ORIGINAL ARTICLE

Effect of vitamin C supplementation on serum Cotinine, MDA and vitamin Clevels in smokeless Tobacco chewers

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ABSTRACT

Smokeless tobacco is a commonly used drug in the world. Tobacco use in any form is a rising crisis for the population in developing countries. Tobacco contributes significantly to oxidative stress induced morbidity and mortality. The study was aimed to evaluate the effect of vitamin C supplementation on serum cotinine, malondialdehyde (MDA) and serum vitamin C level in smokeless tobacco (ST) chewers. In addition, the study was aimed to evaluate the effect of duration of tobacco chewing on above-said parameters. A total number of 338 healthy participants of aged between 31 to 60 years were classified into two groups comprising of tobacco chewers and tobacco non-chewers. Tobacco chewers group further divided into three subgroups with respect to tobacco chewing duration. Participants were asked to take 1000 mg of vitamin C daily for 45 days. Serum cotinine, MDA and serum vitamin C were measured in all the participants before and after supplementation of vitamin C. Serum cotinine (p<0.001) and MDA (p<0.001) were significantly increased whereas serum vitamin C level (p<0.001) was significantly decreased in smokeless tobacco chewers as than to controls. However, serum cotinine (p<0.001) and MDA (p<0.001) were significantly decreased whereas vitamin C (p<0.001) level was significantly increased in both the groups after supplementation of vitamin C as compared to before. It was observed that the changes become more prominent with increased smokeless tobacco consumption duration. Daily intake of vitamin C may be beneficial to restore the oxidant-antioxidant balance in tobacco chewers.

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INTRODUCTION

The most widely spread and commonly used drug in the world is 'Tobacco'. The habit of oral use of smokeless tobacco is becoming a global risk to public health with every passing day due to its various harmful effects. The use of smokeless tobacco was reported to be more rampant in the South Asian countries as compared to the Western world (Robertson, P.B. *et al.,* 1997). Tobacco addiction is leading preventable cause of deaths in India and the World. Tobacco addiction includes addiction to tobacco and tobacco products such as bidis, cigarettes, gutka, and pan masalas. Reactive oxygen species were generated by tobacco leads to enhance oxidative stress (Trueb, R.M., 2003). Tobacco causes an increase in oxidative stress which is duration dependent (Gupta, P.C. *et al.,* 1999).

Nicotine is absorbed from smokeless tobacco and spreads in the body through the circulation within seconds. In humans the main elimination pathway of nicotine oxidation to cotinine by hepatic cytochrome P2A6 and aldehyde oxidase. Cotinine is the

chief metabolite of nicotine, is usually regarded as the finest biomarker for monitoring tobacco exposure in actively and passively exposed individuals (Benowitz, N.L., 1996). The most common sources for cotinine are serum, urine, and saliva (Bernert, J. T.*et al.,* 2000). In vivo half-life of cotinine is approximately 20 hours and is usually detectable for several days (up to a week) after the exposure of tobacco. The level of cotinine in the blood, saliva, and urine are proportionate to the amount of tobacco exposure, so it is an important indicator of tobacco exposure (Florescu, A. *et al.,* 2009).

The recent studies have established that long-term use of tobacco-related products are the possible generators of free radicals. The highly reactive radicals and reactive oxygen species (ROS) can be active initiators of carcinogenesis, DNA damage, activate pro-carcinogens and change the cellular antioxidant defense system (Avti, P.K. *et al.,* 2006) Free radicals are atoms, molecules or ions with unpaired electrons, which are highly reactive to chemical reactions with other molecules. In the biology system, the free radicals are often derived from oxygen, nitrogen and Sulphur molecules. These free radicals are parts of groups of molecules called reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive Sulphur species (RSS). ROS are formed during cellular metabolism and functional activities and have significant roles in cell signalling, gene expression, apoptosis and transport of ions (Vajragupta, O.*et al.,* 2004). However, extreme amounts of ROS can have harmful effects on protein, lipid, RNA and DNA because they are very highly reactive. ROS can hit bases in nucleic acids, amino acid side chains in proteins and double bonds in unsaturated fatty acids, in which ·OH is the strongest oxidant. ROS attacking macromolecules is termed oxidative stress. Cells are usually able to protect themselves against ROS damage through the utilize of intracellular enzymes to maintain the homeostasis of ROS at a minimum level. However, in times of environmental stress and cell dysfunction, ROS levels can increase considerably, and cause major cellular damage in the body. Hence, oxidative stress considerably contributes to the pathogenesis of inflammatory disease, cardiovascular disease, cancer, cataracts, diabetes, Alzheimer's disease, aging and autism (Giles, G.I. 2002, Ames, B.N. *et al.,* 1993, Liu, R.H. *et al.,* 1995, Geier, D.A. *et al.,* 2009, Geier, D.A. *et al.,* 2009).

Nicotine induces oxidative stress both in vivo as well as in vitro to causes a per oxidant/antioxidant imbalance in blood and tissues (Suleyman, H. *et al.,* 2002). Oxidative stress generates free radicals that attack the membrane lipids consequential in the formation of malondialdehyde (MDA) that causes peroxidative tissue damage (Srinivasan, K.N. *et al.,* 2000). Animals' studies have shown significantly increased levels of MDA in serum and liver, conjugated dienes, hydroperoxides, and free fatty acids in rats induced by cigarette smoke (Ashakumary, L. *et al.,* 1996, Zhang, J., *et al.,* 2001). Smokers maintain a sustained free radical load that increases their vitamin C and vitamin E requirement (Helen, A. *et al.,* 1997; Tsuchiya, M., *et al.,* 2002). Supplementation with vitamin C and E is considered safe and easy since these are susceptible to dietary management (Byers, T. *et al.,* 1992). Malondialdehyde [$CH_2 (CHO)_2$] is an end product of lipid peroxidation. This reactive oxygen species occurs naturally and is a biomarker for oxidative stress. Reactive oxygen species degrade polyunsaturated fats present in cell membrane forming malondialdehyde. This malondialdehyde product is used as a biomarker to measure the intensity of oxidative stress in a living organism (http.//en.wikipedia.org/wiki/malondialdehyde, 2015).

Antioxidants molecules can neutralize free radicals by accepting or donating its electron to eliminate the unpaired state of the radical. The antioxidant molecules may directly react with the reactive radicals and demolish them, though they may become new free radicals which are quite active and less dangerous than those radicals they have neutralized. They may be neutralized by other antioxidants or additional mechanisms to finish their radical status. Vitamin C turns to a very stable radical (Asc•), due to its delocalized structure. Furthermore, vitamin C can neutralize the radical form of other antioxidants such as vitamin E and glutathione radical, and restore these antioxidants. Vitamin C itself is voluntarily regenerated from Asc• with NADH and/ or NADPH dependent reductases (Hossain, M.A. *et al.,* 1985). Many antioxidants may directly react with free radical intermediates induced by ROS and terminate the chain reaction, thus stopping the ROS-induced damage (DeFeudis, F.V.*et al.,* 2003). Vitamin C is a potent antioxidant free radical scavenger, be able to scavenge free radicals straight and assist in neutralizing physiological oxidant load produced by exogenous as well as endogenous sources (Rai, R.R., *et al.,* 2006).

In the present study MDA level correlated with serum cotinine in tobacco chewers. Serum vitamin C concentration showed a significant negative correlation with MDA and serum cotinine. The presence of elevated oxidative stress in tobacco chewers seems to be linked with the intensity of complication. The present study is undertaken to evaluate serum cotinine as a biomarker of tobacco consumption, MDA as a biomarker of oxidative stress and serum vitamin C as a biomarker of non-enzymatic antioxidant status in tobacco chewers. The

present study was performed with an objective to assess the effect of vitamin C supplementation on serum cotinine, lipid peroxidation and serum vitamin C levels in smokeless tobacco users. In addition, we have assessed the effects of tobacco chewing duration in years on the above-said parameters in tobacco chewers and compare the findings with tobacco non-chewers.

MATERIALS AND METHODS

The present interventional and comparative study was conducted in the Krishna Institute of Medical Sciences Deemed to be University, Karad, for the duration of 2015–2018. The study protocol was approved by the Ethical Committee of KIMSDU, Karad. Healthy smokeless tobacco users and tobacco non-chewers (controls) of aged 31 to 60 years were selected for the study. The participants having any serious disorder like hypertension, cardiovascular diseases, cancer, any other marked disability or any major endocrinological disorders were excluded from the study. An experimental protocol was explained to all selected participants, and written consent was received from participants articulated willingness to participate in the present study. Complete medical history, family history and personal history with reference to the tobacco-chewing history at present and past was noted in planned pro-forma.

Inclusion criteria

Study group (Tobacco chewers): Adults of the age group of 31 to 60 years who had chewing exclusively smokeless tobacco (dried tobacco leaves crushed with lime paste) at least from last one year or more, non-alcoholic, not suffering from any chronic disease and any regular long time medication.

Healthy controls (Tobacco non-chewers): Age and sex-matched healthy individuals were selected, who had never chewed or smoked any form of tobacco and non-alcoholic in their past life and not suffering from any chronic disease.

Exclusion criteria

Subjects with any type of cardiovascular disease, autonomic dysfunction, endocrinological disorder, metabolic disorder, liver disease, any type of cancer, neurological disorder, any type of a long time and regular medication and alcoholic individuals will be excluded from the present study.

Total of 338 individuals participated in the study. They were classified into smokeless tobacco users (study group) and tobacco non-users (control group). Tobacco users were further distributed into subgroups with respect to tobacco chewing period in years. All the participants' blood was examined for serum cotinine, MDA and serum vitamin C at the study baseline. Study participants enrolled in the study received 90x 500 mg of vitamin C tablets (Celin, Glaxo SmithKline) daily twice for 45 days. The diet, treatment, and physical activity of the study individuals remained unchanged during the course of study. Participants' compliance with the prescribed medicine was monitored by an incessant contact by cell phone. After 45 days of intervention with 1 gm vitamin C, participants were examined, and the tests were repeated.

Blood Collection

Total 5ml venous blood was collected after an overnight fast, in a clot bulb. After a one-hour blood sample was centrifuged at 3500 rpm. Serum was used to analyzed for serum cotinine, MDA and vitamin C concentration.

Investigations

Serum cotinine concentration was determined by cotinine ELISA Calbiotech method on Elisa Reader (calbiotech.com/products/elisa-kits, Accessed, 2013). Principle: The serum and cotinine enzyme conjugate were added to the anti-cotinine antibody-coated wells. Cotinine in the serum competes with a cotinine enzyme conjugate for binding sites. Unbound cotinine and cotinine enzyme conjugate was washed out by washing step. After the addition of the substrate, the color intensity was inversely proportional to the concentration of cotinine in the serum sample. A standard curve was prepared relating color intensity to the concentration of cotinine. Serum cotinine level was measured in ng/ml on the Elisa reader. Serum malondialdehyde estimated by Kei Satoh Method (Ganesh Ghanwat *et al.,* 2015). Trichloroacetic acid and thiobarbituric acid were added to serum, and the mixture was heated to boil in boiling water bath for 30 minutes. The resulting chromogen was extracted with n-butyl alcohol, and the absorbance of the organic phase was determined at the wavelength of 530 nm. The determined results were expressed in terms of malondialdehyde (nmol/ml) which used as a reference standard. Serum vitamin C level determined by Human vitamin (VC) ELISA Kit (Catalog #: BC-EH103623) method (Human Vitamin C (VC) ELISA Kit. Catalog#: BC-EH10362. BICODON Technologies, 2014). Principle: Serum added to pre-coated wells. Anti VC monoclonal antibody with biotin and streptavidin. HRp added to plate resulting in the formation of an immune complex. The unbound enzyme was removed by washing. After incubation substrate, A and B were added. The solution changes to blue and then to yellow with the effect of acid. The colour of the solution and the conc. of vitamin C are directly proportional.

Statistical analysis

Data (min ± SD, min, max) were reported for each study variable. Comparison of study variables between tobacco chewers and tobacco controls were done by using unpaired 't' test. ANOVA test was used for comparison of inter-groups of study variables. In the case of significant F value, Tukey Kramer multiple comparison tests, the post-hoc test were applied, $p < 0.05$ was considered as significant.

RESULTS

Demographic comparison among tobacco non-chewers (controls) and tobacco chewers (study) group were represented in table 1. Age and anthropometric measurement were found to be similar in both groups.
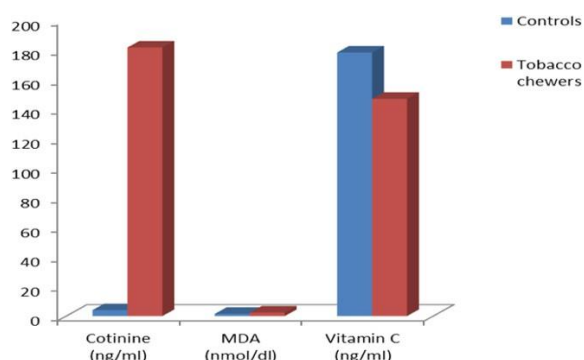
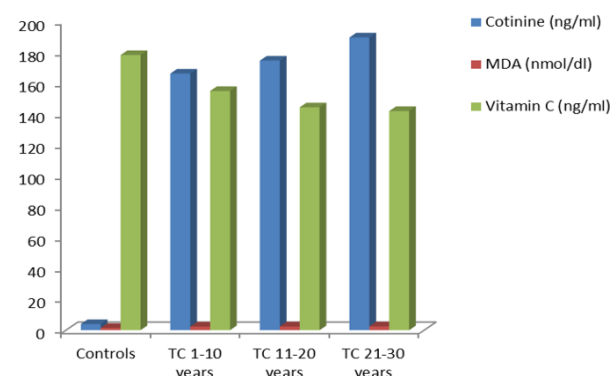**Figure 1: Levels of serum cotinine, MDA and serum vitamin C in controls and chewers**

Table 2 and Figure 1 represented a comparison of serum cotinine, MDA and vitamin C between tobacco non-chewers and tobacco chewers. There was significantly increased concentration of serum cotinine (<0.001), MDA (<0.001), however significantly decreased the concentration of serum vitamin C (<0.001) found in tobacco hewers as compared to tobacco non-chewers.
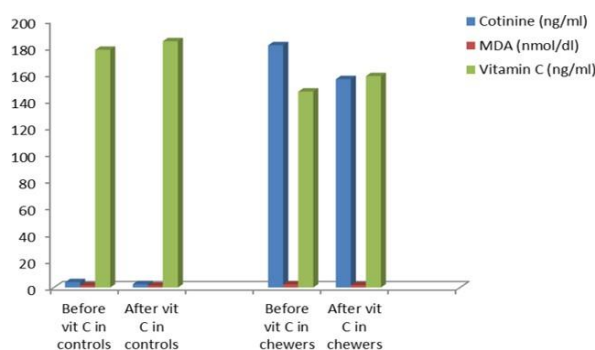
Figure 2: Comparison of serum cotinine, MDA and vitamin C in controls and tobacco chewers before and after supplementation of vitamin C

Table 3 and Graph 2 represented the effect of vitamin C supplementation on mean levels of serum cotinine, MDA and serum vitamin C in both the groups (controls and chewers). There was found to

be significantly decreased levels of serum cotinine (p<0.001) in both the groups, MDA (p<0.001) in both the groups, however, serum vitamin C levels (p<0.001) were significantly increased in both the groups after supplementation of vitamin C as compared to before.

Figure 3: Tobacco chewing duration wise effect on serum cotinine, MDA, Vitamin C in tobacco chewers

Comparison of serum cotinine, MDA and vitamin C according to tobacco chewing duration of 1-10, 11-20 and 21-30 years were represented in Table 4 and Graph 3. We found significantly increased levels of serum cotinine (p<0.001) in all groups, MDA (p<0.001) in all groups, however significantly decreased levels of serum vitamin C (<0.001) in all tobacco chewer groups when compared to tobacco non-chewers.

Table 5 represented correlation of serum cotinine, MDA and vitamin C in controls and tobacco chewers before and after supplementation of vitamin C. Serum cotinine was significantly negatively correlated with vitamin C (<0.01) in tobacco chewers before supplementation and significantly positive correlated with MDA (<0.01) in tobacco chewers before and after supplementation whereas, no significant correlations observed in controls. There were no significant correlations found between vitamin C and MDA in both groups.

DISCUSSION

The significant negative correlations between serum cotinine and MDA with vitamin C reveals about the negative impact of reactive oxygen species on the bioavailability of vitamin C. Oxidative stress might be a common pathway linking varied mechanism meant for the pathogenesis of diseases. ROS be capable for directly damage proteins and lipids as well as alter intracellular signal pathways, such as protein kinases and redox-sensitive transcription factors causing an alteration in protein expression and as a result, irreparable oxidative damage (Fiorentino, T.V. *et al.,* 2013). In this study serum cotinine (<0.001) concentration was found significantly increased in tobacco chewers than

Table 1: Demographics of tobacco non-chewers (controls) and tobacco chewers

Parameters	Control (N=170)	Tobacco chewers (N=168)	Unpaired 't' test (p-value)
Age (years)	44.80±8.56	44.64±8.78	0.16 (0.86)
Height (m)	1.64±0.07	1.65±0.05	1.90 (0.06)
Weight (kg)	63.74±9.49	65.41±10.98	1.50 (0.130)
BMI	23.68±3.03	23.89±3.80	0.56 (0.57)

*p<0.5, **p<0.01, ***p<0.001 compared with controls

Table 2: Mean and SD of serum cotinine, MDA, vitamin C in controls and tobacco chewers

Parameters	Controls Mean±SD (Min-Max) (N=170)	Tobacco chewers Mean±SD (Min-Max) (N=168)	Unpaired 't' test (p-value)
Serum cotinine (ng/ml)	4.00±2.64 (0.00-10.00)	181.55±99.33*** (21.00-603.00)	23.29 (<0.001)
MDA (nmol/dl)	1.50±0.40 (0.87-3.00)	2.37±0.83*** (0.89-4.99)	12.21 (<0.001)
Vitamin C (ng/ml)	178.17±28.64 (96.00-268.00)	146.88±33.06*** (46.90-260.20)	9.30 (<0.001)

*p<0.5, **p<0.01, ***p<0.001 compared with controls

Table 3: Mean and SD of serum cotinine, MDA and vitamin C in controls and tobacco chewers before and after supplementation of vitamin C

Parameters	Tobacco non chewers (Controls)		
	Before N=170	After N=170	Paired 't' test (p value)
Sr. Cotinine (ng/ml)	4.00±2.64 (0.00-10.00)	2.50±1.56*** (0-8.00)	8.62 (<0.001)
MDA (nmol/dl)	1.50±0.40 (0.87-3.00)	1.37±0.33*** (0.76- 2.60)	7.81 (<0.001)
Vitamin C (ng/ml)	178.17±28.64 (96.00-268.00)	184.60±30.52*** (108.00- 274.00)	5.89 (<0.001)

*p<0.05, **p<0.01, ***p<0.001 compared with before treatment of vitamin C

Table 3: Mean and SD of serum cotinine, MDA and vitamin C in controls and tobacco chewers before and after supplementation of vitamin C (Contd....)

Parameters	Tobacco chewers (Subjects)		
	Before N=168	After N=168	Paired 't' test (p value)
Sr. Cotinine (ng/ml)	181.55±99.33 (21.00-603.00)	156.17±82.56*** (18.00- 541.00)	7.86 (<0.001)
MDA (nmol/dl)	2.37±0.83 (0.89-4.99)	1.76±0.52*** (0.68- 3.22)	12.53 (<0.001)
Vitamin C (ng/ml)	146.88±33.06 (46.90-260.20)	158.44±32.47*** (68.60- 264.90)	9.64 (<0.001)

*p<0.05, **p<0.01, ***p<0.001 compared with before treatment of vitamin C

Table 4: Tobacco chewing duration wise effect on serum cotinine, MDA and Vitamin C in tobacco chewers as compared to controls

Para meters	Control (N=170)	Study group (tobacco chewing duration in years)			ANOVA F value (p-value)
		1-10 years (N=50)	11-20 years (N=81)	21-30 years (N=37)	
Sr.cotinine (ng/ml)	4.00±2.64 (0.00-10.00)	166.08±116.05*** (28.0-603.0)	174.48±89.21*** (24.0-457.0)	189.46±96.33*** (21.0-417.0)	169.92 (<0.001)
MDA (nmol/dl)	1.50±0.40 (0.87-3.00)	2.33±0.77*** (0.92-4.15)	2.42±0.88*** (0.89-4.99)	2.48±0.82*** (1.08-4.90)	54.73 (<0.001)
Vitamin C (ng/ml)	178.17±28.64 (96.00-268.00)	154.89±32.98*** (46.9-211.4)	144.24±29.51*** (56.4-197.2)	141.82±39.14*** (58.6-260.2)	30.74 (<0.001)

*p<0.05, **p<0.01, ***p<0.001 compared with control

Table 5: Correlations between serum cotinine, MDA and vitamin C in controls and tobacco chewers before and after supplementation of vitamin C

| | Treatment of vitamin C | Tobacco non-chewers (controls) N=170 | | | | Tobacco chewers (Subjects) N= 168 | | | |
| | | Cotinine | | Vitamin C | | Cotinine | | Vitamin C | |
		r value	p value	r value	p value	r value	p value	r value	p value
Cotinine	Before	--	--	0.09	0.20	--	--	- 0.26**	<0.01
	After	--	--	- 0.003	0.96	--	--	- 0.07	0.30
MDA	Before	0.02	0.79	0.04	0.56	0.41**	<0.01	- 0.87	0.26
	After	-0.09	0.23	0.003	0.96	0.31**	<0.01	0.02	0.76

*p<0.05, **p<0.01, ***p<0.001 compared with other parameter

tobacco non-chewers. Cotinine is the chief metabolite of nicotine and rapidly absorbed in the bloodstream. Cotinine increases oxidative stress in vivo as well as in vitro to causes a peroxidant/antioxidant discrepancy in blood and tissues (Geier, D.A. *et al.,* 2009). Oxidative stress generates free radicals to facilitate an attack on the membrane lipids ensuing in the formation of malondialdehyde (MDA), which causes peroxidative tissue damage (Srinivasan, K.N. *et al.,* 2000).

In our study serum MDA level was significantly higher (P<0.001) in tobacco chewers as compared to tobacco non-chewers. These results are in agreement with the prior studies, showing increased MDA level among smokers (Block, G. *et al.,* 2002, Abdul-Rasheed, O.F, *et al.,* 2013). In the previous study (Chole, R.H. *et al.,* 2010) reported the relationship of lipid peroxidation with the practice of either chewing tobacco or smoking in the controls. In another study (M. Bagchi. *et al.,* 1994) results supported the hypothesis that an aqueous extract of smokeless tobacco (STE) induces the production of reactive oxygen species. Taken collectively with prior studies, the results specify that STE may act at various sites. The reactive oxygen species which are produced may lead to increased lipid peroxidation plus further tissue-damaging effects such as single-strand breaks of DNA, causative to the cytotoxicity of STE. In the present study, tobacco chewers did not differ from tobacco non-chewers in age, so the effect of age on serum MDA levels was omitted. To get rid of the effect of alcohol intake and gender, the individuals consuming alcohol were excluded and all the individuals included in the study were males. The increased level of MDA in tobacco chewers straight reflects elevated oxidative stress as well as lipid peroxidation, which might be due to an interaction of a variety of carcinogenic agents, generating free radicals to a larger amount in tobacco chewers ahead of their protecting capacity or might be due to reduced antioxidant organization accessible in these persons.

In the present study serum, vitamin C (<0.001) level was found to be significantly decreased in tobacco chewers when compared with the controls. Vitamin C is a non-enzymatic free radical scavenger, can scavenge reactive oxygen species and helps to neutralize physiological oxidant burden formed by both exogenous and endogenous sources (Sahin, U., *et al.,* 2001). Vitamin C serves as an antioxidant by protecting other substances from being oxidized by donating its own electrons. However, in this course, vitamin C oxidized itself. The complex produced after the loss of electron is ascorbyl radical which is fairly stable for the half-life of 5-10 seconds and is quite nonreactive (Padayatty, S.J. *et al.,* 2003). The mechanism involved in the decrease of vitamin C level in tobacco chewers might be due to quick oxidation of vitamin by free radicals. The negative association between vitamin C and MDA may be due to the exhaustion of vitamin C when the oxidant load is elevated.

In our study serum cotinine, MDA levels were gradually increased with respect to prolonged tobacco consumption period in years in tobacco chewer groups whereas serum vitamin C level was progressively and gradually decreased with respect to increased tobacco chewing duration, but the changes were not significant. Our findings are in agreement with the previous study (Calikoglu, M. *et al.,* 2002). Another previous study suggested that comparable to the smoking; smokeless tobacco also increased blood nicotine level. Moreover, due to prolonged absorption, elevated levels of nicotine are achieved for a longer duration (Jaywant Thorat *et al.,* 2015). It was observed that the changes become further prominent with increased tobacco consumption period.

In addition, it was observed that daily intake of 1 gm vitamin C for 45 days resulted significantly reduced serum cotinine (<0.001) levels in both (control and chewers) groups, MDA (<0.01) in both the groups, however serum vitamin C (<0.001) level was significantly increased in both the groups. In previous work, 500 mg of vitamin C used to investigate its inhibitory effect on oxidative damage in

smokers (Lee, B.M., *et al.,* 1998). Several studies suggested that ant cancerous effects of vitamin C are elucidated when used at least 80-110 mg of vitamin C per day (Carr, A.C. and Frei, B., 1999). We used 1 gm of Vitamin C supplementation. This amount is a smaller than acceptable higher consumption level for vitamin C which is 2 gms/day (Weinstein, M. *et al.,* 2001). Non-enzymatic antioxidants are reduced in pan masala tobacco users with consequent changes in the biochemical parameters (Raj, Shrestha., *et al.,* 2012). In another study, the researcher demonstrates the elevated oxidative load in smokers than to non-smokers (Nagaraj. *et al.,* 2014). The oxidative stress was increased according to the amount of smoking. This study also demonstrates the lowered antioxidant level in tobacco chewers as compared to controls. Intake of antioxidants might prevent oxidative stress in tobacco consumers. This may suggest that daily intake of vitamin C is beneficial for decrease serum cotinine level as well as restore the oxidant-antioxidant balance in tobacco chewers.

In the present study, significant negative correlation ($p<0.01$) was found between serum cotinine and serum vitamin C, before supplementation of vitamin C whereas, no significant correlation found after supplementation of vitamin C in tobacco chewers. It suggests that increased serum cotinine concentration depleted serum vitamin C level. A significant positive correlation ($p<0.01$) was found between serum cotinine and MDA before and after supplementation of vitamin C in tobacco chewers, it may indicate that increased concentration of serum cotinine, which may enhance reactive oxygen species and in turn serum MDA level. There were no significant correlations found between above said all parameters in tobacco non-chewers (controls). Previous similar studies reported positive correlations in cotinine and lipid peroxide while negative correlations of cotinine and enzymatic antioxidants (Begum, S.F. *et al.,* 2018, Augusta, N., *et al.,* 2018). There was a significant increase in serum cotinine and MDA concentration, however significant decrease in vitamin C level in tobacco chewers than to controls. It shows positive correlations of serum cotinine and MDA whereas negative correlations of cotinine and serum vitamin C. The oxidative stress level was elevated however serum vitamin C level was depleted in accordance to increased tobacco chewing duration in years. Daily intake of 1gm vitamin C is may be beneficial for restoring the balance between oxidative stress and oxidants level in tobacco chewers.

CONCLUSION

The present study concludes that smokeless tobacco chewers are at elevated risk of free radical damage and increase of lipid peroxidation products. Non-enzymatic antioxidants are depleted with a resulting increase in the concentration of MDA in tobacco chewers. Exhaustion of antioxidants is risk-factors for cardiovascular diseases, cancer and other several diseases. Awareness programs concerning the harmful effects of tobacco and its products should be conducted in populations. Antioxidants levels and lipid peroxidation products should be regularly monitored in people consuming tobacco and antioxidants should be supplemented regularly through diet which may positively aid to prevent the oxidative stress caused by tobacco use.

Limitations of the study

This is a small scale study, which does not reflect the actual population and direction pattern in the whole country. However, to substantiate such findings thorough and large scale research is required.

Future Scope

In future detailed and large scale studies investigating nicotine inducible cellular mechanism for understanding the complex pathophysiology of smokeless tobacco and oxidative stress and CVD. Prevention of tobacco use could be an important step in preventing constant amplifies in CVD that is a threat to the worldwide population.

Acknowledgement

Conflict of Interest: The authors insist that they have no conflict of interest.

Abbreviations: CVD- Cardio Vascular Disease; MI- Myocardial Infarction; MDA- Malondialdehyde; RNS- Reactive Nitrogen species; ROS- Reactive Oxygen species; ST- Smokeless Tobacco; STE- Smokeless Tobacco Extract

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