

# 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.,  $O_2^-$ ,  $H_2O_2$ , and  $OH^\bullet$ , from betel-quin 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  $Fe^{2+}$  had an enhancing effect on ROS formation,  $Mg^{2+}$  had a marked inhibitory effect. The cytogenetic effects of ROS generated *in vivo* 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-quin chewers. These results implicate ROS in clastogenesis and probably in the etiology of oral cancer.

## Introduction

The chewing of betel-quin 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-quin 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-quin 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-quin 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-quin 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).

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To samples containing lucigenin and various concentrations of test compounds in a final volume of 200  $\mu\text{L}$ , lime solutions (200  $\mu\text{L}$ ) 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  $\text{O}_2^-$ , 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  $\text{H}_2\text{O}_2$  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  $\text{Mg}^{2+}$ ; about 50% inhibition of  $\text{H}_2\text{O}_2$  formation was seen at a concentration of 250  $\mu\text{M}$   $\text{Mg}^{2+}$  (5).

### 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  $\mu\text{L}$  of 20 mg/mL areca-nut extract or catechu solution, 50  $\mu\text{L}$  of a 4% lime solution and 25  $\mu\text{L}$  of either an areca-nut extract or catechu solution and 25  $\mu\text{L}$  of a 0.3%  $\text{H}_2\text{O}_2$  solution with or without 50  $\mu\text{L}$  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 catechin plus lime, and the effect in these groups was comparable to that in the  $\text{H}_2\text{O}_2$ -treated groups. Lime did not modify micronucleus formation in the

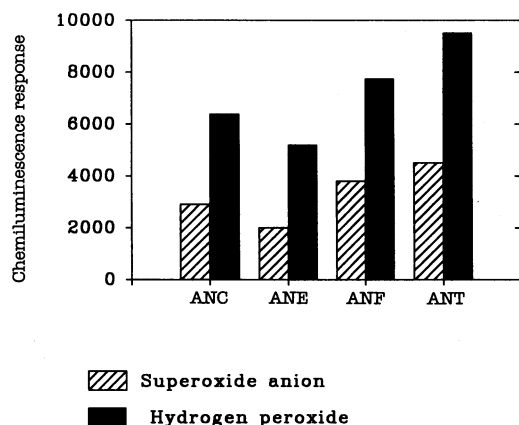


FIGURE 1. Formation of  $\text{O}_2^-$  (hatched bars) and  $\text{H}_2\text{O}_2$  (filled bars) by various areca-nut extracts in the presence of lime at alkaline pH. Chemiluminescence response in the presence of lucigenin is given in arbitrary units. ANC, areca-nut catechins (2  $\mu\text{g}$ ); ANE, areca-nut extract (50  $\mu\text{g}$ ); ANF, areca-nut flavonoids (50  $\mu\text{g}$ ); ANT, areca-nut tannins (50  $\mu\text{g}$ ) [for more details, see text and Nair et al. (6,7)].

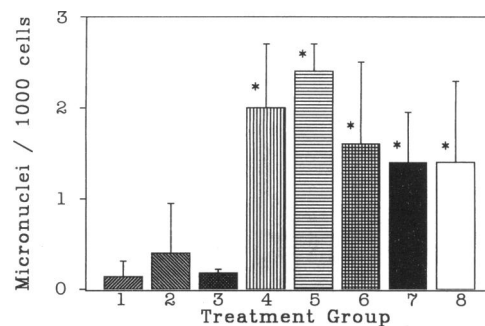


FIGURE 2. Frequency of micronucleated cells in the cheek pouches of groups of five hamsters treated once a day for 5 days with various betel-quid ingredients or hydrogen peroxide: 1, control; 2, areca-nut extract plus catechu; 3, lime; 4, areca-nut extract plus lime; 5, catechu plus lime; 6, areca-nut extract plus catechu plus lime; 7, hydrogen peroxide; 8, hydrogen peroxide plus lime. Micronuclei were scored using a standard procedure (8); means  $\pm$  SD are plotted. Treatment groups that had significantly elevated frequencies of micronuclei ( $p < 0.01$ ; Student's *t*-test) are indicated by an asterisk (\*). The values for the control group with and without atropine treatment were 0.14  $\pm$  0.17 (SD) and 0.16  $\pm$  0.06, respectively.

$\text{H}_2\text{O}_2$ -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  $\text{Ca}(\text{OH})_2$  content of the lime samples and their ability to generate  $\text{H}_2\text{O}_2$  or  $\text{O}_2^-$  or to produce 8-OH-dG in DNA ( $r = 0.8$ ;  $p < 0.005$ ).

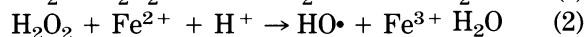
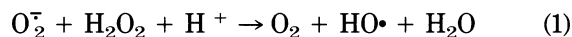
### Frequency of Micronucleated Oral Mucosal Cells in Chewers of Betel-Quid

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  $\text{Ca}(\text{OH})_2$  content of lime, is the major determinant of the generation of ROS from betel-quid components (2,5,7). At pH  $\geq 9.5$ , polyphenols from

areca-nut undergo autoxidation 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):



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).

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