Effect of vitamin C supplementation on autonomic function tests in smokeless tobacco chewers

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Article History:
Received on: 07 Feb 2020
Revised on: 10 Mar 2020
Accepted on: 17 Mar 2020

Keywords:
Cotinine,
Sustained hand grip,
Sympathetic nervous system,
Valsalva ratio

ABSTRACT

According to World Health Organization (WHO) report, tobacco is the single greatest cause of preventable death all over the world. Use of Smokeless Tobacco (ST) increases level of nicotine and autonomic effects throughout the day that are similar to smoking. Aim of the present study was to evaluate the effect of ST on valsalva ratio, Sustained Handgrip test (SHG), serum Cotinine (CTN) level and serum Vitamin C (VC) in ST chewers. Total 338 participants aged between 31 to 60 years were divided into two groups, ST chewers and ST non chewers (controls). ST chewers were further classified with respect to Smokeless Tobacco Chewing (STC) duration (years), frequency (times/day) and quantity (/ day). Participants were asked to consume 1000 mg of vitamin C for 45 days. Valsalva ratio, SHG, serum CTN and serum VC levels were measured at the baseline study and after supplementation of VC. Valsalva ratio (p= 0.006), DBP rise in SHG (p=0.002) and serum VC (p<0.001) levels were significantly decreased however, serum CTN (p<0.001) level was significantly increased in ST chewers as compared to controls. VC supplementation did not show any significant change in valsalva ratio and SHG however, serum CTN (p<0.001) level was significantly decreased and serum VC (p<0.001) level was significantly increased in ST chewers as compared to baseline values. Increased STC duration, frequency and quantity did not show any significant change in valsalva ratio and DBP rise in SHG in ST chewers. The use of ST is associated with decreased sympathetic activity and serum VC level as well as increased serum CTN level in tobacco chewers as compared to controls.

INTRODUCTION

According to World Health Organization (WHO) report, tobacco is the single greatest cause of preventable death all over the world (WHO, 2008). Smokeless tobacco (ST) contains nicotine, which is highly addictive (WHO, 2007). People who consume ST can become addicted to nicotine that might be more likely to become cigarette smokers (Lund and Scheffels, 2014). The various forms of tobacco are smoked, chewed, sniffed or applied to teeth and gums (NSS, 2000). ST products include chewing tobacco, snuff, snus and dissolvable tobacco products (Czoli et al., 2017). Slaked lime is prepared by calcium hydroxide which is obtained from limestone. The mixing of such slaked lime increases the pH of a tobacco and enhanced free nicotine absorption by oral mucosa (Bhisey, 2012).

Global Adult Tobacco Survey-2 (GATS-2) reports that 28.6% of the people consume tobacco in different forms, 10.7% smoking and 21.4% use ST (GATS,
Khaini (11%) and beedis (8%) are the leading forms of tobacco used in India (GATS, 2017). Tobacco consumption annual growth rate is 2% to 3% (WHO, 1997). Smoked form was consumed by 14% of the population (Pawar et al., 2014). Indian men smoke about 6.2 cigarettes per day, this is the lowest of all countries in the world, but among women, the mean cigarettes per day quite high, about 7, higher than the average of men, which was 6.2 (WHO, 2016). ST use is documented in 120 countries of the world (Sinha et al., 2016). India has the largest number of ST users in the world. Of the 346 million worldwide tobacco users, India alone has 152.4 million people and out of that 80.8 million ST users (US NCI and WHO, 2016). There has been considerable rise in ST users among all age groups (Bhan et al., 2016).

The financial burden due to tobacco-related diseases in India was US$ 22.4 billion in year 2011-2012 (MoHFW, 2014). In India, the burden of cardiovascular diseases (CVD), cancers, respiratory diseases, and tuberculosis, is extremely high (Mangesh Pednekar et al., 2016). Mainly, India has highest oral cancer percentage in the world (Rani, 2003). Tobacco is responsible for 90% of oral cancer cases, 46 to 52 % caused due to use of ST (Boffetta et al., 2008).

The cardiovascular system is predisposed by the autonomic nervous system (ANS) (Amrith and CP, 2013). Various studies as well documented on probable acute autonomic effects and hemodynamic changes in tobacco users (Wolk et al., 2005). CVD is the leading cause of death globally, accountable for over 17 million deaths worldwide and tobacco has been expected to directly cause 10% of all CVD (Piano et al., 2010). Since, VC is a natural antioxidant and its action has been studied because of its various important biological effects (Franke et al., 2005). We had supplemented vitamin C to assess the beneficial effects on ANS in ST chewers. The aim of present study is to produce the existing knowledge on ST use in this part of India, with a view to assess the extent of the hazardous health effects of ST use along with evaluation of beneficial effects of supplementation of VC to tobacco chewers.

**MATERIALS AND METHODS**

The present study was carried out in the Krishna Institute of Medical Sciences, Karad during 2016-2019. Study protocol was approved by Institutional Ethical Committee of KIMS DU, Karad (Ref: KIMS DU/IEC/01/2015, Dated: 05/03/2015). Total of 338 subjects, study group (ST chewers) and control group (ST non chewers) enrolled in the study. Participants from both the groups were divided into 3 sub groups according to their age (31-40 yrs, 41-50 yrs and 51-60 yrs).

Each subject was asked to provide information about data demographic, health and tobacco chewing habit. Tobacco chewing habits included smokeless tobacco chewing (STC) duration in yrs, STC frequency/day and STC quantity/time, medical history, family history and personal history with particular reference to the history of tobacco consumption at present and past was recorded in structured proforma. The objectives of study were explained to the participants, after knowing their willingness to participate in the study and written informed consent was obtained.

**INCLUSION CRITERIA**

**ST chewers**

Individuals aged between 31 to 60 yrs who had chewing exclusively smokeless tobacco (dried tobacco leaves mixed with lime paste) at least for last one year and/or more.

**ST non chewers**

Age and sex matched volunteers were selected, who had never chewed or smoked any form of tobacco in their past life.

**EXCLUSION CRITERIA**

Individuals with any type of CVD, autonomic dysfunction, endocrinological disorder, metabolic disorder, liver disease and any type of cancer, neurological disorder, any type of long time and regular medication and alcoholic individuals were excluded from the study.

**Collection of Blood Sample**

2-3 ml of venous blood sample was collected after 12-14 hrs overnight fast from each individual in plain bulb. After one hour, serum was separated and used for estimation of serum cotinine and vitamin C level.

**PARAMETERS**

Valsalva manoeuvre (valsalva) - parasympathetic function test

Valsalva manoeuvre (forced expiration against resistance) is a non invasive and simple test done to assess baroreceptor integrity.

**Principle**

The Valsalva manoeuvre is performed by attempting to forcibly exhale while keeping the mouth and nose closed. The subject is asked to Valsalva for 10-15 seconds. After that the subject is asked to hold the breath for 10-15 seconds. The ratio of the frequency of heart beats to the frequency of Valsalva manoeuvre is measured and recorded. The ratio is calculated by dividing the number of heart beats by the number of Valsalva manoeuvres. The ratio is then compared to the normal values for the age and sex of the subject. A ratio of less than 3 is considered abnormal and is indicative of autonomic dysfunction.
closed. It is used as a diagnostic tool to evaluate the condition of heart.

Valsalva Ratio is taken as ratio of maximum HR during the strain (phase 2) to the minimum heart rate after the strain (phase 4). Alternatively, it can also be calculated as - Longest R-R interval after the strain (phase 4) / Shortest R-R interval during the strain (phase 2). A ratio greater than 1.45 is normal; 1.20-1.45 is border line; and less than 1.20 is abnormal (Jain, 2015).

**Sustained handgrip test**

Isometric Exercise (sympathetic function test).

**Principle**

Sustained hand-grip against resistance causes an increase in heart rate and blood pressure. These responses are detected using ECG and blood pressure monitors.

The value of more than 15 mmHg rise in diastolic BP is taken as normal response, 11-15 mmHg as borderline and less than 10 mmHg or less is abnormal, an indicator of sympathetic insufficiency (Pal, 2013).

Serum cotinine level was estimated by cotinine Elisa Calbiotech kit method on Elisa reader.

**Principle**

The serum and cotinine enzyme conjugate are added to the wells coated with anti-cotinine antibody. Cotinine in the serum competes with a cotinine enzyme conjugate for binding sites. Unbound cotinine and cotinine enzyme conjugate is washed out by washing step. After the addition of the substrate, the intensity of color is inversely proportional to the concentration of cotinine in the samples.

Serum vitamin C level was estimated by the Biocodon Elisa kit method on Elisa reader.

**Principle**

The Biocodon Elisa kits are precoated with capture primary antibody. VC Elisa kit contains precoated human VC monoclonal antibody. Samples were added in precoated wells and incubate. Post incubation anti VC antibody with streptavidin HRP were added to plate resulted formation of immune complex. Unbound enzymes were removed by washing. After incubation substrate A and B were added. The solution was turn blue and change to yellow with the effect of acid. The color of solution and the concentration of vitamin C are positively correlated.

All study participants, ST chewers and controls were asked to consume 1000 mg of VC (Celin tablets) after meal respectively for 45 days. Subjects of both the groups were examined at the baseline study and after supplementation of VC for serum CTN, serum VC, valsalva ratio and DBP rise in SHG after supplementation of VC. The data obtained from both study groups were analyzed and compared by using suitable statistical test.

**RESULTS AND DISCUSSION**

In the present study, valsalva ratio (p=0.006) was significantly decreased in ST chewers group as compared to controls (Table 1). After supplementation of VC, there was not found any significant change for valsalva ratio in both, ST chewers and controls group (Table 2). According to age groups, valsalva ratio (p=0.006) was significantly decreased in ST chewers group (age 51-60 years) as compared to age matched group of controls (Table 3). Comparison of STC with respect to duration, frequency and quantity, valsalva ratio did not show any significant changes in all ST chewer groups as compared controls as well as other ST chewer groups (Table 4).

While in ST chewers, valsalva ratio (p<0.01 and 0.009) showed significant and positive correlation with serum CTN at baseline study and after VC supplementation. However, valsalva ratio (p=0.002 and p<0.001) showed significant and negative correlation at the baseline study and after VC supplementation in ST chewers (Table 5).

Valsalva ratio is dependable indicator of parasympathetic activity which is accountable for recovery of heart rate after strenuous activity similar to Valsalva maneuver. Valsalva maneuver creates an elevated intrathoracic pressure which evokes a complex circulatory response with four different phases. Present study showed that tobacco chewers have lower value of Valsalva ratio as compared to controls, representing derangement of parasympathetic function. Similar findings were observed in the previous studies (Kotamäki, 1995) and (Manzano et al., 2011).

In the Sustained Hand Grip test (SHG), we observed, rise in the DBP at the point, just before the release of the handgrip. DBP rise in SHG (p=0.002) was significantly reduced in tobacco chewers as compared to controls (Table 1). However, supplementation of VC showed insignificant changes for DBP rise in SHG in both controls and tobacco chewers (Table 2). According to age group (31-40 yrs, 41-50 yrs and 51-60 yrs), DBP rise in SHG (p=0.009, 0.003 and <0.001) was significantly decreased in chewer groups as compared to controls (Table 3). With respect to STC duration and frequency, there was not any insignificant change observed in DBP rise in SHG in all ST chewer groups as compared to controls (Table 4). Comparison of STC with respect to quantity (p>1 gm/ time), DBP rise in SHG (p=...
### Table 1: Comparison of Mean and SD of valsala ratio, SHG, sr. CTN and sr. VC between controls and ST chewers

| Parameters               | Controls (N=170) Mean±SD | ST chewers (N=168) Mean±SD | Unpaired t test | p value |
|--------------------------|---------------------------|----------------------------|-----------------|---------|
| Valsalva Ratio           | 1.480±0.09                | 1.451±0.06                 | 3.48            | 0.006   |
| SHG (DBP rise in mmHg)   | 18.97±4.68                | 17.56±3.69                 | 3.07            | 0.002   |
| Sr. CTN (ng/ml)          | 4.00±2.64                 | 181.55±99.33               | 23.29           | <0.001  |
| Sr. VC (ng/ml)           | 178.17±28.64              | 146.88±33.06               | 9.30            | <0.001  |

### Table 2: Comparison of Mean and SD of valsala ratio, SHG, sr. CTN and sr. VC before and after supplementation of vitamin C in controls and ST chewers.

| Parameters               | Controls (ST non chewers) | ST chewers | Paired t test (p value) | Paired t test (p value) |
|--------------------------|---------------------------|------------|-------------------------|-------------------------|
|                          | Before VC                 | After VC   |                          |                         |
|                          | N=170                     | N=168      |                         |                         |
| Valsalva Ratio           | 1.480±0.09                | 1.464±0.09 | 1.63                    | (0.10)                  |
| SHG (DBP rise)           | 18.97±4.68                | 17.86±7.45 | 0.72                    | (0.46)                  |
| Sr. CTN (ng/ml)          | 4.00±2.64                 | 181.55±99.33 | 7.86                  | (<0.001)                |
| Sr. VC (ng/ml)           | 178.17±28.64              | 146.88±33.06 | 9.64                  | (<0.001)                |

### Table 3: Comparison of physiological parameters (Mean±SD) with respect to age matched groups between controls and ST chewers.

| Parameters               | Age Group 31 to 40 years | Age Group 41 to 50 years | Age Group 51 to 60 years |
|--------------------------|--------------------------|--------------------------|--------------------------|
|                          | Controls (N=57)          | ST chewers (N=56)        |                          |
| Valsalva Ratio           | 1.481±0.07               | 1.468±0.05               | 1.13                     | 0.25   |
| SHG (mmHg)               | 21.12±5.62               | 17.98±4.03               | 3.40                     | 0.009  |
| Sr. CTN (ng/ml)          | 4.70±2.90                | 178.21±116.9             | 11.20                    | <0.001 |
| Sr. VC (ng/ml)           | 184.98±26.87             | 150.62±32.70             | 6.10                     | <0.001 |

|                          | Age Group 41 to 50 years | Age Group 51 to 60 years |
|--------------------------|--------------------------|--------------------------|
| Valsalva Ratio           | 1.479±0.10               | 1.464±0.06               | 0.97                     | 0.33   |
| SHG (mmHg)               | 20.03±3.86               | 17.89±3.75               | 3.00                     | 0.003  |
| Sr. CTN (ng/ml)          | 3.78±2.54                | 171.95±87.57             | 14.36                    | <0.001 |
| Sr. VC (ng/ml)           | 177.55±28.76             | 147.57±30.78             | 5.30                     | <0.001 |

|                          | Age Group 51 to 60 years |
|--------------------------|--------------------------|
| Valsalva Ratio           | 1.476±0.09               | 1.421±0.07               | 3.55                     | 0.006  |
| SHG (mmHg)               | 20.25±4.37               | 16.81±3.29               | 4.67                     | <0.001 |
| Sr. CTN (ng/ml)          | 3.50±2.33                | 194.09±91.36             | 15.74                    | <0.001 |
| Sr. VC (ng/ml)           | 171.96±29.22             | 142.53±35.53             | 4.28                     | <0.001 |
### Table 4: Comparison of Mean and SD of Valsalva ratio, SHG, sr. CTN and SR. VC between controls and ST chewers with respect to tobacco chewing duration, frequency and quantity

| Parameters                  | Controls (N=170) | ST chewers with respect to tobacco chewing duration in years (N=50) | ST chewers with respect to tobacco chewing duration in years (N=81) | ST chewers with respect to tobacco chewing duration in years (N=37) | ANOVA F value (p value) |
|-----------------------------|------------------|-------------------------------------------------------------------|-------------------------------------------------------------------|-------------------------------------------------------------------|-------------------------|
|                             |                  | 1-10 years | 11-20 years | 21-30 years |
| Valsalva Ratio              | 1.480±0.09       | 1.460±0.10| 1.452±0.08| 1.501±0.10| 0.000 (0.99) |
| SHG (DBP rise)              | 18.97±9.28       | 17.09±4.19| 18.15±3.45| 17.44±3.74| 1.17 (0.31) |
| Sr. CTN (ng/ml)             | 4.00±2.64        | 166.08±116.05***| 174.48±89.21***| 189.46±96.33***| 169.92 (<0.001) |
| Vitamin C (ng/ml)           | 178.17±28.64     | 154.89±32.98***| 144.24±29.51***| 141.82±39.04***| 30.77 (<0.001) |
|                             |                  | 1-4 times/day | 5-8 times/day | above 9 times/day |
| Valsalva Ratio              | 1.480±0.09       | 1.468±0.10| 1.450±0.11| 1.485±0.08| 1.97 (0.11) |
| SHG (DBP rise)              | 18.97±9.28       | 18.50±3.86| 17.90±3.64| 16.28±3.57| 2.04 (0.10) |
| Sr. CTN (ng/ml)             | 4.00±2.64        | 135.83±78.96***| 181.48±93.77***| 221.43±1.08***| 216.54 (<0.001) |
| Vitamin C (ng/ml)           | 178.17±28.64     | 151.25±27.23***| 143.40±30.5***| 137.28±35.62***| 39.04 (0.001) |
|                             |                  | <0.5 gm/time | 0.5-1.00 gm/time | >1.00 gm/time |
| Valsalva Ratio              | 1.480±0.09       | 1.473±0.09| 1.461±0.10| 1.479±0.11| 0.57 (0.63) |
| SHG (DBP rise)              | 18.97±9.28       | 18.89±3.85| 17.68±3.83| 16.11±3.42*| 2.66 (0.04) |
| Sr. CTN (ng/ml)             | 4.00±2.64        | 141.36±103.63***| 176.48±10.78***| 208.53±10.18***| 159.14 (<0.001) |
| Vitamin C (ng/ml)           | 178.17±28.64     | 154.95±37.29***| 146.48±32.36***| 137.98±34.52***| 31.09 (<0.001) |

*p<0.05, **p<0.01, ***p<0.001 as compared to control
Table 5: Correlations of serum cotinine and vitamin C with physiological parameters (r and p value) in controls and ST chewers before and after supplementation of vitamin C.

| Parameter   | Control (N=170) | ST Chewers (N=168) |
|-------------|-----------------|---------------------|
| Valsalva    |                 |                     |
| Before      | Sr. CTN R value | Sr. VC R value      |
|             | 0.08 0.26       | 0.07 0.36           |
| After       | -0.07 0.31      | -0.07 0.34          |
| SHG (DBP rise) | Sr. CTN R value | Sr. VC R value      |
| Before      | -0.12 0.10      | -0.14 0.06          |
| After       | -0.17 0.02*     | 0.05 0.49           |

*p<0.05, **p<0.01, ***p<0.001 significant correlations of sr. CTN and sr. VC with Valsalva ratio and SHG before and after supplementation of vitamin C.

0.04) was significantly decreased in ST chewers as compared to controls (Table 4). DBP rise in SHG (p=0.02) showed significant and negative correlation with serum CTN in controls after supplementation of VC whereas, DBP rise in SHG showed insignificant correlation with serum CTN in tobacco chewers both the times, at the study baseline and after supplementation of VC (Table 5). The results of present study were in accordance to the previous studies (Kotamäki, 1995) and (Motilal and Tayade, 2016), who also observed that DBP rise in SHG was significantly reduced in the smokers as compared to the non smokers, which suggests that, tobacco use decreases sensitivity of sympathetic nervous system. However, the previous studies, they reported the opposite results to present study, they found that, BP rise in SHG was significantly increased in the smokers as compared to non smokers (Modala et al., 2012) and Benowitz et al. (1988). This might be due to difference in study designs; sample size, study population and some other factors contributing in the variation in results.

In the present study, serum cotinine (CTN) level (p<0.001) was significantly increased in ST chewers as compared to controls (Table 1) however, serum CTN level (p<0.001) was significantly decreased after supplementation of VC in both the ST chewers and controls (Table 2). According to age matched group wise comparison, serum CTN (p<0.001) level showed significant rise in all tobacco chewer groups as compared to age matched controls however, serum CTN level did not show any significant change with increased age between chewers group (Table 3). Comparison of STC with respect to duration, frequency and quantity were represented in Table 4. Serum cotinine (p<0.001) level was significantly and progressively increased with respect to increased STC duration, frequency and quantity. It was suggested that prolonged, frequent and increased quantity of tobacco consumption causes increased impact on serum CTN level. Serum CTN (p<0.01) level showed significant and negative correlation with serum VC in tobacco chewers after supplementation of VC (Table 5). David Siegel reported that, serum CTN level was significantly higher in snuff and ST users and prolonged ST use has an adverse effect on cardiovascular risk factors (Siegel et al., 1992).

In the present study, serum VC (<0.001) level was found to be significantly decreased in ST chewers as compared to the controls (Table 1). Comparison with respect to age matched group, serum VC (p<0.001) level was progressively and significantly decreased with respect to increased age in ST chewers (Table 3). Serum VC (p<0.001) level was significantly and progressively decreased with respect to increased STC duration, frequency and quantity in ST chewers as compared to controls (Table 4). In our study, serum VC (p<0.001) level was significantly increased after supplementation of VC in ST chewers as well as in controls (Table 2). Serum VC (p<0.01) level showed significant and negative correlation with serum CTN level (Table 5).

The current literature supports VC benefits for prevention and treatment of various diseases. Various studies have shown that VC intake higher than the RDA enhances the immune system and reduces the risk of DNA damage (Padayatty et al., 2003). Thus, even if VC requirements differ greatly among individuals, it is suggested that VC supplementation is not only safe but also necessary to achieve optimal health (Deruelle and Baron, 2008). Therefore, in agreement with the current literature, our study advises ST chewers to consume 1000 mg of VC in addition to routine diet daily, in order to ensure an optimal allowance of VC.
CONCLUSION

In conclusion, use of the smokeless tobacco (ST) is significantly associated with traditional cardiovascular risk factors as detected by decreased sympathetic activity. ST use is associated with significantly increased level of serum cotinine and decreased level of serum vitamin C. Therefore, it should be accepted as harmful as cigarette smoking. Supplementation of vitamin C did not show any significant change in valsalva ratio and DBP rise in SHG. However, serum CTN level was found to be significantly decreased and serum VC level was significantly increased in ST chewers after supplementation of vitamin C.

ACKNOWLEDGEMENT

We would like to thank all the study participants and KIMS Deemed to be University who provided fund for conducting the present study.

Conflict of interest

The authors declare that they have no conflict of interest for this study.

Funding source

Krishna institute of Medical Sciences deemed to be University, Karad, India.

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