行政院國家科學委員會專題研究成報告

檳榔及其添加料腫瘤促進作用及機轉之研究（一）
Tumor promoting effect and mechanisms of action of betal quid and additives（一）

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行政院國家科學委員會專題研究計劃成果報告

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一、中文摘要

檳榔是部分國人經常使用的一種嚼塊，也是在引起口腔癌方面最廣被多數人探討的一環。在過往的許多論文中，檳榔嚼塊的各種水溶性及非水溶性的成分已被探討，包括基因毒性、致突變性以及口服後對動物的毒性，但是在口腔癌症生成的角色及過程除了流病調查有絕對的關係性外，其直接證據較薄弱。根據我們初步的觀察，我們認為檳榔嚼塊也許是在癌生成的過程中扮演了癌促進劑(tumor promoter)的作用，因此本研究利用促癌作用模式以benzo(a)pyrene 當作癌起始劑，另外購買二種市售檳榔嚼塊(紅灰及未處理的檳榔子)，抽取其水層萃取物當作癌促進劑塗抹於 CD-1 品系雌性小白鼠皮上觀察其癌症發生的過程，並且利用癌促進作用指標來證明檳榔及其添加物促進癌症生成的作用。

本研究證實檳榔有腫瘤促進作用，經檳榔水萃取物塗抹 CD-1 小白鼠背部，可增加表皮厚度及 leukocyte infiltration，產生 H_2O_2 及上皮 myeloperoxidase 活性，增加 ornithine decarboxylase 活性，在檳榔添加 lime-piper 添加料之水萃取物中有加強之作用，由於腫瘤促進之分子機制已被提出，包括過氧化作用，PKC 活化等，本研究也發現檳榔及添加物 lime-piper 有促進上皮組織 PKC 活化作用及促進 NF-κB 蛋白表現等，此作用在含 lime-piper 之檳榔更顯著。在流病調查上顯示口腔癌與檳榔之密切關係，但在基礎除了間接在基因毒性，突變性上之研究外，缺少了直接之證據說明檳榔在口腔癌作用形成之角色，本研究顯示檳榔有"腫瘤促進作用"之作用，其 lime-piper 有加強此作用之能力。

Abstract

Components of betel quid investigated for genotoxicity, mutagenicity, and animal toxicity. However, little information exists regarding the potential carcinogenicity to form oral betel quid. Considerable attention is focused in tumor promoters that occur environmentally for human uptake. In this study, the tumor promoting effet betel quid in benzo(a)pyrene intiated CD-1 mouse skin was investigated.

In the present study, we found that betel quid (NB) and lime piper betel (LPB) at concentrations of 25, 50, and 75 mg/ml, respectively, not only caused significant induction of hyperplasia, but also epidermal ornithine decarboxylase (ODC). Treatment of mouse skin with LPB caused production of H_2O_2 by 2.41- , 3.90- ,and 3.76- fold, respectively, and marked induction of myeloperexidase (MPO) by1.43-,2.70- ,and2.29- fold. Application of the same amount of LPB also caused significant induction of PKC expression. These results indicated that NB and LPB have the potential as a promoting agent and the LP(lime-piper) play a major role in promoting effect of skin hyperplasia and inflammation. The tumor promoting effect of NB and LPB in mouse skin was associated with the induction of PKC and NF-κB.

二、緣由與目的

Betel is known as *Areca catechu*, a masticatory substance common to several oriental countries. Warm and humid climate are ideal for growth of *Areca* nut. In Taiwan, betel quid (NB) is a natural masticatory, composed of green areca fruit, piper betle and slaked lime paste (1). Fresh areca fruit contains lipids, fiber, alkaloids, polysaccharride and rich polyphonic compounds (2). The alkaloids in areca fruit are arecoline, arecaidine, guvacoline and isoguvacine , with arecoline the most abundant (3). In previous studies, an extraction of NB was found to possess mutagenicity (4-6), induce chromosomal aberrations (7) and increase the frequency of sister chromatin exchange of DNA in Chinese hamaster ovary cells(8). Some animal experiments showed positive results (9-13) and others were negative (14-16) on the NB on its components induced tumors or caused oral diseases. Most investigations of NB focused in its carcinogenic properties,

but little information was available about tumor promoters occurred environmentally and subjected to human. Pathological of betel quid. But up to the present, there has not been direct evidence from animal model to prove the carcinogenesis of NB.

It has been reported that tumor promoters such as 12-o-tetradecanoyl-phorbol-13-acetate (TPA), teleocidin and okadaic acid induced tumor promotion in mouse skin (14-16). The promotion stage has been tested most thoroughly with respect to TPA tumor promoters (17), which produce numerous histological and biochemical alterations in mouse skin (18). Among these, tumor promoting activity is best correlated with epidermal edema, hyperplasia, increased neutrophil infiltration, and formation of H_2O_2 and enhanced MPO, ODC and PKC activities. All these alterations in skin are defined as possible markers of tumor promotion and utilized in this study to assess the promotion effect of NB and LPB.

三、結果與討論

The effects of topical application of NB or LPB induced alteration of cutaneous morphology were examined (Fig.1 and Table1). Topical application of NB or LPB (50,100 and 150 mg) twice a day for 4 days to the dorsal surface of CD-1 mice resulted in 1.5- to 2.5- fold in the epidermal thickness. Inflammatory cell infiltration of the dermis were also observed. Topical application of NB or LPB caused moderately or severe increase respectively in edema in the dermis (Fig.1 and Table 1). Results showed that LP-additive has potential effect on the NB-caused mouse hyperplasia and inflammation.

The effect of topical application of NB or LPB on the H_2O_2 production was examined. As shown in Table 2, NB(25,50 and 75 mg) increased the formation of H_2O_2 by 1.02-, 1.10- and 3.38-fold and LPB increased by 2.41-, 3.90- and 3.76-fold.The effect of betel extract and additives on alteration of MPO activity was examined. Induction of MPO was investigated in NB and LPB-treated mouse skin epidermis. Results also showed that LP-additive has potential effect on the NB-caused mouse epidermis peroxidation. Treatment of NB and LPB also caused the increased ODC activity, especially in LPB. As shown in Table 3, LPB(25, 50 and 75 mg) increased the activity of ODC by 1.49-, 1.66-, and 1.58-fold, but not in NB treatment.

The effects of topical application of NB and LPB on the expressions of PKC was examined. As shown in Fig.2 the expression of protein was increased in PKC on the NB and LPB treated mice epidermis. Treatment with NB and LPB also increased the NF-κB expression significantly in mice epidermis. (Fig.2)

The contents of *Areca* itself includes phenolic compounds, which have been shown to cause mutagenicity (4-6) and to increase sister chromatid exchange (8) of DNA in Chinese hamster. In present, there is no direct evidence to show what relationships exist between betel quid and oral cancer. There are quite complicated possible reasons for the occurrence of oral tumors. First, previous medical reports showed submucosa fibrosis (OSF) in oral mucous. Some people who have a long-time habit of chewing betel do worth oral carcinomas only show OSF at high level. Second, according to earlier studies, two major ingredients of the additives of betel quid (slaked lime) are $Ca(OH)_2$ and euqenol. These compounds could elevate the pH in typical oral situations, especially in pH>8.4 (19), and induce the formation of reactive oxygen species (20-21) to damage the composition of DNA. Third, in chewing process, there is aboudant rough cellulose fiber in the betel quid associated with these additives could hurt the mucosal cells. After a long period of chewing, betel quid would cause injury to gingiva and break the alveoli and oral mucosal cell structures.

In the short-term study, both the extracts of betel quid and additives showed to possess the promoting activity because of not only increasing the production of free radicals but also inducing the activity of ODC. According to previous reports, abundant hydrolyzable tannin and phenolics containing in *Areca* nut played antipodal roles in carcinogenesis. Hydrolyzable tannin was supposed to be an anticarcinogen but phenolics to be a carcinogen (22). Moreover, additives can raise the pH in the oral and induce the production of phenolics to attack normal cells. In human, the absorbency of tannin and phonics is very different, and how the pH of additives influences the absorbency is not yet to be clarified. Taking the reasons together, our study showed that the lime-piper additives may play a more important role in promoting occurrence of oral carcinogenesis. In conclusion, NB and LPB have the potential as a promoting agent. The tumor promoting effect of NB and LPB in mouse skin was associated with the induction of PKC and NF-κB.

四、計劃成果自評

1. 本研究對於檳榔在各種研究中致癌力的存疑，提出一新的證據，確認檳榔為 tumor promoter。
2. 檳榔之添加料紅灰有加強檳榔之腫瘤促進作用。
3. 初步證明檳榔之腫瘤促進作用可能經由活化 PKC 訊息及促進 NF-κB 表

現，進一步之詳細分子機轉，第二年之研究正進行中。

五、參考文獻

1. H.C. Chen, Y.S. Chang Lee. (1984) The mutagenicity of nitrite-treated aqueous extract of *Piper betel* L., Proc. Natl. Sci. counc. ROC (B), 8, 4-10.
2. C.K. Wang, L.S. Hwang , (1993) Analysis of phenolic compounds in betel quid. J. Chinese Agric. Chem. Soc., 31, 623-632.
3. C.K. Wang, L.S., Hwamh (1993) Study on the separation and hydrolysis of alkaloids from betel nut. Food Sci., 20, 514-426.
4. Y. Liu, S. Egyhazi, J. Hansson, S.V. Bhide, P.S. Kulkkarni,R.C. Grafatrom. (1997) O6-methylguanine-DNA methyltransferase activity in human buccal mucosal tissue and cell cultures. Complex mixtures related to habitual use to tobacco and betel quid inhibit the activity in vitro. Carcinogenesis, 18, 1889- 1895.
5. S.E. Lee, Chen, C.L. Chen, L.Y. Ho, P.C. Hsu, J.T. Chang, C.M. Sun, C.W. Cln, T.Y. Liu. (1996) Role of oxidative DNA damage inhydroxychavicol-induced genotoxicity. Mutagenesis, 11, 519-523.
6. C.K.Wang, C.H. Peng. (1996) The mutagenecities of alkaloids and N-nitrosoguvacoline from betel quid. Mutation Res., 40, 165-171.
7. D.S. Rupa, D.D Eastmond. (1997) Chromasomal alterations affecting the 1cen-1q12 region in buccal mucosal cells of betel quid chewers detected using multicolor fluorescence in situ hybridization. Carcinogenesis, 18, 2347-2351.
8. B.J Dave, A.H. Trivrdi, S.G. Adhvaryu. (1992) In virto genotoxic effects of areca nut extract and arecoline, J. Cancer Res. Clin. Oncol., 118, 283-288.
9. K.Goerttler, H. Loehrke, J. Schewizer. (1979) Systemic 2-stage carcinogenesis in the epithelium of the forestomach of mice using 7, 12-dimethylbenz(a)anthracene as initiator and the phornol ester 12-O-tetradecanoylphorbol-13-acetate as promoter. Cancer Res., 39, 1293-1297.
10. M. Suganuma. H. Fujiki, K. Morino. (1987) Tumor promoting activity of teleocidin in skin forestomach of mice initiated transplacentally with 7,12-dimethylbenz(a)anthracene, J. Cancer Res, Clin Oncol., 113, 123-125.
11. M. Suganuma, H. Fujiki, H. Suguri. (1988) Okadaic acid: an additional non-phorbol-12-tertradecanoate-13-acetate-type tumor promoter, Proc. Natl. Acad. Sci. USA., 85, 1786-1771.
12. E. Pick, Y. Keisari. (1980) Asimple colormetric method for the measurement of hydrogen peroxide produced by cells in culture, J. Immunol. Methods, 38, 161-170.
13. R.C. Smart, M.T. Huang, A.H. Conney. (1986) sn-1,2-Diacylglycerols mimic the effects of 12-O-tetradecanoylphorbol-13-acetate in vivo by inducing biochemical changes associated with tumor promotion in mouse epidermis, carcinogenesis, 7, 1865-1870.
14. K. Goerttler, H. Loehrke and J. Schweizer. (1979) systemic 2-stage carcinogenesis in epithelium of the forestomach of mice using DMBA as a initiator and TPA as promoter. Cancer Res., 39, 1293-1297.
15. M. Suganuma, H. Fujiki and K. Morino. (1987) Tumor promoting activity of teleocidin in skin and forestomach of mice initiated transplacentally with DMBA. J. Cancer Res. Clin. Oncol., 113, 123-125.
16. M. Suganuma, H. Fujiki and H. Suguri, (1988) Okadaic acid: An additional TPA-type tumor promoter. Proc. Natl. Acad. Sci. USA. 85, 1768-1771.
17. T.G. O'Brien, R.C. Simsiman and R.K. Boutwell. (1975) Induction of the polyamine-biosynthetic enzyme in mouse epidermis by tumor promoting agents. Cancer Res., 35, 1662-1670.
18. J.S. Lim, K. Frenkel and W.Troll. (1992) Tamoxifen suppresses tumor promoter induced hydrogenperoxide formation by human neutrophils. Cancer Res, 52, 4969-4972.
19. U.J. Nain, J.Nain, M.D. Friesen, N. Bartsch, H. Ohshirua. (1995) Ortho-and meta-tyrosine foremation from phenylalanine in human salica as a marker of hydroxyradical generation during betel quid chewing. Carcinogenesis, 16, 1195-1198.
20. U.J. Nain, R.A. Floyd, J. Nain, V. Bussachin, M. Friesen, N. Bartsch. (1987) Formation of reactive oxygen species and 8-hydroxydroxyguanosine in DNA in vitro with betel quid ingredients. Chem. Biol, Interact., 63, 157-169.
21. U.J. Nain, M.Friesen, I. Richard, R. McLennan, S. Thomas, H. Bartsch. (1990) Effect of line composition on the formation of reactive oxygen

species from areca nut extract in vitro. Carcinogenesis, 11, 2145-2148.
22. C. Peri, C. Pompei. (1971) Estimation of different phenolic groups in vegetable extracts. Phytochem, 10, 2187-2191.

Table 1. Morphological examination of NB- or LPB-treated mouse skin

Treatment[a]	No. of epidermal layer	Epidermal thickness (μm)	leukocyte infiltration[c]
Control	1-2	10.83±2.04	-
NB			
50 mg	2-3	16.43±4.76*,[b]	+
100 mg	2-3	23.57±4.76**	+
150 mg	2-3	20.00±2.67**	+
LPB			
50 mg	2-3	20.00±5.98**	++
100 mg	2-3	24.38±4.95**	++
150 mg	3-4	25.43±4.77**	++

[a] The mice were treated topically with NB or LPB twice a day for 4 days. The animals were killed 18 hours after the last dose and skins were processed for histological tests. Data are expressed as the mean±S.D. from six mice / group.

[b] Statistically different from control group, * $P < 0.01$; ** $P < 0.001$.

[c] Leukocyte infiltration that was moderate (+) or severe (++) was characterized by diffuse infiltration of mononuclear infiltratory cells in the dermis when compared with the controls.

Table 2. The formation of H_2O_2 and induction of MPO activity in mouse skin by topical treatment

Treatment[a]	H_2O_2(nmol/cm^2)	Fold	MPO(units/cm^2)	Fold
Control	12.64±0.45	1	1.30±0.23	1
NB				
25 mg	12.98±1.15	1.02	2.15±0.27*	1.65
50 mg	13.97±10.22	1.10	2.54±0.60*	1.95
75 mg	42.68±11.66**,[b]	3.38	2.37±0.93	1.82
LPB				
25 mg	30.45±11.67	2.41	1.86±0.23	1.43
50 mg	49.32±31.03*	3.90	3.52±0.69***	2.70
75 mg	47.58±30.48*	3.76	2.98±1.29**	2.29

[a] The mice were treated with NB or LPB. A total of 20 h later, the animals were treated again. The mice were killed after the second treatment 1 hour and the skins were removed. The formation of H_2O_2 and MPO activity were determined. The data present the mean±S.D. from six mice / group.

[b] Statistically different from control group,* $P < 0.05$; ** $P < 0.01$.

Table 3. NB- or LPB-induced epidermal ODC in mouse skin.

Treatment[a]	ODC (pmol CO_2/mg protein/h	Fold
Control	264.20±19.70	1
NB		
25 mg	259.16±88.64	0.98
50 mg	229.37±89.31	0.87
75 mg	270.21±90.25	1.02
LPB		
25 mg	394.13±83.17*,[b]	1.49
50 mg	439.37±43.40**	1.66
75 mg	418.64±44.31**	1.58

[a] Mice were treated with NB or LPB. A total of 5 hours later, the animals were killed and the epidermis was isolated. ODC activity was determined. The data present the mean±S.D. from six mice / group.

[b] Statistically different from control group (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$)

Fig. 2