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Cancer-promoting effect of Taiwan betel quid in hamster buccal pouch carcinogenesis

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OBJECTIVE: To investigate the cancer-promoting effect of Taiwan betel quid in hamster buccal pouch carcinogenesis.

MATERIALS AND METHODS: Two hundred and fifty-two non-inbred male adult Syrian golden hamsters were randomly divided into six groups, each containing forty-two animals. A treatment regimen over a 14-week experimental period was employed with six animals per group being killed at seven different periods (every 2 weeks). The right buccal pouch of each animal was painted three times a week with various combinations of 7,12-dimethylbenz[a]anthracene (DMBA), Taiwan betel quid extract, dimethyl sulfoxide (DMSO) and mineral oil.

RESULT: Both the number and size of tumors in animals concurrently treated with DMBA and betel quid were significantly higher than those in animals treated with DMBA alone in each killing period of 8, 10, 12 and 14 weeks. No visible tumors but hyperkeratosis and acanthosis were observed in pouches treated with betel quid alone for all killing periods.

CONCLUSION: Our results indicate Taiwan betel quid may be a co-carcinogen in human oral carcinogenesis, if extrapolation can be made from the current animal study.

Keywords: betel quid; promotion; hamster; DMBA-carcinogenesis

Introduction

Betel quid refers to a combination of areca nut, betel leaf, catechu, slaked lime and tobacco (Nagabhushan *et al*, 1986). There are approximately 2 million betel quid chewers in Taiwan (Ko *et al*, 1992; Chiu *et al*, 1997). In Northern Taiwan, 53.4% of 103 oral cancer patients were betel quid chewers; nearly 80% of all oral cancer deaths related to this habit (Kwan, 1976) whilst 86.2% of 167 oral cancer patients in Southern Taiwan practiced the same habit (Chen, 1987). Furthermore, betel quid chewing has been shown to be statistically significantly associated with oral cancer in Taiwan (Ko *et al*, 1995).

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The International Agency for Research on Cancer (IARC, 1985) stated that there was adequate proof for an association between chewing betel quid together with tobacco use (chewing or smoking) and oral cancer, but insufficient evidence supported a link between betel quid chewing alone and human cancer. It is crucial to further clarify the carcinogenic effect of betel quid alone on oral mucosa, without the combined effect of tobacco or cigarettes. Such carcinogenic effect of betel quid alone on oral mucosa may not be readily determined from individual human biopsy tissue because most betel quid chewers also have tobacco chewing, cigarette smoking and alcohol drinking habits.

The hamster buccal pouch model, first introduced by Salley (1954) and further developed by Morris (1961), is the most widely accepted experimental model of oral carcinogenesis (Lin *et al*, 1989; Lin and Chen, 1991; Chen and Lin, 1996) and closely resembles human lesions (Morris, 1961). Using this model, it should be possible to follow the effects of betel quid during oral carcinogenesis. The aim of the current study was to investigate the cancer promoting effect of Taiwan betel quid on hamster buccal pouch carcinogenesis.

Material and methods

Chemicals

All chemicals including 7,12-dimethylbenz[a]anthracene (DMBA), mineral oil and dimethyl sulfoxide (DMSO), procured from Sigma Chemical Company (St Louis, MO USA), were of the highest purity commercially available.

Extraction of betel quid

The same brand of Taiwan betel quid was purchased from the domestic market of Kaohsiung city for the whole experiment. It comprised a bisected fresh green betel nut sandwiched with an unripe betel fruit and slaked lime. Betel quids were extracted according to the method of Ranadive *et al* (1976), using DMSO as the solvent. Briefly a mixture of betel nut (450 g), unripe betel fruit (120 g) and slaked lime (50 g) was ground together into a paste form with a homogenizer; refrigerated at 4°C for 48 h. Then, the ground material was mixed thoroughly with 300 ml DMSO, strained through a clean cheese-cloth and stored at 4°C in a glass-stoppered, amber bottle until used for the experiment.

Hamsters (Table 1)
Two hundred and fifty-two non-inbred male adult Syrian golden hamsters (8–10 weeks old) weighing approximately 100 g at the commencement of the experiment were obtained from the National Taiwan University Breeding Laboratory. The hamsters were housed under constant conditions (22°C, 12 h light/dark cycle), fed with tap water and standard purina laboratory chow *ad libitum*. After allowing 1 week to acclimatize to the new surroundings, the hamsters were randomly divided into six groups A–F, each containing 42 animals.

Betel quid treatments (Table 1)
A treatment regimen over a 14-week experimental period was employed. The protocol ensured humane practices. The right pouches of hamsters in groups A to C were painted three times a week with DMBA concurrently with betel quid, DMBA alone and betel quid alone. At the end of 2 weeks, six animals from each group A to F were withdrawn randomly from the painting and killed by a lethal dose of diethyl ether in a gas jar at the same time (9 am) of the day to avoid the influence of diurnal variation (Lin and Goepp, 1983). The killed animals were fixed in a supine position with pins. Bilateral pouches were exposed by dissection, cut from their oral openings to their caudal ends along the middle of their lateral walls and examined grossly. As indicated, the tumors were exophytic, well-defined and tended to be spherical in shape (Lin *et al*, 1996). The number of growths was counted and the diameter of the tumors was measured. Similar procedures were performed every second week until all the animals were killed at the end of the experiment. Representative specimens were routinely processed for HE staining. Differences in tumor numbers and dimensions were evaluated by the Student's *t*-test (two-tailed). Statistical significance was indicated when $P<0.05$.

Table 1 Grouping and applications of combinations of betel quid and DMBA to hamsters

Groups	*Right pouches*	*Left pouches*	*Animal numbers*
A	BQ+DMBA	No treatment	42
B	DMBA	No treatment	42
C	BQ	No treatment	42
D	DMSO	No treatment	42
E	Mineral oil	No treatment	42
F	No treatment	No treatment	42

Group A. Concurrently with betel quid (BQ) six times a week and 0.5% DMBA three times a week
Group B: 0.5% DMBA alone three times a week
Group C: BQ alone six times a week
Group D: DMSO six times a week
Group E: Mineral oil six times a week
Group F: No treatment

Results

Gross observations (Table 2)
Hamsters in groups A and B demonstrated erythema and ulceration after 2 weeks painting and by the 4th and 6th weeks, there was thickened mucosa. Papillomatous tumor growths were subsequently seen in the pouches of groups A and B at week 8; exophytic squamous cell carcinomas of various sizes were then induced in pouches at weeks 10, 12 and 14. Both the number and size of tumors in animals of group A were significantly higher than those of group B in each of killing period 8, 10, 12, and 14 weeks ($P<0.05$, Student's *t*-test). Pouches of group C were grossly flat and tumor free with thickened mucosa after 14 weeks treatment. Gross examination of all the pouches in groups D to F as well as the left pouches in all animals revealed no obvious changes in each killing period.

Histological findings
Microscopically, 2-week treated pouches in groups A and B showed an acute inflammatory reaction with engorged blood vessels and severe mucositis. Following the stage of repair in weeks 4 and 6, there was hyperkeratosis, acanthosis and lymphocytic infiltration. The first histologic evidence of overt squamous cell carcinoma was noticed at week 8 in pouches of groups A and B (Table 2). Hyperkeratosis and acanthosis were observed in pouches of group C for all killing periods. No obvious histological changes were noticed in all the pouches of groups D to F. The left pouches of all animals in each killing period appeared to be normal.

Discussion

DMSO was employed as the vehicle of betel quid in this study. It increases the cutaneous and mucous membrane absorption of many chemicals without altering their pharmacological characteristics and possesses the merit of shortening the latency period for tumor production in hamsters treated with DMBA (Suri *et al*, 1971).

Our result is in contrast to the report of Suri *et al* (1971) who demonstrated the carcinogenic effect of a DMSO extract of betel nut alone on the mucosa of the hamster buccal pouch. It is also different from the finding of Ranadive *et al* (1979) who reported that non-tobacco-containing betel quid induces tumors in hamster buccal pouch mucosa. On the other hand, our results are consistent with the findings of Hamner (1972), Mori *et al* (1979) and Wong *et al* (1992); they failed to induce tumors in buccal mucosa of baboon, rat and hamster on exposure to betel quid. These conflicting outcomes may partially be due to the disparities in the constituents or processing techniques of betel quid derived from different geographic areas. A wide variation in arecoline concentrations (an alkaloid of betel nut) among some commercial betel quids from different regions and closely linked to the regional prevalence of oral leukoplakias has been demonstrated (Awang, 1986). Therefore, the understanding of the carcinogenic and mutagenic effects of the various components of Taiwan betel quid are important. Jeng *et al* (1994) investigated the pathological effects of Taiwan betel quid constituents; they found that the extracts

Table 2 Average number and dimension (mm) of tumors in killing periods weeks 8, 10, 12 and 14

Killing periods	*Group A BQ+DMBA*	*Group B DMBA*	*Group C BQ*	*Group D DMSO*	*Group E mineral oil*	*Group F No treatment*
8 weeks	2.00 ± 0.58[a]	0.33 ± 0.47	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	2.33 ± 1.09[b]	0.67 ± 0.15	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10 weeks	3.67 ± 0.75	0.33 ± 0.47	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	6.33 ± 1.70	1.00 ± 0.20	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
12 weeks	5.67 ± 1.49	2.00 ± 1.29	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	10.67 ± 2.14	7.33 ± 2.89	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
14 weeks	6.33 ± 1.11	4.33 ± 1.25	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	12.33 ± 2.45	8.00 ± 2.57	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

[a]Numbers of tumors: means ± standard deviation
[b]Dimensions (mm) of tumors: means ± standard deviation

of betel nut and betel fruit induced DNA strand breakage and reduced cell survival and proliferation in a dose-dependent manner.

The 'co-carcinogenic' effect of betel quid to DMBA is apparent in the present study. Only a few studies have been concerned with the tumor promoting effect of the betel quid. Stich and Tsang (1989) demonstrated a promoting effect of areca nut extracts with an increased number of transformed foci in bovine papillomavirus transformation assay. Tanaka *et al* (1986) reported that betel quid acted synergistically in the process of oral carcinogenesis in rats fed with a betel nut diet. Furthermore, the synergistic effect of betel quid to benzo[a]pyrene-induced carcinogenesis in hamster pouch was demonstrated by Rao (1984). An earlier study, by inserting the quid inside the hamster pouch, concluded that Taiwan betel quid was probably a tumor promoter in oral carcinogenesis (Wong *et al*, 1992). These observations were confirmed in this study. Additionally, the current study also corroborated a previous report that arecaidine (an alkaloid of betel nut) demonstrated a promotion effect in DMBA-induced hamster pouch carcinogenesis (Lin *et al*, 1996).

Thus, if Taiwan betel quid is an effective promoter in oral carcinogenesis as inferred in the current study, a high proportion of oral cancer patients in Taiwan would be betel quid chewers as demonstrated by the epidemiologic surveys (Kwan, 1976; Chen 1987; Ko *et al*, 1995). On the other hand, the finding that Taiwan betel quid is not a significant carcinogen *per se* seems at least partially to agree with the fact that the age-standardized incidence rates of oral cancer per 100 000 people are 3.55 in males and 1.3 in females (Tsang and Chiu, 1990) are lower than in Hong Kong Chinese who do not practice this habit (Wu *et al*, 1986). Therefore, other causative factors including certain viruses such as human papillomaviruses (Steinberg and DiLorenezo, 1996), exogenous/endogenous carcinogens, spontaneous mutation or other unknown aetiologies are likely to initiate oral carcinogenesis.

In conclusion, although the link between betel quid and oral cancer is not a simple one, nevertheless, the results give support to the epidemiological findings (Ko *et al*, 1995) of an association of betel quid chewing and oral cancer and indicate Taiwan betel quid alone cannot induce tumors but it may be a co-carcinogen in human oral carcinogenesis, if extrapolation can be made from the current animal study.

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