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Inhibitory effect of Se-Yeon-Eum on nicotine- and cigarette smoke extract-induced cytotoxicity in human lung fibroblast

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SUMMARY

There are over 4,000 different chemicals in cigarette smoke, including nicotine and tar. These compounds influence on lung tissue directly or indirectly. In this study, we have examined whether an aqueous extract of Se-Yeon-Eum (SYE), composed of Oriental medicine that has been known to be effective to symptom by smoking, inhibits nicotine- or cigarette smoke extract (CSE)-induced cytotoxicity in human embryonic lung fibroblast, MRC-9. Assessment of cell viability using 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay indicated that SYE inhibited not only nicotine-induced cytotoxicity but also CSE-induced cytotoxicity. These results suggest the possibility that the use of SYE may be useful for improvement of many symptoms by smoking.

Key words: Se-Yeon-Eum, Nicotine; Cigarette smoke extract; Human lung fibroblast; Cytotoxicity

INTRODUCTION

Cigarette smoking is a major risk factor in the pathogenesis of lung and oral disease (Vayssier *et al.*, 1998; Hunninghake *et al.*, 1983; Pabst *et al.*, 1995; Benowitz, 1988; Saetta *et al.*, 1994). Cigarette smoking can damage a number of organ system (Snajdar *et al.*, 2000). Smoking increases the incidence and severity of respiratory tract infections and is also a significant risk factor for lung cancer (Hays *et al.*, 1998).

Cigarette smoke is composed of many compounds, both solid and gaseous, and major one is the alkaloid nicotine (Hoffmann *et al.*, 1997). Nicotine is distilled from burning cigarette, and small droplets of tar containing nicotine are inhaled and deposited in the small airways and alveoli. Once nicotine from cigarette smoke reaches the small airways and the alveoli of the lung, it is buffered to physiological pH and rapidly absorbed. Following absorption, nicotine enters the circulation and distributes rapidly to different tissue (Zevin *et al.*, 1998). Through this absorption mechanism, nicotine affects whole body including lung.

Each Chinese character has meaning, and Se-Yeon-Eum (SYE) is divided into three letters 'Se', 'Yeon', and 'Eum', which mean 'wash away', 'smoke', 'liquid medicine', respectively. So it can be interpreted as a drink that washes away dust from smoking. As the name of Se-Yeon-Eum indicates, SYE, composed of Oriental herbs, has been known to be effective to symptom by smoking. Traditionally, in Korea, SYE have been used for lung diseases like asthma, pneumonia and symptoms like cough and phlegm. However, little is known about the mechanism by which SYE rescue the pathological state by cigarette smoke.

In the present study, we selected MRC-9, which is human embryonic fibroblast cell line from normal lung tissue and is used in many cases related to normal lung cell, in order to examine cytotoxicity of nicotine and cigarette smoke extract to lung. The aim of this study focuses on investigating

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the *in vitro* cytotoxic effect of nicotine and cigarette smoke extract (CSE) and whether SYE inhibits nicotine- or CSE- induced cytotoxicity in MRC-9.

MATERIALS AND METHODS

Chemicals

3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and nicotine were purchased from Sigma Chemical Co. (St. Louis, MD, USA). Cell culture medium, RPMI 1640, and Trypsin-EDTA, and penicillin (100 U/ml) and streptomycin (100 μg/ml) were purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from a Cambrex Co. (Walkersville, Maryland, USA).

Cell culture

MRC-9, human embryonic lung fibroblast, was obtained from Korea Research Institute of Bioscience and Biotechnology (Taejon, Korea). MRC-9 cells were grown at 37°C in RPMI medium 1640 supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were grown in a humidified atmosphere of 95% air and 5% CO_2.

Preparation of Se-Yeon-Eum

The plant sample was purchased from the Oriental Pharmacy Store, College Oriental Pharmacy (Iksan, Korea) and authenticated by Professor Y.S. Lyu, College of Oriental Medicine, Wonkwang University. A voucher specimen (number 82-10-30) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University. Oriental medicines in SYE was washed three times with distilled water for getting rid of impures and dried in the shade. SYE was extracted with distilled water (100 g/1000 ml) at 100°C for 2 h. The extract was filtered through a 0.45 μm microfilter (Millipore, France) and lyophilized. The ingredients of 100 g of SYE include 10 g of Liriopsis Tuber, 7.5 g of Platycodi Radix, etc. The w/w yield of the extract of SYE was about 17.5%. The dried extracts were dissolved and diluted in sterilized phosphate-buffered saline (PBS, NaCl 136 mM, KCl 2.6 mM, Na_2HPO_4 3.2 mM, and NaH_2PO_4 1.8 mM) before used.

Cigarette smoke extract

Ten grams of cigarette (Marlboro medium, Philip Morris) were mixed with 100 ml of culture media. This mixture was centrifuged for 10 min at 450×g. The supernatant was collected and re-centrifuged for 2 h at 13,000×g. The supernatant was adjusted to pH of 7.4 with 5N NaOH and then filter sterilized through a 0.22 μm microfilter before kept at 4°C. To test optimal cytotoxic effect of CSE, we carried out using CSE extract in serial dilutions (~1/6).

MTT assay

Cells were plated out at a density of 1×10^5 cells/ml in 4-well plates (Nunc, Sweden) and allowed an overnight period for attachment. Then the medium was removed and fresh medium, along with various concentrations of SYE was added to cultures in parallel. After incubation for 2 h, nicotine and CSE were treated in respective concentrations. Control cells without agents were cultured using the same conditions with comparable media changes. After incubating for 24 h or 48 h, MTT assay was performed by the method of Mosman *et al.* (1983) and Scudiero *et al.* (1998). To determine the cell viability, 50 μl of MTT was added to each well and cells were additionally incubated for 4 h. After washing the supernatant out, the insoluble formazan product was dissolved in DMSO. Then, optical density (OD) of 96-well culture plates was measured using an enzyme-linked immunosorbent assay (ELISA) reader at 540 nm. The optical density of formazan formed in untreated control cells was taken as 100% of viability.

Statistical analysis

Each datum was represented as the mean±SEM of two dependent experiments under the same conditions. The Students *t*-test was used to make a statistical comparison. Results with $P<0.05$ were considered statistically significant.

RESULTS

Inhibitory effect of SYE on nicotine-induced cytotoxicity

To examine the inhibitory effect of SYE on nicotine-induced cytotoxicity, cells were pretreated with

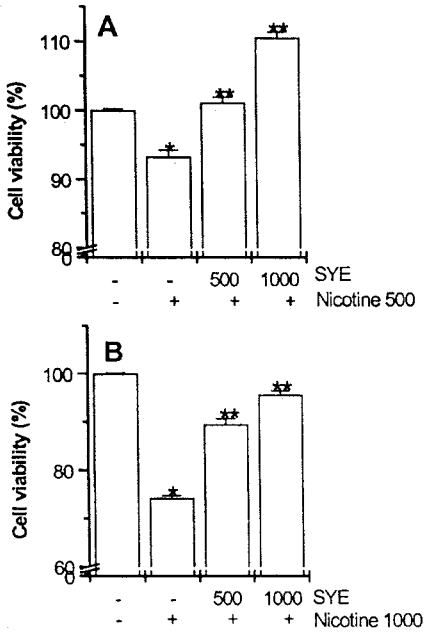

Fig. 1. (A) Inhibitory effect of SYE (500, 1000 μg/ml) on nicotine (1000 μg/ml)-induced cytotoxicity in 24 h. (B) Inhibitory effect of SYE (500, 1000 μg/ml) on nicotine (500 μg/ml)-induced cytotoxicity in 24 h. Each value is presented as the mean ± SEM of two independent experiments. *P<0.05; significantly different from the control value. **P<0.05; significantly different from the nicotine treated value.

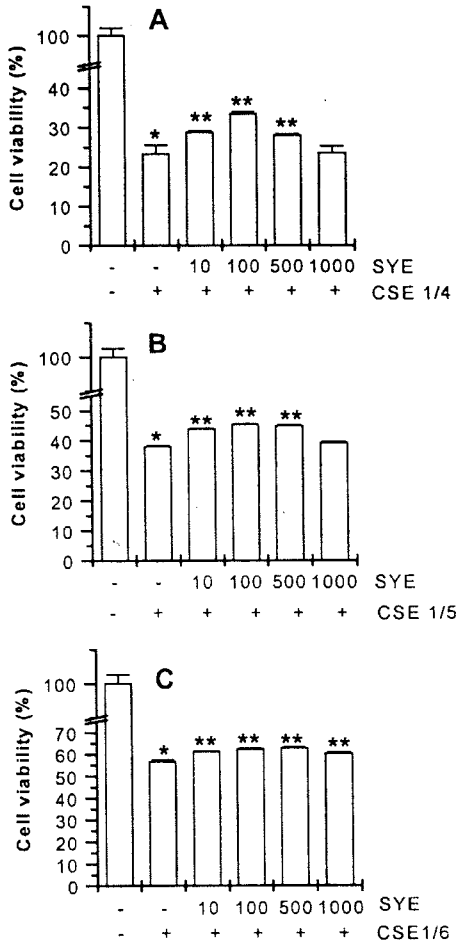

Fig. 2. (A) Inhibitory effect of SYE (10, 100, 500, 1000 μg/ml) on CSE (1/4 diluted)-induced cytotoxicity. (B) Inhibitory effect of SYE (10, 100, 500, 1000 μg/ml) on CSE (1/5 diluted)-induced cytotoxicity. (C) Inhibitory effect of SYE (10, 100, 500, 1000 μg/ml) on CSE (1/6 diluted)-induced cytotoxicity. Each value is presented as the mean ± SEM of two independent experiments. *P<0.05; significantly different from the control value. **P<0.05; significantly different from the CSE treated value.

SYE (500, 1000 μg/ml) for 2 h and then nicotine (500, 1000 μg/ml) was treated. After incubating for 24 h, cell viability was measured by using MTT assay. SYE inhibited the cytotoxic effect significantly in both nicotine concentrations (Fig. 1). In addition, SYE not only showed the inhibitory effect of nicotine (500 μg/ml)-induced cytotoxicity, but also increased cell viability over 100% (Fig. 1).

Inhibitory effect of SYE on CSE-induced cytotoxicity

Next, in order to determine the inhibitory effect of SYE on CSE-induced cytotoxicity, we treated SYE (10, 100, 500, 1000 μg/ml). Two hours later, the medium was removed and changed with flesh medium including 1/4, 1/5, 1/6 diluted CSE and SYE (10, 100, 500, 1000 μg/ml). MTT assay showed that SYE increased the dropped cell viability by CSE (Fig. 2). But SYE did not inhibit CSE-induced cytotoxicity dose-dependently differently from nicotine-induced cytotoxicity.

DISCUSSION

There are over 4,000 different chemicals in cigarette smoke, and it is quite likely that a combined insult from several chemical constituents is responsible for the injury (Snajdar *et al.*, 2000). Several studies have shown that cigarette smoke extract has harmful effect to our body. CSE suppress anti-tumor cytokines IL-1β, IL-2, IFN-γ, TNF-α (Ouyang *et al.*, 2000). These 4 cytokines has been known to play important roles in the host defense against infection and cancer. And CSE inhibits human

bronchial epithelial cell repair processes (Wang *et al.*, 2001). In addition, CSE impairs dilatation of resistance arterioles in response to activation of important cellular dilator pathways (Mayhan *et al.*, 1996). Besides many studies related to a baneful influence of CSE were reported.

Absorbed nicotine affects whole body. Nicotine has a variety of endocrine effects, including ACTH and cortisol release (Baron *et al.*, 1995), and beta endorphin release (Seyler *et al.*, 1986). Nicotine has been reported to affect the hypothalamic-pituitary events that read to ovulation, mainly the ovulatory surge of luteinizing hormone (Blake *et al.*, 1972).

Besides, nicotine treatment of responding T cells decreased production of IL-2 and IFN-γ. These Th1 cytokines are involved in the generation of lymphokine-activated killer cells and cytotoxic T cell that are capable of destroying lung tumors (McAdam *et al.*, 1995; Sadanaga *et al.*, 1999). Several studies have shown that progressive tumor growth is associated with decreased antitumor immunity and lower levels of IL-2 and IFN-γ (Yamamura *et al.*, 1993; Ghosh *et al.*, 1995). Namely, nicotine has immunosuppressive effect as nicotine acts on all over the body through the circulatory system.

Nicotine influences on brain as well. Nicotine reaches the brain in about 10 to 20 seconds and the brain is exposed to high level (Gourlay *et al.*, 1997). Through such process nicotine has direct effects on the brainstem and spinal cord (Su, 1982).

Nicotine affects the cardiovascular system. During smoking, effects of nicotine mediated by the central nervous system follow activation of peripheral chmoreceptors, particularly the carotid chemoreceptor. Central and peripheral mechanisms like this result in the release of catecholamines from the adrenal grands and from vascular nerve ending. As a result, there is an acute increase in heart rate and blood pressure when nicotine is delivered via cigarette smoking (Benowitz *et al.*, 1982).

In spite of nicotines harmful influences, it is true that many smokers cant give up smoking because of addictive character of nicotine. So therapeutic use of nicotine like nicotine gum, patch, nasal spray, and mouthpiece apply to smoking cessation. However, such a nicotine absorption has many side effects (Balfour *et al.*, 1996).

Nicotianae Folium (cigarette) has been used as a medicine for lung disease in Oriental medicine in Korea. It dehumidifies moisture in lung, so it can cure many symptoms caused by moisture. For cigarettes dry characteristic, however, excessive smoking causes diseases like asthma, pneumonia and symptoms like cough, and phlegm. In Oriental medicine in order to add moisture to lung many herbal medicines like *Liriopsis Tuber* (Liliaceae) have been used.

In this study, we demonstrated that SYE, has been known to be effective to lung diseases like asthma, pneumonia and symptoms like cough and phlegm, exhibited significant inhibitory effect on nicotine- and CSE-induced cytotoxicity in MRC-9. It seems that SYE not only have effect on injury induced by smoking, but are also useful to smoking cessation without side effects. SYE, as well, appear to mitigate every symptom induced by cigarette smoking.

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