Pergamon

Oral Oncol, Eur J Cancer Vol. 32B, No. 5, pp. 343–346, 1996

Printed in Great Britain
0964-1955/96 $15.00 + 0.00

PII: S0964-1955(96)00018-8

Studies on Promoting Activity of Taiwan Betel Quid Ingredients in Hamster Buccal Pouch Carcinogenesis

Y.-T. Jin,[1] S.-T. Tsai,[2] T.-Y. Wong,[3] F.-F. Chen[1] and R. M.-Y. Chen[1]

[1]Department of Pathology; [2]Department of Otolaryngology, National Cheng Kung University Medical Center; and [3]Division of Oral and Maxillofacial Surgery, National Cheng Kung University Hospital, Tainan, Taiwan

Previous studies indicated that Taiwan betel quid is a promoter rather than an initiator during the carcinogenesis of hamster buccal pouch carcinoma. The maximum promoting activity can be demonstrated 24 weeks after betel quid implantation, following an initial application of 0.5% DMBA (7,12-dimethyl benzanthracene) three times per week for 4 weeks. In the present study, components of Taiwan betel quid and its additives (dry betel nut fibre, piper betel, slaked lime, cold aqueous extract, and hot aqueous extract) were applied respectively or in various combinations to investigate their promoting activity. One hundred and thirty Syrian hamsters were divided into 13 groups based on different combinations of the betel quid ingredients applied. The incidence of tumours in the hamster buccal pouch was significantly higher in groups exposed to dry betel nut fibre ($P < 0.01$) and cold aqueous extract ($P < 0.05$). The results indicate that betel nut fibre and cold aqueous extract are the major components of betel quid that may promote carcinogenesis in the hamster buccal pouch. Copyright © 1996 Elsevier Science Ltd

Keywords: betel quid, hamster buccal pouch, carcinogenesis

Oral Oncol, Eur J Cancer, Vol. 32B, No. 5, pp. 343–346, 1996.

INTRODUCTION

The high incidence of oral cancer in South Asia has long been attributed to the betel quid chewing habit [1, 2]. Epidemiological studies and animal experiments using extracts from betel quid have both suggested that there is a statistically significant correlation between betel quid chewing and oral cancer [3–7]. However, the mechanism or biochemical processes from betel quid chewing to oral cancer, the kinetics of neoplastic transformation in terms of the quantity, frequency and duration of exposure to betel quid versus the inception of neoplasm in humans, are not clearly elucidated.

The hamster buccal pouch has been used as an experimental model for oral cancer for many years [8]. Squamous cell carcinoma can be induced by continued application of certain chemical carcinogens, such as dimethyl benzanthracene. This model has subsequently been refined [9] and it is established that application of a 0.5% solution of 7,12-dimethyl benzanthracene (DMBA) three times per week in heavy mineral oil to the buccal pouch mucosa will produce, histologically, areas of hyperkeratosis and dysplasia at 6–8 weeks, early squamous cell carcinoma at 8–10 weeks, and, finally invasive carcinoma at 10–12 weeks. Traditionally the carcinogen application is continued until the termination of the experiment. Eisenberg has, however, demonstrated that it is not necessary to apply the carcinogen throughout. In an alternative regime pathological changes occur by 10–12 weeks of application, and are irreversible leading to cancer formation despite cessation of the DMBA applications [10].

Carcinogenesis is a multistep process which is most readily demonstrated in experimental models by chemical carcinogenesis [11]. The cancer induction can be broadly divided into two stages: initiation and promotion [12]. Initiation and promotion by DMBA may be demonstrated in the hamster buccal pouch model system [13], using 0.1% DMBA as an initiator and 0.5% DMBA as a promoter. Previous reports also demonstrate that 2-acetyl-aminofluorene can act as an initiator when croton oil is used as a promoter [14].

It has not yet been possible to induce cancer in the hamster buccal pouch by betel quid alone [15, 16]. However, a few studies have shown that carcinomas could be induced by betel quid together with other carcinogens in

Correspondence to Y.-T. Jin.
Received 11 Jan. 1996; provisionally accepted 14 Feb. 1996; revised manuscript received 7 Mar. 1996.

the hamster buccal pouch [4, 17]. Unfortunately, most of these effects are either statistically insignificant or insufficient to draw firm conclusions.

We have used the hamster check pouch model to elucidate the role of Taiwan betel quid in oral carcinogenesis [7]. In that study, 160 Syrian hamsters were divided into eight experimental and control groups, each group composed of 10 animals. The incidence of tumour in cheek pouches was recorded after short-term (12 weeks) or mid-term (24 weeks) insertion of betel quid subsequent to varied periods (2 weeks, 4 weeks, or 6 weeks, respectively) of 0.5% DMBA (7,12-dimethyl benz[a]anthracene) topical application three times a week. The tumour development rate was significantly higher in the group in which betel quid was used as a promoter for 24 weeks as compared with that in the control group, using DMBA as the initiator only for 4 weeks. Treatment by betel quid alone for 36 weeks or 52 weeks, or 0.1% DMBA applied for 10 weeks after betel quid treatment did not lead to tumour formation. These results indicated that Taiwanese betel quid was probably a promoting factor in oral carcinogenesis, rather than a cancer initiator or a significant carcinogen by itself [7, 18].

In this study, we used the same animal model to investigate the carcinogenic effect of the various components of Taiwan betel quid. Consideration was also applied to parameters such as mucosal trauma due to betel nut fibres [19], quantity of betel quid, frequency and duration of areca nut extract application, and the possible carcinogenic activity of additives (slaked lime or piper betel). The ultimate goal of this study was to assess or identify the carcinogenicity or promoting potency of various ingredients of betel quid.

MATERIALS AND METHODS

Animals

One hundred and thirty male Syrian hamsters, aged 2 months, were used as experimental animals and divided into 13 equal groups. The hamsters were fed standard Purina laboratory pellets and water *ad libitum*.

Ripe, unprocessed betel nuts (*Areca catechu*), were each cut into four pieces, and ground in a pestle and mortar and immersed in distilled water at 4°C overnight to extract water soluble components. The insoluble fraction was oven-dried at 45°C overnight to extract water soluble components. The insoluble fraction was oven-dried at 45°C and used as betel nut fibre (BNF) [17]

One hundred grams of betel nut was ground with 200 ml of distilled water and left at 4°C for 24 h. The mixture was then removed, magnetically stirred for 4 h and again left at 4°C for 24 h. Then it was filtered under vacuum and the filtrate stored at 4°C as cold aqueous extract of betel nut (CEB) [16].

One hundred grams of betel nut was cut into pieces and boiled in 200 ml of distilled water for 4 h in a round-bottomed flask fitted with reflux after which it was filtered under vacuum and the filtrate (hot aqueous extract of betel nut (HEB)) stored at 4°C [16]

Piper betel (PB) was cut into pieces and stored at 4°C.

Slaked lime (SL) was stored at 4°C before use.

All components of betel quid were obtained freshly from retail source and prepared weekly.

The carcinogen (initiator) DMBA (7,12-dimethyl benzanthracene) was dissolved in heavy mineral oil at 0.5% concentration. Both were purchased from Sigma Chemical Ltd (Missouri, U.S.A.).

Experimental procedures

The right buccal pouch of each hamster was painted with 0.5% DMBA solution in heavy mineral oil with a No. 4 camel brush three times weekly. The DMBA application was continued for 4 weeks and the animals then remained untreated for 1 week. They were then randomly divided into 13 groups of 10 animals. Twelve groups served as experimental groups and their right buccal pouches were filled or painted with components of betel quid thrice weekly for 24 weeks as previously described [7]. Animals were untreated for a further 6 weeks before sacrifice. The 13th group served as the control, and was left untreated after the initial 4 weeks' application of DMBA and sacrificed at the same time as the experimental groups. The ingredients or combination of ingredients of betel quid used in the experimental groups are shown in Table 1. At the end of the experiment, the animals were killed by inhalation of an overdose of ethyl ether. Both right and left buccal pouches were excised, examined grossly for evidence of pathological lesions, and photographed. Tumours, if present, were measured and recorded. The pouches were then fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with haematoxylin and eosin for microscopic study. Dysplasia was diagnosed based on the presence of cellular pleomorphism, nuclear atypicality, hyperchromatism, loss of polarity, and individual cell dyskeratosis. Carcinoma was defined by the presence of stromal invasion on top of cytological dysplastic features, including those pure exophytic tumours invading their own stroma.

Statistical analysis

The difference in tumour incidence dysplasia and carcinoma between the experimental groups and the control group was evaluated by Student's *t*-test (two-tailed).

Table 1. Experimental period and ingredients of betel quid used in each experimental group

Week	Treatment
0–4	DMBA three times weekly
5	No treatment
6–29	Three times weekly application of Group 1, SL; Group 2, BNF; Group 3, PB; Group 4, PB + SL; Group 5, HEB; Group 6, HEB + SL; Group 7, CEB; Group 8, CEB + SL; Group 9, HEB + PB; Group 10, CEB + PB; Group 11, HEB + PB + SL; Group 12, CEB + PB + SL; Group 13, no treatment (control).
30–35	No application
36	Sacrifice

SL, slaked lime; BNF, betel nut fibre; PB, piper betle; HEB, hot aqueous extract of betel nut; CEB, cold aqueous extract of betel nut.

Table 2. Incidence of dysplasia or carcinoma in hamster buccal pouch treated with ingredient alone or combination of ingredients of Taiwan betel quid as promoting factor after the initiation of DMBA

Group	Ingredients	Effective no. of animals	No. of animals with dysplasia or carcinoma	No. of animals with carcinoma	Average tumour size (mm^3) ± S.D.
1	SL	8	4	2	183.3 ± 506.5
2	BNF	10	10**	9**	478.0 ± 1463.2
3	PB	9	5	5	132.3 ± 358.7
4	PB + SL	9	6	4	147.6 ± 276.0
5	HEB	10	6	5	697.9 ± 1551.5
6	HEB + SL	9	5	4	277.2 ± 454.2
7	CEB	10	9**	7*	1310.1 ± 2763.6
8	CEB + SL	9	6	5	962.6 ± 2895.7
9	HEB + PB	9	5	4	303.9 ± 613.9
10	CEB + PB	10	7	5	1111.2 ± 2076.9
11	HEB + PB + SL	9	5	4	353.3 ± 594.8
12	CEB + PB + SL	10	6	6	804.0 ± 1579.0
13	Control	9	4	2	132.8 ± 210.5

**$P < 0.01$ versus control.

*$P < 0.05$ versus control.

SL, slaked lime; BNF, betel nut fibre; PB, piper betle; HEB, hot aqueous extract of betel nut; CEB, cold aqueous extract of betel nut.

RESULTS

The left buccal pouches of all animals and some right pouches of experimental animals appeared to be normal, with no visible tumours. The others showed between one and eight tumours per pouch ranging in diameter from 1 to 13 mm. Most of them were exophytic. The average tumour volume in each group was calculated. The largest were in the group using cold aqueous extract of betel nut. There was no statistically significant difference in tumour volume between groups (Table 2).

Microscopic observation revealed that the left buccal pouches of all animals were essentially normal. The right buccal pouches showed variable histopathological changes from focal hyperkeratosis, dysplasia, to carcinoma. In the control group, two of nine animals developed carcinoma. The tumour incidence was significantly high in the group using dry betel nut fibre and in the group using cold aqueous extract as the promoter for 24 weeks ($P < 0.05$). There was no significant difference in tumour incidence between the control group and other experimental groups (Table 2).

DISCUSSION

In animal studies on the carcinogenicity of betel quid, one of the serious defects has been that the investigators could not make hamsters or other animals chew or retain betel quid in their oral cavity, simulating the situation in man. Therefore, studies in the hamster buccal pouch have mostly used extracts from betel nut, such as betel alkaloids or their derivatives, by direct application, feeding or peritoneal injection, which are different in terms of composition, concentration and duration. In our previous experiments [7], using a newly devised external wire collar, we can retain a solid piece of betel quid in the buccal pouch of hamster for a period of time, closely simulating the situation in man. We showed that betel quid may play a promoter role in hamster buccal pouch carcinogenesis although we could not ascertain which of the major components in betel quid, or rather what combination of these components, might be responsible.

The present study is an attempt to test the potency of various Taiwanese betel quid ingredients or their combination in promoting carcinogenicity. The advantage of studying Taiwanese quid compared to the quid chewed in other Asian countries is its simplicity. For example, the complex composition of an Indian betel quid, which consists of three main ingredients (areca nut, betel leaf and slaked lime) and a great variety of additives, such as tobacco, catechu, seeds, spices and metal sheets (e.g. silver, gold), makes it difficult to trace the carcinogenic activity to a particular ingredient or compound [20]. The results of this study showed that the incidence of tumours in the hamster buccal pouch was significantly higher in the group exposed to dry betel nut fibre ($P < 0.01$) and in the group using cold aqueous extract ($P < 0.05$) as promoters for 24 weeks. It thus indicates that during the promoting phase of carcinogenesis, the betel nut fibre and cold aqueous extract play an important role. The average tumour size varied greatly in each group, however, due to the wide range of the tumour size (1–13 mm in diameter), the standard deviation is too large to show any statistical significance among the groups (Table 2).

During the change of betel nut fibre three times weekly, traumatic bleeding or oozing of hamster buccal pouch mucosa was seen, presumably a result of mechanical friction produced by the rough fibre. The promoting effect of chronic trauma has been identified previously in experiments using DMBA treatment with subsequent wounding of the hamster buccal pouch mucosa [19]. Our results suggest that the promoting capacity of betel nut fibre is due to chronic trauma.

The cold aqueous extract of betel nut also showed significant promoting activity in our study. The exact composition and its promoting mechanism is under investigation. Our results are consistent with a previous report which demonstrated that in the areca nuts, besides known major alkaloids, there are other water extractable substances. Such substances are genotoxic in Chinese hamster ovary cells [21].

In the present study, we have also shown that the cancer promoting potency varies between mixtures of different composition. For example, cold aqueous extract alone will produce significant promoting activity while its combined application with slaked lime or piper betel will not. We speculate that slaked lime or components in piper betel might interact with ingredients in the betel cold aqueous extract, and thus neutralise or counteract its promoting effect.

Slaked lime and piper betel are both reported to have genotoxic or carcinogenic potential [22–24]. This potential was not confirmed in our animal model.

There are two major betel alkaloids, arecoline and arecaidine, present in high concentration in boiled betel nut extracts [25]. These alkaloids have been demonstrated to inhibit fibroblast proliferation in a concentration dependent manner. However, no significant promoting activity of hot aqueous extracts of betel nut was detected.

Reports indicate that the habit of betel quid chewing is increasing in Taiwan, particularly among the young [26, 27]. It appears that this unhealthy habit will not be voluntarily eradicated in the near future. It has been proposed that individual constituents shown to be carcinogenic might be identified and eliminated. Unfortunately, the results of these experiments indicate that fibre and cold aqueous extracts are the two most potent promoters and both are nearly impossible to remove. Therefore, it seems that elimination of the betel quid chewing habit by public health measures may be the only way to reduce the oral cancer incidence in Taiwan.

Finally, this study used over 100 hamsters for the entire experiment. However, due to the many groups involved in the study comparison, only moderate numbers of animals were allowed in each group. Some of the insignificant differences among the groups might turn out to be of biological importance in a larger study. This report points out the most remarkable differences that might implicate the most promising carcinogenic ingredients worth further study.

1. Hirayama T. An epidemiological study of oral and pharyngeal cancer in central and south-east Asia. *Bull WHO* 1966, **34**, 41–69.
2. Kwan HW. A statistical study of oral carcinomas in Taiwan with emphasis on the relationship with betel nut chewing: a preliminary report. *J Formosan Med Assoc* 1976, **75**, 497–505.
3. Suri K, Goldman HM, Wells H. Carcinogenic effect of a dimethylsulphoxide extract of betel nut on the mucosa of the hamster buccal pouch. *Nature* 1972, **230**, 383–384.
4. Ranadive KJ, Ranadive SN, Shivapurkar NM, Gothskar SV. Betel quid chewing and oral cancer: experimental studies on hamsters. *Int J Cancer* 1979, **24**, 835–843.
5. Chen CH, Lin CZ, Chang P. A statistical study on 234 cases of oral carcinoma. *J Otolaryngol Soc ROC* 1984, **19**, 20–25.
6. Nishikawa A, Prokopczyk B, Rivenson A, Zang E, Hoffmann D. A study of betel quid carcinogenesis—VIII. Carcinogenicity of 3-(methylnitrosamino) propionaldehyde in F344 rats. *Carcinogenesis* 1992, **13**, 369–372.
7. Wong TY, Jin YT, Chen HO, Lin LM. Studies on Taiwan betel quid carcinogenicity in hamster cheek pouch. *Chinese Dent J* 1992, **11**, 155–162.
8. Salley JJ. Experimental carcinogenesis in the cheek pouch of the Syrian hamster. *J Dent Res* 1954, **33**, 253–262.
9. Silberman S, Shklar G. The effect of a carcinogen (DMBA) applied to the hamster's buccal pouch in combination with croton oil. *Oral Surg* 1963, **16**, 1344–1365.
10. Eisenberg E. Neoplasia following cessation of DMBA application to hamster buccal pouch. *J Dent Res* 1977, **56**, 1430–1431.
11. Vogelstein B, Fearon ER, Hamilton SR, *et al.* Genetic alterations during colorectal-tumor development. *New Eng J Med* 1988, **319**, 525–532.
12. Harris CC. Chemical and physical carcinogenesis: advance and perspectives for the 1990s. *Cancer Res* 1991, **51** *(suppl)*, 5023s.
13. Odukoya O, Shklar G. Two-phase carcinogenesis in hamster buccal pouch. *Oral Surg* 1982, **54**, 547–552.
14. Ritchie AC, Saffiotti U. Orally administered 2-acetylaminofluorene as an initiator and as a promoter of epidermal carcinogenesis in the mouse. *Cancer Res* 1955, **15**, 84–93.
15. Dunham LJ, Herrold KM. Failure to produce tumors in the hamster cheek pouch by exposure to ingredients of betel quid; histopathological changes in the pouch and other organs by exposure to known carcinogens. *J Natl Cancer Inst* 1962, **29**, 1047–1057.
16. Ranadive KJ, Gothoskar SV, Rao AR, Tezabwalla BU, Ambaye RY. Experimental studies on betel nut and tobacco carcinogenicity. *Int J Cancer* 1976, **17**, 469–476.
17. Rao AR. Modifying influences of betel quid ingredients on B(a)P-induced carcinogenesis in the buccal pouch of hamster. *Int J Cancer* 1984, **33**, 581–586.
18. Jin YT, Wong TY, Lin LM. Experimental studies on Taiwan betel quid carcinogenicity. *J Dent Res (Abstract of IADR Annual Meeting)* 1991, **70**, 272.
19. Maeda H, Nakane S, Takehana S, Sato E. Epithelial dysplasia produced by carcinogen pretreatment and subsequent wounding. *Oral Surg Oral Med Oral Pathol* 1989, **68**, 50–56.
20. Stich HF, Rosin MP, Brunnemann KD. Oral lesions, genotoxicity and nitrosamines in betel quid chewers with no obvious increase in oral cancer risk. *Cancer Lett* 1986, **31**, 15–25.
21. Dave BJ, Trivedi AH, Adhvaryu SG. *In vitro* genotoxic effects of areca nut extract and arecoline. *J Cancer Res Clin Oncol* 1992, **118**, 283–288.
22. Nair UJ, Obe G, Friesen M, Goldberg MT, Bartsch H. Role of lime in the generation of reactive oxygen species from betel-quid ingredients. *Environ Health Pers* 1992, **98**, 203–205.
23. Thomas SJ, MacLennan R. Slaked lime and betel nut cancer in Papua New Guinea. *Lancet* 1992, **340**, 577–578.
24. Jeng JH, Hahn LJ, Lu FJ, Wang YJ, Kuo MY. Eugenol triggers different pathobiological effects on human oral mucosal fibroblasts. *J Dent Res* 1994, **73**, 1050–1055.
25. Wyk CW van, Olivier A, Miranda CM de, Bijl P van der, Grobler-Rabie AF. Observation on the effect of areca nut extracts on oral fibroblast proliferation. *J Oral Path Med* 1994, **23**, 145–148.
26. Ko YC, Chiang TA, Chang SJ, Hsieh SF. Prevalence of betel quid chewing habit in Taiwan and related sociodemographic factors. *J Oral Path Med* 1992, **21**, 261–264.
27. Lu CT, Lan SJ, Hsieh CC, *et al.* Prevalence and characteristics of areca nut chewers among junior high school students in Changhua county, Taiwan. *Commun Dent Oral Epidemiol* 1993, **21**, 370–373.

Acknowledgement—This research was supported in part by National Science Council of Republic of China grants (NSC80-0412-B006-70).