行政院國家科學委員會專題研究計畫 成果報告

抗氧化蛋白在嚼食檳榔引發口腔癌前病變及口腔癌的探討
研究成果報告(精簡版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 95-2314-B-040-021-
執 行 期 間 ：95 年 08 月 01 日至 96 年 07 月 31 日
執 行 單 位 ：中山醫學大學牙醫學系

計 畫 主 持 人：蔡崇弘
共 同 主 持 人：張育超、何永全
計畫參與人員：博士班研究生-兼任助理：李宣信

處 理 方 式 ：本計畫可公開查詢

中 華 民 國 96 年 10 月 21 日

The upregulation of heme oxygenase-1 expression in areca quid chewing-associated oral squamous cell carcinomas

Shiuan-Shinn Lee, [a] Shun-Fa Yang, [a] Yung-Chuan Ho, [b]

Chung-Hung Tsai, [a,c] Ming-Yung Chou, [d] and Yu-Chao Chang [d,e]

[a] Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan

[b] School of Applied Chemistry, Chung Shan Medical University, Taichung, Taiwan

[c] Department of Oral Pathology, Chung Shan Medical University Hospital, Taichung, Taiwan

[d] Oral Medicine Center, Chung Shan Medical University Hospital, Taichung, Taiwan

[e] Institute of Stomatology, College of Oral Medicine, Chung Shan Medical University, Taichung, Taiwan

Please send all correspondence to:

Dr. Yu-Chao Chang, Oral Medicine Center, Chung Shan Medical University Hospital, 110, Sec. 1, Chien-Kuo N. Rd., Taichung, Taiwan.

TEL: 886-4-22015111 Ext. 66268

Fax: 886-4-24759065

E-mail:cyc@csmu.edu.tw

BACKGROUND: Heme oxygenase-1 (HO-1) is known as an oxidative stress responsive protein upregulated by various physiological and endogenous stimuli. The aim of this study was to investigate the difference of HO-1 expression in normal human oral epithelium and areca quid-associated oral squamous cell carcinoma (OSCC) and further explore the potential mechanism that may lead to induce HO-1 expression.

METHODS: Thirty five OSCC and 10 normal epithelium specimens were examined by immunohistochemistry and analyzed by the clinico-pathological profiles. The oral epithelial cell line GNM cells were challenged with arecoline, a major areca nut alkaloid, by reverse-transcriptase polymerase chain reaction. Furthermore, tobacco smoke carcinogen benzo[*a*]pyrene (BaP) and glutathione (GSH) precursor N-acetyl-L-cysteine were added to find the possible regulatory mechanisms.

RESULTS: HO-1 expression was significantly higher in OSCC specimens ($p<0.05$). No significant difference in HO-1 expression was observed with respect to age, sex, T category, and stage ($p>0.05$). The high HO-1 expression was associated with lymph node metastasis ($p=0.005$). Arecoline was found to elevate HO-1 mRNA in a dose-dependent manner ($p<0.05$). The addition of BaP enhanced the arecoline-induced HO-1 expression ($p<0.05$). Moreover, the addition of NAC markedly inhibited the arecoline-induced HO-1 expression ($p<0.05$).

CONCLUSION: These results suggest that HO-1 expression is significantly upregulated in OSCC from areca quid chewers and arecoline may be responsible for the enhanced HO-1 expression *in vivo*. The compounds of cigarette smoke may act synergistically in the pathogenesis of areca quid chewing associated-OSCC. The regulation of HO-1 expression induced by arecoline is critically dependent on the intracellular GSH

concentration.

Key words: areca quid; oral squamous cell carcinoma; heme oxygenase-1; arecoline; benzo[a]pyrene; N-acetyl-L-cysteine

Introduction

Known risk factors for oral squamous cell carcinoma (OSCC), the most common form of oral cancer, are long-term tobacco, alcohol, and areca quid use (1). The habit of areca quid chewing is wide spread in Taiwan, Southwest Asia and India and also with potential links to the occurrence of OSCC (2). It was estimated that approximately 2 million people with the habit of areca quid chewing in Taiwan (3). The areca quid in Taiwan consists of two halves of a fresh areca nut sandwiched with a piece of inflorescence *Piper betle* and lime mixture, but no tobacco consisted in the quid. Epidemiological studies have demonstrated the relationship between areca quid chewing and the incidence of OSCC in Taiwan (4,5).

Heme oxygenase-1 (HO-1), a 32-kDa microsomal enzyme (6), participates in maintaining the cellular homeostasis and plays an important protective role in the tissues due to reducing oxidative injury and attenuating the inflammatory response whose expression is upregulated by a variety of stimuli, including heme, oxidative stresses, heavy metals, and inflammatory cytokines (7,8). HO-1 catalyzes the oxidative degradation of heme to biliverdin, which in turn is reduced to bilirudin (9). HO-1 functions as an antioxidant enzyme because locally produced bilirudin works as an efficient scavenger of reactive oxygen species (ROS). Many studies have shown that the increased expression of HO-1 was found in a wide variety of human tumors such as prostate, pancreatic, and oral cancers (10-13).

Previous studies have demonstrated that polymorphism in HO-1 promoter is related to the risk of OSCC occurring on areca quid chewers (14,15). These suggest that HO-1 may play an important role in the pathogenesis of areca quid chewing-associated OSCC.

However, the authors do not take into account the cellular source of the HO-1 in OSCC as well as normal oral epithelium. On the basis of these observations, the present work was undertaken to identify the *in situ* localization of HO-1 expression in normal oral epithelium and OSCC specimens. More specifically, we also explored whether HO-1 expression can be triggered in human epithelial cell line GNM cells stimulated by arecoline, the major areca nut alkaloid, in vitro.

In Taiwan, about 86% of the areca quid chewers are also smokers (2). Tobacco smoke contains several classes of carcinogens, such as polycyclic aromatic hydrocarbons, volatile and tobacco-specific nitrosamines, that affect humans (16). Little is known about the association between areca quid chewing and cigarette smoking. Furthermore, benzo[a]pyrene (BaP), a polycyclic aromatic hydrocarbon produced from cigarette smoking, was added to test how it modulated the arecoline-induced HO-1 expression in GNM cells. In addition, antioxidants are substances that, when existing at low concentrations compared with those of the oxidizable substrate, significantly delay or prevent oxidation of that substrate (17). To determine whether oxidative stress could modulate the induction of HO-1 expression by areca quid chewing, a cell-permeable glutathione (GSH) precursor N-acetyl-L-cysteine (NAC) was added to find the possible mechanism and its protective effect on arecoline-induced HO-1 expression in GNM cells.

Materials and methods

Sample collection

Ten normal oral epithelium specimens from non-areca quid chewers and 35 OSCC specimens from areca quid chewers with available clinical stage (TNM system), were drawn from the files of the Chung Shan Medical University Hospital. Diagnosis was based on histological examination of hematoxylin- and eosin stained sections. Institutional Review Board permission at the Chung Shan Medical University Hospital was obtained for the use of discarded human tissue. The clinico-pathological profiles of the patients are shown in Table 1.

Immunohistochemistry

Five micron sections from formalin-fixed, paraffin-embedded specimens were stained with the monoclonal anti-HO-1 antibody (Santa Cruz Biotechnology, CA, USA) (1:50 dilution) using a standard avidin-biotin-peroxidase complex method as described previously (18,19). Diaminobenzidine (Zymed, South San Francisco, CA, USA) was then used as the substrate for localizing the antibody binding. Negative controls included serial sections from which either the primary or secondary antibodies were excluded. The preparations were counterstained with hematoxylin, mounted with Permount (Merck, Darmstadt, Germany) and examined by light microscopy. Processed immunohistochemically for HO-1 expression, sections graded as “low” were represented by positive stained cells less than 50%; sections graded “high” exhibited positive stained cells over 50% on three sections/tissue at 200× magnification.

Cell culture

The oral epithelial cell line GNM cells, derived from a patient with $T_2N_{2a}M_0$

gingival carcinoma and metastasis to the cervical lymph node, were grown in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10 % fetal calf serum (FCS) (Gibco Laboratories, Grand Island, NY, USA) and antibiotics (100 U/ml of penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml of fungizone). Cultures were maintained at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air. Confluent cells were detached with 0.25 % trypsin and 0.05 % EDTA for 5 min, and aliquots of separated cells were subcultured.

HO-1 expression analysis

Cells arrested in G_0 by serum deprivation (0.5 % FCS; 48 h) were used in the experiments (19). Nearly confluent monolayers of GNM cells were washed with serum free medium and immediately thereafter exposed at the various concentrations (0, 10, 20, 40, 80, and 160 μg/ml) of arecoline (Sigma, St. Louis, MO, USA). Cell lysates were collected at 6 h for reverse transcriptase polymerase chain reaction. Cultures without FCS were used as negative controls. Subsequently, 1 μM BaP (Merck, Darmstadt, Germany) and 1 mM NAC (Sigma, St. Louis, MO, USA) were added to test its regulatory effects during a 6 h incubation period.

Reverse-transcriptase polymerase chain reaction

Total RNA was prepared using TRIzol reagent (Gibco Laboratories, Grand Island, NY, USA) following the manufacturer's instructions. Single-stranded DNA was synthesized from RNA in a 15 μl reaction mixture containing 100 mg random hexamer and 200 units of Moloney murine leukemia virus reverse transcriptase (Gibco Laboratories, Grand Island, NY, USA). The reaction mixture was diluted with 20 μl of water and 3 μl of the diluted reaction mixture was used for the polymerase chain reaction

(PCR). PCR reaction mixture contains 10 μmol of forward and reverse primers and two units of Tag DNA polymerase. Amplification was performed at 25 cycles for GAPDH and 30 cycles for HO-1 in a thermal cycle. Each cycle consisted of 1 min of denaturation at 94 ℃, 1 min of annealing at 58 ℃, and 1 min of extension at 72℃. The sequences of primers used were as follows (20):

(A) GAPDH

Forward: 5'-TCCTCTGACTTCAACAGCGACACC-3'

Reverse: 5'-TCTCTCTTCCTCTTGTGCTCTTGG-3'

(B) HO-1

Forward: 5'-AAGGCTTTAAGCTGGTGATGG-3'

Reverse: 5'-AGCGGTGTCTGGGATGAACTA-3'

The PCR products were analyzed by agarose gel electrophoresis and a 527 bp band for HO-1 was noted. When the band densities were measured and compared with the density of the band obtained for the housekeeping gene GAPDH, relative proportions of mRNA synthesis could be determined within each experiment. The intensity of each band after normalization with GAPDH mRNA was quantified by the photographed gels with a densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, CA, USA).

Statistical analysis

The statistical significance of differences between HO-1 staining grade and various clinico-pathologic features (T-categories, N-categories, sex, staging of the patients, cell differentiation of OSCC) were assessed by the Fisher's exact test for independence. For RT-PCR, triplicate or more separate experiments were performed throughout this study. The significance of the results obtained from control and treated groups was statistically

analyzed by the paired Student t-test. A *p*-value of <0.05 was considered to be statistically significant.

Results

Weak HO-1 expression was observed in the specimens of normal epithelium (Fig. 1a). However, the HO-1 staining was mainly expressed in the epithelium OSCCs and the intensity of HO-1 was significantly higher than normal epithelium specimens ($p < 0.05$). In well differentiated OSCCs, only cells located at the periphery of epithelial pearl were labeled, while centrally situated cells remained negative (Fig. 1b). In moderate differentiated OSCCs, homogenous and intensive staining for HO-1 was labeled in the nest (Fig. 1c). A mosaic pattern was observed for HO-1 labeling in poorly differentiated OSCCs (Fig. 1d). In addition, HO-1 expression was also observed in the cytoplasm of fibroblasts, endothelial cells, and inflammatory cells.

As shown in Table 2, subjects were divided into two age groups, greater than or less than the median (51 years). The immunostaining of HO-1 in OSCC could be classified into two groups: one was low grade, 51.42% (18/35) and the other was high grade, 48.57% (17/35). No significant difference in HO-1 expression was observed with respect to other factors, such as age, sex, T category, and stage ($p> 0.05$). The high HO-1 expression level was significantly associated with lymph nodes metastasis ($p = 0.005$).

To examine the effect of arecoline on the HO-1 expression, GNM cells were treated with arecoline and the levels of mRNA were measured. RT-PCR was used to verify whether arecoline could affect HO-1 mRNA gene expression in GNM cells. Figure 2a reveals a dose-dependent change following treatment of GNM cells with various concentrations of arecoline. Arecoline was found to elevate HO-1 mRNA gene expression in a dose-dependent manner ($p<0.05$). From the AlphaImager 2000 (Fig. 2b), the amount of HO-1 was elevated about 1.6, 2.3, 2.9, 2.7, and 1.8 fold after exposure to

10, 20, 40, 80, and 160 μg/ml arecoline ($p<0.05$), respectively. Moreover, the peak of HO-1 mRNA levels induced by arecoline was 40 μg/ml. On the basis of these results, experiments described below were performed at a concentration of 40 μg/ml arecoline.

Subsequently, 1 μM BaP and 1 mM NAC were added to test their regulatory effects during a 6-h incubation period. BaP was found to increase HO-1 mRNA expression (Fig. 3a). From the AlphaImager 2000 (Fig. 3b), the amount of HO-1 was elevated about 2.4 fold after exposure to BaP ($p<0.05$). BaP enhanced the arecoline-induced HO-1 mRNA levels up to 5 fold ($p<0.05$). In addition, OTZ was found to reduced the arecoline-induced HO-1 expression by lowering about 1.2 fold ($p<0.05$).

Discussion

HO-1 is known as a stress-inducible protein. The immunohistochemical expression of HO-1 in neoplastic tissue has been demonstrated in many human cancers (10-13). In this study, HO-1 expression was found to overexpress in areca quid chewing associated-OSCCs as compared with normal epithelium tissues. HO-1 was also noted in the cytoplasm of fibroblasts, endothelial cells, and inflammatory cells. Our results are similar to the previous studies that HO-1 was highly expressed on OSCCs (11,13).

In the present study, we also investigate the relationship between HO-1 expression and the clinical features of areca quid chewing associated-OSCCs. The level of HO-1 expression was inversely correlated to the histological differentiation, T category, and stage. There is significantly higher level of HO-1 expression in the metastasis group than in non-metastasis group of areca quid chewing associated OSCC. Our results were generally in agreement with previous studies that there was no correlation between HO-1 expression and T category as well as stage (11,13). Consistently, Lin *et al*. (15) who found that areca quid chewing associated-OSCC with lymph node metastasis had significantly higher frequency of HO-1 L allelotypes. However, our results differed from that of Tsuji *et al*. (11) who reported that high HO-1 expression in OSCC was detected in non-metastasis group and Yanagawa *et al*. (13) who also found that low HO-1 expression in tongue squamous cell carcinoma was associated with lymph node metastasis. The reason for this contrary result is not clear. It may be result from different origins of the specimens, different causation of OSCC, or different experimental protocols used in each laboratory. The HO-1 expression in OSCCs may not necessarily be comparable in all specimens.

The mechanism responsible for the HO-1 expression in areca quid chewing-associated OSCCS might be explained as follows. HO-1 is upregulated by oxidative stress and a variety of heavy metals, such as iron (9). Earlier studies have demonstrated that areca nut contains high iron content (2) and generates ROS during chewing (21,22). It is therefore feasible to suggest that high levels of iron released and ROS generated may at least in part be responsible for the upregulation of HO-1 in areca quid chewing-associated OSCCs.

The etiology of OSCC is well known that an association exists with the habit of areca quid chewing. In this study, we first reported the upregulation of HO-1 mRNA expression in GNM cells stimulated by arecoline. This suggests that one of the pathogenetic mechanisms of OSCC may be the synthesis of HO-1 expression by epithelial cells in response to areca quid challenge.

Cigarette smoking is also a major independent risk factor for the development of oral cancer. In this study, BaP was found to induce HO-1 expression in human epithelial cell line GNM cells. Our results were in agreement with Vayssier-Taussat *et al*. (23) who report that BaP was found to induce HO-1 expression in human monocytes and vascular endothelial cells. Consistently, Chang *et al*. (24) who reported that HO-1 was found to upregulate in the gingiva of cigarette smokers. In this study, BaP has a synergistic effect on arecoline-induced HO-1 expression on GNM cells. This result may partly explain why patients who combined the habits of areca quid chewing with cigarette smoking were at greater risk of contracting oral cancer in Taiwan (5). Earlier reports have demonstrated consistently that a synergistic increase in risk of oral cancer has been shown among people who chew areca quid and smoke cigarettes (25-27).

Accumulated evidence indicates a role for HO protein in cellular or tissue damage and suggests that the induction of HO-1 is a protective response against oxidative stress (6). Previously, we have demonstrated that arecoline significantly depleted intracellular GSH in human buccal mucosal fibroblasts (28). To test the contribution of ROS to the induction of stress responses by arecoline in GMN cells, we treated GMN cells with NAC during exposure to arecoline. It is known that GSH plays a role in cellular protection from damage produced by free radicals and electrophiles. When cellular GSH is depleted, cells become extremely prone to oxidative damage. NAC is easily deacetylated inside the cells and provides cysteine for cellular GSH synthesis and thus stimulates the cellular GSH system (29). In the present study, the addition of NAC leads to decrease in the induction of HO-1 expression by arecoline. The results indicated that NAC exhibits a protective effect against arecoline. Taken together, arecoline-induced HO-1 expression is influenced by intracellular GSH levels.

This study represents, to the best of our knowledge, the first systemic attempt to evaluate the role of HO-1 expression in areca quid chewing-associated OSCC in human both *in vitro* and *in vivo*. We demonstrated that HO-1 is elevated in OSCC specimens obtained from areca quid chewers. The high HO-1 expression was associated with lymph node metastasis. The expression profile suggests HO-1 could be used clinically as a marker for tumors possessing the potential for lymph node metastasis. Arecoline-induced HO-1 expression suggests that areca quid chewing may contribute the pathogenesis of OSCC by HO-1 expression. BaP has a synergistic effect of arecoline-induced HO-1 expression. Thus, daily consumption of milligrams of arecoline or BaP, culminating in a cumulative dose, should be considered for its adverse effects. We suggest that people who

combine the habits of areca quid chewing and cigarette smoking are more susceptible to suffer from OSCC than people who chew areca quid alone. In addition, the regulation of HO-1 expression induced by arecoline is critically dependent on the intracellular GSH concentration.

Acknowledgments

This study was supported by a research grant from National Science Council, Taiwan (NSC95-2314-B-040-021).

References

1. Silverman S Jr. Oral cancer. 4th ed. Hamilton, Ontario: *American Cancer Society*, B.C. Decker, 1998; p.1-40.
2. IARC. Betel-quid and areca-nut chewing. *Lyon: International Agency for Research on Cancer, Monographs* 1986; **37**: 141-202.
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**: 261-264.
4. Kwan HW. A statistical study on oral carcinomas in Taiwan with emphasis on the relationships with betel nut chewing: a preliminary report. *J Formos Med Assoc 1976*; **75**: 497-505.
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**: 450-453.
6. Choi AM, Alam J. Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. *Am J Respir Cell Mol Biol* 1996; **15**: 9-19.
7. Ryter SW, Choi AM. Heme oxygenase-1: redox regulation of a stress protein in lung and cell culture models. *Antioxid Redox Signal* 2005; 7: 80-91.
8. Maines MD, Gibbs PE. 30 some years of heme oxygenase: from a "molecular wrecking ball" to a "mesmerizing" trigger of cellular events. *Biochem Biophys Res Commun* 2005; **338**: 568-577.
9. Maines MD. Heme oxygenase: function, multiplicity, regulatory mechanisms, and

clinical applications. *FASEB J* 1988; **2**: 257-268

10. Maines MD, Abrahamsson PA. Expression of heme oxygenase-1 (HSP32) in human prostate: normal, hyperplastic, and tumor tissue distribution. *Urology* 1996; **47**: 727-733.
11. Tsuji MH, Yanagawa T, Iwasa S, Tabuchi K, Onizawa K, Bannai S, Toyooka H, Yoshida H. Heme oxygenase-1 expression in oral squamous cell carcinoma as involved in lymph node metastasis. *Cancer Lett* 1999; **138**: 53-59.
12. Sunamura M, Duda DG, Ghattas MH, Lozonschi L, Motoi F, Yamauchi J, Matsuno S, Shibahara S, Abraham NG. Heme oxygenase-1 accelerates tumor angiogenesis of human pancreatic cancer. *Angiogenesis* 2003; **6**: 15-24.
13. Yanagawa T, Omura K, Harada H, Nakaso K, Iwasa S, Koyama Y, Onizawa K, Yusa H, Yoshida H. Heme oxygenase-1 expression predicts cervical lymph node metastasis of tongue squamous cell carcinomas. *Oral Oncol* 2004; **40**: 21-27.
14. Chang KW, Lee TC, Yeh WI, Chung MY, Liu CJ, Lin SC. Polymorphism in heme oxygenase-1 (HO-1) promoter is related to the risk of oral squamous cell carcinoma occurring on male areca chewers. *Br J Cancer* 2004; **91**: 1551-1555.
15. Lin SC, Liu CJ, Yeh Wi, Lui MT, Chang KW, Chang CS. Functional polymorphism in *NFKB*1 promoter is related to the risks of oral squamous cell carcinoma occurring on older male areca (betel) chewers. *Cancer Lett* 2006; **243**: 47-54.
16. Hoffmann D, Lavoie EJ, Hocht SS. Nicotine: A precursor for carcinogens. *Cancer Lett* 1985; **26**: 67-75.
17. Halliwell B. Antioxidants: the basics-what they are and how to evaluate them. *Adv Pharmacol* 1997; **38**: 3-20.

18. Tsai CH, Hsieh YS, Yang SF, Chou MY, Chang YC. Matrix metalloproteinase 2 and 9 expression in human oral squamous cell carcinoma and the effect of protein kinase C inhibitors: preliminary observations. *Oral Surg Oral Med Oral Pathol* 2003; **95**: 710-716.
19. Lee SS, Yang SF, Ho YC, Tsai CH, Chang YC. The upregulation of metallothionein-1 expression in areca quid chewing-associated oral squamous cell carcinomas. *Oral Oncol* (In press).
20. Salinas M, Diaz R, Abraham NG, Ruiz de Galarreta CM, Cuadrado A. Nerve growth factor protects against 6-hydroxydopamine-induced oxidative stress by increasing expression of heme oxygenase-1 in a phosphatidylinositol 3-kinase-dependent manner. *J Biol Chem* 2003; **278**: 13898-13904.
21. 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 Perspect* 1992; **98**: 203-205.
22. Chen CL, Chi CW, Liu TY. Hydroxyl radical formation and oxidative DNA damage induced by areca quid in vivo. *J Toxicol Enviro Health A* 2002; **65**: 327-336.
23. Vayssier-Taussat M, Camilli T, Aron Y, Meplan C, Hainaut P, Polla BS, Weksler B. Effects of tobacco smoke and benzo[*a*]pyrene on human endothelial cell and monocyte stress responses. *Am J Physiol Heat Circ Physiol* 2001; **280**: 1293-1300.
24. Chang YC, Lai CC, Lin LF, Ni WF, Tsai CH. The up-regulation of heme oxygenase-1 expression in human gingival fibroblasts stimulated with nicotine. *J Periodont Res* 2005; **40**: 252-257.
25. Shanta V, Krishnamurthy S. Further study in aetiology of carcinomas of the upper

alimentary tract. *Br J Cancer* 1963; **17**: 8-23.

26. Hirayama T. An epidemiological study of oral and pharyngeal cancer in central and south-east Asia. *Bull. World Health Organ* 1966; **34**: 41-69.
27. Jafarey NA, Mahmood Z, Zaidi SHM. Habits and dietary pattern of cases of carcinoma of oral cavity and oropharynx. *J Pakistan Med Assoc* 1977; **27**: 340-343.
28. Chang YC, Tai KW, Chou MY, Tseng TH. Synergistic effects of peroxynitrite on arecoline-induced cytotoxicity in human buccal mucosal fibroblasts. *Toxicol Lett* 2000; **118**: 61-68.
29. Gillissen A, Novak D. Characterization of *N*-acetyl-cysteine and ambroxol in anti-oxidant therapy. *Res Med* 1998; **92**: 609-623.

Legends for figures

Figure 1 (a) Weak immunoreactivity of HO-1 was observed in normal epithelium specimen. (200x) (b) In well differentiated OSCCs, only cells located at the periphery of epithelial pearl were labeled, while centrally situated cells remained negative. (200x) (c) In moderate differentiated OSCCs, homogenous and intensive staining for HO-1 was labeled in the nest. (200x) (d) In poorly differentiated OSCCs, a mosaic pattern was observed for HO-1 labeling. (200x)

Figure 2 (a) Expression of HO-1 mRNA gene in arecoline-treated GNM cells by RT-PCR assay. Cells were exposed for 6 h in serum free medium containing arecoline concentration as indicated. M=DNA molecular size marker. GAPDH gene was performed in order to monitor equal RNA loading. (b) Levels of HO-1 mRNA gene treated with arecoline were measured by AlphaImager 2000. The relative level of HO-1 gene expression for each sample was normalized against GAPDH mRNA signal and the control was set as 1.0. Optical density values represent the means±standard deviations. * represents significant difference from control values with $p < 0.05$.

Figure 3 (a) The regulatory effects of BaP and NAC on arecoline induced HO-1 mRNA activity expression in GNM cells. Cells were coincubation with BaP for 6 h in the presence of 40 μg/ ml arecoline. M=DNA molecular size maker. GAPDH gene was performed in order to monitor equal RNA loading. (b) Levels of

HO-1 gene in GNM cells after exposure to BaP alone and to 40 μg/ml arecoline. Quantitation was achieved by AlphaImager 2000 as described in Fig. 2b. * represents significant difference from control values with $p < 0.05$. # represents statistically significant between arecoline alone and arecoline with BaP or NAC; $p < 0.05$.