Role of Lime in the Generation of Reactive Oxygen Species from Betel-Quid Ingredients

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The role of lime in the formation of reactive oxygen species (ROS), i.e., O₂⁻, H₂O₂, and OH⁻, from betel-quet components (extracts of areca nut and catechu) was investigated in vitro using a chemiluminescence technique and an assay for oxidative DNA damage involving analysis of 8-hydroxy-2'-deoxyguanosine. Of the various areca-nut extracts, the catechin fraction, at alkaline pH, was shown to be the most active producer of ROS. The free Ca(OH)₂ content and pH of lime samples (a component of betel quid and chewing tobacco) were highly correlated with the generation of ROS from areca-nut extract in vitro and with oxidative base damage to DNA in vitro. While Fe²⁺ had an enhancing effect on ROS formation, Mg²⁺ had a marked inhibitory effect. The cytogenetic effects of ROS generated in vitro were measured in Syrian golden hamsters in which the cheek pouch had been painted with lime and an areca-nut extract or catechu, singly or in combination. The frequency of micronucleated cells was increased only in animals that had received both the areca-nut extract and lime. The frequency of micronucleated cells in exfoliated oral mucosal cells from Indian chewers of betel quid with tobacco containing lime or of tobacco with lime was significantly higher than in a control (no habit) group. These studies demonstrate that addition of lime to betel quid constituents generates ROS, which induce cytogenetic damage in hamster cheek pouch and may contribute to the cytogenetic damage observed in the oral cavity of betel-quid chewers. These results implicate ROS in clastogenesis and probably in the etiology of oral cancer.

Introduction

The chewing of betel-quid with tobacco has been established as the principal etiological factor for the high incidence of oral cancer in India and some other Asian countries. Potent carcinogenic agents have been derived from tobacco and areca-nut, in particular, tobacco- and areca-nut-specific nitrosamines (1). Less attention has been paid to the phenolic compounds in areca-nut and catechu, to which betel-quid chewers are exposed in relatively large quantities. Several polyphenols have been shown to be genotoxic at alkaline pH in Saccharomyces cerevisiae, probably by formation of reactive oxygen species [ROS (2)]. ROS have been postulated to induce oxidative and chromosomal damage which could be involved in several stages of the carcinogenic process in oral mucosa (2). The use of lime by betel-quid chewers to achieve an alkaline pH may thus play a crucial role in the genesis of oral cancer (3), particularly in areas of the world where no tobacco is used in the quid. Therefore, we have studied the role of lime in the formation of ROS by three approaches: a) using a chemiluminescence assay to measure 8-hydroxy-deoxyguanosine (8-OH-dG) formation in vitro from betel-quid ingredients; b) using the micronucleus test to assess chromosomal damage in the hamster cheek pouch in vivo, and c) measuring the frequency of micronucleated, exfoliated oral mucosal cells in chewers of betel-quid with lime. Except for the study on hamster cheek pouch, details of this work have been published in detail elsewhere, as cited in the text.

Experimental Methods and Results

Preparation of Samples

Areca-nut extract, areca-nut tannin, areca-nut catechin, and areca-nut flavonoids were prepared as described earlier (4). Lime suspensions, prepared in water at a concentration of 0.05% (w/v), were centrifuged and used immediately. Catechu was powdered and suspended in distilled water.

Formation of ROS in Vitro

The chemiluminescent responses due to ROS were measured in a luminometer linked to an Apple IIe computer (5).
To samples containing lucigenin and various concentrations of test compounds in a final volume of 200 µL, lime solutions (200 µL) were added by autodispenser with constant stirring and the chemiluminescence response monitored at 30°C. A sharp peak at 2-2.5 sec was identified as due to $O_2^-$, indicating inhibition of its generation by superoxide dismutase. Another chemiluminescent response at 60 sec, which could be inhibited by catalase, was taken to be proportional to the amount of $H_2O_2$ generated. The four areca-nut extracts were capable of generating ROS at alkaline pH (Fig. 1); the catechin fraction was the most active on a weight basis. In these assays, lime-mediated ROS formation was inhibited in a dose-dependent manner by $Mg^{2+}$; about 50% inhibition of $H_2O_2$ formation was seen at a concentration of 250 µM $Mg^{2+}$.

Micronucleus Formation in the Hamster Cheek Pouch

To examine the effect of lime on ROS generation in vivo, 6- to 8-week old Syrian golden hamsters, in groups of five, were given 0.01% atropine in drinking water for 2 hr prior to treatment to decrease salivation. Cheek pouches were then painted once a day for 5 days with various components of betel-quid at the following concentrations: 50 µL of 20 mg/mL areca-nut extract or catechu solution, 50 µL of a 4% lime solution and 25 µL of either an areca-nut extract or catechu solution and 25 µL of a 0.3% $H_2O_2$ solution with or without 50 µL lime solution. A control group that had no atropine treatment showed that atropine did not affect micronucleus formation. As seen in Figure 2, significantly elevated frequencies of micronuclei were observed in groups that received areca-nut extract plus lime or catechu plus lime, and the effect in these groups was comparable to that in the $H_2O_2$-treated groups. Lime did not modify micronucleus formation in the $H_2O_2$-treated group (Fig. 2). Thus, in this hamster model, the clastogenic effects are due to ROS that are generated from areca-nut extracts in the presence of the alkaline pH resulting from application of lime.

Effect of Lime Composition on ROS Formation

Twenty-five lime samples, collected in Papua New Guinea from regions where the incidence of oral cancer is high were assayed in vitro for their ability to generate ROS and 8-OH-dG in DNA in the presence of areca-nut extracts (5). Positive and highly significant correlations were shown to exist between the $Ca(OH)_2$ content of the lime samples and their ability to generate $H_2O_2$ or $O_2^-$ or to produce 8-OH-dG in DNA ($r = 0.8; p < 0.005$).

Frequency of Micronucleated Oral Mucosal Cells in Chewers of Betel-Quid

Exfoliated human oral mucosal cells were collected as described earlier (6) from chewers of betel-quid with tobacco and lime ($n = 35$), chewers of tobacco plus lime ($n = 35$), and a control (no habit) group ($n = 27$). The frequency of micronucleated cells was significantly elevated in the two exposed groups: $4.83 \pm 0.70$ ($p < 0.02$) and $5.20 \pm 0.66$ ($p < 0.005$) as compared to the control group ($2.59 \pm 0.37$). No correlation was seen between age, duration, or frequency of habit and the frequency of micronucleated cells in either of the habit groups.

Discussion

A number of studies have demonstrated that pH, as determined by the free $Ca(OH)_2$ content of lime, is the major determinant of the generation of ROS from betel-quid components (2,5,7). At pH ≥ 9.5, polyphenols from
areca-nut undergo autodestruction and yield $O_2^-$, which has been detected in our assays in vitro (7). The presence of iron in betel-quid, also detected in lime samples (5), could play an important role in the formation of OH radicals, the principal DNA-damaging species implicated in the formation of 8-OH-dG. These may arise from $O_2^-$ via a transition metal-catalyzed Haber-Weiss reaction (Eq. 1) or via the Fenton reaction (Eq. 2):

$$O_2^- + H_2O_2 \rightarrow O_2 + HO^- + H_2O \quad (1)$$

$$H_2O_2 + Fe^{2+} + H^+ \rightarrow HO^- + Fe^{3+} + H_2O \quad (2)$$

The use of lime together with areca-nut was found to elevate the pH in the oral cavity into the alkaline range, as measured in chewers' saliva in Papua New Guinea (R. McLennan, personal communication). The likelihood that ROS are formed at the site where lime is placed in the oral cavity during the chewing process is supported by our experimental data and human observations. We observed an increased frequency of micronucleated cells in the cheek pouch of hamsters treated with both lime and betel-quid ingredients but not in those treated with any of the agents alone. Chewing of betel-quid with lime or of tobacco with lime increased the number of micronucleated oral mucosa cells over that in a no-habit control group (6). Oxidative DNA base damage, such as 8-OH-dG, and chromosomal damage in buccal mucosal cells of chewers of betel-quid with lime may be partly responsible for the genesis of oral cancer in these people. 8-OH-dG in DNA, if not repaired, is a miscoding lesion that leads to G to T transversions (9,10). Sequence analysis of DNA from oral cancer tissue to detect the prevalence of point mutations, for example in the p53 tumor-suppressor gene (11), may give further clues to the causative etiological agent(s).

This manuscript was presented as a poster at the Conference on Biomonitoring and Susceptibility Markers in Human Cancer: Applications in Molecular Epidemiology and Risk Assessment that was held in Kailua-Kona, Hawaii, 26 October–1 November 1991.

This work was supported by U.S. National Institutes of Health grant no. CA 43176.

REFERENCES

1. IARC. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Vol. 37. Tobacco Habits Other than Smoking: Betel-quid and Areca-nut Chewing, and Some Related Nitrosamines. International Agency for Research on Cancer, Lyon, 1985.

2. Stich, H. F., and Andrews, F. The involvement of reactive oxygen species in oral cancers of betel-quid/tobacco chewers. Mutat. Res. 214: 47–61 (1989).

3. Atkinson, L., Chester, I. C., Smyth, F. G., and Seldam, R. E. J. Oral cancer in New Guinea. Cancer 17: 1289–1298 (1964).

4. Stich, H. F., Ohshima, H., Pignatelli, B., Michelon, J., and Bartsch, H. Inhibitory effect of betel nut extracts on endogenous nitrosation in humans. J. Natl. Cancer Inst. 70: 1047–1050 (1983).

5. Nair, U. J., Friesen, M., Richard, I., McLennan, R., Thomas, S., and Bartsch, H. Effect of lime composition on the formation of reactive oxygen species from areca-nut extracts in vitro. Carcinogenesis 11: 2145–2148 (1990).

6. Nair, U., Obe, G., Nair, J., Maru, G. B., Bhide, S. V., Pieper, R., and Bartsch, H. Evaluation of frequency of micronucleated oral mucosal cells as a marker for genotoxic damage in chewers of betel-quid with or without tobacco. Mutat. Res. 261: 163–168 (1991).

7. Nair, U. J., Floyd, R. A., Nair, J., Bussanchini, V., Friesen, M., and Bartsch, H. Formation of reactive oxygen species and of 8-hydroxydeoxyguanosine in DNA in vitro with betel-quid. Chem.-Biol. Interact. 63: 157–169 (1987).

8. Heddle, J. A. A rapid in vitro test for chromosomal damage. Mutat. Res. 18: 187–190 (1973).

9. Wood, M. L., Dizdaroglu, M., Gajewski, F., and Essigmann, J. M. Mechanistic studies of ionizing radiation and oxidative mutagenesis: genetic effects of a single 8-hydroxyguanine (7-hydro-8-oxoguanine) residue inserted at a unique site in a viral genome. Biochemistry 34: 7024–7032 (1990).

10. Shibutani, S., Takeshita, M., and Grollman, A. P. Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxoG. Nature 349: 431 (1991).

11. Levine, A. J., Monand, J., and Finlay, C. A. The p53 tumour suppression gene. Nature 361: 459–466 (1993).