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Effect of areca nut on salivary copper concentration in chronic chewers

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Abstract

The chewing of areca nut is associated with the development of oral submucous fibrosis (OSF), a condition predominantly encountered in Asians indulging in the habit. The pathogenesis of this condition is however, unclear, though several mechanisms have been proposed. Copper has previously been implicated as a possible aetiological factor. In this study, total copper concentration was measured via atomic absorption spectrophotometry in whole mouth saliva of 15 volunteers who were regular chewers, before and after their habitual chew. An aliquot of the latter was also analysed for copper. Six non-chewing volunteers acted as controls. Salivary copper concentrations were corrected for protein content. Over 50% of the subjects had basal salivary copper concentration higher than the range seen in normal controls. All but two subjects demonstrated an increase in the salivary [Cu] following their habitual chew. Marked changes were seen in those with low basal salivary concentrations. These data indicate that soluble copper found in areca nut is released into the oral environment of habitual chewers. Its buccal absorption may contribute to the oral fibrosis in Asians who regularly chew this nut.

Introduction

The chewing of areca nut (seed of the fruit of the oriental palm, *Areca Catechu*; often referred to as betel nut) is widespread in the Asian Subcontinent, Pacific Islands and in communities migrating to western countries there from (Warnakulasuriya 2002). Apart from tobacco, alcohol and caffeine, it is the most commonly used substance. Indeed, globally, an estimated 600 million people are believed to indulge in the habit. Areca nut is usually consumed alone, in its natural form, as a refined (processed) product, or as a key ingredient of the chewing quid: the latter consists of several constituents including slaked lime, aniseed and grated coconut, wrapped in a betel leaf (Reichart & Philipsen 1996; Warnakulasuriya 2002). Many individuals/communities also use tobacco as a component of the quid.

Chronic areca nut chewing has been strongly linked to the development of oral submucous fibrosis (OSF) (Murti *et al.* 1995; Warnakulasuriya *et al.* 1997; Gupta *et al.* 1998), a chronic progressive pre-cancerous condition characterised by fibrosis of the mucosal lining of the oral cavity and occasionally the upper digestive tract (Maher *et al.* 1991). The oral fibrosis often progresses into the submucosal regions and the underlying muscle, leading to deposition of dense fibrous bands (Johnson *et al.* 1997) and consequently restricts the opening of the mouth (trismus), a hallmark of the condition. The risk of cancer development in the upper aero-digestive tract is increased markedly in patients with OSF (Warnakulasuriya 2002; IARC 2004).

The pathogenesis of OSF is however, unclear (Warnakulasuriya *et al.* 1997; Tilakaratne *et al.* 2006). The disease is now epidemic in the Indian subcontinent (Gupta & Nandakumar 1999), and also emerging in the heavy users within the Asian community in the UK (Winstock *et al.* 2000). Areca nuts have a higher copper content than other commonly consumed nuts (Trivedy *et al.* 1999), and upon chewing by volunteer subjects (non-chewers), have been shown experimentally to result in the release of appreciable amounts of copper into the oral fluid (Trivedy *et al.* 1997). Copper has been implicated in the pathogenesis of several fibrotic conditions (Britton 1996) and mucosal biopsies from OSF subjects have also revealed a higher copper content (Trivedy *et al.* 2000). These investigations are indicative of copper being an aetiological factor for OSF. As copper levels in oral fluids in habitual areca chewers in any South Asian country, have to date not been investigated, we set out to examine salivary copper levels in chronic chewers before and after a challenge with their habitual areca chew.

Materials and methods

Subjects

Fifteen males (age: 29 ± 14 year) with no previous history of ill health, and attending an outpatient Clinic at Nagpur Dental Hospital in India, were recruited for the study. Each subject completed a medical questionnaire, was questioned regarding chewing habits (i.e. brand of areca nut preparation(s) used, the frequency of chewing and the duration of the habit) and asked to provide a sample of their areca nut product. Whole mouth salivary samples were obtained from each volunteer, before and after chewing his habitual areca nut product. Fluid samples were kept cold prior to transportation to the laboratory. There after they were stored at −20 °C until analysis. Three of the subjects were regular smokers (5–10 cigarettes/d) and all bar one consumed no alcohol. Salivary samples obtained from six non-chewing Asian volunteers (age: 24 ± 3 year), constituted the control group. Ethical permission for the study was obtained from the Institutional Ethics Review Board.

Analysis of samples/saliva for copper

The analyses were performed by the Supraregional Assay and Advisory Service (SAAS) Trace-Metal laboratory at King's College Hospital. Metal-free tubes from Elkay (Basingstoke, Hampshire) were used for collection and analysis of samples. Reagents, of suitable quality for Atomic Absorption Spectrometry were from BDH Merck Ltd (Poole, Dorset). Palladium chloride was from Sigma-Aldrich Co. Ltd (Poole, Dorset).

A small sample of each areca nut product (300–400 mg) was ground to a powder using a pestle and mortar, weighed, and digested overnight with concentrated nitric acid in a metal-free tube. The digest was then diluted appropriately, briefly centrifuged and analysed for copper using graphite furnace (electrothermal) atomic absorption spectrophotometry (Perkin Elmer Model 4100ZL), with Zeeman background correction. The actual concentrations in the samples (run in duplicate) were extrapolated from a calibration curve established by analysing similarly, a range of aqueous standards of known concentration. Palladium chloride was used as the stabilising (modifier) reagent during the analysis. The accuracy of the measurements was assessed by analysing digests of the National Bureau of Standards bovine liver (Lot 1577A).

Salivary samples were thawed, vortexed, briefly centrifuged and an aliquot of the supernatant deproteinated with 10% Trichloroacetic acid. After centrifugation (3000 rpm for 10 min), the resulting supernatant was analysed (in duplicate) for copper using the above-mentioned instrument. A matrix-matched calibration curve was created by adding aqueous standards (25–200 μg/l) to a salivary sample (with low copper content) treated in a similar fashion. The protein content of the salivary samples was measured via an automated colorimetric method, within the Diagnostic Biochemistry section at King's.

Statistical analysis

Data are shown as mean ± SD. Statistical comparisons were carried out by Students '*t*' test or regression analysis, using Microsoft Excel software.

Results

Table 1 provides the ages, areca nut chewing frequency and the duration of the habit of the volunteer subjects. There were marked differences in not only the commercial preparations used by subjects but also in the frequency of chewing (3–16 per day). The duration of the habit also varied from 2 to 29 years, with the average age of starting being 19 year. It is noteworthy that two subjects started their habit at a very young age (5–6 years). The table also gives the mean values for the copper content in the habitual areca nut product and in the corresponding salivary samples. The mean copper content in the areca nut products was $26.7 \pm 8.1 \mu g/g$ dry wt (range: 18.5–42.0 $\mu g/g$). The mean copper concentration for the NSB bovine liver standard was $149 \pm 6 \mu g/g$ (certified range 158 ± 7).

The mean salivary copper in the chewers at entry was $21.3 \pm 11.5 \mu g/l$ (range 6–42): Corrected for protein, the copper content was $0.032 \pm 0.013 \mu g/mg$. Although the mean corrected copper content was higher than in the control group ($0.028 \pm 0.006 \mu g/mg$), the differences failed to reach statistical significance ($p > 0.05$). Eight of the 15 volunteers (53%) did however, exhibit values higher than the range seen in the control group. The correlation between the initial salivary copper concentration and the copper content in the product used ($r = -0.37$, Figure 1) or the duration of habit ($r = -0.05$) was weak and failed to reach statistical significance ($p > 0.05$).

After their habitual chew, 13 subjects (87%) demonstrated an increase in salivary copper concentration by 21–250%: Most marked changes were seen in those subjects whose initial salivary copper concentration was low ($r = -0.65$, $p < 0.02$; Figure 2). One subject showed little change, whilst one actually showed a decrease. There was a small, but statistically insignificant correlation, between salivary copper concentration post-chew and the copper content of the areca product ($r = -0.24$).

Discussion

Areca nut usage has escalated markedly in the past two decades in South-East Asia owing to the wide availability of various commercial products/brands. Consequently, the risk of developing oral submucous fibrosis (OSF) and oral cancer has increased. The pathogenesis of OSF, a condition associated with increased collagen deposition in the oral submucosa however, remains unclear,

Table 1. Chewing habits, copper in areca nut and in salivary samples pre and post chewing.

Subject	Age (y)	Chewing Frequency/day	Duration (y)	Areca [Cu] (μg/g)	Salivary [Cu] * (μg/mg protein)		Δ Change (%)
					Before	After	
1	28	5	4	20.0	0.044	0.054	22.7
2	28	7	10	18.5	0.033	0.040	21.2
3	21	10	8	27.5	0.049	0.045	−8.2
4	18	16	4	31.0	0.044	0.060	36.4
5	21	12	2	37.0	0.035	0.073	108.6
6	75	6	35	22.0	0.038	0.076	99.9
7	35	12	18	19.5	0.038	0.073	92.1
8	N/K	N/K	N/K	15.0	0.029	0.038	31.0
9	23	12	18	31.0	0.013	0.045	246.2
10	35	5	29	39.3	0.029	0.042	44.8
11	22	3	2	42.0	0.012	0.029	141.7
12	21	3	2	27.0	0.023	0.041	78.3
13	23	6	8	27.0	0.015	0.041	173.3
14	26	5	3	23.5	0.052	0.040	−23.1
15	33	15	4	20.5	0.031	0.093	200

*Salivary samples (collected before and after the habitual chew) and a portion of the product were analysed for copper using atomic absorption spectrophotometry. The salivary values are corrected for the protein content. N/K = not known.

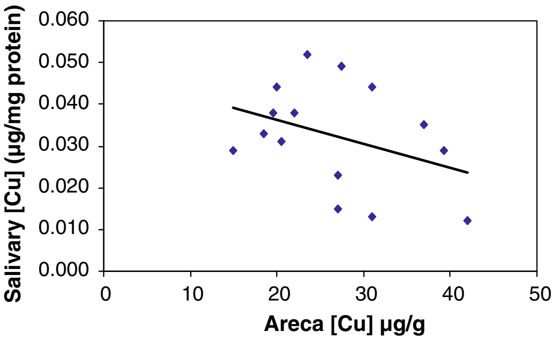

Figure 1. Relationship between copper content in areca product and initial salivary copper concentration, in habitual chewers.

though several mechanisms have been proposed (Tilakaratne *et al.* 2006). The fibrotic changes seen in OSF resemble that seen in Wilson's disease and in Indian childhood cirrhosis, both of which are associated with alterations in copper metabolism (Baker *et al.* 1995; Schilsky 1996), thus indicating that copper may have an aetiological role. The mechanisms of fibrosis and collagen types involved in these diseases could however, differ.

Copper was found in all the areca product samples provided by the volunteers. There was however, a marked variation in the concentration in the different brands used. A trend of higher salivary copper concentrations was evident in a large proportion (8/15) of the subjects. Of these, all but one had been using areca nut products regularly for over 3 years. The one volunteer that had been in the habit for <3 year did however, use the product frequently (12$\times$ daily): Also, the copper concentration in the product used by this subject was high

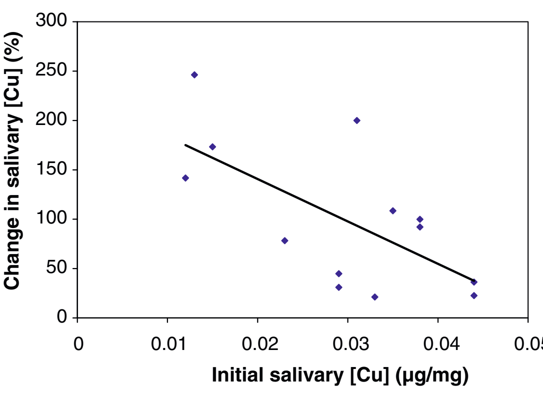

Figure 2. Relationship between change in salivary copper concentration (expressed as %) and the initial [Cu]. All subjects but two showed a positive change in salivary copper concentration post-chewing of their habitual product.

(37 μg/g). It is noteworthy that 7 members out of this group of 8, had indulged in a chew within the last hour of their appointment. Thus, it is not easy to ascertain if the measured initial concentration is a true reflection of the basal level.

What was however, very evident in this study was the release of copper into the oral fluid following the chewing of the areca nut product. Almost all members of the group exhibited an increase, with most marked changes seen in those subjects who initially had a low salivary copper concentration. Changes were less marked in subjects with a high basal concentration: This finding is interesting and suggests that the flow rate of saliva or the rate of absorption of copper from the chew may well be higher in these subjects.

The absence of a strong relationship between copper concentration in the product and in the saliva (post-chew) is not altogether surprising, as various factors will govern the release of copper from each preparation (and thus the salivary concentration) including: interaction of copper with the other constituents, the amount of preparation chewed at anyone time and the copper concentration within the preparation, the pH of saliva and its flow rate, as well as the time the product is actually in contact with the oral mucosa. As habits such as spitting or holding the quid in the mouth are common among users, copper concentrations could markedly fluctuate.

The manner in which copper may be associated in the aetiology is unclear. A possible mechanism is the stimulation of the copper dependent enzyme, lysyl oxidase, resulting in upregulation of collagen synthesis and its cross-linking. Indeed, lysyl oxidase activity has been demonstrated to be increased in fibroblasts cultured from OSF patients (Ma *et al.* 1995). Although areca nut usage is known to be a risk factor for OSF, not all users develop the disease, thus indicating that not only individual susceptibility but the presence of other factors (multigenic process) maybe important for the onset of the disease (Rajalalitha & Vali 2005). With migrant populations using areca nut as a chewing/masticatory substance, this product is now freely available in western markets and via the internet. OSF is thus increasingly recognised in migrant populations addicted to the product (Avon 2004).

This study provides evidence of excess concentrations of copper in the saliva of heavy users and of its release into oral fluids following chewing of areca nut products. The presence of copper in saliva following areca chewing and in turn its contact with oral tissues and buccal absorption warrants further study.

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