行政院國家科學委員會專題研究計畫成果報告

計畫編號：NSC 90-2314-B-040-018
執行期限：90 年 8 月 1 日至 91 年 7 月 31 日
主持人：張育超 中山醫學院牙醫學系
共同主持人：周明勇 中山醫學院牙醫學系

一、中文摘要

由流行病學研究報告指出吸煙會促進牙周病的進行及延遲牙周治療的癒合反應，但從文獻回顧中發現鮮少有研究探討香煙對牙周組織的破壞機轉，所以本研究以組織培養法，培養人類牙周韌帶造纖維母細胞，探討香煙的主要成份尼古丁對牙周韌帶造纖維母細胞的抑制機轉，結果顯示，尼古丁會對人類牙周韌帶造纖維母細胞產生細胞毒性作用，此一現象會隨著濃度、作用的時間增加而增加($p<0.05$)；且尼古丁也會明顯地抑制細胞增殖及降低蛋白質的合成，尼古丁的濃度在 50 μM 及 200 μM 時分別會抑制 48 % 及 86 % 人類牙周韌帶造纖維母細胞的生長，在 10 mM 濃度的尼古丁會明顯地抑制蛋白質合成量約為控制組的 44 % ($p<0.05$)；加入細胞外 2-oxothiazolidine-4-carboxylic acid (OTZ)可以保護細胞免於尼古丁誘發的細胞毒性，但在加入 50 μMBSO (buthionine sulfoximine) 後其可加強尼古丁造成的細胞毒性；superoxide dismutase 及 catalase 並不會影響由尼古丁引起的細胞毒性反應；由這結果顯示是硫醇(thiol)的消耗，而非因氧自由基引起尼古丁的細胞毒性機制。

關鍵詞：牙周病、尼古丁、人類牙周韌帶造纖維母細胞、抑制機轉、硫醇

Abstract

The use of tobacco products significantly contributes to the progression of periodontal disease and poor response to healing following periodontal therapy. The purpose of this study was to determine the effects of nicotine, a major component of cigarette smoking, on human periodontal ligament fibroblasts (PDLFs) growth, proliferation, and protein synthesis to elucidate its role in periodontal destruction associated with its use. Human PDLFs were derived from three healthy individuals undergoing extraction for orthodontic reasons. At a concentration higher than 2.5mMnicotine was found to exhibit cytotoxic to human PDLFs ($p<0.05$). Nicotine also significantly inhibited cell proliferation and decreased protein synthesis in a dose-dependent manner. At concentrations of 50 and 200 μM, nicotine suppressed the growth of PDLF with 48% and 86% ($p<0.05$), respectively. A 10-mM concentration level of nicotine significantly inhibited the protein synthesis to only 44 % of these in the untreated control ($p<0.05$).

Furthermore, the effects of anti-oxidants (superoxidedismutase (SOD); catalase and 2-oxothiazolidine-4-carboxylic acid (OTZ) and buthionine sulfoximine (BSO) were added to search for the possible mechanism of action, as well as a method for the prevention, of cigarette smoking-associated periodontal diseases. The addition of OTZ, a precursor of cysteine that metabolically promotes GSH synthesis, acted as a protective effect on the nicotine-induced cytotoxicity. However, SOD and catalase did not decrease the nicotine-inducedcytotoxicity. In contrast, the addition of BSO, a cellular GSH synthesis inhibitor, enhanced the nicotine-induced cytotoxicity. These results indicate that thiol depletion could be the mechanism for nicotine cytotoxicity. The levels of nicotine tested inhibited cell growth, proliferation, and protein synthesis on human PDLFs. This suggests that nicotine itself might augment the destruction of periodontium associated with cigarette smoking. In addition, these inhibitory effects were associated with intracellular thiol levels. Factors that induce glutathione synthesis of human PDLF may be used for further chemoprevention of cigarette smoking-related periodontal diseases.

Keywords : periodontal disease; nicotine; human periodontal ligament fibroblasts; inhibitory mechanisms

二、緣由與目的

Tobacco contains a complex mixture of substances including nicotine, various nitrosamines, trace-elements, and a variety of poorly characterized substances. Nicotine is one of the over 2,000 potentially toxic substances in tobacco smoke. The previous study has shown that tobacco smokers have saliva concentrations of nicotine as high as 1.56 mg/mL, which is more than 100,000 times higher than the level in blood (Hoffmann & Adams 1981). *In vitro* study also has shown that nicotine can be detected on the root surface of periodontally involved teeth (Cuff *et al.* 1989). Nicotine has been shown to be able to alter some cellular functions. For example, exposure of human fibroblasts derived from periodontium to nicotine affects cell growth, as well as attachment (Peacock *et al.* 1993; Tipton & Dabbous 1995; James *et al.* 1999; Giannopoulou *et al.* 1999; Chang *et al.* 2001). In addition, human gingival fibroblasts rapidly take up and accumulate high levels of nicotine *in vitro*, most of which remains inside the fibroblasts, where it can affect cell metabolism or functions (Hanes *et al.* 1991).

Previous clinical findings indicate that cigarette smoking is a risk factor in periodontal disease (Genco 1996). The potential toxicological implications of nicotine, a reaction product from cigarette smoking, on periodontium remains to be elucidated. In this study, cell growth, proliferation, and protein synthesis assays were performed to

elucidate the pathobiological effects of nicotine on cultured human PDLFs. In addition, little is known about whether chemical interactions can modulate the nicotine-induced cytotoxicity. Furthermore, the effects of anti-oxidants (superoxide dismutase (SOD); catalase and 2-oxothiazolidine-4-carboxylic acid (OTZ) were added to discover for the possible mechanism of action, as well as a method for the prevention, of cigarette smoking-associated periodontal diseases.

三、結果與討論

Nicotine demonstrated a cytotoxic effect on human PDLFs (Fig. 1). Nicotine reduced the activity of dehydrogenase of cells over a 24-h culture period in a dose-dependent manner. The 50% inhibition concentration of nicotine was about 12.6 mM. Fig.2 shows the effect of nicotine on incorporation of [^{3}H]-thymidine for cell proliferation. Nicotine inhibited the DNA synthesis of PDLF in a dose-dependent manner ($p<0.05$) and was cytotoxic at the concentration levels $\geq$ 25 μM. Nicotine at the concentration level of 50 μM inhibited the DNA synthesis to only 52 % of these in the untreated control. Elevating the nicotine concentration to 400 μM completely inhibited DNA synthesis. Effect of nicotine on protein synthesis of human PDLF is shown in Fig. 3. Nicotine inhibited protein synthesis at 5 mM and higher concentrations in a dose-dependent manner, as determined by [^{3}H]-leucine incorporation. A 15 mM concentration level of nicotine significantly inhibited the protein synthesis to only 23 % of these in the untreated control Three anti-oxidants were added to investigate whether they could protect cells from nicotine cytotoxicity. The concentrations of SOD (10-100 μg/ml), catalase (5-50 μg/ml) and OTZ (0.5-10 mM) were not cytotoxic to human PDLF by MTT assay ($p>0.05$)(data not shown). The combination effects of nicotine and OTZ on human PDLF by MTT assay is shown in Fig.4. Addition of OTZ extracellularly could protect the cells from nicotine-induced cytotoxicity. When the OTZ concentration was elevated up to 5 mM, nicotine cytotoxicity was almost completely blocked. However, SOD or catalase did not show any protective effects on nicotine-induced cytotoxicity (Table 1 and 2). To further elucidate the roles of GSH in nicotine-associated cytotoxicity, BSO was used to deplete the cellular GSH level as reported by Mulder & Ouwerkerk-Mahadevan (1997). The combination effects of nicotine and BSO on human PDLF by MTT assay is shown in Fig. 5. BSO (5 and 50 μM) was not cytotoxic to human PDLF by MTT assay ($p>0.05$). Nicotine at a concentration of 2.5 mM caused about 15 % of cell death over the 24h incubation period. Addition of 50 μM

BSO enhanced the cytotoxic response to cause about 35 % of cell death on nicotine-induced cytotoxicity.

We concluded that nicotine significantly inhibited human PDLF growth, proliferation and protein synthesis. Depletion of cellular thiol activity might render the cells more vulnerable to other reactive agents present in the dental plaque within cigarette smoking. This may partly explain the role of nicotine to be an important at-risk factor regarding the etiology, progression, and the outcome of the treatment of inflammatory periodontal diseases. In addition, GSH-related agents were antagonistsfor the cytotoxicity of nicotine. Thus, increasing dietary intake of GSH-rich foods or dietary supplements of GSH may have chemopreventive potential to reduce cigarette smoking-associated periodontal disease. Therefore, development of agents that induce the cellular synthesis of GSH level might be useful for chemoprevention of cigarette smoking-related periodontal disease.

五、參考文獻

Chang YC, Lii CK, Tai KW, Chou MY. Adverse effects of arecoline and nicotine on human periodontal ligament fibroblasts *in vitro*. *J Clin Periodontol* 2001; 28: 277-282.

Cuff MJ, McQuade MJ, Scheidt MJ, Sutherland DE, Van Dyke TE. The presence of nicotine on root surfaces of periodontally diseased teeth in smokers. *J Periodontol* 1989; **60**: 564-569.

Genco RJ. Current view of risk factors for periodontal diseases. *J Periodontol* 1996; **67**: 1041-1049. Giannopoulou C, Geinoz A, Cimasoni G. Effects of nicotine on periodontal ligament fibroblasts *in vitro*. *J Clin Periodontol* 1999; **26**: 49-55.

Hanes PJ, Schuster GS, Lubas S. Binding uptake and releases of nicotine by human gingival fibroblasts. *J Periodontol* 1991; **62**: 147-152.

Hoffmann D, Adams JD. Carcinogenic tobacco-specific N-nitrosamines in snuff and in the saliva of snuff dippers. *Cancer Res* 1981; **41**: 4305-4308.

James JA, Sayers NM, Drucker DB, Hull PS. Effects on the attachment and growth of periodontal ligament fibroblasts. *J Periodontol* 1999; **70**: 518-525.

Peacock ME, Sutherland DE, Schuster GS, Brennan WA, O'Neal RB, Strong SL, Van Dyke TE. The effect of human gingival fibroblasts *in vitro*. *J Periodontol* 1993; **64**: 658-665.

Tipton DA, Dabbous Mkh. Effects of nicotine on proliferation and extracellular matrix production of human gingival fibroblasts *in vitro*. *J Periodontol* 1995; **56**: 1056-1064.

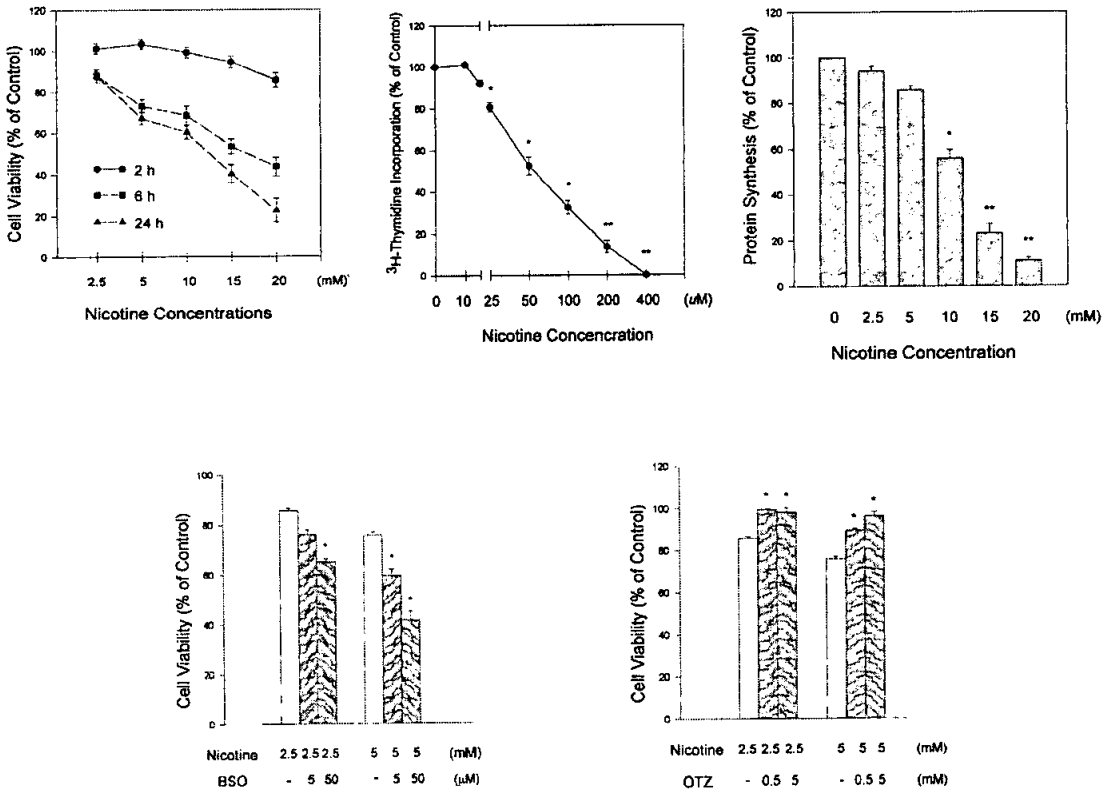

Table 1. Effects of SOD on nicotine-induced cytotoxicity

Chemicals	Cell viability (% of control)
2.5 mM nicotine	82.6 ± 1.3
50 μg/ml SOD + 2.5 mM nicotine	80.3 ± 2.1
100 μg/ml SOD + 2.5 mM nicotine	83.5 ± 1.6
5 mM nicotine	77.2 ± 1.5
50 μg/ml SOD + 5 mM nicotine	78.0 ± 2.3
100 μg/ml SOD + 5 mM nicotine	75.7 ± 3.9

Table 2. Effects of catalase on nicotine-induced cytotoxicity

Chemicals	Cell viability (% of control)
2.5 mM nicotine	81.4 ± 2.0
10 μg/ml catalase + 2.5 mM nicotine	82.4 ± 0.8
50 μg/ml catalase + 2.5 mM nicotine	83.0 ± 1.7
5 mM nicotine	76.0 ± 1.4
10 μg/ml catalase + 5 mM nicotine	77.4 ± 1.2
50 μg/ml catalase + 5 mM nicotine	75.3 ± 3.4