Original Article

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Serum Cotinine Concentration and Serum Lipid Profile: Risk for Cardiovascular Disease in Smokeless Tobacco Users

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Abstract

Background: The objective of the study is to find out the effect of long time oral nicotine consumption on serum lipid profile in exclusive smokeless tobacco consumers and compare the findings with healthy tobacco non-users.

Materials and Methods: This case-control study was performed in 100 subjects in which 50 were exclusively smokeless tobacco consumers (cases) and 50 were age and sex matched tobacco non-users (controls). Age group was 30-50 years, Individuals if found to be a smoker, have any associated co-morbid illness or taking regular medication including vitamin/mineral supplements/herbal/native medicines were excluded. Enzymatic methods were used to estimate serum lipid parameters including total cholesterol level, high-density lipid (HDL) cholesterol level and triglycerides (TGL) level using commercial kits. Low-density lipid (LDL) cholesterol level was then calculated. Serum cotinine (CTN) level was estimated using enzyme linked immunosorbent assay kit.

Results: In the present study, mean serum CTN level was found to be raised in smokeless tobacco users as compared to non-users. Also, the lipid parameters were also deranged in cases as compare to control group. A significant association between raised mean serum CTN levels and raised cholesterol, low HDL, raised LDL, TGL, and LDL/HDL ratios was observed ($P < 0.001$) in cases. A strong positive correlation was observed between serum CTN level and deranged lipid profile.

Conclusion: Thus, the present study shows that people who are smokeless tobacco users for the long duration have accumulated more nicotine metabolites as compared to tobacco non-user subjects. Long-term use of smokeless tobacco results deranged lipid profile which is an independent marker for cardiovascular disease.

Key words: Cotinine, Enzyme-linked immunosorbent assay, High-density lipid, Low-density lipid, Smokeless tobacco

INTRODUCTION

Tobacco addiction is a major health concern in our country. The most recognized health risk associated with any tobacco product is the carcinogenic side-effect. However, tobacco has other deleterious effects including its adverse effect on lipid profile.[1,2]

Nicotine is the major addicting substance in tobacco and is thought to be responsible for most of the adverse effects associated with its use. Smokeless tobacco extract has been shown to increase oxidative stress as a result of reactive oxygen free radicals. Overall increased oxidative stress may promote lipid peroxidation and increased deposition in vessel wall. This can lead to atherosclerosis in future.[3,4]

Nicotine is the major addicting substance in tobacco is metabolized in the liver by cytochrome P450 enzymes (mostly cytochrome P450 Type 2A6, and also by Type 2B6). A major metabolite is cotinine (CTN), concentration of which can be measured in urine and serum.[5] Since half-life of CTN is more than nicotine, the level of CTN is used to assess the tobacco exposure.

Because of the pharmacological properties of nicotine and other constituents of smokeless tobacco there is concern that smokeless tobacco products may lead to

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cardiovascular disease or death from cardiovascular causes.[6] In short, nicotine modifies cell structure in a way that facilitates migration and invasion of cells that line the blood vessels. This enables a change in structures called podosomes, which lead to poor vessels and can cause the formation of plaque. Over time, plaque can cause arteries to harden, a form of heart disease called atherosclerosis. It can also block blood flow to the heart or brain, keeping oxygen from reaching those organs and causing heart attack or stroke.[7]

As per global adult tobacco survey India Report, Khaini is the most common smokeless tobacco product in use in India, and also the most common smokeless tobacco in use by men. This is followed by gutkha. The use of smokeless tobacco in India is peculiar as there are large no. of smokeless tobacco products in use e.g. paan with tobacco, paan masala with tobacco, Gul, Mawa, Mishri, Bajjar, Gudakhu etc.

In view of all these facts, this study is designed to assess the effect of smokeless tobacco (mainly gutkha) on lipid profile.

MATERIALS AND METHODS

A case-control study was designed. Ethical clearance was taken from Institutional Ethics Committee before the start of the research. An informed consent was taken from each subject on prescribed consent form obtained from research cell. A total of 100 subjects age group of 30-50 years, were included in the study of which 50 smokeless tobacco consumers (cases) and 50 age and sex matched tobacco non-users (controls) were enrolled. Individuals if found to have any associated co-morbid illness or taking regular medication including vitamin/mineral supplements/herbal/native medicines, was excluded.

Inclusion Criteria

1. Subjects of 30-50 years of age having history of tobacco chewing and gutkha eating (only smokeless tobacco)
2. Control group was comprised of age and sex matched subjects who did not use tobacco in any form.

Exclusion Criteria

1. Age <30 and >50 years
2. Smokers (cigarettes, beedi, hookah etc.)
3. Smokeless tobacco chewers but smokers too
4. Chronic systemic disease
5. Diabetes mellitus
6. Obese persons (World Health Organization Criteria: Body mass index)
7. Family history of hypertension and diabetes mellitus
8. Pregnancy
9. Oral carcinoma.

A working proforma (case sheet) was filled for every subject. It included demographic data (particulars of a subject such as a name, age, sex, address, contact number), past history, and family history of chronic illness. Further addiction history was taken which included type, amount, and duration of consumption of smokeless tobacco.

Blood Sample Collection

After overnight fasting, 5 ml of blood samples were collected in a syringe from all the subjects in the morning. Serum was separated by centrifugation at 3000 rotation per minute.

Biochemical Investigation

Lipid profile

Lipid profile was done the same day in the molecular lab by autoanalyzer.

A total of 6 parameters *viz*. total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride (TGL), very LDL (VLDL) and LDL/HDL ratio were assessed. The criteria for determination of derangement was shown in Table 1.[8]

Serum CTN Level

Exposure to tobacco can be detected by measuring nicotine and its metabolites. Nicotine has a short half-life and is not used as a marker for tobacco exposure. CTN due to its longer half-life has been used in research as a reliable marker of tobacco exposure.

Serum CTN levels were measured by CTN enzyme-linked immunosorbent assay (ELISA) kit (Blue Gene Biotech), catalogue number E01C0050.

Principle of the Assay

CTN ELISA kit applies the competitive enzyme immunoassay technique utilizing a monoclonal anti-CTN antibody and a CTN-high reactive protein (CTN-HRP) conjugate. The assay sample and buffer are incubated together with CTN-HRP conjugate in the pre-coated plate for the one hour. After the incubation period, the wells are decanted and washed five times. The wells are then incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction forms a blue colored complex. Finally, a stop solution is added to stop the reaction, which will then turn the solution yellow. The intensity of the color is measured spectrophotometrically at 450 nm in a microplate reader. The intensity of the color is inversely proportional to the CTN concentration since CTN from samples and CTN-HRP conjugate compete for the anti-CTN antibody binding site. Since, the number of sites is limited, as more

sites are occupied by CTN from the sample, fewer sites are left to bind CTN-HRP conjugate. A standard curve is plotted relating the intensity of the color (optical density) to the concentration of standards. The CTN concentration in each sample is interpolated from this standard curve. In a non-smoker mean serum CTN concentration is <12 ng/ml. Higher value is expected in passive smokers. The statistical analysis was done using Statistical Package for Social Sciences Version 15.0 statistical Analysis Software.

OBSERVATIONS AND RESULTS

The present study was carried out with an aim to find out the association of serum CTN level with lipid profile in smokeless tobacco users. Comparison of two groups for mean value of lipid parameters has been shown in Table 2 (Group 1-cases, Group 2-controls).

None of the subjects in Group II had total cholesterol and serum TGL levels >200 mg/dl and >150 mg/dl respectively as against 56% and 58% of subjects in Group I, thus showing a significant difference between two groups ($P < 0.001$). Proportion of subjects with low HDL was significantly higher ($P < 0.001$) in Group I (72%) as compared to that in Group II (26.0%). A total of 49 (98%) subjects in Group I and 7 (14.0%) in Group II had serum LDL levels >100 mg/dl, thus showing a significant difference between two groups and a total of 40 (80%) of Group I and 2 (4.0%) of Group II subjects had LDL/HDL ratio >3. For all the lipid parameters, the differences between two groups were significant statistically ($P < 0.001$) (Table 3).

Serum CTN levels ranged from 56.9 to 210.5 ng/ml in Group I and from 23.1 to 68.7 ng/ml in Group II. Mean serum CTN level in Group I was 146.89 ± 33.21 ng/ml and 38.66 ± 10.66 ng/ml in Group II. On comparing the data statistically, the differences between two groups were found to be significant statistically ($P < 0.001$). (Table 4).

A significant association between raised mean serum CTN levels and raised cholesterol, low HDL, raised LDL, TGL, and LDL/HDL ratios was observed ($P < 0.001$) (Table 5).

Table 1: Criteria for determination of deranged lipid profile values[8]

Parameter	Derangement criteria
Total cholesterol (mg/dl)	>200
HDL (mg/dl)	<40
LDL (mg/dl)	>130
VLDL (mg/dl)	>30
TGL (mg/dl)	>150
LDL/HDL	>3.5

Source: National cholesterol education program-Adult treatment panel-III, LDL: Low-density lipid, HDL: High-density lipid, VLDL: Very low-density lipid, TGL: Triglyceride

Table 2: Comparison of lipid parameters in two groups

Lipid parameters	*n*=50				Significance of difference	
	Group I		Group II			
	Mean	SD	Mean	SD	*t*	*P*
Total cholesterol	200.88	15.63	148.12	16.22	16.642	<0.001
HDL	39.44	2.29	50.50	8.09	9.315	<0.001
LDL	131.07	13.66	75.90	19.96	16.182	<0.001
Triglycerides	151.91	11.63	108.64	16.12	15.443	<0.001
VLDL	30.38	2.33	21.73	3.22	15.443	<0.001
LDL/HDL ratio	3.33	0.38	1.59	0.64	16.691	<0.001

For all the parameters except HDL, mean value in Group I was significantly higher as compared to that in Group II (*P*<0.001), LDL: Low-density lipid, HDL: High-density lipid, VLDL: Very low-density lipid, TGL: Triglyceride

Table 3: Comparison of derangement of lipid parameters in two groups

Lipid parameters	*n*=50				Significance of difference	
	Group I		Group II			
	No.	%	No.	%	"χ^2"	*P*
Total cholesterol >200 mg/dl	28	56.0	0	0	38.889	<0.001
HDL (<40 mg/dl in males, <50 mg/dl in females)	36	72.0	13	26.0	21.168	<0.001
LDL>100 mg/dl	49	98.0	7	14.0	71.591	<0.001
TGL>150 mg/dl	29	58.0	0	0	41.494	<0.001
LDL/HDL ratio>3	40	80.0	2	4.0	59.78	<0.001

LDL: Low-density lipid, HDL: High-density lipid, VLDL: Very low-density lipid, TGL: Triglyceride

Table 4: Comparison of serum cotinine levels (ng/ml) between two groups

Variable	Group I (*n*=50)	Group II (*n*=50)
Minimum	56.9	23.1
Maximum	210.5	68.7
Mean	146.89	38.66
SD	33.21	10.66

t=22.14, *P*<0.001, SD: Standard deviation

Table 5: Association of serum cotinine levels with different lipid parameters (overall) (*n*=100)

Variable	No. of cases	Mean serum cotinine	SD	Significance of association
Total cholesterol				
Normal	72	73.16	56.23	*t*=6.046; *P*<0.001
Deranged	28	141.99	34.41	
HDL				
Normal	51	67.54	53.50	*t*=4.725; *P*<0.001
Deranged	49	118.46	54.79	
LDL				
Normal	44	40.41	21.27	*t*=12.534; *P*<0.001
Deranged	56	133.90	46.22	
Triglyceride				
Normal	71	69.94	53.57	*t*=7.313; *P*<0.001
Deranged	29	147.60	31.10	
LDL/HDL ratio				
Normal	58	59.02	48.67	*t*=8.829; *P*<0.001
Deranged	42	138.91	38.73	

HDL: High-density lipid, LDL: Low-density lipid, SD: Standard deviation

DISCUSSION

Effect of smokeless tobacco use on serum CTN levels and its association with cardiovascular risk factors were analyzed.

The 100 subjects were enrolled in the study of which 50 subjects were exclusive smokeless tobacco users (study group), and remaining 50 subjects were age and sex matched controls (non-tobacco users).

Statistically, no significant difference was found between the two groups for any demographic characteristic.

Smokeless tobacco (gutkha) remains in contact of buccal mucosa for a longer duration, and a large amount of nicotine is absorbed into the blood stream. Nicotine then binds to acetylcholine receptors present on endothelial cells; it is metabolized in the liver by cytochrome P450 enzymes. A major metabolite is CTN which has a much longer half-life than nicotine, the reason for it being used as a biochemical marker of average daily intake of nicotine.

In the present study, a high mean serum CTN level was found in cases (146.89 ± 33.21 ng/ml) as compared to controls (38.66 ± 10.66 ng/ml) (Figure 1).

Longer duration of ST exposure resulted in high level of serum CTN levels. CTN is found in blood, serum, urine, and saliva. It is accumulated in hair, brain, and probably other parts of body.[3]

In the control group mean serum CTN level was 38.66 ± 10.66 ng/ml. Maximum level was 68.7 ng/ml, and minimum level was 23.1 ng/ml. Since our controls were not using tobacco in any form, it is assumed that the level above the expected value was due to passive exposure of tobacco smoke.

In the present study, raised mean serum CTN level was found to be significantly associated with many parameters of dyslipidemia.

Dyslipidemia in ST users has been reported by many past studies like Khurana *et al.*[9] and Gupta *et al.*[10] On contrary, in some other studies of healthy baseball players and firemen, smokeless tobacco users demonstrated no significant differences in total cholesterol levels, LDL and HDL levels compared with non-users.[5,11]

In the present study, the control group had lower total cholesterol (mean 148.12 ± 16.22 mg/dl) than cases (mean 200.88 ± 15.63 mg/dl) and this difference was statistically significant ($P < 0.001$). Tucker[12] found higher cholesterol level in ST users (>240 mg/dl).

The HDL cholesterol level in the control group was higher (mean 50.50 ± 8.09 mg/dl) than those in study group (mean 39.44 ± 2.29 mg/dl) and the difference was statistically significant ($P < 0.001$). Khurana *et al.*[9] also found similar HDL level in ST users (38 mg/dl) (Figure 2 and 3).

The LDL cholesterol in the study group was higher (mean 131.07 ± 113.66 mg/dl) as compared to control group (mean 75.90 ± 19.96 mg/dl) and the difference was statistically significant ($P < 0.001$).

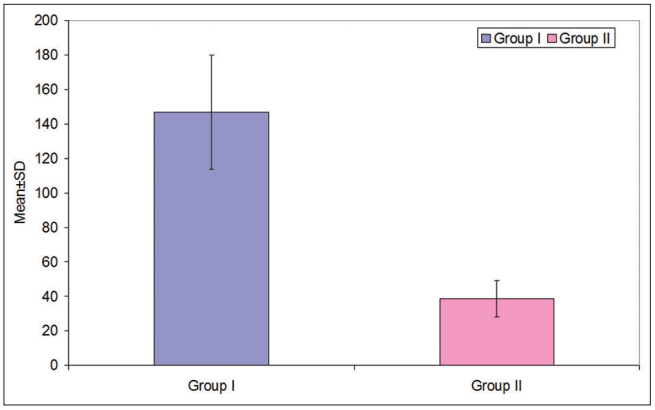

Figure 1: Comparison of serum cotinine levels (ng/ml) between two groups

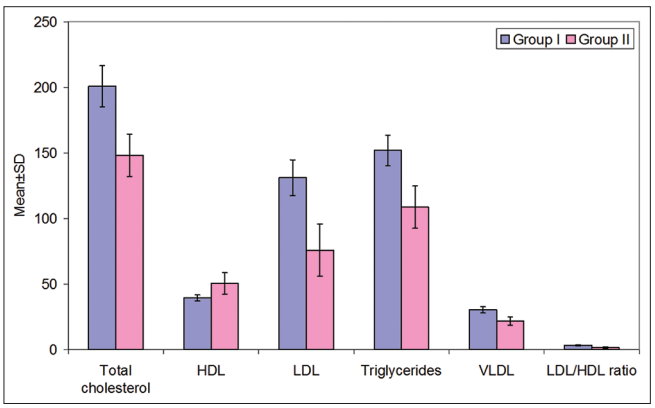

Figure 2: Comparison of lipid parameters in two groups

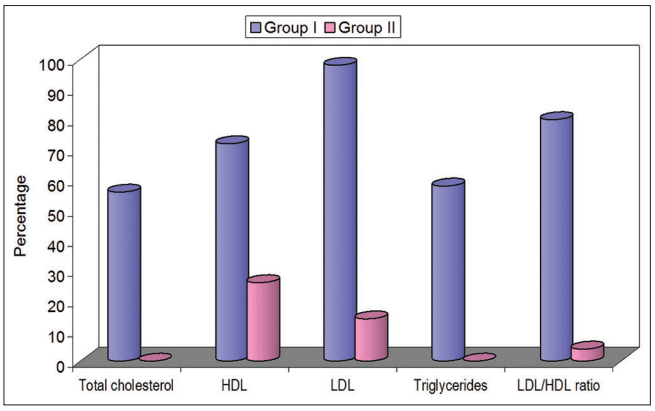

Figure 3: Comparison of derangement of lipid parameters in two groups

TG levels in the study group were also higher (mean 151.91 ± 11.63 mg/dl) than levels in control group (mean 108.64 ± 16.12 mg/dl) and the difference was statistically significant ($P < 0.001$). Similar TGL levels were obtained by Khurana *et al*.[9] They found TGL level of 160 mg/dl in ST users and 96 mg/dl in controls.

Similarly, VLDL levels were also higher in the study group (mean 30.38 ± 2.33 mg/dl) as compared to control group (mean 21.73 ± 3.22 mg/dl) and difference was statistically significant ($P < 0.001$).

LDL/HDL ratio also follows the above trend and shows higher value in study group (mean 3.33 ± 0.38) than in controls (mean 1.59 ± 0.64) and the difference was statistically significant ($P < 0.001$).

In ST users and non-users the deranged lipid profile were significantly associated with high mean serum CTN level. Hence, long-term consumption of ST probably results in deranged lipid profile.

Above findings could be explained by the fact that nicotine after absorption binds to acetylcholine receptors on the endothelial cell surface where it promotes atherogenesis thrombotic and vascular occlusion by promoting formation of plaque in vessel wall. It causes direct injury to endothelial cells which act as a nidus for plaque formation. Nicotine also affects lipid peroxidation which results in dyslipidemia.[13]

These sympathoadrenergic and hemostatic mechanisms affect vascular system most importantly coronary system and could lead to stroke.[14]

A significant association was observed between serum CTN levels with duration of its use and not with daily consumed amount. Hence, duration of use of tobacco seems to be a better predictor of risk outcome than the amount of tobacco consumed.

LDL/HDL ratio shows a higher mean value in gutkha consumers as compared to non-consumers - an independent risk factor for cardiovascular diseases.

Therefore, consumption of gutkha on a long-term basis imposes a serious threat to overall health precisely to cardiovascular health.

CONCLUSION

High serum CTN level in smokeless tobacco consumers probably results in deranged lipid profile which is an independent marker for cardiovascular disease. Intense education program about adverse health events of smokeless tobacco should be under taken through all means including audio-visual media to the public and to students through their curriculum. Hence, national policy makers must be enlightened by the findings and efforts to curb the use of smokeless tobacco by the society must be their priority.

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