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Minimal arecaidine concentrations showing a promotion effect during DMBA-induced hamster cheek pouch carcinogenesis

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The purpose of the present study was to determine the minimal arecaidine concentrations showing a synergistic effect on DMBA-induced hamster cheek pouch carcinogenesis. One hundred and twelve male adult Syrian golden hamsters were divided into 16 groups, each containing seven animals. After eight weeks of DMBA initiation and then four weeks of arecaidine promotion, 100% tumor incidence was found with arecaidine concentrations of 400 μg/ml and 500 μg/ml; average tumor numbers were 1.86±0.63 and 1.86±0.93 respectively ($P<0.05$). After four weeks of DMBA and a subsequent eight weeks of arecaidine painting, all hamsters developed visible tumors with arecaidine concentrations of 900 μg/ml and 1000 μg/ml; average tumor numbers were 1.86±0.82 and 2.14±1.09 respectively ($P<0.05$). The tumor dimensions varied little and differences were not statistically significant. Without DMBA pretreatment, regardless of the high arecaidine concentrations (1000 μg/ml, 2000 μg/ml and 3000 μg/ml) applied, no visible tumor growth was observed; only hyperkeratosis and inflammation could be discerned histologically. Thus, the minimal concentrations of arecaidine displaying a synergistic effect in the DMBA-induced hamster cheek pouch of carcinogenesis were found to be 400 μg/ml applied for four weeks after eight weeks of DMBA application, and 900 μg/ml applied for eight weeks after four weeks of DMBA painting. These findings may be useful for other studies concerning the tumorgenicity of arecaidine.

Key words: arecaidine; DMBA-carcinogenesis; hamster; oral; promotion

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Betel quid chewing is a common habit in Asia and is related to the high frequency of oral cancers in many Asian countries, including Taiwan (1). Arecaidine is regarded as one of the main constituents of betel nut contributing to its role in the aetiology of oral cancers (2). It is mutagenic in bacteria (3) and, using the technique of *in vitro* cell transformation assay, induces transformed cells in baby hamster kidney (4). In addition, using the SOS chromotest, arecaidine has been found to be mutagenic (5). Thus, the mutagenic properties of arecaidine *in vitro* have been fully established. However, very little is currently known about the effects of arecaidine *in vivo* (6).

Thus far, only a few studies (7, 8) on the tumorgenicity of arecaidine in the hamster cheek pouch model of carcinogenicity (9) have been reported. McDonald (7) failed to induce squamous cell carcinoma by painting arecaidine on the buccal pouch mucosa of hamsters. This may have been due to the low concentration (25 μg/ml) and pH value (pH=3) applied. Later, our laboratory (8) studied the promotion effect of arecaidine on hamster buccal pouch mucosa with DMBA as the initiator for four and eight weeks respectively. By increasing the pH value to 7.2 and the arecaidine concentration to 500 μg/ml, exophytic squamous cell papillomas or carcinomas were successfully induced in 7,12-dimethylbenz(a)anthracene (DMBA)-treated hamster buccal pouches (8). We found that after eight weeks of DMBA initiation and subsequent four weeks of arecaidine promotion, 100% tumor incidence was obtained with an arecaidine concentration of 500 μg/ml. On the other hand, only 30% tumor incidence was found after four weeks of DMBA and then eight weeks of arecaidine applications at a concentration of 500 μg/ml (8). Thus, the synergis-

tic effect of arecaidine in DMBA-induced hamster cheek pouch carcinogenesis has been demonstrated, with the minimal concentration being less than 500 μg/ml after eight weeks or more than 500 μg/ml after four weeks of DMBA initiation. However, the exact minimal arecaidine concentrations required have not been determined.

The aim of the present study was to confirm our previous work (8) and further to determine the minimal concentrations of arecaidine showing a synergistic effect in hamster cheek pouch tissue in DMBA-induced squamous cell carcinogenesis. The minimal concentration is defined as the concentration that produces a 100% incidence of histologically proven tumors that are either squamous cell papillomas or carcinomas.

Material and methods

A total of 112 non-inbred male adult Syrian golden hamsters (10–12 weeks old) weighing approximately 120g were obtained from the National Taiwan University Breeding Laboratory. On arrival, the animals were housed in a temperature-controlled room, fed on a commercial diet with tap water. After allowing a week to adjust to the new surroundings, the hamsters were divided into 16 groups (A to P), each containing seven animals. With reference to our previous study (8), a treatment regimen over a 12 weeks experimental period was employed in the present study (Table 1).

The arecaidine concentrations in groups H to L ranged from 600 μg/ml to 1000 μg/ml in increments of 100 μg/ml.

The right buccal pouch of each hamster in group A was painted three times weekly for 12 consecutive weeks with a heavy mineral oil containing 0.5% DMBA (Sigma Chemical Co., St. Louis, Missouri); the same pouch of groups B to F was painted three times weekly for eight weeks with the same oil, using a No. 4 camel-hair brush. After eight weeks, DMBA application to group B animals was discontinued and the animals were left untreated for four weeks. Concentrations of arecaidine solution (Sigma Chemical Co., St. Louis, Missouri) in polyethylene glycol (PEG, #400:#4000=1:2), ranging from 200 μg/ml up to 500 μg/ml in increments of 100 μg/ml, were applied to the right buccal pouch of hamsters in groups C to F respectively six times a week for a further period of four weeks. The left pouches of animals in groups A to G were untreated throughout the whole trial, serving as a control. The right buccal pouches of hamsters in groups G to L were painted with DMBA three times a week for four weeks. Following this period, DMBA painting of group G animals was terminated and the animals were left untreated for eight weeks. Arecaidine solutions, ranging in concentration from 600 μg/ml to 1000 μg/ml in increments of 100 μg/ml, were painted on the right buccal pouches of hamsters in groups H to L respectively six times weekly for a further eight weeks. The left pouches of animals in groups G to L were left untreated throughout the whole experiment. Arecaidine solution in three different concentrations (1000 μg/ml, 2000 μg/ml and 3000 μg/ml) was applied to the right buccal pouches of hamsters (untreated with DMBA) in groups M to O six times weekly for 12 weeks. Group P animals, that were left untreated throughout the trial, served as further controls. The treatment protocol is summarized in Table 1.

Table 1. Protocol to determine the minimal arecaidine concentrations showing promotion effect during DMBA-induced hamster buccal pouch carcinogenesis

Exp. Gp	Treatment protocol (12wk)
A. DMBA	12wk →
B. DMBA	8wk → No treatment, 4wk →
C. DMBA	8wk → (200 μg/ml), Arecaidine 4wk →
D. DMBA	8wk → (300 μg/ml), Arecaidine 4wk →
E. DMBA	8wk → (400 μg/ml) Arecaidine 4wk →
F. DMBA	8wk → (500 μg/ml) Arecaidine 4wk →
G. DMBA	4wk → No treatment 8wk →
H. DMBA	4wk → (600 μg/ml), Arecaidine 8wk →
I. DMBA	4wk → (700 μg/ml), Arecaidine 8wk →
J. DMBA	4wk → (800 μg/ml), Arecaidine 8wk →
K. DMBA	4wk → (900 μg/ml), Arecaidine 8wk →
L. DMBA	4wk → (1000 μg/ml), Arecaidine 8wk →
M. (1000 μg/ml) Arecaidine	12wk →
N. (2000 μg/ml) Arecaidine	12wk →
O. (3000 μg/ml) Arecaidine	12wk →
P. no treatment	12wk →

At the end of 12 weeks, all the animals were killed by a lethal dose of diethyl ether. As indicated, the tumors were exophytic, well-defined and tended to be spherical in shape. Each buccal pouch was exposed and examined grossly; the number of growths was counted and the diameter of the tumors was measured. The mucosa was excised. Samples were taken from areas where lesions were visible; otherwise they were taken longitudinally, at random. The samples were immersed for 12 h in 10% neutral buffered formalin, dehydrated in ascending alcohols and cleared in xylene, before embedding in paraffin. Sections of 4 μm thickness were cut, stained with hematoxylin and eosin (H&E) and examined under a light microscope. Particulars concerning the animals in groups B to F and G to L and the number and size of their tumors are summarized in Tables 2 and 3.

Table 2. Effect of DMBA (8wk) and arecaidine (4wk) on hamster buccal pouch carcinogenesis

Group	No. of hamsters	No. of hamsters with tumor	Total no. of tumors	Average no. of tumors	Average dimension (mm) of tumors
B(0)[a]	7	5	7	1±0.76	0.7±0.2
C(200)[a]	7	5	9	1.09±1.02	0.85±0.15
D(300)[a]	7	5	8	1.14±0.99	1.2±0.36
E(400)[a]	7	7[b]	13	1.86±0.63[c]	1.4±0.4
F(500)[a]	7	7[b]	13	1.86±0.93[c]	1.33±0.5

[a] No. in brackets represents arecaidine concentration in μg/ml
[b] One hundred percent tumor incidence
[c] Statistical significance as compared with group B ($P<0.05$, *t*-test)

Table 3. Effect of DMBA (4wk) and arecaidine (8wk) on hamster buccal pouch carcinogenesis

Group	No. of hamsters	No. of hamsters with tumor	Total no. of tumors	Average no. of tumors	Average dimension (mm) of tumors
G(0)[a]	7	0	0	0	0
H(600)[a]	7	5	7	1±0.75	2±1.2
I(700)[a]	7	5	8	1.14±0.99	2.57±1.7
J(800)[a]	7	4	7	1±0.65	2.45±1.6
K(900)[a]	7	7[b]	13	1.86±0.82[c]	2.89±1.4
L(1000)[a]	7	7[b]	15	2.14±1.09[c]	2.53±1.5

[a] No. in brackets represents arecaidine concentration in μg/ml
[b] One hundred percent tumor incidence
[c] Statistical significance as compared with group H ($P<0.05$, *t*-test)

Animals in groups C to F were compared with those in group B and animals in groups I to J were contrasted with those in group H. Differences were tested by the Student's *t*-test.

Results

Histologically, the tumors yielded in the present study were either exophytic squamous cell papillomas or carcinomas. All animals in the positive control group A developed visible tumors (a total number of 15; 2.33±1.05 tumor per animal with dimensions of 2.75±1.35), while those of negative control group P were free of tumors as demonstrated under the light microscope using H&E staining. After eight weeks of DMBA initiation and a subsequent four weeks of arecaidine promotion, the incidence of tumor growth was 100% in groups E (arecaidine concentration: 400 μg/ml) and F (arecaidine concentration: 500 μg/ml), with the average tumor numbers of 1.86±0.63 and 1.86±0.93 respectively (Student's *t*-test, $P<0.05$; Table 2). After four weeks of DMBA initiation followed by eight weeks of arecaidine promotion, all hamsters in groups K (arecaidine concentration: 900 μg/ml) and L (arecaidine concentration: 1000 μg/ml) demonstrated visible tumors, with average tumor numbers of 1.82±0.82 and 2.14±1.09 respectively (Student's *t*-test, $P<0.05$; Table 3). Differences in tumor dimensions were not statistically significant (Tables 2, 3). Without DMBA pretreatment, regardless of the high arecaidine concentrations (1000 μg/ml, 2000 μg/ml and 3000 μg/ml) applied, no visible tumor growths were noted in groups M to O; only hyperkeratosis and inflammation could be seen histologically. Therefore, the minimal concentration of arecaidine showing synergistic effect in DMBA-induced hamster buccal pouch carcinogenesis was found to be 400 μg/ml if applied for four weeks after eight weeks of DMBA initiation, and 900 μg/ml if applied for eight weeks after four weeks of DMBA application.

Discussion

The results of these experiments suggest that under the synergistic effect of arecaidine, a shorter period of DMBA initiation may be needed to attain an average tumor number comparable to the positive control group.

Cellular mechanisms of promotion may involve an increase in cellular DNA activity (10). This is in accord with the facts that arecaidine may cause chromosome breakdown and an increase in sister-chromatid-exchange (SCE) (11). It is possible that the DMBA-initiated epithelial cells of hamster buccal pouch were enhanced by the action of arecaidine at suitable concentrations. It may be reasonable to predict that the shorter the DMBA initiation period, the greater arecaidine concentration may be needed to display its synergistic effect. A similar concentration-dependent action of arecaidine on oral fibroblasts has been reported recently (12).

For the groups M to O, without DMBA pretreatment, arecaidine concentrations (1000 μg/ml, 2000 μg/ml and 3000 μg/ml) in the present study have been elevated to at least 80 times that of McDonald (7). Regardless of such high arecaidine concentrations, no visible tumor growth was noted after 12 weeks of arecaidine painting. Only hyperkeratosis and inflammation can be observed histologically. This microscopic finding is similar to that of McDonald (7). However, the effects of an application period significantly longer than 12 weeks remain unknown and further studies may be required.

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