Role of Areca Nut Consumption in the Cause of Oral Cancers

A Cytogenetic Assessment

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Background. Cytogenetic studies, framed to assess the possible genomic damage caused by areca nut consumption (without tobacco and not as a component of betel quid), were performed among areca nut chewers, which included normal people who chew areca nuts, patients with oral submucous fibrosis, and patients with oral cancer, and healthy nonchewing controls.

Results. The analysis showed statistically significant increases in the frequencies of sister chromatid exchanges and chromosome aberrations in peripheral blood lymphocytes and the percentage of micronucleated cells in exfoliated cells of buccal mucosa among all three groups of chewers when compared with those of the controls.

Conclusions. The current data, the first of this type among only areca nut chewers, highlight that this popular masticatory is erroneously considered "safe" and that it increases the genomic damage even when chewed without tobacco. The data also signify that, henceforth, in cytogenetic biomonitoring, areca nut consumption also should be considered as one of the confounding factors. *Cancer* 1992; 70:1017–1023.

Key words: areca nut consumption, genotoxicity, oral cancers, cytogenetic study, CA, SCE, MNC.

The areca nut, popularly known as "betel nut," is almost symbolic of the culture of some Oriental nations and is one of the oldest known masticatories among Asians. It accounts for a major portion, by weight, of betel quid, which is composed of betel leaf, areca nut, catechu, and lime, to which tobacco may or may not be added.[1] The occurrence of oral premalignant and malignant diseases among betel quid chewers needs no introduction. However, because of the strong proven association between the manner of tobacco consumption and the development of site-specific cancers, only tobacco has been held responsible for the harmful effects. This has eclipsed the possible harmful effects of areca nut. It has been reported that, compared with those who do not chew betel quid at all, the risk of oral cancer (OC) is higher among those who chew betel quid even without tobacco; however, the relative risk associated with each ingredient used in the quid has not been established clearly.[2,3] Investigations have been directed toward elucidating the carcinogenic potential of the individual ingredients of betel quid.

The in vitro experiments performed in our laboratory, with the use of a mammalian test system, provided evidence regarding the genotoxic effects of areca nut.[4] The mutagenic/carcinogenic properties have been reported by other investigators.[1] However, the extrapolation of carcinogenesis data generated from in vitro assays and laboratory animals to the heterogenous human population often is complicated. The altered pharmacokinetics and metabolic conversions may prevent the substance from eliciting a comparable response in humans. Furthermore, when predicting genotoxic carcinogenicity, it has been suggested that the in vivo genotoxicity data would serve well to substantiate the in vitro data.[5] Because human beings may be exposed to a vast array of agents that might participate in the carcinogenic process, long-term studies for carcinogenicity in experimental animals also may not necessarily reflect a realistic situation. Moreover, the genotoxic effects of chronic low-level exposure to areca nut might well be estimated with cytogenetic analysis in people with a habit of chewing areca nut. A positive result clearly would demonstrate its carcinogenic/genotoxic potential in human beings and eliminate the expense and

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work involved in performing animal bioassays. Thus, to substantiate the information gained by in vitro assays, and in consideration of the fact that there are no reports on cytogenetic studies in human beings regarding only areca nut consumption, the current investigation was extended in this sphere.

In the current study, the in vivo DNA damage caused by areca nut consumption was estimated, with three short-term assays, in the target and nontarget tissues. The most extensively used method to assess the genetic effects in human beings exposed to potential mutagens or carcinogens has been the analysis of chromosomal aberrations (CA) and sister chromatid exchanges (SCE) in phytohemagglutinin-stimulated peripheral blood lymphocytes. Here, it was also possible to study the DNA damage in the target tissue (i.e., the oral mucosa) by estimating the micronucleated cell (MNC) frequency in exfoliated cells of buccal mucosa. The study thus was made more comprehensive by analysis of the DNA damage in peripheral blood lymphocytes, the tissue indirectly exposed to the substance, and the exfoliated buccal mucosa cells, the tissue coming in direct contact with the substance, with the use of three cytogenetic markers among people consuming areca nut and among normal healthy controls. OC generally are preceded by premalignant lesions or conditions.[2] Oral submucous fibrosis (OSMF) now is accepted as a premalignant condition, and its potency in being converted into OC has been well established by detailed studies.[6,7] Areca nut chewing is considered to be the major etiologic factor responsible for the development of OSMF.[8,9] Hence, so that we could learn more about the habit-induced diseases and the utility of the parameters in detecting the changes at premalignant or malignant stages, the chewers also included patients with OSMF or OC.

Patients and Methods

Selection of Subjects

In all, 43 vegetarian age-matched and sex-matched teetotalers, not engaged in any hazardous occupation, were included in the study. They were categorized according to the habit of areca nut consumption and the condition of their oral mucosa; the group included the following: (1) controls, which included only people who had never consumed areca nut or tobacco in any form and had no viral disease or antibiotic therapy during the preceding 6 months; (2) normal people who chewed areca nut (NAC) were those who consumed at least one areca nut per day for no less than 2 years and did not have clinically detectable changes in their oral mucosa; (3) patients with OSMF who chewed areca nut; and (4) patients with OC (ICD 140-145) who had oral squamous cell carcinomas (histologically confirmed) and chewed areca nut. Even among the three groups of chewers, only those who had no concurrent habit of consuming tobacco in any form were selected.

Samples were collected before the patients received therapy and with informed consent of all the subjects. The peripheral blood lymphocyte collection, culture, staining, and scoring protocols for SCE and CA analysis and the sample collection and staining for micronucleus assay have been detailed earlier.[10,11] Because of the poor hygienic condition of the oral mucosa among those with OC and because of abundant necrotic cells in the preparations, it was not feasible to obtain smears that could be scored; hence, the micronucleus assay was not performed among these patients. The slides were coded, and blind scoring was performed for all three parameters by people who were not involved in coding. Slides from all four groups were distributed evenly among the scorers. Student's t test was used to determine the statistical significance of the results.

Results

Figure 1 provides comparative data of SCE per cell frequency among controls and three groups of areca nut chewers. As observed in the figure, the controls, with a

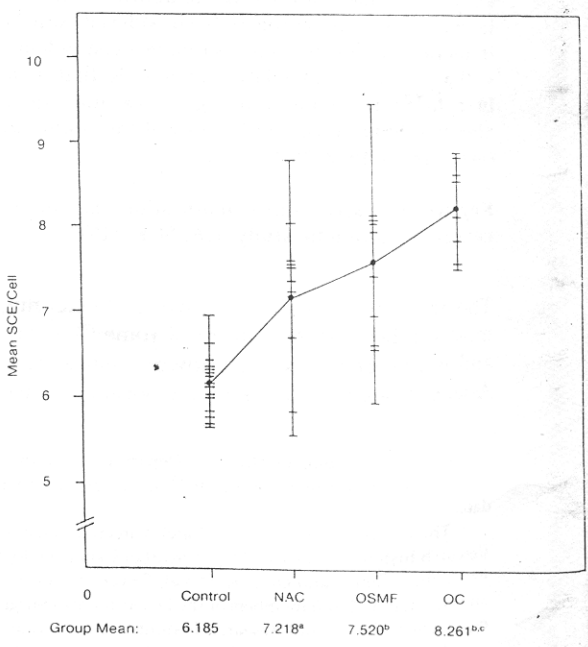

Figure 1. Frequency of individual mean SCE/cell values among controls and different groups of areca nut chewers. a: $P < 0.01$ when compared with controls, b: $P < 0.001$ when compared with controls, c: $P < 0.01$ when compared with NAC.

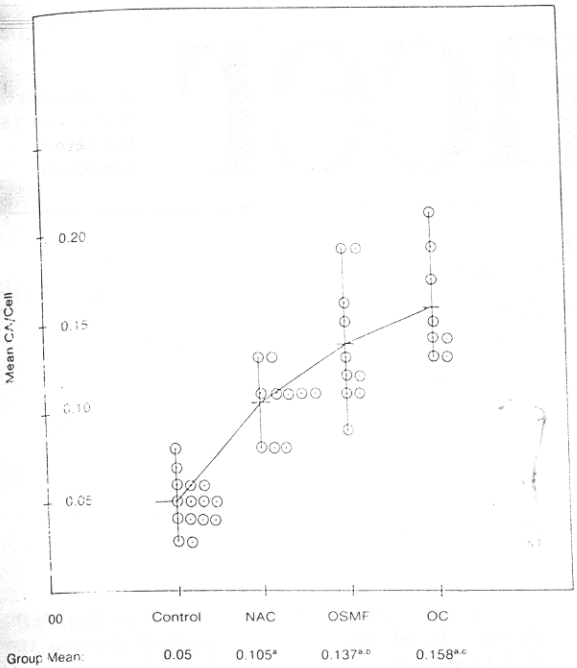

Figure 2. Expression of individual mean CA/cell values among controls and different groups of areca nut chewers. a: $P < 0.001$ when compared with controls, b: $P < 0.02$ when compared with NAC, c: $P < 0.001$ when compared with NAC.

range of 5.66–6.95, had a mean value of 6.185 ± 0.008 (mean ± standard error of the mean). However, in the NAC, the SCE frequencies varied from 5.59 to 8.79, providing a mean value of 7.128 ± 0.288. The patients with OSMF exhibited a variation from 5.98 to 9.45, with a mean of 7.404 ± 0.367, whereas in the patients with OC, a mean of 8.126 ± 0.184 SCE per cell was computed from the values ranging from 7.51 to 8.90. On comparison of the mean values of different groups, it was observed that all three groups of chewers showed a statistically significant elevation in SCE frequencies compared with the controls. Among the chewers, a marginal increase in SCE was observed in patients with OSMF compared with NAC, but when the values of NAC were compared with those of patients with OC, the difference was significant ($P < 0.01$). Although a clear increase was demonstrated between the mean values of patients with OSMF and those with OC, the difference was not statistically significant.

The mean values of CA observed in the controls and in different groups of areca nut chewers are shown in Figure 2. The mean CA value in controls was found to be 0.05 CA per cell, which increased to 0.105 in NAC, 0.137 in patients with OSMF, and 0.158 among patients with OC. All three groups of areca nut chewers showed a highly significant elevation in CA frequency when compared with that of the controls ($P < 0.001$). Within the groups of chewers, when the values for NAC were compared with those of the two groups with disease, the increase was found to be statistically significant. Among the areca nut chewers, despite an apparent increase in CA frequencies in patients with OC as compared with those with OSMF, the increase was not statistically significant. The 5.0% CA observed in the controls almost doubled (10.5%) in NAC and escalated to 13.6% in patients with OSMF and 15.8% in those with OC. Table 1 shows the various types of CA observed in all of the groups.

Figure 3 shows the details of percentages of MNC observed in controls, NAC, and patients with OSMF. As witnessed in the scattergram, the controls had a mean percentage of MNC as low as 0.193%; the mean percentage was 0.73% in NAC and 0.74% in patients with OSMF. In controls, the percentage of MNC ranged from 0.1% to 0.3%. In most of the chewers, the percentages of MNC were observed to be higher than the highest value among the controls. The frequency of MNC was more than three times greater among the chewers as compared with the controls, which was statistically significant ($P < 0.001$). It was noteworthy that, in patients with OSMF, although the percentage of MNC remained analogous to that of NAC, the percentage of micronuclei was higher than that observed in NAC (i.e., 0.87% in NAC and 1.06% in patients with OSMF).

Table 1. Variations in Types of Chromosome Aberrations Observed in Peripheral Blood Lymphocytes of Controls and Three Groups of Areca Nut Chewers

Group	Total aberrations (%)	Chromatid (%)			Chromosome (%)				
		G	*B/F*	*I*	*G*	*B/F*	*Dm*	*D*	*R*
Controls (n = 15)	5.0	3.7	0.9	—	0.3	0.1	—	—	—
NAC (n = 10)	10.5	7.3	2.1	0.3	0.6	0.1	—	—	—
Patients with OSMF (n = 10)	13.7	8.8	3.5	0.1	0.9	0.6	—	—	—
Patients with OC (n = 8)	15.8	9.5	4.3	0.1	1.3	0.3	0.1	0.3	—

G: gap; B: break; F: fragment; I: interchange; Dm: double minutes; D: dicentric; R: ring; OC: oral cancer; OSMF: oral submucous fibrosis; NAC: normal people who chewed areca nut.

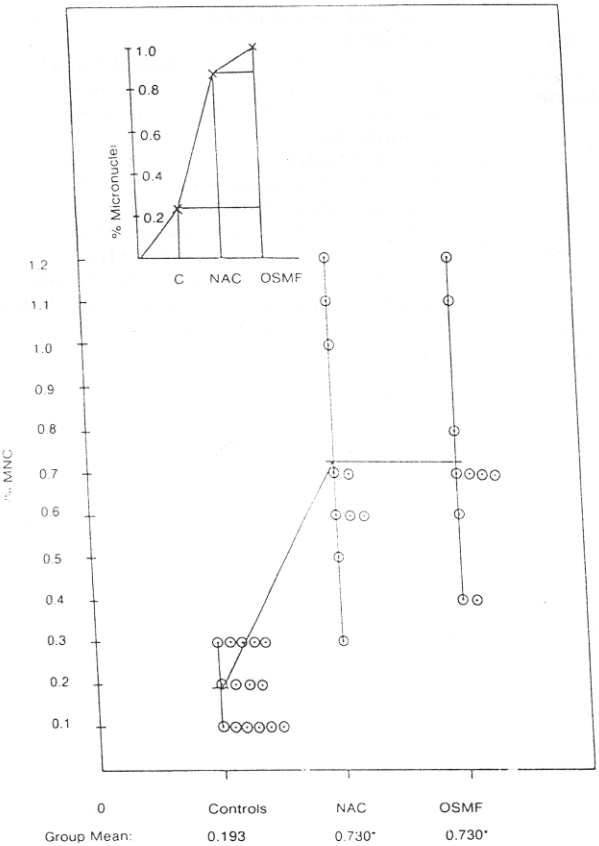

Figure 3. Frequency of individual percentages of MNC and group mean frequency of micronuclei among controls and areca nut chewers. *$P < 0.001$.

The distinct increase exhibited by all the cytogenetic markers among all three groups of areca nut chewers compared with the controls is shown clearly in the collective data presented in Table 2.

Discussion

The multifactorial origin of cancer makes its control more challenging than that of other diseases. With approximately 80–90% of cancers determined environmentally, it would be of interest to identify substances that might damage DNA and ultimately increase the risk of cancer. The causal relationship of chewing betel quid with tobacco and OC is well documented. The literary references to the habit of chewing betel quid (which always contains areca nut) in India are at least 2000 years old.[12] OC have been described elaborately in "Sushruta Samhita," a treatise on Indian surgery, written in 600 B.C., indicating that OC were common even in those days.[13] This was well before the entry of tobacco into the Indian culture approximately during 16th century.[1] These reports focus our attention on the possible carcinogenic effects of betel quid ingredients other than tobacco.

Tobacco and areca nut alkaloids have been detected in the saliva and urine samples of tobacco–areca nut chewers.[14–16] It has been suggested that tobacco-specific nitrosamines play a major role in the origin of OC.[17,18] Currently there is growing concern regarding the carcinogenic potential of the nitrosation products of the areca nut alkaloid, arecoline.[16] Recent reports have indicated that, apart from the preformed N-nitroso compounds in betel quid with or without tobacco, a substantial fraction of N-nitroso compounds are synthesized in vivo in the oral cavity.[19] Numerous recent reports have proven that the saliva of betel quid chewers contains nitrosamines derived from areca nut alkaloids.[14–16,19] Traces of 3-(methyl nitrosamino)propionitrile, a powerful carcinogen in F344 rats,[20] have been detected in saliva of those who chew betel quid without tobacco,[19] and have been found to have organ-specific carcinogenic activity.[21] Stich and Anders[22] recently reported the appearance of N-nitrosoguvacine and rela-

Table 2. Comprehensive Data on the Cytogenetic End Points Among Controls and Three Groups of Areca Nut Chewers

Group	SCE/cell (mean ± SE)	CA/cell (mean ± SE)	% MNC (mean ± SE)
Controls (n = 15)	6.185 ± 0.088	0.050 ± 0.004	0.193 ± 0.022
NAC (n = 10)	7.218 ± 0.288*	0.105 ± 0.006†‡	0.730 ± 0.085‡
Patients with OSMF (n = 10)	7.520 ± 0.303‡	0.137 ± 0.010‡§	0.730 ± 0.078‡
Patients with OC (n = 8)	8.261 ± 0.184‡	0.158 ± 0.010‡§	—

SE: standard error; OC: oral cancer; OSMF: oral submucous fibrosis; NAC: normal people who chewed areca nut; SCE: sister chromatid exchanges; CA: chromosomal aberrations; MNC: micronucleated cell.

* $P < 0.01$.

† $P < 0.01$.

‡ $P < 0.001$.

§ $P < 0.001$, excluding gaps.

tively large amounts of tannins in the saliva of betel quid chewers and that the saliva was genotoxic to Chinese hamster ovary cells. The carcinogenicity of N-nitrosoguvacine is an open question with two contradictory reports.[21,23] The genotoxic effects of several areca nut–specific N-nitroso compounds have been investigated in cultured human epithelial cells.[24] Earlier, we reported increased genomic damage in the peripheral blood lymphocytes and oral epithelial lining among mava (tobacco plus areca nut) chewers.[25] To understand the relationship between chewing of only areca nut and oral carcinogenesis, an effort was made to analyze DNA damage in the same tissues with the use of the same cytogenetic parameters among areca nut chewers.

A statistically significant increase in SCE and CA was found in the lymphocytes of areca nut chewers. SCE are induced efficiently by substances that form covalent adducts to the DNA or interfere with DNA precursor metabolism or repair. Hemoglobin adducts of arecoline, the major areca nut alkaloid, have been reported.[26] Talaska et al.[27] have found a correlation between DNA adducts and CA that may lead to neoplasia. The clastogenic action of the aqueous extract of areca nut and the areca nut alkaloids have been observed by us and other investigators.[4,28–31] Thus, the chromosome-breaking ability of areca nut–derived alkaloids, polyphenols/tannins, and nitrosamines may be jointly responsible for chromosome breakage observed among the areca nut chewers.

Previously we reported increases in SCE and CA among people chewing a mixture of areca nut and tobacco.[25] It was interesting that, in the current study (i.e., among only areca nut chewers), the SCE and CA remained comparable to those in people who chewed areca nut with tobacco. This lack of difference, which one would not have expected, might result from the variation in chewing pattern. The areca nut chewers generally swallow the saliva completely, thus bathing the esophageal lining with the genotoxins released during chewing. However, those who chew areca nut plus tobacco, because of the bitter taste of tobacco, expectorate the saliva periodically. This might be the reason for the analogous DNA damage observed in both groups. The swallowing of saliva has been blamed for the increased rate of esophageal cancer among these people.[32,33] Because areca nut, when chewed alone, always is consumed in toto, it might increase the body burden of tannins, alkaloids, and the in vivo formation of nitrosamines from the alkaloids. The genotoxic effects of the saliva of the chewers have been reported.[34] Tannins also have been found to induce SCE in human lymphocytes.[35] The increase in SCE and CA frequency among chewers with diseased oral mucosa might be attributed to the effect of the habit, the occurrence of the disease, or a combined effect of both. SCE increases have been reported in patients with precancerous conditions.[11,36] Contradictory findings have been reported regarding SCE among patients with OC.[23,37] Hence, the exact reason for additional SCE increases in patients with OC remains to be determined.

The increase in DNA damage among areca nut chewers signifies the in vivo genotoxic effects of areca nut on the nontarget tissue. This indicates the possible role of areca nut in increasing the risk of cancers at sites other than the oral cavity. Bhide et al.[38] have shown that areca nut extract can induce liver neoplasms in mice. The enhancing effects of dietary administration of areca nut on carcinogenesis in the liver and upper digestive tract was observed by Tanaka et al.[39] The modifying influence of areca nut ingredients on benzo(a)pyrene-induced carcinogenesis also has been reported.[40] Tumor-promoting activity of areca nut also has been documented.[22,41]

The target organ for the genotoxic action of areca nut is the oral epithelium. The difficulty in obtaining a dividing cell population from human epithelial tissue has been resolved by adapting the micronucleus test. Poor oral hygiene in patients with OC hampered the collection of smears from all these patients; hence, this test was not performed among them. The percentages of MNC were found to increase significantly among the chewers. An increased frequency among Indians chewing areca nut and betel quid has been reported.[42] It is speculated that the release of areca nut alkaloids, tannins, nitrite, and thiocyanate during chewing and the formation of N-nitroso compounds in the saliva[14] contribute to the nearly threefold greater frequency of MNC among normal chewers and patients with OSMF. Micronuclei represent an early marker reflecting areca nut–initiated epithelial carcinogenesis in the oral mucosa. Although the percentages of MNC among NAC and patients with OSMF remained comparable, the numbers of micronuclei were found to be higher among areca nut chewers with OSMF (Fig. 3). This suggests that it serves as an easily quantifiable marker, not only in the assessment of genotoxic rates, but also in indicating the early premalignant changes in habit-associated oral carcinogenesis.

Thus, in the current investigation, a combined application of three cytogenetic end points proved to be a highly sensitive indicator in quantitating genetic injury caused by areca nut consumption among humans. This signals the need for new investigations precisely delineating the use of chewing tobacco and areca nut separately and not just as components of betel quid.

Interindividual differences have been observed in different markers, and interpretations should be made cautiously. This also may reflect the variation in the risk of cancer, and only long-term follow-up studies of these people can resolve the enigma. Nevertheless, in view of the in vivo DNA damage, henceforth during population monitoring with cytogenetic parameters, the habit of chewing areca nut also should be taken into account and be considered equally harmful as tobacco.

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