Study of salivary arecoline in areca nut chewers

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Abstract

Aims: Arecoline, a predominant alkaloid present in arecanut, has been implicated in the pathogenesis of several oral diseases because of its mutagenic and carcinogenic potential. The response of cultured cells to arecoline is highly dependent on its concentration; arecoline stimulates cultured cells above 0.1 µg/ml and is cytotoxic above 10 µg/ml. Although this alkaloid seems important for areca nut induced oral diseases and carcinogenesis, little is known of the levels achieved before, during and after chewing. Also, it is prudent to understand its effects in arecanut chewers for a comprehensive understanding of its pathogenesis. Accordingly, the present study quantified the salivary arecoline levels in arecanut chewers.

Materials and Methods: The study participants were divided into Study Group A & B and Control Group C; unstimulated whole saliva was collected by spitting method for a period of 5 min. Then, participants in Group A and C chewed 0.5 g of areca nut without any other additives while in Group B were asked to chew 0.5 g of inert rubber base impression material. Stimulated whole saliva from all three groups was collected into graduated tubes during chewing at time intervals of 1, 3, 5, 10, 15, 20 and 25 min. Then, all participants were asked to remove nut particles or inert rubber base material from the mouth, and saliva samples were collected further up to 20 min, changing tubes at 5 min interval. Salivary arecoline was quantitated by HPLC-MS. The tabulation and descriptive statistics of the study were carried out.

Results: In the present study, baseline levels of arecoline were zero in all three groups, whereas mean salivary arecoline levels during chewing were 76.93 ng/ml, 129.83 ng/ml and 64.83 ng/ml and after chewing were 196.17 ng/ml, 321.12 ng/ml and 43.75 ng/ml in Groups A, B and Control respectively, which were significantly higher than reported threshold levels.

Conclusions: The data from this study reveals that a significant amount of arecoline would be trapped in oral cavity, or being re-circulated between blood and saliva might have resulted in surprisingly high levels of arecoline even 10 mins after chewing in both groups after which the levels started declining. The higher levels of salivary arecoline achieved during and after chewing are enough to cause cytotoxic and genotoxic effects on oral tissues over a period of time in chronic chewers. The great differences in salivary arecoline levels achieved during chewing, may contribute to the variable response to areca nut seen in communities where this habit is widespread. Areca nut users have persistent background salivary arecoline levels long after chewing, whereas concentrations achieved are highly variable and consistent with a role in oral pre-malignancy and malignancy.

Keywords: Arecanut, arecoline, HPLC-MS, OSMF, OSCC, SALIVA

INTRODUCTION

Areca nut is the fourth most commonly used social drug, ranking after nicotine, ethanol and caffeine. Around 600 million people are estimated to chew betel quid in...
India and Southeast Asia (prevalence of up to 80% in parts of India). The International Agency for Research on Cancer (IARC) notes that in some part of India, almost one out of three children and teenagers regularly or occasionally chew these products (WHO, 2003).

In 2004, the IARC confirmed areca nut as human carcinogen. Carcinogenic nitrosamines derived from the areca nut are formed in the saliva of chewers. The major constituents of the nut are carbohydrates, fats, proteins, crude fiber, polyphenols (flavonols and tannins), alkaloids and mineral matter. It contains at least nine structurally related pyridine alkaloids including arecoline, arecaidine, arecaaine, arecolidine, guvacine, isoguvacine, guvacoline and coniine. Biologically, they are most important and have a stimulating effect. Arecoline, the predominant alkaloid present in areca nut, has shown to be implicated in the pathogenesis of many oral diseases because of its genomic, mutagenic and carcinogenic potential. Fibroblasts and keratinocytes are reported to respond to arecoline with: depressed DNA synthesis, cell cycle arrest, cell death, both increased and decreased collagen production and cytokine synthesis. Of importance is determination of biologically relevant concentrations for arecoline, whereas two concentration thresholds appear critical, being 0.1 µg/mL for collagen stimulation and 10 µg/mL for cytotoxicity.

The response of cultured cells to arecoline is highly dependent on concentration and suggests that knowledge of arecoline concentration in the oral cavity achieved during chewing is important to understand pathogenesis of oral disease in nut chewers.

Despite the established role of areca nut in oral diseases and carcinogenesis and the sensitivity of oral epithelial cells to arecoline, only two studies have been reported in the literature describing salivary arecoline levels associated with nut chewing. Accordingly, the present study was taken up to expand upon previously published data and to demonstrate individual variation in salivary arecoline levels in areca nut chewers potentially contributing to individual clinical outcomes using high-performance liquid chromatography-tandem mass spectrometry (HPLC/MS/MS) which is the most sensitive method than the earlier described methods.

MATERIALS AND METHODS

Patients and sample collection
Study participants were areca nut chewers identified from the Outpatient Department of P. M. N. M Dental College and Hospital, Bagalkot, Karnataka, India. The inclusion criteria were as follows: (1) patients who were consuming commercially available areca nut alone or with other ingredients and (2) the duration of consumption of areca nut was 3 years or more. Participants with any of the following were excluded from the study: (1) patients with history of previous treatment for oral squamous cell carcinoma (OSCC) and (2) patients with systemic disorders or using any medications affecting salivary composition and flow. Informed consent was obtained from all patients participating in the study; ethical clearance was obtained from the institutional ethics review board.

The study participants were divided into three groups – A, B and C – after recording a brief history of the habit along with clinical findings. Following this, each participant was asked to rinse his/her mouth with deionized water to facilitate unstimulated whole saliva was collection by spitting method for a period of 5 min. Then, participants in Group A and C chewed 0.5 g of areca nut without any other additives while in Group B were asked to chew 0.5 g of inert rubber-base impression material. Stimulated whole saliva from all three groups was collected into graduated tubes during chewing at time intervals of 1, 3, 5, 10, 15, 20 and 25 min. Then, all participants were asked to remove nut particles or inert rubber-base material from the mouth, and saliva samples were collected further up to 20 min, changing tubes at 5 min interval.

Sample preparation and examination
Saliva samples were cold centrifuged at 402 ×g (3000 rpm) and decanted into fresh tubes for snap freezing in liquid nitrogen and stored at −20°C. Arecoline level quantification was done using high-performance LC-MS/MS method, using following materials: AB SCIEX API 4000™ LC-MS/MS System, Foster City, CA/Concord, Ontario, Canada, Arecoline hydrobromide, Arecoline hydrochloride, Arecoline hydrobromide and Triethylamine from Sigma-Aldrich (Germany), HPLC grade – Acetonitrile, Methanol and Water and Research grade sodium hydrogen phosphate from Spectrochem Pvt., Ltd. (India). A procedure based on LC-MS/MS was used for arecoline quantification. Liquid/liquid extraction with chloroform/isopropanol (95:5 v/v) was used for extraction procedure.

Chromatography was performed on a C8 reversed-phase column using a gradient of 50-mM ammonium formate, pH 5.0 and acetonitrile as a mobile phase at a flow rate of 0.5 mL/min. Separated analytes were determined by electrospay ionization MS/MS in the positive ion mode using multiple reaction monitoring.

Stock solution (1 mg/mL concentration)
About 1 mg of compound, exactly weighed, was transferred to a 1-mL volumetric flask. A few milliliters of water was
added and the compound was dissolved using menthol at room temperature. After substance dissolution, the volume was made up with menthol. Primary stock 1 mg/mL was prepared with the water and rest of the stocks was prepared in the 100% methanol. Human saliva sample (50 μL): About 10 μL (500 ng/mL) of niacin internal standard working solution was added and diluted with 50 μL 0.1 M phosphate buffer to obtain the pH 7.4. To this aliquot, 2.5 mL of chloroform/isopropanol (95:5, v/v) was added. The aqueous phase containing saliva and phosphate buffer at pH 7.4 for this solution was added with 100 μL of ammonium chloride to get the pH 9.5, to extract the arecoline. The organic phase was evaporated to dryness under a stream of nitrogen and re-dissolved in 200 μL of mobile phase; a10 μL volume was injected into the LC column.

Chromatographic conditions
Agilent 1200 HPLC system was used. Chromatography was performed on an X-terra, C8, (4.6 mm i.d. ×50 mm) analytical column (Waters, Milford, MA, USA) and operated at 40°C.

The mobile phase was an isocratic elution 50-mM ammonium formate, pH 5.0 and acetonitrile (10:90). Under these conditions, retention time (RT) were typically 1.64 min for arecoline and 1.50 min for niacin. Column effluent was introduced into the mass. The temperature of the autosampler was kept 4°C, and the runtime was 2.2 min.

Mass spectrometric conditions
An API-4000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA/Concord, Ontario, Canada) was equipped with an electrospray source, operating in the positive ion mode. Data were collected and processed using Sciex Analyst 1.4.2 data collection and integration software on a DELL compatible computer.

MRM parameters
- Molecular weight: 155
- Q1: 156
- Q3: 141.

The optimized parameters were as follows: curtain gas, gas 1 and gas 2 (nitrogen) of 40, 40 and 60 units, respectively; dwell time: 330 ms; source temperature: 550°C and ion spray voltage: 5500V. Unit mass resolution was set in both mass-resolving quadrupole Q1 and Q3. Data were acquired on a Dell Precision 370 workstation and were processed using the Analyst 1.4.1 software package (MDS Sciex).

Statistical analysis
Kruskal–Wallis test was used to evaluate the difference in mean arecoline/pH/salivary flow between the three groups. 
P < 0.05 was considered as statistically significant. Multiple comparisons were carried out using Mann–Whitney U-test.

RESULTS
The study group consisted of Group A & B with 20 individuals in each group having areca nut chewing habit for at least for 3 years while control group (Group C), consisted of 10 individuals, who occasionally chewed areca nut. Of the 40 study participants, 26 (52%) were female and 24 (48%) were male. Female participants tended to be older (mean age: 37.96 ± 10.5 years) than males (mean age: 29.52 ± 10.1 years).

Arecoline levels in saliva before, during and after chewing fresh areca nut or placebo
Baseline levels of arecoline (i.e., before chewing areca nut or placebo) were undetectable in all the three groups. The maximum arecoline concentrations achieved during chewing nut (Group A) ranged from 49 ng/ml to 280 ng/ml with a mean salivary arecoline level of 76.93 ng/ml (P < 0.001). No clear difference was seen between male and female participants with regard to peak concentrations of arecoline achieved. Peak levels of salivary arecoline levels were always achieved during the 1st min with mean salivary arecoline level of 175.75 ng/ml (P = 0.006); then, there was a fall in salivary arecoline levels further up to the 25th min with a mean value of 24.95 ng/ml (P = 0.002).

Whereas, the maximum salivary arecoline concentrations achieved during chewing placebo (Group B) ranged from 79 ng/ml to 386 ng/ml with a mean level of 129.83 ng/ml (P < 0.001). Similar to Group A, highest peak was achieved at the 1st min of chewing with a mean value of 240.75 ng/ml (P < 0.001) further followed a downward trend up to the 25th min with a value of 45.75 ng/ml (P = 0.002). When these values were compared with control group (Group C), it showed a statistically significant difference; maximum levels achieved ranged from 153 ng/ml to 13 ng/ml with a mean of 64.63 ng/ml, but arecoline peaks followed a similar trend where at the 1st min, mean arecoline level was 139.80 ng/ml (P < 0.001) and, at the 25th min, level was 7 ng/ml (P = 0.002), and these fluctuations in arecoline levels can be ascribed to the kinetics of arecoline diffusion and salivary flow rate.

The maximum arecoline concentrations achieved after chewing nut (Group A) were substantially higher than during chewing, ranging from 154 ng/ml to 333 ng/ml with
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a mean salivary arecoline level of 196.18 ng/ml ($P < 0.001$). High peak levels were always achieved after the removal of nut particles with mean salivary arecoline level of 293.75 ng/ml ($P < 0.001$) which then followed a downward trend further up to the 20th min with a mean value of 24.95 ng/ml ($P = 0.002$). Surprisingly, participants in Group B after chewing placebo showed much higher levels of salivary arecoline where maximum levels ranged from 426 ng/ml to 926 ng/ml with a mean of 321.13 ng/ml. High peak being achieved after removal of a placebo with a mean level of 489.25 ng/ml ($P < 0.001$). Further, there was a fall in salivary arecoline level up to the 20th min with a mean value of 172.0 ng/ml. When these were compared with a control group, maximum levels achieved ranged from 21.20 ng/ml to 74.80 ng/ml with a mean of 43.75 ng/ml which showed a statistically significant difference, but arecoline peaks followed a similar trend where at after removal of nut particles, mean arecoline level was 74.80 ng/ml ($P < 0.001$) and, at the 20th min, level was 21.20 ng/ml ($P < 0.001$) [Graphs 1 and 2].

DISCUSSION

The results described in this study showed a surprisingly higher level of arecoline after chewing than during chewing; notably, the concentration of arecoline was generally above 100 ng/ml and then following a downward trend; these results are in contrast to study done by Cox et al. Detection of arecoline after chewing suggests its persistence in the oral cavity long after exposure to the nut that has pleiotropic responses on a variety of tissue types that together account for its carcinogenic properties. It is possible that there are mechanisms that either trap arecoline in the oral cavity or perhaps re-circulate arecoline between blood and saliva. These possibilities are both supported by appreciable salivary arecoline levels 25 min after removal nut particles from the mouth.

There are at least three possible explanations for this$^{17-31}$– first, the residual arecoline is possibly not secreted completely by the kidneys. The reason could possibly be due to the chemical structure of arecoline preventing movement of the drug into a high-salt fluid such as urine but instead establishing “steady-state” equilibrium and allowing arecoline to move from systemic blood into saliva in the salivary glands and from a tissue bank back into the saliva. Second, arecoline has a pKa of 7.64 so that its uncharged and lipophilic form predominates in alkaline conditions, and it is the electrically neutral form of arecoline which is best able to penetrate the mucosal barrier through “simple diffusion.” In the light of this, the near neutral pH of unstimulated saliva suggests that much of the background salivary arecoline detected in the current study would be in the charged form having little penetrative ability. However, the more alkaline pH of saliva upon stimulated secretion may result in a preponderance of the uncharged form of arecoline, and hence, episodes of greater mucosal penetration where it follows the downward trend. It is also possible that the nonionized form of arecoline becomes sequestered in fat and is slowly released into circulation between chewing episodes. Finally, it is also possible that small amount of arecoline is absorbed into dental plaque and slowly released; hence, the level of arecoline in saliva might reflect the oral hygiene state of the patient.

This appears to be the first time that a substance absorbed across the oral mucosa has been documented to be present in the saliva. Analogous to the present study, other studies have shown the occurrence of prolonged salivary fluoride levels after cessation of fluoride exposure as well as entero-salivary recirculation that has been observed with a number of substances including heavy metals and alkaloids. This provides precedent for the idea that the salivary glands may concentrate arecoline for re-secretion long after areca nut chewing has ceased. The correlation of drug between saliva and blood depends on several factors including pH of saliva, salivary flow rate, rate of chewing, protein binding of the drug and its pK. For acidic drugs, the concentration is lower in saliva, whereas for basic drugs, the concentration is higher in saliva.
The uniqueness of the present study is that we made an attempt to quantify salivary arecoline levels in chronic nut chewers using nonnut components – something that has not been described in earlier studies. Hence, it can be stated that in chronic nut chewers, the “mere act of chewing” even the nonnut components may redirect arecoline from blood or tissue banks to saliva. This was clearly evident in the present study where the high levels of salivary arecoline were not only achieved during chewing nut but also immediately after chewing nut as well as chewing of nonnut components; this “adaptive salivary reflex” makes arecoline available to affect the oral tissues in during intervals. Hence, the high arecoline concentrations reported in the present study would appear to be sufficient to stimulate collagen synthesis and also to reduce fibroblast proliferation, while there may also be keratinocyte cytotoxicity. Interestingly, however, most of the participants in the present study did not show clinical signs of oral submucous fibrosis (OSMF) or any other habit-associated lesions. This is consistent with the notion that the period of exposure is important, as well as perhaps individual, and potentially idiosyncratic responses to areca nut. It is further possible that although substantial cellular injury occurs in many individuals using areca nut, there is often no apparent detectable clinical change, with the consequence that epidemiological surveys for detectable oral disease may not fully assess the impact of areca nut use.

This idea is consistent with the reported increased incidence of OSCC among areca nut users, despite relatively modest detectable premalignant changes. With regard to this, it is important to note that increased keratinocyte “turnover” would be expected in response to arecoline cytotoxicity and that such accelerated cellular turnover is consistent with an increased incidence of malignant change. [32-46]

While acknowledging the concentration-dependent effects of arecoline upon fibroblasts and keratinocytes outlined above, it is important to recognize that this can only have biological meaning if arecoline is able to penetrate the mucosal barriers to enter the tissues. The potential of arecoline to enter the tissues, and hence, the circulation through the oral epithelium is important to properly interpret data presented in this study. The reported arecoline threshold level for stimulation of collagen synthesis is 100 ng/ml. In the present study, baseline levels of arecoline were undetectable in all the three groups, whereas mean salivary arecoline levels during chewing were 76.93 ng/ml, 129.83 ng/ml and 64.83 ng/ml and after chewing were 196.17 ng/ml, 321.12 ng/ml and 43.75 ng/ml in Groups A, B and C, respectively [Graph 1], which were significantly higher than reported threshold levels. Arecoline trapped in oral cavity or being re-circulated between blood and saliva might have resulted in surprisingly high levels of arecoline even 10 min after chewing in both groups after which the levels started declining.

The monitoring of the arecoline concentration levels, presented in this study, provides a dynamic picture of the fluctuation of these levels. For the first time, there is documentation supporting the idea that chronic chewers are more at risk of developing an unusual response to the chewing of the nonnut components. This could reflect a number of variables including pH of the saliva, rate of chewing of the areca nut, which in turn may reflect different personalities and stress levels.

Most of the burden of disease related to the use of the areca nut is within the developing countries of Asia including India, so that research into areca nut use is facilitated by the development of relevant laboratory techniques that may be more readily available in these countries. Previously reported studies used “gas chromatography” and “HPLC” to determine arecoline concentration or to distinguish arecoline from other alkaloids in saliva. The present study has developed an alternative approach to arecoline measurement which was of comparable accuracy and high sensitivity to earlier described methods. As described in this study, “HPLC/MS/MS” was found to be an effective alternative technology because of its precise nature of measuring the mass of the arecoline while at the same time being more widely available in the developing world. Hence, this is a means of enabling basic research in countries that experience a heavy disease burden that is linked to this habit. [33-46]

The salivary arecoline levels observed in the present study both during and after chewing demonstrate that the concentration level of arecoline in the mouth varies significantly over time, and these levels are generally well above those required for stimulation of collagen synthesis as well as being cytotoxic for the cell. While the period in the study was over a 45-min period, it can be accepted that the possibility exists for very high concentrations to be present in the mouth if the nut is used over extended periods of time.

Therefore, assuming that, arecoline is a significant psychoactive ingredient, and then, appreciable levels of circulating arecoline are likely achieved by means of absorption across the oral mucosa before expectoration for most users. Changes in salivary arecoline levels were highly idiosyncratic, consistent with differences in specific chewing habits between individuals. Areca nut users have persistent background salivary arecoline levels long after chewing, whereas concentrations achieved are highly
variable and consistent with a role in oral premalignancy and malignancy. [47-50]

CONCLUSION

The general effects of areca nut chewing have much impact not only on the regional tissues like oral mucosa but also on the systemic health of an individual. However, many times, the deleterious effects instigated on the oral mucosa can outweigh the systemic effects to a large extent when it becomes a habit. In conjunction with many previous epidemiological and case-control studies conducted in Asia, the present study reaffirms the role of areca nut in the pathogenesis of potentially malignant and malignant disorders such as OSMF and OSCC.

The present study corroborates that salivary arecoline levels sufficient to cause stimulation and cytotoxicity were often achieved for significant periods during and after chewing; further, these levels were achieved not only during chewing nut components but also during chewing non-nut components, and these levels were significantly higher after chewing than during chewing, thus strengthening the role of arecoline in oral diseases associated with this habit.

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Conflicts of interest

There are no conflicts of interest.

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