per cent in controls and smokers respectively, and smoking has not affected the monocyte count in the smokers chosen for the present study.

As given in the above table the mean level of polymorphs in controls and smokers was found to be 69.7 ± 4.8 per cent and 74.9 ± 4.7 per cent respectively. Polymorphs count was found to be high in smokers and the difference is statistically significant ($P<0.05$).

The elevation in the total leukocyte count of the smokers was found to be due to the elevated polymorphs. With regard to lymphocytes, eosinophils and monocytes statistically significant

difference was not observed between the controls and smokers

Table-V shows the mean cholesterol, triglycerides, free fatty acid levels and lipase activity in controls, betel-nut chewers and betel-nut and tobacco chewers and the individual values are given in Appendix - X.

TABLE-V

SERUM CHOLESTEROL, TRIGLYCERIDES, FREE FATTY ACIDS AND LIPASE ACTIVITY IN CONTROLS, BETEL-NUT CHEWERS AND BETEL-NUT AND TOBACCO CHEWERS

Parameters	Controls (n=8) Mean ± S.D	betel-nut chewers (n=8) Mean ± S.D	betel-nut and tobacco chewers (n=8) Mean ± S.D	Groups compared	't' value
Cholesterol (mg/100ml)	188.1 ± 16.5	204.3 ± 21.6	228.1 ± 1.2	1 Vs 2	1.580NS
				1 Vs 3	6.397**
Triglycerides (mg/100ml)	105.8 ± 15.5	130.7 ± 12.4	138.2 ± 185.0	1 Vs 2	2.397*
				1 Vs 3	3.004**
Free fatty acids (m.mol/lit)	0.9 ± 0.2	1.1 ± 0.2	1.2 ± 0.3	1 Vs 2	1.620NS
				1 Vs 3	2.000NS
Lipase activity (U/lit)	160.0 ± 29.2	195 ± 8.0	406.3 ± 117.8	1 Vs 2	3.050**
				1 Vs 3	5.068**
	1	2	3		

NS - NOT SIGNIFICANT

* - $P < 0.05$

** - $P < 0.01$

The mean cholesterol level in controls and betel-nut chewers were 188.1 ± 16.5mg/100ml and 204.3 ± 21.6mg per 100ml respectively. The serum cholesterol level of betel-nut and tobacco chewers was found to be 228.1 ± 1.2mg per 100ml. Though the serum cholesterol level was found to he higher in betel-nut chewers and betel-nut and tobacco chewers than that found in controls it is well within the normal range as per the standard of Varley et al (1980).

Mean triglyceride level in controls, betel-nut chewers and betel-nut and tobacco chewers were 105.8 ± 15.5, 130.7 + 12.4 and 138.2 ± 185.0mg per 100ml respectively. The results indicate a significant increase in the serum triglyceride level of betel-nut chewers and betel-nut and tobacco chewers when compared to that of controls, ($P < 0.05$ and $P < 0.01$ respectively). But when compared with the normal range suggested by Varley et al (1980) the triglyceride levels in the above cases is within the normal range only. An elevated triglyceride concentration in betel-nut chewers was reported by Archer (1985) also.

The above table shows the mean level of free fatty acids of controls, betel-nut chewers and betel-nut and tobacco chewers as 0.9 ± 0.2, 1.1 ± 0.2 and 1.1 ± 0.3m.mol/lit respectively. Betel-nut and tobacco chewers have slightly elevated serum free fatty acids and the difference is statistically insignificant. When the mean level of free fatty acids in control and betel-nut chewers were compared, the difference is statistically insignificant. The serum considered was found to be higher than the normal range 0.1 to 0.9m.mol/lit suggested by Varley et al (1980).

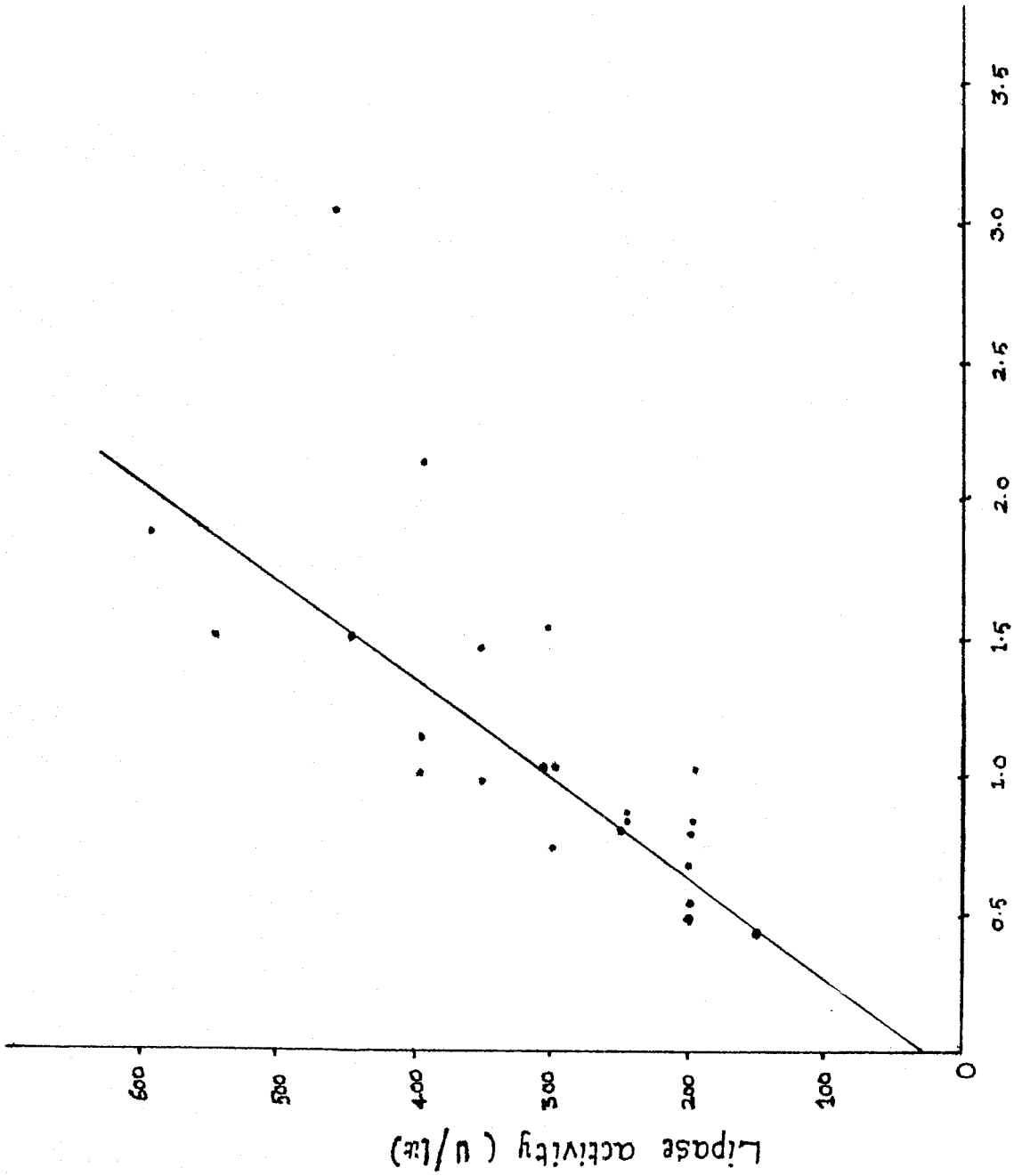


FIG-2. CORRELATION BETWEEN FREE FATTY ACIDS AND LIPASE ACTIVITY IN SMOKERS AND BETEL-NUT AND TOBACCO CHEWERS

Lipase activity in serum of betel-nut chewers, betel-nut and tobacco chewers and controls was found to be 160 ± 29.2U/lit, 195 ± 8.0U/lit and 406.3 + 117.8U/lit respectively. Betel-nut chewers and betel-nut and tobacco chewers have elevated mean lipase activity in serum compared to controls and the difference is statistically significant ($P < 0.01$). The normal serum lipase activity in Cherry Crandall units is 20-270U/lit (Tietz, 1976). Betel-nut chewers were found to have serum lipase activity within the normal limits and it was highly elevated in betel-nut and tobacco chewers when compared with controls and the normal values.

A positive correlation (+0.691) was found between the serum free fatty acid and lipase activity of the selected subjects indicating that the elevation in serum free fatty acid might be due to the increased lipase activity. Figure- I illustates the correlation between the serum free fatty acid and lipase activity in smokers and betel-nut and tobacco chewers.

Serum creatinine and total protein levels in controls, betel-nut chewers and betel-nut and tobacco chewers are shown in Table-VI. The individual values are given in Appendix-X.

TABLE - VI

SERUM CREATININE AND TOTAL PROTEIN LEVELS IN CONTROLS, BETEL NUT CHEWERS AND BETEL NUT AND TOBACCO CHEWERS

Parameters	Controls (n=8) Mean ± S.D	betel-nut chewers (n=8) Mean ± S.D	betel-nut and tobacco chewers (n=8) Mean ± S.D	Groups compared	't' value
Creatinine mg/100ml	0.8 ± 0.4	0.7 ± 0.3	0.7 ± 0.2	1 Vs 2	1.628NS
				1 Vs 3	0.815NS
Total protein g/100ml	7.5 ± 0.5	6.5 ± 0.3	6.1 ± 0.3	1 Vs 2	4.388**
				1 Vs3	5.869**
	1	2	3		

NS - NOT SIGNIFICANT

** - $P < 0.01$

As seen from table-VI the serum creatinine in controls, betel-nut chewers and betel-nut and tobacco chewers was found to be 0.8 ± 0.4, 0.7 ± 0.3 and 0.7 ± 0.2mg per 100ml. The difference in serum creatinine between controls and the two groups of chewers is not found to be statistically significant.

The serum total protein value was 7.5 ± 0.5, 6.5 ± 0.3 and 6.1 ± 0.3g per 100ml in controls, betel-nut chewers and betel-nut and tobacco-chewers respectively. Both betel-nut chewers and betel-nut and tobacco chewers have less serum total protein than that found in controls and the differences in the mean level of serum total protein between betel nut chewers and controls and that between controls and betel-nut and tobacco chewers was found to be statistically significant ($P < 0.01$). According to Varley et al (1980) the normal range of serum total protein is 6.0 to 8.0g per 100ml. The total protein in serum of both betel-nut chewers and betel-nut and tobacco chewers was found to be within the normal range indicating that betel-nut chewing or betel-nut and tobacco chewing has not affected the serum total protein content of the volunteers selected for the study.

The normal range of serum creatinine is 0.2 to 0.9mg per 100ml (Tietz 1976). As per this standard, the serum creatinine level was found to be well within the normal range even in the two classes of chewers investigated.

Table-VII gives the mean levels of blood carboxyhaemoglobin and serum thiocynate in controls, betel-nut chewers and betel-nut and tobacco chewers, and individual values are given in Appendix-X.

TABLE - VII

CARBOXYHAEMOGLOBIN AND THIOCYANATE LEVELS IN CONTROLS, BETEL-NUT CHEWERS AND BETEL-NUT AND TOBACCO CHEWERS

Persons	Carboxy haemoglobin Mean ± S.D	Thiocyanate mg/100ml Mean ± S.D	Groups compared	't'values
Controls-1 (n=8)	1.2 ± 0.3	2.1 ± 0.1	1 Vs 2	2.064NS
			1 Vs 3	1.640NS
Betel-nut chewers-2 (n=8)	1.5 ± 0.3	2.3 ±0.3	1 Vs 2	1.623NS
			1 Vs 3	5.875**
Betel-nut and tobacco chewers-3 (n=8)	1.4 ± 0.3	2.9 ± 0.3	1 Vs 2	1.623NS
			2 Vs 3	5.875**

NS - NOT SIGNIFICANT

** - $P < 0.01$

The mean values of carboxyhaemoglobin in controls, betel-nut chewers and betel-nut and tobacco chewers was 1.2 ± 0.3, 1.5 ± 0.3 and 1.4 + 0.3 per cent respectively. Difference in the carboxyhaemoglobin level of blood in betel-nut chewers and betel-nut and tobacco chewers was found to be statistically insignificant when compared to that of controls. The normal level of blood carboxyhaemoglobin is less than 1.5 per cent, (Varley,1980). Betel-nut chewing and betel-nut and tobacco chewing does not found to increase the blood carboxyhaemoglobin level.

Controls, betel-nut chewers and betel-nut and tobacco chewers showed 2.1 ± 0.1, 2.3 ± 0.3 and 2.9 ± 0.3 mg of thiocynate per 100ml of serum respectively. Mean thiocyanate level in the betel-nut chewers was slightly more than that in controls; but the difference was not statistically significant, Betel-nut and tobacco chewers showed a statistically significant ($P < 0.01$) high concentration of thiocyanate when compared to the controls.

Table-VIII shows the mean value of leukocyte counts in controls, betel-nut chewers and betel-nut and tobacco chewers and individual values are given in Appendix-XI.

TABLE - VIII

TOTAL AND DIFFERENTIAL COUNT OF LEUKOCYTES IN CONTROLS, BETEL-NUT CHEWERS AND BETEL NUT AND TOBACCO CHEWERS

Parameters	Controls (n=8) Mean±S.D	Betel-nut chewers (n=8) Mean ± S.D	Betel-nut and tobacco chewers Mean ± S.D	Groups comapred	't' values
Total count per cu.mm	7237.500±184.0	7706.2±407.0	7905±212.6	1 Vs 2	2.8**
				1 Vs 3	2.5*
Eosinophils per cent	4.875± 1.3	6.0±0.9	6.8± 1.6	1 Vs 2	1.5NS
				1 Vs 3	2.9**
Lymphocytes per cent	25.120± 1.6	23.0±5.2	22.3± 3.8	1 Vs 2	1.1NS
				1 Vs 3	1.9NS
Monocytes per cent	1.425±0.0.6	1.0±0.2	1.3± 0.8	1 Vs 2	1.7NS
				1 Vs 3	0.4NS
Polymorphs per cent	67.600± 1.9	69.0±3.8	70.6± 5.3	1 Vs 2	1.1NS
				1 Vs 3	1.4NS
	1	2	3		

NS - NOT SIGNIFICANT

* - $P < 0.05$

** - $P < 0.01$

The total leukocyte count in controls betel-nut chewers and betel-nut and tobacco chewers was 7237.5 ± 184, 7706.2 ± 407, and 7905 + 212.6/cu.mm respectively. Mean level of leukocytes count was found to be high in betel-nut and tobacco chewers compared to that of controls and the difference was found to be significant statistically ($P < 0.05$). Betel-nut chewers have raised leukocyte count compared to controls and the difference was found to be statistically significant ($P < 0.01$).

Mean level of eosinophils in controls, betel-nut chewers and betel-nut and tobacco chewers were 4.875 ± 1.3 per cent, 6.0 ± 0.9 per cent, and 6.8 ± 1.6 per cent respectively, Betel-nut chewers and betel-nut and tobacco chewers have higher eosinophil count compared to controls. The difference in the mean value of eosinophil in betel-nut chewers and controls was statistically significant ($P < 0.01$). Betel-nut chewing was found to increase the eosinophil count in the betel-nut chewers chosen for the present study.

The mean level of monocytes in controls, betel nut chewers and betel-nut and tobacco chewers was 1.425 ± 0.6, 1.0 ± 0.2 and 1.3 ± 0.8 per cent respectively and there was no significant change in the monocyte count due to chewing of betel-nuts with or without tobacco.

There was no significant change in the mean polymorphs level in controls, betel-nut chewers and betel-nut and tobacco chewers. The mean value of polymorph was found to be 67.6 ± 1.9, 69.0 ± 6.2 and 70.6 ± 5.3 respectively in the above mentioned group.

It is clear from Table-VIII that there is no significant difference in the differential leukocytes (other than eosinophils) between the controls and the two categories of chewers in question.

Table-IX gives the mean serum cholesterol, triglycerides free fatty acids, lipase activity, total protein and creatinine in smokers, betel-nut chewers and betel-nut and tobacco chewers. Figure-2 illustrates the mean serum cholesterol triglyceride and free fatty acid levels in controls, smokers, betel-nut chewers and betel-nut and tobacco chewers.

TABLE - IX

SERUM CHOLESTEROL, TRIGLYCERIDE, FREE FATTY ACIDS, LIPASE ACTIVITY, TOTAL PROTEIN AND CREATININE IN SMOKERS BETEL NUT AND BETEL NUT AND TOBACCO CHEWERS

Parameters	Smokers (n=13) Mean ± S.D	Betel-nut chewers (n=8) Mean ± S.D	Betel-nut and tobacco chewers (n=8) Mean±S.D	Groups Compared	't' values
Cholesterol (mg/100ml)	240.5 ± 34.3	204.3 ± 21.6	228.1 ± 34.2	1 Vs 2 1 Vs 3 2 Vs 3	2.545* 0.219NS 1.600NS
Triglycerides (mg/100ml)	146.3 ± 17.6	130.7 ± 12.4	138.2 ± 18.5	1 Vs 2 1 Vs 3 2 Vs 3	2.080NS 1.546NS 0.181NS
Free fatty acids (m.mol/lit)	1.1 ± 0.8	1.1 ± 0.2	1.2 ± 0.3	1 Vs 2 1 Vs 3 2 Vs 3	0.035NS 0.108NS 0.857NS
Lipase activity (U/lit)	261.5 ± 79.5	195.0 ± 8.0	406.3 ±117.8	1 Vs 2 1 Vs 3 2 Vs 3	2.245* 3.198** 4.485**
Creatinine (mg/100ml)	0.6 ± 0.2	0.7 ± 0.3	0.7 ± 0.2	1 Vs 2 1 Vs 3 2 Vs 3	1.071NS 1.633NS 3.124**
Total Protein (g/100ml)	7.0 ± 0.4	6.5 ± 0.3	6.1 ± 0.3	1 Vs 2 1 Vs 3 2 Vs 3	2.930** 4.836** 1.940NS
	1	2	3		

NS - NOT SIGNIFICANT
* - $P < 0.05$
** - $P < 0.01$

As seen in table-IX the mean cholesterol level in smokers betel-nut chewers and betel-nut and tobacco chewers were 240.5 ± 34.3, 204.3 ± 21.6 and 228.1 ± 34.2mg/100ml respectively. Among the above three categories, smokers have increased level of cholesterol and the difference is statistically significant ($P < 0.05$). There is no statistically significant difference in the level of cholesterol in betel-nut chewers and betel-nut and tobacco chewers. The difference in the mean cholesterol level in smokers and betel-nut and tobacco chewers is also not statistically significant. The normal cholesterol level as stated by Varley (1980) is 140-250mg/100ml. Smokers, betel-nut chewers and betel-nut and tobacco chewers have the cholesterol level was in the normal range only. But in smokers the cholesterol level is in the extremity of the range.

The mean triglyceride level in smokers is as 146.3 ± 17.6 compared to 130.7 ± 12.4mg/100ml and 138.2 ± 18.5mg/100ml in betel-nut chewers and betel-nut and tobacco chewers. Smokers ware found to have slightly elevated serum triglyceride level compared to the other two and the differences were not statistically significant. Smokers, betel-nut chewers and betel-nut tobacco chewers all found to have the serum triglyceride level within the normal range suggested by Varley (1980).

The results indicated the mean level of free fatty acid levels in smokers, betel-nut chewers and betel-nut and tobacco chewers as 1.1 ± 0.8m.mol/lit, 1.1 ± 0.2m.mol/lit and 1.2 ± 0.3 m.mol/lit respectively. Betel-nut chewer and betel-nut and tobacco chewere have slightly increased serum free fatty acids

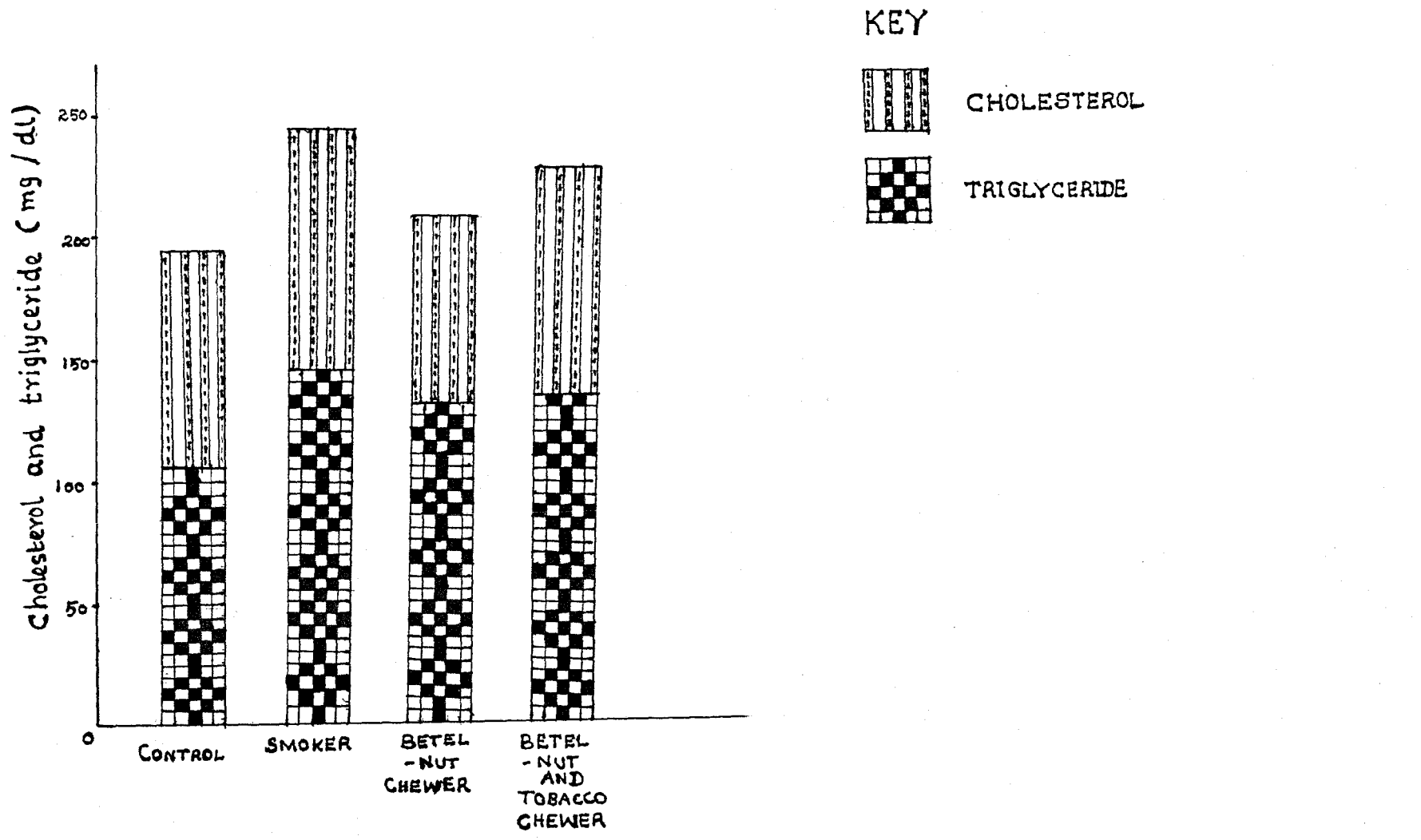


FIG 3 CHOLESTEROL AND TRIGLYCERIDE LEVELS IN CONTROLS, SMOKERS, BETEL-NUT CHEWERS AND BETEL-NUT AND TOBACCO CHEWERS

compared to smokers but the differences are not statistically significant. Among betel-nut chewers and betel-nut and tobacco chewer, the latter has increased serum free fatty acids compared to the former but the difference is statistically insignificant. The normal level of serum free fatty acids as represented by Varley (1975) is 0.1 to 0.9m.mol/lit.

As represented in the Table-IX the mean serum level of lipase activity in smokers is 261.5 ± 79.5U/lit and that of betel-nut chewer and betel-nut and tobacco chewer are 195 ± 8U/lit and 406.3 ± 117.8U/lit respectively. Smokers showed increase in the level of lipase activity compared to betel-nut chewers and the difference is statistically significant ($P < 0.05$). When the lipase acitivity in the betel-nut chewers and betel-nut and tobacco chewers are compared the difference in their value was found to be statistically significant ($P < 0.01$). Betel-nut and tobacco chewers have highly elevated lipase activity in serum than smokers and the difference is statistically significant at one per cent level. Smokers have the serum lipase activity in the upper limit of the normal range. i.e., 20-270U/lit as formulated by Tietz (1976). In betel-nut chewers and betel-nut and tobacco chewers the serum lipase activity is elevated compared to controls and the differences are statistically significant ($P < 0.01$).

The mean level of creatinine in smokers betel-nut chewers and betel-nut and tobacco chewers were 0.6±0.2, 0.7±0.3 and 0.7±0.2mg/100ml respectively. No statistically significant decrease in the serum creatinine concentration in the betel-nut

chewers and betel-nut and tobacco chewers compared to smokers. The difference in the mean level of serum creatinine in betel-nut chewers and betel-nut and tobacco chewers is statistically significant ($P < 0.01$).

The mean level of serum total protein in smokers, betel-nut chewers and betel-nut and tobacco chewers were 7 ± 0.4, 6.5 ± 0.3 and 6.1 ± 0.3g per 100ml respectively. Mean value of total protein in betel-nut and tobacco chewer is less than found in the other two groups. Between the betel-nut chewer and betel-nut and tobacco chewers the difference in serum total protein is significant statistically ($P < 0.01$).

Carboxyhaemoglobin and thiocyanate levels in smokers, betel-nut chewers and betel-nut and tobacco chewers are given in Table-X, and figure-3 represents the same.

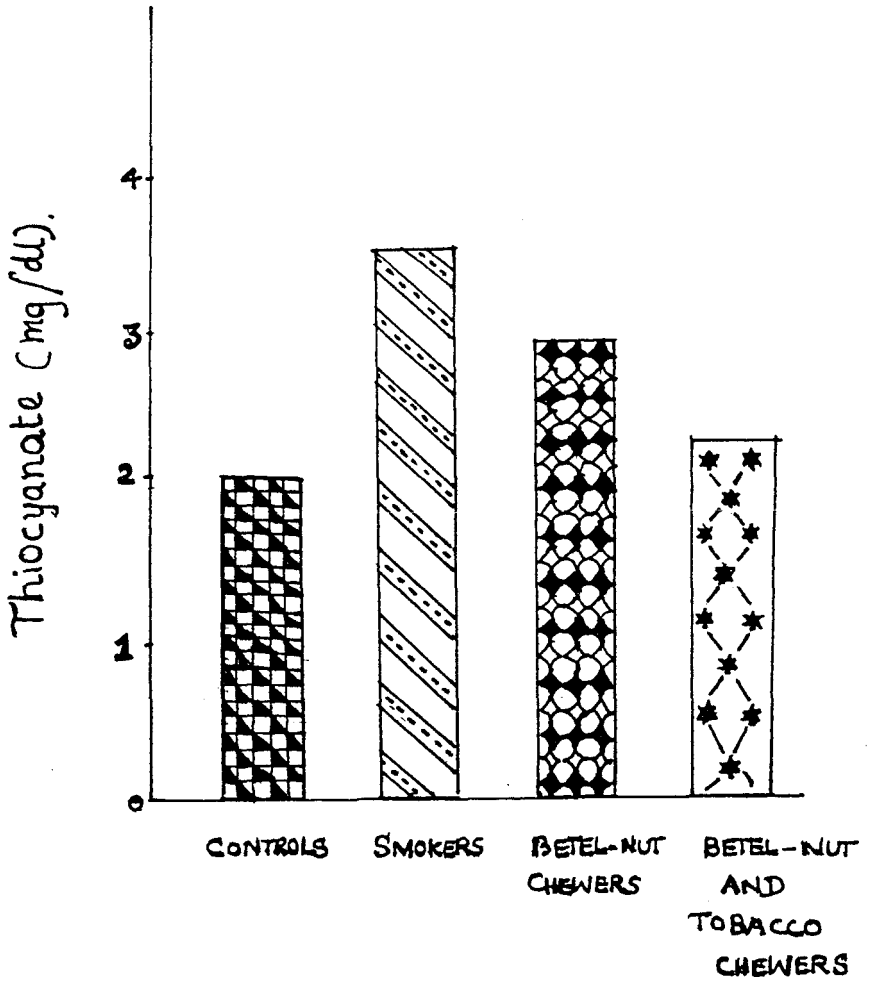
TABLE - X

CARBOXYHAEMOGLOBIN AND THIOCYANATE LEVEL IN SMOKERS BETEL NUT CHEWERS AND BETEL NUT AND TOBACCO CHEWERS

Parameter	Smokers (n=13) Mean ± S.D	Betel-nut chewers (n=8) Mean ± S.D	Betel-nut and tobacco chewers (n=8) Mean ± s.D	Groups compared	't' values
Carboxyhaemoglobin (%)	2.3 ± 0.5	1.5 ± 0.3	1.4 ± 0.3	1 Vs 2	3.455**
				1 Vs 3	3.943**
				2 Vs 3	0.409NS
Thiocyanate (mg/100ml)	3.6 ± 0.6	2.3 ± 0.3	2.9 ± 0.4	1 Vs 2	4.950**
				1 Vs 3	2.346**
				2 Vs 3	3.630**
	1	2	3		

NS - NOT SIGNIFICANT

** - $P < 0.01$



FIG·4. SERUM THIOCYANATE LEVEL IN CONTROLS, SMOKERS, BETEL-NUT CHEWERS AND BETEL-NUT AND TOBACCO CHEWERS

From the above table it is clear that the mean value of carboxyhaemoglobin in smokers (2.3 ± 0.5 g/100ml) was higher than that found in the two types of chewers chosen for the study. This differences was statistically significant ($P < 0.01$) indicating that while smoking increases the carboxyhaemoglobin level. Chewing of betel-nut with or without tobacco, does not increase the carboxyhaemoglobin level.

The mean serum thiocyanate level in smokers, betel-nut chewers and betel-nut and tobacco chewers was found to be 3.6 ± 0.6, 2.3 ± 0.3 and 2.9 ± 0.4mg/100ml respectively. Smokers have a highly elevated thiocyanate level compared to the other two groups and the difference in the values was significant statistically ($P < 0.01$). Amoung the two types of chewers, the chewers of betel-nut and tobacco showed a significantly elevated level of thiocyanate ($P < 0.01$). The mean carboxyhaemoglobin in controls and smokers is illustrated in figure - 3. Figure - 4 represents the mean thiocyanate level in smokers, betel-nut chewers and betel-nut and tobacco chewers.

Total and differential count of leukocytes in smokers, betel-nut chewers and betel-nut and tobacco chewers are given in Table-XI and the individual values are given in Appendix-XI.

TABLE - XI

TOTAL AND DIFFERENTIAL COUNT OF LEUKOCYTES IN SMOKERS, BETEL-NUT CHEWERS AND BETEL-NUT AND TOBACCO CHEWERS

Parameter	Smokers (n=13) Mean ± S.D	Betel-nut chewer (n=8) Mean ± S.D	Betel-nut and tobacco chewer (n=8) Mean ± S.D	Groups compared	't' values
Total count (cu.mm)	7868 ± 942	7706.0 ± 407.4	7905 ± 212.60	1 Vs 2 1 Vs 3 2 Vs 3	0.302NS 0.098NS 1.147NS
Eosinophil (per cent)	2.38 ± 1.78	6.0 ± 0.9	6.75 ± 1.28	1 Vs 2 1 Vs 3 2 Vs 3	5.028** 5.961** 1.302NS
Monocytes (per cent)	1.04 ± 0.96	1.0 ± 0.2	1.25 ± 0.84	1 Vs 2 1 Vs 3 2 Vs 3	0.110NS 0.500NS 0.867NS
Lymphocytes (per cent)	22.70 ± 4.50	23.0 ± 5.2	22.25 ± 3.50	1 Vs 2 1 Vs 3 2 Vs 3	0.741NS 0.063NS 0.460NS
Polymorphs (per cent)	74.98 ± 4.70	69.0 ± 3.8	70.60 ± 5.30	1 Vs 2 1 Vs 3 2 Vs 3	3.074** 1.905NS 0.453NS
	1	2	3		

NS - NOT SIGNIFICANT

** - P<0.01

There was no significant difference noticed in the total and differential leukocyte count of smokers, betel-nut chewers and betel-nut and tobacco chewers. But the difference in the eosinophil count was found to be statistically significant ($P < 0.01$). It was elevated in both betel-nut chewers and betel-nut and tobacco chewers. This showed that chewing betel-nut with or without tobacco elevates the eosinophil count somehow.

The results of this study revealed that smoking is more harmful than betel-nut and betel-nut and tobacco chewing.

Summary and Conclusion

V - SUMMARY AND CONCLUSION

The present study was undertaken to find out the "Changes in blood constituents due to smoking and chewing of betel-nuts and tobacco". The results of the study are summarised below:

Twenty nine persons participated in this study who were grouped as smokers, betel-nut chewers and betel-nut and tobacco chewers. Seven men served as controls for smokers and four normal men and four normal women served as controls for the two types of chewers. Their blood and serum were analysed for biochemical parameters along with total and differtial leukocytecounts. The estimated parameters were serum total cholesterol, triglycerides, lipase activity, free fatty acids, total protein, creatinine, Carboxyhaemoglobin, thiocyanate and total and differntial count of leukocytes.

Smoking

Smokers were found to have increased serum cholesterol. The serum total cholesterol in smokers was 240.5 ± 34.3 mg per 100 ml against the level of 192.1 ± 15.5 mg per 100 ml in controls and the difference is statistically significant ($P<0.01$). The serum cholesterol level of smokers was found to be closer to the upper limits of the normal range whereas that of the controls was found closer to the lower limit of the normal range. This increase in serum cholesterol may be due to the Carboxyhaemoglobin present in the smoke which enhances the lipid ladden cells.

Mean triglyceride level in smokers was 146.3 ± 17.6 and that of controls was 104.7 ± 15 mg per 100 ml. This difference in the serum triglyceride level of smokers and controls was found to be statistically significant. ($P < 0.01$).

The serum free fatty acid level was found to be 1.065 ± 0.75 and 0.81 ± 0.19 m.mol/litre in smokers and controls respectively and this difference is not statistically significant. This slightly raised free fatty acids level in smokers may be due to the increased lipase activity in the smokers.

The mean lipase activity in the serum of the smokers was found to be 261.5 ± 79.5 U/lit against the value of 182.8 ±22.9 U/lit in the controls. Increased lipase activity was seen in the smokers compared to that of controls and was found to be statistically significant ($P<0.01$).

Total protein level in the serum of smokers was found to be 7.0 ± 0.426 g per 100 ml while it was 7.8 ± 0.3 g per 100 ml in controls. Smoking was found to decrease the serum total protein level and the difference was statistically significant ($P<0.01$).

Controls and smokers had the mean level of serum creatinine as 1.0 ± 0.37 mg per 100 ml and 0.55 ± 0.23 mg/100 ml respecively and the difference was statistically significant, ($P<0.01$).

The mean carboxyhaemoglobin level in smokers was 2.29 ± 0.54 percent while that of the controls was found to be 1.2 ± 0.28 per cent. The results showed that in smokers a significantly ($P<0.01$) high level of carboxyhaemoglobin was found compared to that of controls.

The serum thiocyanate level in smokers and controls were 3.5 ± 0.64 and 2.02 ± 0.22 mg per 100 ml respectively. This difference in the level of serum thiocyanate was statistically significant, ($P<0.01$).

The mean total leukocyte count in smokers and controls were found to be 7768 ± 942/cu.mm and 7040 ± 478.7/cu.mm respectively. Total leukocyte count was slightly elevated in smokers compared to controls and was statistically significant ($P<0.05$).

The eosinophil count in smokers was found to be within the normal range indicating that smoking has not affected the eosinophil count of the blood in the smokers chosen for the study, the eosinophil count of the smokers was 2.38 ± 1.7 per cent and that of controls was 2.57 ± 0.98 per cent. The mean value of lymphocytes in smokers and controls was found to be 22.7 ± 4.5 per cent and 26.29 ± 4.39 per cent respectively and the difference was not statistically significant.

The monocyte count was found to be 1.4 ± 0.55 and 1.04 ± 0.96 per cent respectively in controls and smokers and the difference was not statistically significant.

Mean level polymorphs was found to be raised in smokers compared to controls. The mean level of polymorphs was 69.7 ± 4.8 per cent in controls and 74.98 ± 4.74 per cent in smokers. Thus the slight elevation in the total leukocyte count of the smokers was found to be due to elevated polymorphs.

BETEL-NUT CHEWING AND BETEL-NUT AND TOBACCO CHEWING

Mean total cholesterol level in controls, betel-nut chewers and betel-nut and tobacco chewers were 188.12 ± 164.6 mg/100 ml, 204.3 ± 21.6 mg per 100 ml and 228.12 ± 1.2 mg per 100 ml respectively. Though the serum cholestrol level was found to be higher in betel-nut chewers and betel-nut and tobacco chewers than that found in controls it was well within the normal range as per the standard of Varley et al, (1980).

Controls, betel-nut chewers and betel-nut and tobacco chewers had the mean level of serum triglycerides as 105.8 ± 15.45, 130.7 ± 12.4 and 138.2 ± 18.5 mg per 100 ml respectively. The results indicated a significant increase in the serum triglyceride level of betel-nut chewers and betel-nut and tobacco chewers when compared to that of controls ($P<0.05$ and $P<0.01$ respectively).

The mean level of free fatty acids in controls, betel-nut chewers and betel-nut and tobacco chewers were 0.91 ± 0.18, 1.075 ± 0.2 and 1.195 ± 0.33 m.mol/lit respectively. Betel-nut and tobacco chewers had slightly elevated serum free fatty acids and the difference was not statistically significant. When the mean level of free fatty acids in controls and betel-nut chewers are compared the difference was not statistically significant. The serum free fatty acid level of the two categories of chewers considered was found to be higher than the normal range 0.1 to 0.9 m.mol/litre suggested by Varley et al, (1980).

Lipase activity in serum of betel-nut chewers, betel-nut and tobacco chewers and controls was found to be 160 ± 29.2 U/lit,

195 ± 8 U/lit and 406.25 ± 117.8 U/lit respectively. Betel-nut chewers and betel-nut and tobacco chewers had elevated mean lipase activity in serum compared to controls and the difference was statistically significant, ($P < 0.01$). The normal serum lipase activity in Cherry-Crandall units is 20-270 U/lit (Tietz, 1976). Betel-nut chewers were found to have serum lipase activity within the normal limits and was highly elevated in betel-nut and tobacco chewers when compared with controls and normal values. A positive correlation (+0.691) was found between the serum free fatty acids and lipase activity of the selected subjects indcating that the elevation in serum free fatty acids might be due to the increased lipase activity.

The serum creatinine in controls, betel-nut chewers and betel-nut and tobacco chewers was found to be 0.848 ± 0.39, 0.67 ± 0.25 and 0.715 ± 0.19 mg per 100 ml. The difference in the serum creatinine between controls and the two groups of chewers was not found to be statistically significant.

The serum total protein value was 7.5 ± 0.55, 6.46 ± 0.34 and 6.11 ± 0.34 g per 100 ml in controls, betel-nut chewers and betel-nut and tobacco chewers respectively. Both betel-nut chewers and betel-nut and tobacco chewers had less serum total protein than that found in control and the difference was statistically significant, ($P < 0.01$). Both betel-nut chewers and betel-nut and, tobacco chewers were found to have normal total protein in serum indicating that betel-nut chewing or betel-nut and tobacco chewing did not affect the serum total protein.

Mean level of carboxyhaemoglobin and thiocyanate in controls, betel-nut chewers and betel-nut and tobacco chewers was found to be 1.21 ± 0.26, 1.53 ± 0.32 and 1.44 ± 0.44 per cent respectively. There was no statistically significant difference in the carboxyhaemoglobin level of blood in betel-nut chewers and betel-nut and tobacco chewers, compared to that of controls. The normal level of blood carboxyhaemoglobin is less than 1.5 per cent (Varley, 1980). Betel-nut chewing and betel-nut and tobacco chewing did not found to increase the blood carboxyhaemoglobin level.

Controls, betel-nut chewers and betel-nut and tobacco chewers had the mean level of serum thiocyanate as 2.12 ± 0.09, 2.31 ± 0.298 and 2.94 ± 0.36 mg per 100 ml respectively. Mean thiocyanate level in the betel-nut chewers was slightly more than that in controls but the difference was not statistically significant. Betel-nut and tobacco chewers showed statistically significant ($P<0.01$) high level of thiocyanate when compared to the controls.

The total leukocyte count in controls, betel-nut chewers and betel-nut and tobacco chewers were 7237.5 ± 184, 7706.2 ± 407, and 7905 ± 212.6/cu.mm respectively. Total leukocyte count was increased in betel-nut and tobacco chewers compared to normals and the difference was statistically significant ($P<0.05$). Betel-nut chewers had raised leukocyte count compared to controls and the difference was statistically significant ($P<0.01$).

Mean levels of eosinophils in controls, betel-nut chewers and betel-nut and tobacco chewers were 4.875 ± 1.25, 6.0 ± 0.9 and 6.75 ± 1.50 per cent respectively. Betel-nut chewers and betel-nut and tobacco chewers had higher eosinophil count compared to controls.

Mean level of lymphocytes in controls, betel-nut chewers and betel-nut and tobacco chewers were 25.12 ± 1.5, 23 ± 5.2 and 22.25 ± 3.82 per cent respectively. There was no significant difference in the lymphocyte count of the above three cases chosen for the study. The mean level of monocytes in controls, betel-nut chewers and betel-nut and tobacco chewers were 1.425 ± 0.64, 1.0 ± 0.2 and 1.25 ± 0.84 per cent respectively and there was no significant change in the monocyte count due to chewing of betel-nut or/and betel-nut and tobacco.

There was no significant change in the mean polymorphs level in controls, betel-nut chewers and betel-nut and tobacco chewers as shown by the values of 67.6 ± 1.87, 69 ± 6.2 and 70.6 ± 5.3 per cent respectively.

When the serum total cholesterol in controls, betel-nut chewers and betel-nut and tobacco chewers were compared, smokers had increased level of cholesterol and the difference was statistically significant. Smokers, betel-nut chewers and betel-nut and tobacco chewers have the total cholesterol within the normal range only. But in smokers the cholesterol level was found closer to the upper limit of the normal range.

Smokers were found to have slightly elevated triglycerides

compared to betel-nut chewers and betel-nut and tobacco chewers and this may be due to the increased content of nicotine in cigarettes smoked.

Betel-nut chewer and betel-nut and tobacco chewers had slightly increased serum free fatty acids compared to smokers and this may be due to the increased lipase activity in the chewers. Anyhow the difference was not statistically significant.

The serum lipase activity was found to be within the normal range in all the three groups of experimentals. But the betel-nut and tobacco chewers had a highly elevated serum lipase activity among the three groups investigated.

Smokers had increased carboxyhaemoglobin level compared to betel-nut chewers and betel-nut and tobacco chewers and the difference was statistically significant ($P < 0.01$).

Smokers had highly elevated serum thiocyanate compared to betel-nut chewers and betel-nut and tobacco chewers and the difference was statistically significant ($P < 0.01$).

Among the betel-nut chewers, betel-nut and tobacco chewers and smokers, betel-nut and tobacco chewers showed increased eosinophil count compared to smokers and betel-but chewers. The difference in the level of eosinophil in the betel-nut and tobacco chewers and smokers was statistically significant ($P < 0.01$). When betel-nut chewers and betel-nut and tobacco chewers were compared, the difference in the eosinophil count was statistically significant ($P < 0.01$).

Betel-nut and tobacco chewing was somehow found to cause an increase in the eosinophil count of the chewers.

The results indicated that smokers had increased levels of serum total cholesterol, triglyceride, free fatty acids, lipase activity, carboxyhaemoglobin and thiocyanate. Smoking was also found to be associated with increased leukocyte count. Total protein and creatinine were slightly decreased in smokers compared to controls.

Betel-nut chewing and betel-nut and tobacco chewing were found to be associated with slight increase in cholesterol, triglycerides, free fatty acids and lipase activity compared to controls. Eosinophil count was also found to be increased in betel-nut chewers and betel-nut and tobacco chewers.

Although statistically significant difference in the various parameters selected for the study was found between those of controls and the experimentals, (Smokers and the two types of chewers), drastic deviation from the normal range of the parameters was not observed. Due to lack of time, further probe into the investigation could not be made. It would be better to categorise the experimentals as mild, moderate and severe smokers/chewers and carry out the investigation.

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Appendices

APPENDIX - I

ESTIMATION OF TOTAL PROTEIN : BIURET METHOD (TIETZ, 1976)

PRINCIPLE

The colorimetric method for protein estimation makes use of biuret reaction. Substances which contain - $CONH_2$ group joined directly or through a single carbon or nitrogen atom give a blue purple colour with alkaline copper sulphate solution. Proteins thus give a purple colour which is different for different proteins. The reaction takes its name from the complex formed i.e., biuret.

REAGENTS

1. Stock biuret reagent

Dissolved 45g of Rochelle's Salt in about 400ml of 0.2N Sodium hydroxide and 15g of Copper sulfate $5H_2O$, stirring continuously until the solution was complete. Added 5g of Potassium iodide and made upto a litre with 0.2N Sodium hydroxide.

2. Dilute biuret reagent

Diluted 200ml of Stock reagent to a litre with 0.2N Sodium hydroxide which contained 5g of potassium iodide per litre.

3. Standard protein solution

Weighed 400mg of albumin and dissolved in 0.9% Saline Solution. So that 1.0ml of this solution contains 4mg of protein.

4. 0.9% Saline Solution.

5. 22.5% Sodium Sulphate Solution.

PROCEDURE

Into a series of test tubes pipetted out 0.5 to 2.5 ml of standard protein solution. The volume was then made upto 2.5ml with water. Into another test tube added 0.4 ml of the serum and diluted with 0.9% Sodium hydroxide to 10ml. From this 2.5 ml of the solution was taken. Now added 3.0ml of dilute biuret reagent to all the tubes. Along with these blank was also taken. The colour developed was read at 500 mμ after 30 minutes in a colorimeter.

A standard graph was drawn by plotting concentration on X-axis and colorimeter readings on Y-axis and the amount of protein present was calculated.

APPENDIX - II

ESTIMATION OF CHOLESTEROL (Zak's method).

PRINCIPLE:

Cholesterol reacts with Ferric cholride in the presence of concentrated sulphuric acid to give a pink colour. The intensity of the colour produced is directly proportional to the amount of cholesterol present and is read at 540 mμ in a colorimeter.

REAGENTS:

1. Stock Ferric chloride reagent.

840mg of pure dry Ferric chloride was weighed and dissolved in 100ml of glacial acetic acid.

2. Ferric chloride precipitating reagent.

10ml of the stock Ferric chloride was taken in a 100ml standard flask and made upto the mark with glacial acetic acid.

3. Ferric chloride diluting reagent.

8.5ml of the stock Ferric chloride was diluted to 100ml with pure glacial acetic acid in a 100ml standard flask.

4. Stock cholesterol solution.

100mg of pure dry cholesterol was taken in a 100ml standard flask and dissolved in 100ml of glacial acetic acid.

5. Working standard solution.

10ml of the stock standard was made upto 100ml with pure glacial acetic acid. 1.0ml of this solution contains 100 μg of cholesterol.

PROCEDURE:

0.5 to 2.5ml of the working standard cholesterol was pipetted put into a series of clean dry test tubes. The total volume of each tube was made upto 5.0ml with Ferric chloride diluting reagent.

To 0.1ml of serum added 4.9ml of ferric chloride precipitating reagent and mixed well. Allowed to stand for a while and centrifuged. Transferred 2.5ml of the clear supernatant into a dry test tube and added 2.5ml of diluting reagent. Mixed well. The tubes were kept in cold water and to each tube added 4.0ml of concentrated sulphuric acid drop by drop. The solution were mixed well. The tubes were allowed to come to room temperature. A blank was also simultaneously prepared by taking 5.0ml of the diluting reagent and 4.0ml of concentrated sulphuric acid. After 30 minutes the colour developed was read at 540mμ.

APPENDIX - III

ESTIMATION OF TRIGLYCERIDES IN SERUM (CHIN et al METHOD)

PRINCIPLE

Triglyceride is measured after hydrolysis by estimating the glycerol content. The commenest procedure involves oxidation of glycerol to formaldehyde which is measured colorimetrically with chromotropic acid. The lipid extract of serum must be freed from other sources which yields formaldehyde. Florosil may be used for this purpose.

REAGENTS

1. Chloroform : Redistilled grade

2. Florosil

Activated by heating for 4 hours at 124°c. Stored in a tightly stoppered bottle. Reactivated with 1N hydrochloric acid.

3. Alcoholic Potassium hydroxide

Dissolved reagent grade Potassium hydroxide (about ¼th of a pellet) in 2.0ml of redistilled 95% ethyl alcohol. Diluted 0.5ml stock Potassium hydroxide solution to 2.5ml with 95% ethanol (this was freshly prepared before use).

4. 0.2N Sulphuric acid

5. Sodium arsenite (0.5M)

Dissolved 2.25g sodium hydroxide and 5.0g of reagent grade Arsenious trioxide in distilled water and diluted to 100ml with

distilled water.

6. Sodium metaperiodate (0.05M)

Dissolved 1.07g of Sodium metaperiodate in 100ml of distilled water.

7. Chromotropic acid

1.12g of Sodium salt of chromotropic acid in 100ml of water. Added 300ml of concentracted sulphuric acid to 150ml distilled water. Cooled in ice. When cooled added this chromotropic acid.

8. Stock Standard Solution

50mg of Olive oil distilled in 100ml of chloroform in a volumetric flask. Standard was checked often.

9. Working Standard Solution

Diluted one in hundred with chloroform or 0.1 to 10ml (1ml = 0.05mg).

PROCEDURE

Added 2.0g of florosil in a glass stoppered tube and 10ml of chloroform. Added 0.5ml plasma. Shook well. Filtered through a filter paper and pipetted out 0.5ml portions of the filtrate into two test tubes. Pipetted out 0.5ml of standard into two test tubes. Evaporated both tests and standards at 60°-70°c.

To one set of tubes (test and standard) added 0.5ml of alcoholic Potassium hydroxide and to the other 0.5ml of alcohol.

Evaporated at 60°c to 70°c. To all the test tubes added 0.5ml of 0.2N sulphuric acid. Boiled for 10 minutes in a gently boiling waterbath. Cooled all the tubes, added 0.1ml of sodium metaperiodate solution kept for 10 minutes, then added 0.1ml of sodium arsenite. A yellow colour appeared and vanished in a few minutes. Added 5.0ml of chromotropic acid reagent to each tube. Mixed well and heated in a boiling waterbath for 10 minutes. After cooled determined the optical density at 570mμ.

CALCULATION

$$\text{mg of triglyceride/100ml} = \frac{\text{Reading of test x concentration of standard}}{\text{Reading of standard x volume of serum taken}}$$

$$= \frac{\text{Test}}{\text{Standard}} \times 100\text{mg per cent.}$$

APPENDIX - IV

ESTIMATION OF FREE FATTY ACIDS (FALHOLT et al, 1973)

PRINCIPLE

Serum was extracted with chloroform - heptane methanol mixture in the presence of phosphate buffer to eliminate interference from phospholipids and the extract shaken with a high density reagent at pH 8. The copper soaps remained in the upper organic layer from which an aliquot was removed and the copper content determined colorimetrically with diphenyl carbazide.

REAGENTS

1. Extraction Solvent containing heptane, chloroform and methanol (5:5:1).

2. Phosphate buffer pH 6.4

33mmol/lit. Mixed two volumes of potassium dihydrogen phosphate (4.539/g/l) and one volume of Disodium hydrogen phosphate dihydrate (5.938g/l).

3. Stock copper solution. 500m.mol/litre.

12.07g of copper nitrate ($Cu(NO_3)_2$ $3H_2O$) was made upto 100ml with water.

4. Triethanolamine solution : 1mol/litre

Diluted 10ml of triethanolamine to 100ml with water.

5. Sodium hydroxide 1 mol/litre.

6. Copper reagent

Mixed 10ml of copper solution, 10ml of triethanolamine and 6.6ml of sodium hydroxide and diluted to 100ml. Added 33g of Sodium chloride and adjusted to pH 8.1. Prepared daily.

7. 1.5 Diphenyl carbazide solution

4g/litre in ethanol prepared as required immediately before use by adding 40mg in 10ml ethanol to 0.1ml triethanolamine solution.

8. Stock standard palmitic acid solution.

Dissolved 51.2mg palmitic acid in the extraction solvent and made upto 100ml. Stored in a tightly stoppered container.

9. Working standard solution

Diluted 5.0ml stock standard to 20ml with extraction solvent to give a solution containing 500μmol per litre. To put up with each batch for a standard curve diluted 2,4,6,8ml stock standard to 20ml. These are equivalent to 200, 400, 600 and 800 micromoles per litre respectively.

PROCEDURE

Collected a fasting venous specimen separated off the cell as soon as possible and determined the nonesterified fatty acid or stored at - 20°c without delay.

To 50 microlitre serum in a suitable stoppered centrifuge tube added 1.0ml of phosphate buffer and 6.0ml of extraction solvent. At the same time prepared tube with 50 microlitre water and 50 microlitre working standard respectively. Shook vigorously for 90 seconds. Stood for 15 minutes. Then centrifuged at 4000rpm for 10 minutes. Carefully removed the buffer by suction and transferred 5.0ml extract to a similar dry centrifuge tube, added 2.0ml of copper reagent and shook vigorously for 5 minutes. Centrifuged at 4000rpm for 5 minutes and transferred 3.0ml of the upper layer to a tube containing 0.5ml diphenyl carbazide solution and mixed carefully. Read after 15 minutes at 550mμ.

CALCULATION

$$\text{Serum nonesterified fatty acid (μmol/litre)} = \frac{\text{Reading of unknown-Reading of blank}}{\text{Reading of standard-Reading of blank}} \times 500$$

APPENDIX - V

DETERMINATION OF LIPASE ACTIVITY IN SERUM (TIETZ 1976)

PRINCIPLE

An aliquot of serum is incubated with a stablized olive oil emulsion at a reaction pH of 7.8 for 3 hours at 37°c. The liberated fatty acids are titrated to pH 10.5 with sodium hydroxide 0.050 mol/lit to a light blue colour with thymolphthalein as indicator.

REAGENTS

1. Purified Olive Oil

Add 300ml of the best quality olive oil to 60g of chromatographic grade alumina ($Al_2 O_3$ Merch No.71207) with stirring. Stir the suspension at frequent intervals over the course of an hour. Permit the alumina to settle out and filter the supernatant, through a qualitative filter paper (Whatmann No.1 or equivalent) using a Buchner funnel and suction.

2. Oil emulsion

Add 7.0g Gummaccacia (emulsifier) and 0.2g of Sodium benzoate (Preservative) to 100ml of water in a high speed blender and dissolve with gentle blender action. Then add 100ml of purified olive oil and emulsify the mixture by operating the blender at top speed for 10 minutes. Store the emulsion at 4 to 10°c. Emulsions are stable for a month at 4 to 10°c.

3. Buffer base : Stock solution

0.80mol/litre tris (hydroxy methyl amino methane) Dissolve 48.55g of Tris in 500ml of the water keep the reagent refrigerated.

4. Tris - hydrochloride buffer.

0.20mol/litre pH 8.0 at 27°c (pH 7.75 at 37°c). To 500ml of buffer base in a beaker add 21ml of Hcl (0.2mol/litre) and dilute with water to 150 to 160ml. Permit the solution to cool to 25°c. Check the pH with a pH meter then adjust to pH 8.0 by careful addition of more acid. Adjust the volume to 200ml.

5. Sodium hydroxide standard.

0.05mol/litre prepare by diluting any laboratory stock sodium hydroxide to exactly 0.05mol/litre.

6. Thymolphthalein indicator

1 per cent in 95 per cent ethanol (V/V).

7. Ethanol 95 per cent (V/V)

PROCEDURE

1. Use a pair of 40 to 50ml contrifuge tubes labelled T(test) and B (blank) for each unknown. Into each tube place 25ml of water 10ml of olive oil emulsion and 1.0ml of Tris-hydrochloride buffer. The test should be done in duplicate if sufficient serum is available.

2. Place the tubes in a water bath at 37°c and permit to warm up for 5 minutes.

3. Add 1.0ml of unknown serum (other specimen) to the 'T' tube. Cover the tube with parafilm. Mix vigorously and return to the water bath. Permit the reaction to proceed for 3 hour at 37°c.

4. At the end of the incubation period, remove the tubes from the water bath and add 3.0ml of 95 per cent ethanol to all the tubes to stop the enzyme reaction. Then add 1.0ml of the unknown sera to B tubes, and mix. Add 5 drops of thymolphthalein and titrate the contents of the tubes with 0.05M sodium hydroxide. Add the sodium hydroxide rapidly dropwise until the first tint of blue is seen and then more cautiously until a definite blue colour is obtained.

5. To calculate the difference $(V_T - V_B) = \Delta V$. This difference gives the value of lipase activity in the customary Cherry-Crandall units. The unit of activity is defined as the quantity of enzyme in 1.0ml of serum which will produce fatty acids equvalent to 1.0ml of 0.050mol/litre sodium hydroxide (50μmol) under the conditions of the test.

APPENDIX - VI

ESTIMATION OF CREATININE IN SERUM : (VARLEY, 1975)

PRINCIPLE

Creatinine when treated with an alkaline picrate solution forms a red coloured complex. The intensity of the colour developed is compared in the colorimeter at 540 mμ.

Blood contains both creatinine and creatine. Creatine is converted to creatinine by boiling for one hour in the presence of picric acid. The total amount of creatinine is found out from which the amount of creatine is calculated.

PROCEDURE

Took 4.0ml of water, added 1.0ml of serum, 1.0ml of 10%. Sodium tungstate solution and 2.0ml of 2/3N sulphuric acid. Kept for 10 minutes and centrifuged. 3.0ml of the supernatant was pipetted out into a test tube.

0.5 to 2.5ml of the working standard solutions were taken and volumes of these were made upto 3.0ml with water. Along with these a blank was also prepared. Added 1.0ml of 0.04M picric acid to all the tubes and 1.0ml of 0.75N sodium hydroxide and let it stand for 20 minutes for colour to develop. Shook well and colour developed was compared with the standard against reagent blank at 540 mμ.

Determination of creatine

To 3.0ml of supernatant in a test tube added 1.0ml of 0.04M

picric acid, noted the level of liquid and placed in beaker of briskly boiling water for one hour. The mouth of the tubes were closed with marbles in order to minimise evaporation. Then added 1.0ml of 0.75N Sodium hydroxide and completed the estimation as for creatinine.

APPENDIX - VII

ESTIMATION OF BLOOD CARBOXY HAEMOGLOBIN (WHITEHEAD AND WORTHINGTON METHOD)

The pH in the technique is raised to 5.82, the period of heating increased to 8 minutes and the temperature used is 57.0 to 57.5°c. This completely precipitates oxyhaemoglobin while approximately 80% of the carboxyhaemoglobin remains in solution.

REAGENTS

1. Sodium acetate solution

Dissolve 40.8g sodium acetate $3H_2O$ in water and make to 100ml.

2. Acetic acid

Dilute 28ml glacial acetic acid to 100ml with water.

3. Sodium chloride solution

9g/litre.

PROCEDURE

Dilute 1ml whole blood with 9ml saline in a centrifuge tube and centrifuge for 5 minutes. Remove the supernatant fluid as completely as possible. Repeat the washings with a further 9ml saline and again removed the supernatant fluid. Add 4.5ml water and mix well. Place 1.0ml of this solution in a 15 x 1.2cm test tube. Pour the remainder into a conical

flask and bubble carbon monoxide through for 1 minute while shaking well. If necessary prepare the gas from sulphuric acid and oxalic acid. Transfer 1ml of treated blood to a similar test tube. To both tubes add with mixing 3ml sodium acetate solution then 0.75ml acetic acid. Place the tubes in a water bath at 57 to 57.5°c for exactly 8 minutes. Cool and filter through 7cm Whatman No:1 paper. Read at 555nm. Dilute the 100% carboxyhaemoglobin specimen to 10 before reading and if necessary the test solution enough to give about the same reading.

CALCULATION

Blood carboxyhaemoglobin (per cent) =

$$= \frac{\text{Reading of Unknown - Diluting factor}}{\text{Reading of 100\% control x 10}} \times 100.$$

APPENDIX - VIII

ESTIMATION OF THIOCYANATE IN SERUM - BOWLER'S METHOD

PRINCIPLE

The serum proteins are precipitated with trichloro acetic acid. The clear filtrate treated with Ferric nitrate and the orange red colour measured.

REAGENT

Ferric nitrate-nitric acid reagent is made by dissolving 80g of Ferric nitrate, $(Fe(NO_3)_3 . 9H_2O$ in 250ml of 2N nitric acid, diluting to 500ml with water and filtering.

PROCEDURE

Serum 1.0ml is measured into a test tube and mixed with 6.5ml of water and 2.5ml of a 20% trichloro acetic acid. After 10 minutes, the proteins are filtered off with a Whatman No.40 filter paper (9cm). Then 5.0ml of the filtrate is mixed with 5.0ml of Ferric nitrate reagent in the absence of day light and the colour compared with that of a standard within 15 minutes. The standard is prepared by treating in the same way as serum. 1.0ml of a solution containing 0.1mg of thiocyanate iron is prepared by diluting 8.62ml of 0.1N Sodium or Potassium thiocyanate with water to 500ml. A control test which yield practically no colour is carried out by treating in the same way, 1.0ml of a serum from a person who has not received thiocyanate. A blue filter is used.

APPENDIX - IX

ESTIMATION OF WHITE BLOOD CELLS

Apparatus

Microscope, WBC pipette, Neubaver ruling.

WBC pipette

It has got markings 0.5 and 1.0mm on the stem and so the dilution made is 1/20 and 1/10 respectively. The bore of the capilary tube is greater than that of the R.B.C. pipette.

Slide : NEUBAVER RULING

The counting slide consists of a thick glass slide with a central platform divided by a short tranverse gutter into two portions each of which is ruled with the counting grid. The counting platform for the Nevbaver type is engraved with ruling which measures 3 x 3mm. This area is divided into 5 large squares. First divided by 5 horizontal and 5 vertical lines that is 25 x 6400. We use the first 5 lines and then 4 lines. So the capacity of the small square is 1/20 x 1/20cm and the depath of the counting chamber between the coverglass, and ruling is 1/10mm.

The white cells are counted from big squares at the corners each measuring 1 sq.mm.

Diluting fluid

This is composed of gention violet to stain the nucleus

and acetic acid to destroy the RBC and distilled water for dilution.

PROCEDURE

Cleaned the finger tip with spirit. Allowed it to dry. Pricked the finger with surgical needle. A sudden prick was given so that a free flow of blood was obtained. Drew the blood upto 0.5 mark and diluted upto the mark. The pipette was held firmly by its ends between the four fingers and thumb and roated so that the content was mixed well. The mixture was then applied to the narrow slit between the counting chamber and the cover slip. The fluid runs under the cover slip. The slide was set aside for 2 minutes for the cells to set.

Counting

Counting was done under a high power objective. Each medium sized square contains 16 small squares. The cells were counted from 5 sets of 16 small squares. The squares were counted simultaneously in horizontal rows of 4 at a time.

Differential count of leukocytes

PROCEDURE

Blood stains can be made either on slides (glass) or cover glasses. To get a thin uniform blood smear, the slide must be free from grease. Hence the slides were cleaned with glacial acetic acid followed by water and alcohol. Three such slides were taken, one was used as a spreader and the other two were

covered with blood film. The ends of the spreader were pinched off to make the film narrower than the slide.

PREPARATION OF BLOOD SMEAR

Cleaned the figer with spirit. Allowed it to dry and pricked it with a clean needle to obtain a drop of blood. Applied one end of the slide to the drop of blood. Placed the slide on some smooth surface and held it in position with the thumb and the index finger of the left hand. The narrow edge of the spreader was placed in the drop and held there until the blood had spread across it. It was then drawn over the whole length of the first slide. The inclination of the spreader should be 45° and hence it was held between thumb index and middle finger, With their tips resting on the table to avoid undue pressure. Allowed the film to dry in air.

Staining the Film by Leishman's Stain

This is a simplification of the method of staining introduced by Romanowsky. The stain consists of a mixture of methylene blue and eosin in methyl alcohol. The dry film was covered with this stain for 1 minute. Double the quantity of distilled water was added to the stain so as to stay on the slide without spilling. Mixed it thoroughly with rocking movement. The diluted stain was allowed to stain for 6 minutes. The mixture was then poured off. This was then covered with distilled water and washed with it until the pink colour appeared. Allowed it to dry in oil. Covered the smear with cidar wood oil and examined it under a microscope using oil immersion objective.

Estimation of the Film

Methyl alcohol acts as a fixative. In a properly stained film the R.B.C.'S are pink, the cytoplasm of the white cell blue, small neutrophil granules purple, large eosinophil granules brick red and basophil granules dark blue.

A differential count can be carried out efficiently using a mechanical stage and oil immersion objective. The differential count is done to ascertain the relative number of different varieties of leukocytes. Counted at least a hundred leukocytes and tabulated in a column, using the mechanical stage travelled in full width of the film, moved the film a little and examined in the reverse direction. This avoids repetition of counting the same fluid. A stained film also shows morphological characterstics of red and white cells.

APPENDIX - X

BIOCHEMICAL PARAMETERS OF CONTROLS, SMOKERS, BETEL-NUT CHEWERS AND BETEL-NUT AND TOBACCO CHEWERS

Volenteer No.	Cholesterol mg/100ml	Triglyceride mg/100ml	Free fatty acids m.mol/lit	Lipase acitivity U/lit	Total Protein g/100ml	Creatinine mg/100ml	COHB %	Thiocyanate mg/100 ml
Controls:1								
1	180	90.0	0.90	190	7.6	1.24	1.00	1.90
2	205	85.0	0.70	210	7.6	0.80	0.90	1.80
3	165	100.0	1.00	170	7.9	1.25	1.60	1.80
4	200	120.0	0.70	140	8.3	0.53	1.20	2.10
5	195	98.0	0.50	180	7.5	1.60	1.50	2.20
6	210	115.0	1.00	200	7.9	0.80	0.90	2.40
7	190	125.0	0.90	190	8.0	0.80	1.30	2.00
Smokers								
1	250	140.5	0.75	200	6.6	0.44	2.80	4.05
2	255	127.8	1.50	300	6.2	0.67	1.90	4.05
3	220	162.6	2.10	400	7.4	0.42	1.65	3.12
4	200	140.0	3.05	500	6.8	0.47	3.20	2.80
5	245	124.6	0.87	250	7.6	0.39	2.70	4.00

Contd..

6	200	149.2	0.95	200	7.0	0.27	2.50	4.00
7	280	122.7	0.77	200	7.4	0.29	2.70	5.00
8	285	125.4	0.55	200	6.6	0.57	2.40	3.00
9	200	170.5	0.50	200	7.2	0.50	2.05	3.50
10	286	146.7	0.42	150	7.0	0.42	2.90	3.50
11	270	163.1	1.00	300	7.6	1.00	2.40	2.85
12	196	166.0	0.75	295	7.4	0.75	2.70	3.12
13	240	165.2	0.95	200	6.9	0.95	1.90	3.21
Controls-II								
1	170	89.2	1.00	170	6.9	0.66	0.89	2.00
2	185	91.8	0.60	160	7.9	0.42	1.20	2.10
3	170	95.0	1.00	150	8.1	0.69	1.30	2.30
4	190	105.0	0.80	170	7.8	1.25	1.00	2.10
5	180	120.0	0.90	100	6.9	0.80	1.40	2.15
6	190	95.0	1.20	150	7.8	0.53	1.00	2.10
7	220	120.0	1.00	200	6.9	1.60	1.20	2.00
8	200	130.0	0.80	180	8.0	0.84	1.70	2.20

Contd..

Chewers								
1	185	122.7	0.90	190	6.3	0.90	1.30	2.75
2	185	126.0	1.20	200	6.7	0.50	1.90	2.50
3	195	167.3	0.82	185	6,1	0.63	2.00	2.30
4	250	133.0	1.20	190	5.9	0.40	1.50	2.50
5	200	126.0	0.90	200	6.7	0.84	1.50	1.90
6	220	121.7	0.98	210	6.8	0.27	1.77	2.00
7	205	126.0	1.40	190	6.5	0.80	1.00	2.20
8	195	130.4	1.20	195	6.7	1.00	1.40	2.40
Betel-nut and Tobacco chewers								
1	210	123.8	1.00	300	5.9	0.47	1.40	3.10
2	255	96.5	1.40	350	5.7	0.80	1.70	3.50
3	180	160.0	0.85	250	6.7	0.82	1.00	3.20
4	190	134.0	1.80	600	6.3	0.70	1.20	3.10
5	280	134.7	1.49	550	5.9	0.45	1.80	2.40
6	270	139.0	0.92	350	6.5	0.60	1.30	2.90
7	240	148.0	1.00	400	6.0	1.00	1.40	2.60
8	250	130.0	1.10	400	5.9	0.90	1.70	2.70

APPENDIX-XI

TOTAL AND DIFFERENTIAL LEUKOCYTE COUNT OF CONTROLS SMOKERS BETEL NUT CHEWERS AND BETEL NUT AND TOBACCO CHEWERS

Volunteer No.	WBC Total count	Eosinophil per cent	Lymphocytes per cent	Monocytes per cent	Polymorphs per cent
Controls-I					
1	6,600	3	32	4	61
2	7,200	2	33	0	65
3	7,000	2	22	1	75
4	7,100	2	24	2	72
5	6,400	3	25	0	72
6	7,900	4	23	2	71
7	7,080	2	25	1	72
Smokers					
1	6,850	3	25	2	70
2	8,000	4	24	1	71
3	7,300	6	30	2	62
4	7,260	0	20	1	79
5	8,100	1	26	0	73

Contd..

6	8,900	1	21	3	75
7	9,050	1	22	2	76
8	7,025	3	31	1	65
9	7,950	4	24	2	70
10	6,750	2	20	0	80
11	7,100	2	19	3	75
12	8,200	2	18	2	78
13	9,800	2	16	1	81
Controls-II					
1	7,200	4	26	1	69
2	7,400	5	24	1	70
3	7,200	3	29	1	67
4	7,600	4	26	1	69
5	7,100	4	28	2	66
6	7,200	6	26	2	66
7	7,000	3	32	0	65
8	7,200	2	28	1	69

Contd..

Betel-nut Chewers					
1	7,800	6	28	1	65
2	7,000	6	25	2	67
3	8,200	5	30	0	65
4	7,600	7	17	1	72
5	7,200	7	26	1	66
6	8,100	5	28	0	67
7	8,000	6	18	2	74
8	7,750	6	20	1	73
Betel-nut and Tobacco chewers					
1	8,000	7	25	0	68
2	8,200	5	19	1	75
3	7,800	7	28	2	63
4	8,400	5	17	2	76
5	8,300	7	19	2	80
6	8,000	8	20	1	72
7	8,250	8	25	1	76
8	7,890	5	23	0	72