Journal of the Formosan Medical Association (2016) 115, 108–112

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.jfma-online.com

ORIGINAL ARTICLE

Cytotoxicity and transformation of C3H10T1/2 cells induced by areca nut components

Chien-Yang Yeh [a,f], Hsin-Ming Chen [a,f], Mei-Chi Chang [b,**], Seng-Heng Kok [a], Jang-Jaer Lee [a], Bei-En Chang [c], Po-Yuan Jeng [a,d], Chiu-Po Chan [e], Jiiang-Huei Jeng [a,*]

[a] *School of Dentistry and Department of Dentistry, National Taiwan University Medical College and National Taiwan University Hospital, Taipei, Taiwan*
[b] *Biomedical Science Team, Chang Gung University of Science and Technology, Kwei-Shan, Taoyuan, Taiwan*
[c] *Graduate Institute of Oral Biology, National Taiwan University Medical College, Taipei, Taiwan*
[d] *School of Dentistry, University of Cardenal Herrera, CEU, Valencia, Spain*
[e] *Department of Dentistry, Chang Gung Memorial Hospital, Taipei, Taiwan*

Received 11 October 2014; received in revised form 2 November 2014; accepted 6 January 2015

KEYWORDS
areca nut;
betel quid;
chemical carcinogenesis;
cytotoxicity;
oral cancer;
transformation;
tumor promotion

Background/purpose: Betel quid (BQ) chewing is popular in Taiwan and many other countries. There are about 200–600 million BQ chewers in the world. BQ chewing is one major risk factor of oral cancer and oral submucous fibrosis (OSF). While areca nut (AN), a main component of BQ, exhibits genotoxicity, its transformation capacity and its role in the initiation and promotion stages of carcinogenesis are not fully clear.
Methods: Mouse C3H10T1/2 cells were exposed to AN extract (ANE) for 24 hours. Cytotoxicity was evaluated by colony forming efficiency. For the transformation assay, C3H10T1/2 cells were exposed to ANE for 24 hours and then incubated in medium with/without 12-O-tetradecanolylphorbol-13-acetate (TPA; a tumor promoter) for 42 days. Cells were stained with Giemsa and type II and type III transformed foci were counted for analysis of the transformation capacity of ANE.
Results: ANE exhibited cytotoxicity to C3H10T/12 cells at concentrations higher than 320 μg/mL as shown by a decrease in colony numbers. ANE (80–640 μg/mL) alone mildly stimulated the transformed foci formation ($p > 0.05$). In the presence of TPA, ANE (80–640 μg/mL) markedly stimulated the transformed foci formation. The percentage of dishes with foci increased

Conflicts of interest: The authors have no conflicts of interest relevant to this article.

* Corresponding author. Jiiang-Huei Jeng, Department of Dentistry and School of Dentistry, National Taiwan University Hospital and National Taiwan University Medical College, Number 1, Chang-Te Street, Taipei, Taiwan.
** Corresponding author. Mei-Chi Chang, Chang Gung University of Science and Technology, 261, Wen-Hua 1st Road, Kwei-Shan, Taoyuan, Taiwan.
E-mail addresses: mcchang@mail.cgust.edu.tw (M.-C. Chang), jhjeng@ntu.edu.tw (J.-H. Jeng).
[f] These authors contributed equally to this study.

http://dx.doi.org/10.1016/j.jfma.2015.01.004

from 0% in controls to 20% in ANE (80 μg/mL and 320 μg/mL)-treated groups and further increased to 65—94% in ANE plus TPA groups.
Conclusion: These results indicate that ANE is a weak complete carcinogen. ANE is an effective tumor initiator and can induce malignant transformation of C3H10T1/2 cells in the presence of a tumor promoter. ANE may be involved in multistep chemical carcinogenesis by its malignant transformation capacity.

Introduction

Betel quid (BQ) chewing is a popular oral habit in Taiwan, India, Sri Lanka, South Africa, and many other Southeast Asian countries. Previous studies have indicated that BQ chewing habit is the major risk factor of oral leukoplakia, oral submucous fibrosis (OSF), and oral squamous cell carcinoma (OSCC).[1,2] There are about 2.0 million BQ chewers in Taiwan.[3] Recently, oral cancer has become the fifth most common cancer in both males and females and the fourth most common cancer in males in Taiwan, and has been a critical health issue.[4] Moreover, the incidence of oral cancer is 123-fold higher in patients who smoked, drank alcohol, and chewed BQ than abstainers.[5] These results suggest the presence of chemical carcinogens in the BQ.

Areca nut (AN) as the main component of BQ is considered to be the major etiologic factor of oral cancer. AN extract (ANE) contains mainly tannin and areca alkaloids (such as arecoline and arecaidine) that are potential carcinogens. During the period of chewing BQ, carcinogenic substances are released from BQ, irritate the oral mucosa, and finally lead to the malignant transformation of normal oral epithelium to OSCC.

Many prior studies have revealed that ANE exhibits genotoxic and mutagenic activities to different kinds of cells such as oral keratinocytes, Chinese hamster ovary cells, and oral fibroblasts.[1,2] Our recent study further showed that BQ chewing may affect the wound healing, inflammatory condition, and fibrotic processes in oral mucosa via inducing prostanoids and cytokines production in oral keratinocytes, as well as buccal fibroblasts contraction by ANE and areca alkaloids. Persistent buccal fibroblast contraction may induce fibrotic contracture of oral mucosal tissue, leading to OSF.[6—8]

Chemical carcinogenesis has been shown to be a multistep process which includes tumor initiation, promotion, progression, and many others.[9,10] Various carcinogens may be involved in specific steps of carcinogenesis by inducing DNA damage, cytotoxicity, cell proliferation, tissue inflammation, and impairment of cell—cell communication.[11—13] However, limited studies are focused on whether AN components may induce malignant transformation of cultured cells. C3H10T1/2 mouse embryonic fibroblastic cells have been widely used to clarify the involvement of chemicals in different steps of carcinogenesis.[14] C3H10T1/2 cells may be transformed by chemical carcinogens to generate transformed foci. These transformed cells, when inoculated into subcutaneous tissue of mice, may induce tumor formation, showing the capacity of toxic chemicals to stimulate malignant transformation of cells.[14—16] We therefore designed this study to further delineate the transformation capacity of AN components using C3H10T1/2 cells.

Materials and methods

Materials

Basal Medium Eagles, fetal calf serum, phosphate-buffered saline (PBS), penicillin, and streptomycin were from Life Technology (Gibco, Life Technologies, Grand Island, NY, USA). Mouse C3H10T1/2 cells were from American Type Culture Collection (ATCC, Manassas, VA, USA). They were cultured in Basal Medium Eagles with 10% fetal calf serum and penicillin/streptomycin. 12-O-Tetradecanolylphorbol-13-acetate (TPA) and 3-methylcholanthrene (MCA) were from Sigma Chemical Company, St Louis, MO, USA. ANE was extracted, prepared, weighed, and used as outlined in previous studies.[8,17] In short, raw AN was bought from the market, cleansed with double-distilled water, cut into small pieces, and lyophilized. The dried AN pieces (50 g) were ground by a motor-driven coffee blender to produce fiber and powder. They were then extracted by 1 L of double-distilled water at 4°C for 4 hours and centrifuged at 5000 rpm for 15 minutes. The supernatant was filtered by Advantec filter papers (Toyo Roshi Kaisha Ltd, Tokyo, Japan), lyophilized again, and weighed prior to use.

Cytotoxicity of ANE on C3H10T1/2 cells

Briefly, 200 C3H10T1/2 cells were seeded into 6 cm culture dishes. After 24 hours of attachment, they were exposed to 5 mL fresh culture medium containing ANE (80 μg/mL, 320 μg/mL, or 640 μg/mL) for 24 hours. Culture medium was decanted. Cells were further cultured in fresh medium for 10 days with a change of medium at Day 5. The medium was then decanted and cells were washed with PBS, fixed, and stained with 5% Giemsa solution and finally rinsed by water before taking pictures and the colonies were counted as outlined in a previous study.[18]

Transformation activity of ANE with/without TPA

Briefly 2×10^3 C3H10T1/2 cells were seeded into 6 cm culture dishes. After 24 hours of attachment, they were exposed to 5 mL fresh medium containing ANE (80 μg/mL, 320 μg/mL, or 640 μg/mL) for 24 hours. Culture medium was decanted. Cells were further cultured with/without TPA for 42 days, with a change of culture medium and TPA

(0.25 μg/mL) every 7 days. MCA (2 μg/mL) was used as a positive control.

Finally, the culture medium was then decanted and cells were washed with PBS, fixed and stained with 5% Giemsa solution, and finally rinsed by water before taking pictures and observation under a microscope. For scoring of type II and type III transformed foci, the characteristics and definition of foci were defined by Reznikoff et al.[15] Briefly, type I foci was defined as the presence of tightly packed cells. The type II foci were defined as a focus showing a massive build-up of cells to an opaque multilayer with a criss-cross pattern. Type III foci showed the presence of highly polar and multilayer criss-cross arrays of stained cells.

Statistical analysis

Three separate experiments were performed and the accumulative data of transformed foci were calculated for analysis. The statistically significant difference between two groups was analyzed by the Student t test. A p value < 0.05 was considered to be statistically significant.

Results

Cytotoxicity of ANE on C3H10T1/2 cells

Clonal growth of C3H10T1/2 cells will form a cell colony after Giemsa staining as shown in Fig. 1A. The number and size of colony formation of C3H10T1/2 cells decreased after exposure to ANE 640 μg/mL for 24 hours (Fig. 1A). Quantitatively, the plating efficiency of C3H10T1/2 cells was about 25—30%. After 24 hours of exposure to 320 μg/mL and 640 μg/mL ANE, the number of colonies was obviously inhibited (Fig. 1B).

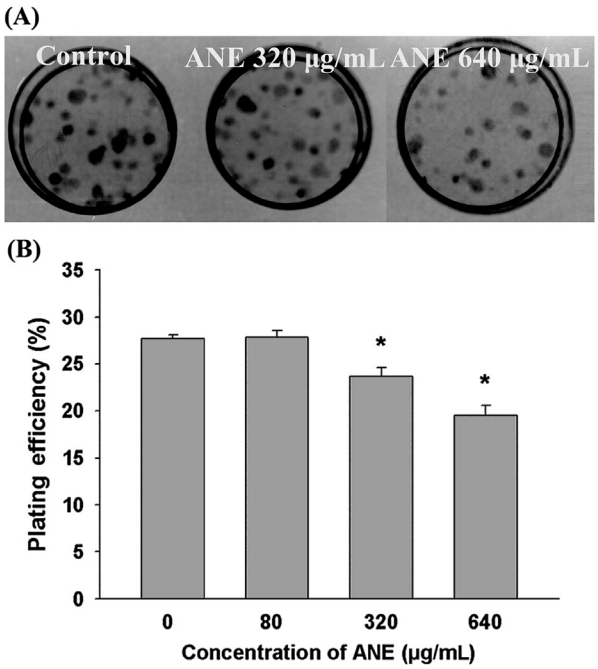

Figure 1 Cytotoxicity of areca nut extract (ANE) on C3H10T1/2 cells. (A) Colony formation of C3H10T1/2 cells after exposure to different concentrations of ANE. One representative staining picture is shown; (B) quantitative analysis in plating efficiency of C3H10T1/2 cells after exposure to different concentrations of ANE. Results are expressed as mean ± SEM. * Statistically significant difference ($p < 0.05$) when compared with the control.

Transformation of C3H10T1/2 cells induced by MCA and ANE with/without TPA

Various carcinogens have been shown to induce the transformation of C3H10T1/2 cells.[15] As shown in Fig. 2A (left), untreated cells (control) show no marked transformed foci after staining. Transformed foci of C3H10T1/2 cells were observed after exposure to ANE plus TPA (Fig. 2A, middle) and MCA plus TPA (Fig. 2A, right).

Under microscopic examination, untreated C3H10T1/2 cells showed monolayer culture (Fig. 2B, left). The transformed C3H10T1/2 cells induced by ANE plus TPA showed a multilayer and criss-cross appearance (Fig. 2B, right).

Quantitative analysis of transformed foci

Quantitatively, no transformed foci were noted in solvent treated controls. Exposure to ANE (80 μg/mL and 320 μg/mL) alone slightly elevated the number of transformed foci/dish to 0.2 and 0.25 ($p > 0.05$; Table 1). By the addition of TPA (a tumor promoter), the number of ANE-induced transformed foci were markedly elevated to 1.75, 1.1, and 1.69 transformed foci/dish ($p < 0.05$; Table 1). MCA, a well-known carcinogen and positive control, also obviously stimulated the transformed foci formation in the presence of TPA. The percentage (%) of dishes with foci also increased from 0% in controls to 20% in ANE (80 μg/mL and 320 μg/mL)-treated groups and further to 65—94% in ANE (80—640 μg/mL) plus TPA groups (Table 1).

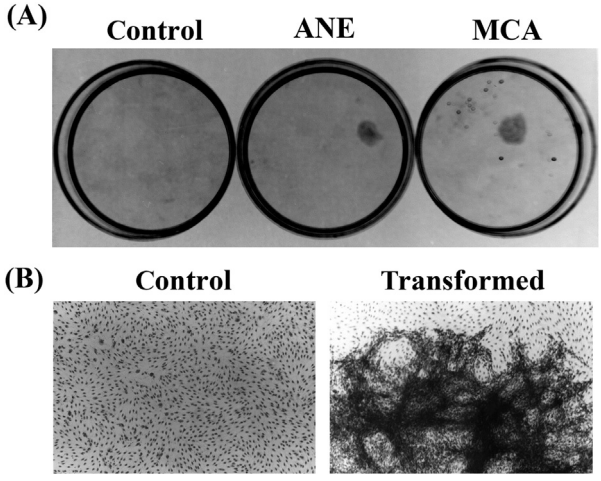

Figure 2 Effect of areca nut extract (ANE) on the transformed foci formation of C3H10T1/2 cells. (A) In control dishes, no transformed foci are noted after staining (left), transformed foci are noted in C3H10T1/2 cells induced by ANE plus 12-O-tetradecanolylphorbol-13-acetate (TPA) (middle), and transformed foci are also noted in C3H10T1/2 cells transformed by 3-methylcholanthrene (MCA) plus TPA (right). One representative staining result of the culture dish is shown; (B) control cells are monolayer in culture (left), whereas transformed cells show multilayers and are criss-cross in appearance under a microscope.

Table 1 Inducing the transformation of C3H10T1/2 cells by areca nut extract (ANE) with/without 12-O-tetradecanolylphorbol-13-acetate (TPA).

Treatment groups	No. of dishes with transformed foci		No. of transformed foci/dish	Dishes with foci (%)
	Type II	Type III		
Solvent control	0	0	0	0/14 (0)
ANE 80 μg/mL	2	2	0.20 ± 0.09	4/20 (20)
ANE 320 μg/mL	5	0	0.25 ± 0.12	4/20 (20)
ANE 640 μg/mL	0	0	0	0/16 (0)
TPA 0.25 μg/mL	2	4	0.43 ± 0.17[a]	5/15 (33)
ANE 80 μg/mL + TPA	19	16	1.75 ± 0.33[a,b,c]	14/20 (70)
ANE 320 μg/mL + TPA	17	5	1.10 ± 0.25[a,b,c]	13/20 (65)
ANE 640 μg/mL + TPA	19	8	1.69 ± 0.25[a,b,c]	15/16 (94)
MCA 2 μg/mL + TPA	24	4	2.00 ± 0.41[a,b]	10/14 (71)

MCA = 3-methylcholanthrene.
[a] Statistically significant difference when compared with control group.
[b] Statistically significant difference when compared with TPA group.
[c] Statistically significant difference when compared with respective ANE-treated group.

Discussion

In Taiwan and many other Southeast Asian countries, BQ chewing is a popular oral habit that increases the risk of oral cancer and OSF, a precancerous condition showing epithelial atrophy and tissue fibrosis.[1,2] In addition, long-term BQ chewing may also cause inflammatory periodontitis and root fracture.[19,20] More seriously, ANE-induced inflammatory reactions are important for human response to infection, irritation, and injury, as well as to oral cancer.[7,21] AN not only induces oral epithelial hyperplasia and carcinogenesis, but also has an impact on subcutaneous connective tissue resulting in OSF. This can be due to genotoxicity and nongenotoxicity of BQ components. In Taiwan, the major components of BQ include AN, lime, *piper betle* inflorescence with/without betel leaf.[1,2] In this study, we found that with increasing concentration of ANE, the number and size of colony formation of C3H10T1/2 cells obviously decreased, suggesting the cytotoxic effect of ANE. Similarly, ANE also caused cytotoxicity to oral keratinocytes and fibroblasts.[6,7,22] Cytotoxicity and compensatory tissue regenerative cell proliferation may lead to the fixation of DNA damaged cells and promote carcinogenesis.[9,23] During carcinogenesis, the cytotoxic effect by chemicals may also cause the clonal selection of malignant cells, leading to progression of cancer.[24,25] Toxicity of ANE may therefore contribute to oral carcinogenesis by inducing cytotoxicity.

To know more about the role of AN in oral carcinogenesis, we found that treatment by ANE alone without TPA showed only a mild transformation capacity toward C3H10T1/2 cells ($p > 0.05$). ANE has been shown to exhibit genotoxicity to different kinds of cells.[1,2] This result suggests that ANE is not a strong complete carcinogen, not simulating the well-known complete carcinogen MCA that may induce transformed foci alone. BQ components have been suggested to be tumor promoters by using both *in vitro* and *in vivo* models.[26–29] Previous studies have found that BQ extract may promote the transformation of JB6 cells and can be a tumor promoter.[27] This is possibly due to AN components or other toxic components in the BQ (e.g., AN) or toxic species (such as reactive oxygen species) generated during interaction of BQ components. ANE was also shown to promote the transformation of bovine papilloma virus-transfected C3H10T1/2 cells.[28] Accordingly, AN components have been shown to induce reactive oxygen species (ROS), prostanoids, and other cytokines (interleukin-6, Granulocyte-macrophage colony-stimulating factor, etc.) productions that are potential tumor promoters.[7,12,21,30,31] Prostaglandin and ROS have been demonstrated to promote the transformation of carcinogen and radiation-treated C3H10T1/2 cells.[32,33]

Moreover, we found that ANE markedly stimulated the transformation of C3H10T1/2 cells, especially in the presence of the tumor promoter, TPA. These results suggest that AN components have tumor initiating properties, even at a concentration of 80 μg/mL. BQ components have been shown to induce DNA breaks, micronuclei formation, and chromosomal aberrations that are important for tumor initiation.[1,2] ANE with/without TPA may induce type II and type III transformed foci formation with typical morphologic changes in C3H10T1/2 cells. Injection of these transformed cells into subcutaneous tissue of mice may elicit tumor formation *in vivo*.[14–16] These results demonstrate the evident transformation capacity and tumorigenicity of AN components.

In conclusion, BQ chewing may contribute to the pathogenesis of OSF and OSCC, possibly due to BQ components-induced cytotoxicity and malignant cell transformation. AN components may be involved in the multistep chemical carcinogenesis by their tumor initiation and promotion properties. More studies on the cellular and molecular changes in different steps of the BQ-induced carcinogenesis are necessary to further improve the prevention and treatment strategies of oral cancer and OSF.

Acknowledgments

This study is supported by grants from the National Science Council (NSC102-2314-B-255-003-MY2, NSC102-2628-B-255-001-MY3, NSC101-2320-B-255-002, NSC-100-2314-B-002-094,

and NSC-101-2320-B-255-002) and Chang Gung Memorial Hospital (CMRPF1C0011, CMRPF1C0012, NMRPF3C0091, and NMRPF3C0061).

References

1. Jeng JH, Chang MC, Hahn LJ. Role of areca nut in betel quid-associated chemical carcinogenesis: current awareness and future perspectives. *Oral Oncol* 2001;**37**:477—92.
2. IARC. Betel-quid and areca-nut chewing and some areca-nut derived nitrosamines. *IARC Working Group on the Evaluation of Carcinogenic Risks to Humans* 2004;**85**:1—334.
3. Ko YC, Chiang TA, Chang SJ, Hsieh SF. Prevalence of betel quid chewing habit in Taiwan and related sociodemographic factors. *J Oral Pathol Med* 1992;**21**:261—4.
4. Chen YJ, Chang JT, Liao CT, Wang HM, Yen TC, Chiu CC, et al. Head and neck cancer in the betel quid chewing area: recent advances in molecular carcinogenesis. *Cancer Sci* 2008;**99**:1507—14.
5. Ko YC, Huang YL, Lee CH, Chen MJ, Lin LM, Tsai CC. Betel quid chewing, cigarette smoking and alcohol consumption related to oral cancer in Taiwan. *J Oral Pathol Med* 1995;**24**:450—3.
6. Jeng JH, Ho YS, Chan CP, Wang YJ, Hahn LJ, Lei D, et al. Areca nut extract up-regulates prostaglandin production, cyclooxygenase-2 mRNA and protein expression of human oral keratinocytes. *Carcinogenesis* 2000;**21**:1365—70.
7. Chang MC, Wu HL, Lee JJ, Lee PH, Chang HH, Hahn LJ, et al. The induction of prostaglandin E2 production, interleukin-6 production, cell cycle arrest and cytotoxicity in primary oral keratinocytes and KB cancer cells by areca nut ingredients is differentially regulated by MEK/ERK activation. *J Biol Chem* 2004;**279**:50676—83.
8. Chang MC, Lin LD, Wu HL, Hsien HC, Wang TM, Jeng PY, et al. Areca nut-induced buccal mucosa fibroblast contraction and its signaling: a potential role in oral submucous fibrosis — a pre-cancer condition. *Carcinogenesis* 2013;**34**:1096—104.
9. Barrett JC. Mechanisms of multistep carcinogenesis and carcinogen risk assessment. *Environ Health Perspect* 1993;**100**:9—20.
10. Minamoto T, Mai M, Ronai Z. Environmental factors as regulators and effectors if multistep carcinogenesis. *Carcinogenesis* 1999;**20**:519—27.
11. Short BG. Cell proliferation and renal carcinogenesis. *Environ Health Perspect* 1993;**101**(Suppl. 5):115—20.
12. Grisham MB, Jourd'heuil D, Wink DA. Review article: chronic inflammation and reactive oxygen and nitrogen metabolism-implications in DNA damage and mutagenesis. *Aliment Pharmacol Ther* 2000;**14**(Suppl. 1):3—9.
13. Klaunig JE, Kamendulis LM, Xu Y. Epigenetic mechanisms of chemical carcinogenesis. *Human Exp Toxicol* 2000;**19**:543—55.
14. Boreiko CJ. Mechanistic aspects of initiation and promotion in C3H10T1/2 cells. *Carcinog Compr Surv* 1985;**9**:153—65.
15. Reznikoff CA, Bertram JS, Brankow DW, Heidelberger C. Quantitative and qualitative studies of chemical transformation of cloned C3H embryo cells sensitive to post-confluence inhibition of cell division. *Cancer Res* 1973;**33**:3239—49.
16. Lehane MM, Bryant PE, Riches AC, Allen LA, Briscoe CV, Meiville J, et al. 238Pu alpha-particle-induced C3H10T1/2 transformants are less tumorigenic than the X-ray-induced equivalent. *Carcinogenesis* 1999;**20**:35—40.
17. Chang MC, Chan CP, Wang WT, Chang BE, Lee JJ, Tseng SK, et al. Areca nut ingredients activate Chk1/Chk2, induce cell cycle arrest and regulate MMP-9 and TIMPs production in SAS epithelial cells. *Head Neck* 2013;**35**:1295—302.
18. Chang MC, Wang YJ, Lee PH, Chen E, Tsai YL, Lin BR, et al. Induction of necrosis and apoptosis to KB cancer cells by sanguinarine is associated with reactive oxygen species production and mitochondrial membrane depolarization. *Toxicol Appl Pharmacol* 2007;**218**:143—51.
19. Ling LJ, Hung SL, Tseng SC, Chen YT, Chi L, Wu KM, et al. Association between betel quid chewing, periodontal status and periodontal pathogens. *Oral Microbiol Immunol* 2001;**16**:364—9.
20. Gao YJ, Yin XM, Wu HJ. Relationship between vertical root fracture and the habits of chewing betel nut. *Hunan Yi Ke Da Xue Bao* 2001;**26**:161—2.
21. Chang MC, Chiang CP, Lin CL, Lee JJ, Hahn LJ, Jeng JH. Cell-mediated immunity and head and neck cancer: with special emphasis on betel quid chewing habit. *Oral Oncol* 2005;**41**:757—75.
22. Jeng JH, Kuo ML, Hahn LJ, Kuo MY. Genotoxic and non-genotoxic effects of betel quid ingredients on oral mucosal fibroblasts in vitro. *J Dent Res* 1994;**73**:1043—9.
23. Bogdanffy MS, Valentine R. Differentiating between local cytotoxicity, mitogenesis, and genotoxicity in carcinogen risk assessments: the case of vinyl acetate. *Toxicol Lett* 2003;**140—141**:83—98.
24. Eriksson L, Ahluwalia M, Spiewak J, Lee G, Sarma DS, Rooni MJ, et al. Distinctive biochemical pattern associated with resistance of hepatocytes in hepatocyte nodules during liver carcinogenesis. *Environ Health Perspect* 1983;**49**:171—4.
25. Kohle C, Schwarz M, Bock KW. Promotion of hepatocarcinogenesis in humans and animal models. *Arch Toxicol* 2008;**82**:623—31.
26. Lin MH, Chou FP, Husng HP, Hsu JD, Chou MY, Wang CJ. The tumor promoting effect of lime-piper betel quid in JB6 cells. *Food Chem Toxicol* 2003;**41**:1463—71.
27. Lin MH, Wang CJ, Huang HP, Chou MY, Chou FP. The tumorigenic characteristics of lime-piper betel quid-transformed JB6 cells. *Arch Toxicol* 2004;**78**:167—73.
28. Tsang SS, Stich HF, Fujiki H. Cell transformation induced by bovine papillomavirus DNA as an assay for tumor promoters and chemopreventive agents. *Cancer Detect Prev* 1991;**15**:423—7.
29. Jin YT, Tsai ST, Wong TY, Chen FF, Chen RM. Studies on promoting activity of Taiwan betel quid ingredients in hamster buccal pouch carcinogenesis. *Eur J Cancer B Oral Oncol* 1996;**32**(B):343—6.
30. Chang MC, Chen YJ, Chang HH, Chan CP, Yeh CY, Wang YL, et al. Areca nut components affect COX-2, cyclin B1/cdc25C and keratin expression, PGE2 product in keratinocyte is related to reactive oxygen species, CYP1A1, Src, EGFR and Ras signaling. *PLoS One* 2014;**9**:e101959.
31. Ji WT, Lee CI, Chen JY, Cheng YP, Yang SR, Chen JH, et al. Areca nut extract induces pyknotic necrosis in serum-starved oral cells via increasing reactive oxygen species and inhibiting GSK3beta: an implication for cytopathic effects in betel quid chewers. *PLoS One* 2013;**8**:e63295.
32. Parfett C, Macmillan J, Pilon R. Oxidative stress mediates tumor promoter-induced proliferin gene expression in C3H10T1/2 cells. *Int J Oncol* 1993;**3**:917—25.
33. Wolfle D. Enhancement of carcinogen-induced malignant cell transformation by prostaglandin F(2alpha). *Toxicology* 2003;**188**:139—47.