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Ciga-X inhibits nicotine-induced human lung fibroblasts cytotoxicity and craving for cigarettes

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SUMMARY

Cigarette smoking contributes to lung cancer, cardiovascular diseases, oral diseases, *etc*. In desire to reduce their risk of disease, many cigarette smokers have tried to quit smoking. Sensory aspects of cigarette smoke are important for providing smoking satisfaction. Previously it was reported that citric acid aerosol significantly reduced craving for cigarettes and enhances smoking reduction and cessation. In this study, we tested whether a newly combined product Ciga-X, an aerosol for cessation aid, had toxicity in human embryonic lung fibroblast (MRC-9). The inhibitory effect of Ciga-X on cytotoxicity induced by cigarette smoke extract (CSE) or nicotine was examined in MRC-9, and craving for cigarettes and smorkers satisfaction after using Ciga-X was estimated. Ciga-X did not affect cell viability and had no toxicity in MRC-9. Ciga-X significantly inhibited not only CSE-induced cytotoxicity but also nicotine-induced cytotoxicity in MRC-9. One hundred and forty smokers rated the satisfaction for Ciga-X aerosol and craving reduction for cigarettes after using Ciga-X. The percentage of over 5 rating was 71.0% and 50.0% of subjects in satisfaction test for Ciga-X compared to their own brand and in craving reduction for cigarette, respectively. Besides, craving reduction for cigarette was highly correlated with the duration of smoking. Subjects have smoked under 10 years were more reduced in craving for cigarettes after using Ciga-X as compared to over 10 years ($p=0.049$). These results suggest that Ciga-X may be effective in promoting smoking abstinence with the reduction of CSE- or nicotine-induced human lung fibroblasts cytotoxicity.

Key words: Ciga-X; Cytotoxicity; Smoking cessation; Cigarette smoke extract; Nicotine

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INTRODUCTION

Smoking contributes to coronary heart disease, stroke, pulmonary disease, peripheral vascular disease, and lung cancer (Hays *et al*., 1998). Cigarette smoking has been believed major risk factor in the pathogenesis of lung and oral diseases (Benowitz, 1988). In desire to reduce their risk of disease, many cigarette smokers have tried to quit smoking (Rose, 1996). Smokers attempting to quit often experience overall withdrawal discomfort and unpleasant abstinence symptoms including craving for cigarettes, irritability, anxiety, sleep disturbances, weight gain, depression, and difficulty

concentrating (Sommese and Patterson, 1995; West and Shiffman, 2001). Although some of these withdrawal symptoms can be alleviated by nicotine replacement such as nicotine chewing gum, skin patch and nasal spray, craving for cigarettes does not appear to be abated substantially (Hughes *et al*., 1984; Bobrie *et al*., 1993).

The satisfaction derived from smoking depends on not only the pharmarcological effects of nicotine but also the sensory stimulation from smoke inhalation. Specifically, sensory aspects of cigarette smoke are critical for providing smoking satisfaction. Smokers report enjoying the feel of cigarette smoke as it is inhaled, and when these sensory effects are blocked, smoking satisfaction is blunted (Rose, 1988; Westman *et al*., 1996). It was reported that citric acid aerosol inhaler might produce a tracheal scratch and provide a useful tool for smokers while trying to quit smoking (Rose and Hickman, 1987; Levin *et al*., 1990; Behm *et al*., 1993). Local anesthesia of respiratory airway significantly blocked craving (Rose *et al*., 1985), and the adequacy of airway anesthesia was assessed by the inhalation of citric acid solution (Burki *et al*., 1983). Citric acid has no known toxic effects and quenches smokers craving for cigarettes.

Ciga-X is an aerosol for cessation aid that composed of citric acid, vitamin C, tartaric acid, *etc*. The inhibitory effect of Ciga-X on cytotoxicity induced by cigarette smoke extract (CSE) or nicotine was examined in human embryonic lung fibroblast (MRC-9), and craving for cigarettes and smorkers satisfaction were investigated after using Ciga-X.

MATERIALS AND METHODS

Reagents

Cell culture medium, RPMI 1640 was purchased from Gibco BRL (Grand Island, NY USA). Chemicals including of 3-[4,5-dimetylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and nicotine were obtained from Sigma (St. Louis, MO, USA). Ciga-X was kindly provided by HUMANBIO Co., Ltd. (Iksan, Korea). Cigarette smoke extract (CSE) was prepared. First, 10 g of cigarette (Marlboro medium, Philip Morris Co., Ltd.) was 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 7.4 with 5 N NaOH and then filter sterilized through a 0.22 μm filter before kept at 4°C. To test optimal cytotoxic effect of CSE, the CSE was employed to the cells in serial dilutions (~1/8)

Cell culture

MRC-9 was obtained from Korea Research Institute of Bioscience and Biotechnology (Taejon, Korea). MRC-9 was grown in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum (FBS) at 37°C in 5% CO_2 and 95% humidity.

MTT assay

To investigate the viability of cells, MTT assay was performed (Mosman *et al*., 1983; Kim *et al*., 2001). Briefly, MRC-9 was 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 along with medium change, the various concentrations of Ciga-X was treated. After incubation for 10 min, nicotine (1 mg/ml) and CSE (1/8 dilution) were treated. After incubation for 24 h, MTT solution (500 μg/ml) was added and the cells were incubated at 37°C for additional 4 h. The crystallized MTT was dissolved in DMSO and the amount of dark blue formazan was determined by measuring the absorbance at 540 nm. The optical density of formazan formed in untreated control cells was taken as 100% of viability.

Craving for cigarettes and satisfaction test

One hundred and forty smokers (men) were participated in craving for cigarettes and satisfaction test. The mean age of subjects was 36.1 ± 9.5 yr, and average duration they had been smoking for 14.7 ± 8.5 yr. The mean daily cigarette consumption of subjects was 17.2 ± 6.3 cigarettes/day. In the test of craving for cigarettes, subjects were asked to rate how much they felt craving reduction for their brand of cigarette after using Ciga-X. Subjects rated using 10-point scales ranging from 1 ("not at all") to 10 ("very much"). In the satisfaction test, subjects were asked to rate how much they felt satisfaction for Ciga-X compared to their usual brand of cigarette after using Ciga-X. The degree of satisfaction was

rated a 10-point scales from 1 ("not at all") to 10 ("own brands").

Statistical analysis

Results were expressed as the mean ± SD from four independent experiments. The significance of the differences between two groups was determined by the Mann-Whitney U test. In craving for cigarettes and satisfaction test, the correlation was analyzed by Chi-square test (Pearson χ^2-test). Subjects were divided into two groups according to the duration they had been smoking (under or over 10 yr), and the degree of decrease craving and satisfaction was "5" as a datum point in analysis. For all tests, *P* values less than 0.05 were considered significant.

RESULTS

No cytotoxic effect of Ciga-X in MRC-9

To test cytotoxic effects of Ciga-X, aerosol for cessation aid, we performed MTT assay in MRC-9 cells. Fig. 1 shows the viability of cells at 24 h incubation after treatment with appropriately diluted Ciga-X. In cells treated with Ciga-X of various concentrations, Ciga-X did not affect cell viability in each concentration and had no toxicity on MRC-9 cells.

Inhibitory effect on CSE- and nicotine-induced cytotoxicity

In order to determine the inhibitory effect of Ciga-X on CSE- or nicotine-induced cytotoxicity, we pretreated with Ciga-X (10^{-5}-10^{-3} of an undiluted solution) and then CSE or nicotine were treated. After incubating for 24 h, cell viability was measured by MTT colorimetric assay. As shown in Fig. 2, CSE had cytotoxic effect in comparison with absence of CSE (about 63% cell death). Ciga-X significantly inhibited CSE-induced cytotoxic effect

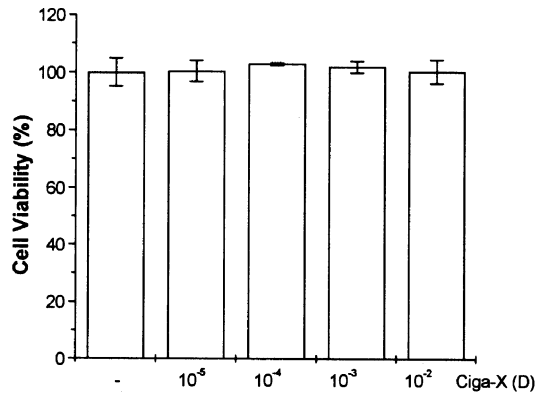

Fig. 1. Effect of Ciga-X on cell viability in MRC-9 cells. Cell viability was evaluated by MTT colorimetric assay at 24 h incubation after treatment with 10^{-5}-10^{-2} dilution of Ciga-X. All data represent mean ± SD of four independent experiments.

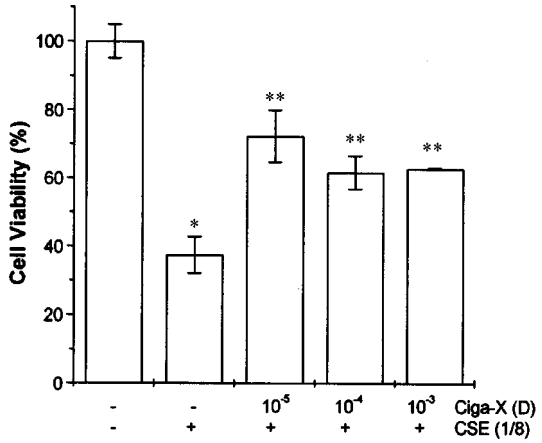

Fig. 2. Inhibitory effect of Ciga-X on CSE-induced cytotoxicity in MRC-9 cells. Cells were incubated in the absence or presence of Ciga-X (10^{-5}-10^{-3} dilution) for 10 min prior to CSE (1/8 dilution) treatment. Cell viability was evaluated by MTT colorimetric assay, and all data represent mean ± SD of four independent experiments. **P*<0.05; significantly different from the saline value. ***P*<0.05; significantly different from the CSE-treated value (Mann-Whitney U test).

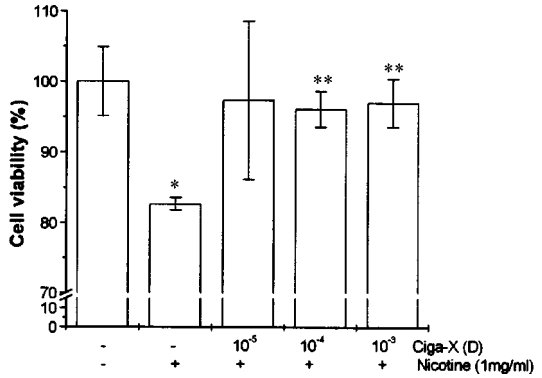

Fig. 3. Inhibitory effect of Ciga-X on nicotine-induced cytotoxicity in MRC-9 cells. Cells were incubated in the absence or presence of Ciga-X (10^{-5}-10^{-3} dilution) for 10 min prior to nicotine (1 mg/ml) treatment. Cell viability was evaluated by MTT colorimetric assay, and all data represent mean ± SD of four independent experiments. **P*<0.05; significantly different from the saline value. ***P*<0.05; significantly different from the nicotine-treated value (Mann-Whitney U test).

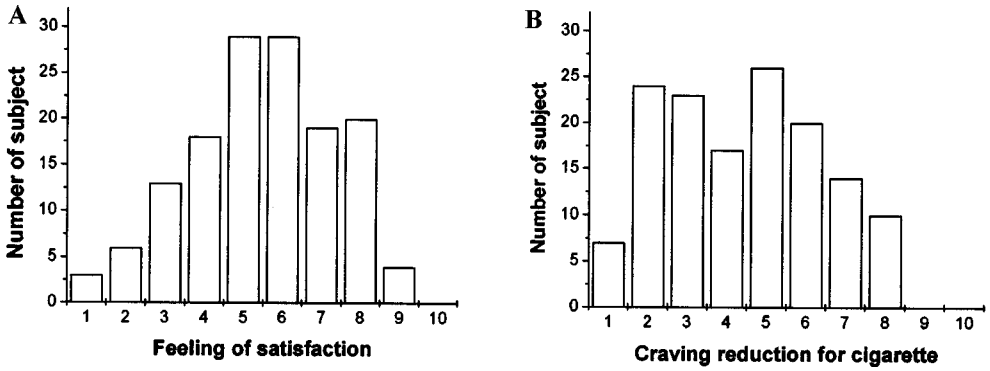

Fig. 4. (A) The satisfaction test. Subjects were asked to rate how much they felt satisfaction for Ciga-X compared to their usual brand of cigarette after using Ciga-X. The degree of satisfaction was rated a 10-point scales from 1 ("not at all") to 10 ("own brands"). (B) Craving reduction test. Subjects were asked to rate how much they felt craving reduction for cigarette after using Ciga-X. Subjects rated using 10-point scales ranging from 1 ("not at all") to 10 ("very much").

in MRC-9 (about 50% recovery in cell death). Nicotine also showed cytotoxicity in Fig. 3 even in somewhat (about 18% cell death). Ciga-X blocked nicotine-induced cytotoxic effect and increased cell viability near 100% in MRC-9.

Inhibitory effect on craving for cigarettes and satisfaction test

One hundred and forty smokers were participated in craving for cigarettes and satisfaction test (see materials and methods). As shown in Fig. 4A, the percentage of over 5 rating was 71.0% in satisfaction test for Ciga-X compared to their own brand. The 50.0% of subjects also reported over 5 rating in craving reduction for cigarette by using Ciga-X (Fig. 4B). Subjects were divided into two groups according to the duration they had been smoking (under or over 10 yr), and the degree of craving reduction and satisfaction was 5 as a datum point in analysis (Fig. 5). There was a significant interaction of reduction craving and smoking duration. Subjects have smoked under 10 yr were more reduced in craving for cigarettes after using Ciga-X as compared to over 10 yr (p=0.049, Fig. 5B). It showed that the trend for cigarette craving after using Ciga-X in subjects smoked for a short time was lower than in subjects smoked for a long time. But, satisfaction was not significant (P=0.579, Fig. 5A) according to smoking duration.

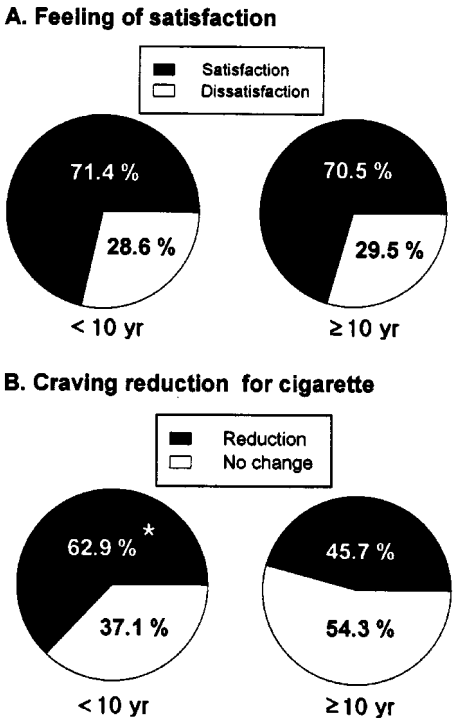

Fig. 5. (A) The feeling of satisfaction according to the smoking duration (under or over 10 yrs). The datum point of satisfaction and dissatisfaction was rating "5". B) Craving reduction for cigarettes according to the smoking duration. The datum point of craving reduction was rating "5". *P<0.05; significantly different from the subjects smoked over 10 years (Pearson χ^2-test).

DISCUSSION

Cigarette smoke contains more than 4,000 components of which at least 50 are carcinogenic, both solid and gaseous (Hoffmann *et al.*, 1997; Rodgman *et al.*, 2000). The major inducer of cigarette dependence for smoker and tobacco chewers is nicotine. In

respect to lung cancer, the most important agents are the carcinogenic polynuclear aromatic hydrocarbons and tobacco-specific N-nitrosamines. The increased risk for cardiovascular diseases among cigarette smokers is likely related to the exposure to tar, carbon monoxide, nitrogen oxides, and hydrogen cyanide. Nicotine is distilled from burning cigarette, and small droplets of tar containing nicotine are inhaled and deposited in the small airways and alveoli. Following absorption, nicotine enters the circulation and distributes rapidly to different tissues, including the brain, affects whole body (Zevin *et al*., 1998; Waldum *et al*., 1996). Recently, several studies have shown that CSE had harmful effect to body. CSE suppressed anti-tumor cytokines IL-1β, IL-2, IFN-γ, and TNF-α, which have been known to play important roles in the host defense against infection and cancer (Ouyang *et al*., 2000). CES also inhibited human bronchial epithelial cell repair processes (Wang *et al*., 2001), and impaired dilatation of resistance arterioles in response to activation of important cellular dilator pathways (Mayhan and Sharpe, 1996).

Although harmful influence of smoking, it is true that many smokers can't give up smoking because of addictive character of nicotine (Rose, 1996). Addiction of nicotine has led to the development of smoking cessation treatment methods that provide nicotine replacement. Most people trying to quit smoking eventually relapse even when provided with nicotine in the form of gum, patches, nasal spray, or inhaler (Hajek *et al*., 1999). The failure of nicotine replacement methods was due to lack important sensory/behavioral components of cigarette smoking, such as taste, aroma, and respiratory tract sensations accompanying each puff of smoke. In several studies, these sensations are especially important in relieving craving for cigarettes (Rose *et al*., 2000). It was reported that citric acid aerosol inhaler might produce a tracheal sensation and provide a useful tool for smokers while trying to quit smoking (Rose and Hickman, 1987; Levin *et al*., 1990; Behm *et al*., 1993). The citric acid aerosol also alleviated craving for cigarettes and negative affect. Besides, local anesthesia of respiratory airway significantly blocked craving and was assessed by the inhalation of citric acid solution (Burki *et al*., 1983). The combination of airway sensory replacement and nicotine replacement may prove beneficial for smoking cessation (Westman *et al*., 1995).

In the present study, we demonstrated that Ciga-X had no toxic effects on human lung fibroblasts and exhibited significant inhibitory effect on CSE- and nicotine-induced cytotoxicity in MRC-9. Our question study was not limited subjects desired to quit smoking. It is probably one factor which accounts for the lower ratings of craving reduction and satisfaction in our study. Nevertheless, more than half of subjects felt satisfaction after using Ciga-X and craving reduction. Ciga-X delivered from a cigarette substitute may be effective in reducing smoking and promoting smoking abstinence through tracheal stimuli and local anesthesia.

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