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The Scavenger Effects of Various Antioxidants in Cigarette Filters on the Free Radicals in Mainstream Smoke

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ABSTRACT : This study was conducted to evaluate the effect of additives (antioxidants for free radicals reduction) in cigarette filter treated with various antioxidants (three types of proanthocyanidins and ascorbic acid) and various concentrations of ascorbic acid and loaded with activated carbon on the delivery of free radicals of mainstream smoke (MS) by ESR. Also, we analyzed Hoffmann's analytes andscavenger activity according to the storage time and *in vitro* cytotoxicity. The analysis of spin number of vapor and particulate phase free radicals in MS are decreased to 14~24 % and 16~40 %, respectively. As aresult of antioxidant potential for inactivity of vapor and particulate phase free radicals, natural antioxidants were more effective than ascorbic acid. Based on the result of the analysis of Hoffmann's analytes for various antioxidantstreated cigarette filters during the smoking, cigarette filter treated with ascorbic acid showed the lower amount of the deliveries of hydroquinone, isoprene and quinoline in MS than those treated with the other antioxidants. In the significant t-test on the difference of the cytotoxicity among the various antioxidants treated-cigarette filters, there are no significant differences at the 95 % confidence level. Those results indicated that the antioxidants were useful for reducing free radicals in MS because of the fast reaction between antioxidant and free radicals.

Key words **: Antioxidants, free radicals, scavenger effect, proanthocyanidin, a scorbic acid**

Cigarette smoke is composed of more than 4,000 compounds, and some of them are identified toxic of an amount of 10^{16}. Free radicals is well known as they may bind to DNA and could lead to damage of cells (Pryor *et. al.*, 1985, Zang *et. al.*, 1995 and Baum *et. al.*, 2003)). Free radicals in the gas phase, however, are highly reactive and are extremely difficult to inactivate in cigarette filters. Free radicals from cigarette smoke have been indicated in the pathogenesis of smoking induced lung diseases, such as chronic obstructive pulmonary disease, emphysema and lung cancer (Rahman and MacNee, 1996). The carcinogenic mechanism of tobacco smoking is complex and results from various constituents one major group being free radicals (Hecht, 1999). The major research trends regarding reduction of free radicals were widely classified (Zang *et. al.*, 1996)

- The use of cigarette filter treated with antioxidants.

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- Formation restraint of free radicals by the chemicals such as potassium nitrate
- Control of free radicals in mainstream smoke using the low nitrogen content in tobacco leaves.

Therefore in those studies, to confirm scavenger effect according to the various antioxidants in cigarette filter on free radicals in mainstream smoke, prototype cigarette filters treated with various antioxidants were prepared by syringe injection and, to confirm a scavenger effect of activated carbon and optimum concentration of ascorbic acid in cigarette filter on free radicals in mainstream smoke, the prototype cigarette filters loaded with activated carbon and treated with ascorbic acid as changes concentration were done. We confirmed a change of surface morphology according to the treatment of antioxidant, and after we investigated reduction rate of free radicals in mainstream smoke, correlation between delivery of free radicals and some smoke constituents and safety evaluation by antioxidant and activated carbon.

MATERIALS AND METHODS

Materials

Proanthocyanidins used as antioxidants, were two types of pine bark extracts (Pycnogenol® and PineXol®) and grape seed extract (Grape seed PE OPC). Pycnogenol®, 99 % grade extract of the bark of the French maritime pine, was provided by Horphag Research Inc. (Geneva, Switzerland), PineXol®, 99 % grade extract of the bark of the Korean red pine, was provided by Nutrapharm inc. (Namyangju, Korea) and grape seed extract, 99 % grade extract of the grape seed, was provided by Naturex inc. (NJ. USA). Ascorbic acid was purchased from Merck (NJ, USA) Agents for determination of spin number of free radicals, spin trap *N*-tertbutyl-2-phenyl nitrone (PBN) and n-Hexane were purchased from Sigma (MO, USA). Neutral red, dimethylsulfoxide (DMSO) and sodium dodecyl sulfate (SDS) were purchased from the Sigma-Aldrich Company (St Louis, USA). Phosphate-buffered saline (PBS, pH 7.4), fetal bovine serum (FBS), L-glutamine, penicillin/streptomycine solution, Ham's F-12 medium and trypsin were acquired from GIBCO (Grand Island, USA). All other chemicals and reagents used were of the best available grade. Other agents for analysis of smoke constituents were used after being purchased from Duksan Chemical Co., Korea. All reagents and chemicals used in these studies were of analytical grade.

Preparation of prototype cigarettes

To confirm the scavenger effect according to the various antioxidants in cigarette filter on free radicals in mainstream smoke, prototype cigarette filters treated with various antioxidants were prepared by syringe injection. Table 1. shows

Table 1. Treatment conditions for investigation of various antioxidants on free radicals in MS

Sample name	Antioxidant	Tobacco Weight (mg)	Filter EPD (mmH_2O)	Cigarette EPD (mmH_2O)	Loading amount (mg/cig.)	Tar / Nicotine
Control	-	665±10	98±2	157±4	0 mg/50 uL Ethanol	8.4 / 0.78
PSE-Fra	Pycnogenol				0.4mg/50 uL Ethanol	8.5 / 0.74
PSE-Kor	PineXol					8.8 / 0.80
GSE	Grape seed					8.5 / 0.76
AA	Ascorbic acid					8.4 / 0.78

cigarette filter treatment conditions of various antioxidants.

To confirm a scavenger effect of activated carbon and optimum concentration of ascorbic acid in cigarette filter on free radicals in mainstream smoke, we prepared two types of cigarette filter such as acetate mono filter and carbon mono filter. Acetate mono filter group was composed of four types of ascorbic acid concentration in cigarette filters and carbon mono filter group was composed of two types of ascorbic acid concentration and same carbon amount in cigarette filters.

Table 2 shows cigarette filter treatment conditions of ascorbic acid concentration and carbon amount.

All of samples were sorted out on the same physical properties such as tobacco column, filter EPD and tobacco UPD.

Observation of surface morphology by treatment of antioxidants

To observe the surface morphology of the acetate fiber and activated carbons according to the treatment of antioxidant using SEM, the acetate fibers and activated carbons were respectively placed onto a bronze stud, and sputter coated with gold before observation.

Determination of spin number of free radicals in MS

Vapor phase free radicals in MS were detected by an electron spin resonance (ESR) spin trapping technique (Müller and Teufel, 2003). Vapor phase smoke was passed through four 5 mL-impinger filled 0.1 mol/L *N*-tertbutyl-2-phenyl nitrone (PBN) in hexane spin trapping solution at 10 °C. Prototype cigarettes were prepared at 22 °C room temperature and 60% humidity (ISO standard, 1999). In this study, mainstream smoke constituentswere collected under the conditions of a 35-mLpuff volume, 2 sec puff duration, and 60 sec puff interval according to ISO machine-smoking conditions (ISO standard, 1999). Smoking of 20 cigarettes according to ISO using a Borgwaldt RM 20/CS smoking machine. ESR analysis of all samples was analyzed in 15 minutes after lighting the first cigarette. ESR spectra of the spin adducts were measured using JEOL spectrometer at 238 °C. Table 3 shows the ESR conditions.

The ESR instrument was adjusted to the same condition by a standard marker using the spin probe, TEMPO (Tetramethylpiperidino-oxy). Percentage of delivery ratio and reduction ratio for free radicals were calculated by the following equation:

Table 2. Treatment conditions for comparative evaluation of cigarette filters loaded with activated carbon and treated with ascorbic acid as changes concentration

Classification / Sample name		Tobacco Weight (mg)	Filter EPD (mmH_2O)	Cigarette EPD (mmH_2O)	Loading amount per filter tip (mg/cig.)		Tar / Nicotine
					Activated Carbon	Ascorbic acid	
Acetate mono	AC (control)	665±10	90±2	140±5	-	-	10.3 / 0.92
	AV-1				-	0.27	10.0 / 0.96
	AV-2				-	0.38	10.4 / 0.95
Carbon mono	AV-3				-	0.56	10.7 / 0.93
	CC (Control)				30	-	8.8 / 0.90
	CV				30	0.28	9.0 / 0.88

$$\text{Percentage of delivery ratio} = \frac{\#\text{ of free radicals for sample}}{\#\text{ of free radicals for control}} \times 100$$

$$\text{Percentage of redcution ratio} = 100 - \text{delivery ratio}$$

Table 3. ESR conditions for analysis of vapor phase free radicals

X-band, microwave power	20 mW
Modulation frequency	100 kHz
Modulation amplitude	1 G
Quantification	Double integration

Analysis of some compounds in MS

Prototype cigarettes were prepared at 22 °C room temperature and 60% humidity (ISO Standard 3402, 1999). Also, mainstream smoke constituents were collected under the conditions of a 35-mL puff volume, 2 sec puff duration, and 60 sec puff interval according to ISO machine-smoking conditions (ISO Standard 3308, 2000). Analysis of hydroquinone is based on Health Canada method T114 (Determination of Phenolic Compounds in Mainstream Tobacco Smoke). Analysis of isoprene is based on Health Canada method T116 (Determination of 1,3-Butadiene, Isoprene, Acrylonitrile, Benzene and Toluene in Mainstream Tobacco Smoke). And analysis of quinoline is based on the Health Canada T 112 (Determination of Pyridine, Quinoline and Styrene in Mainstream Tobacco Smoke).

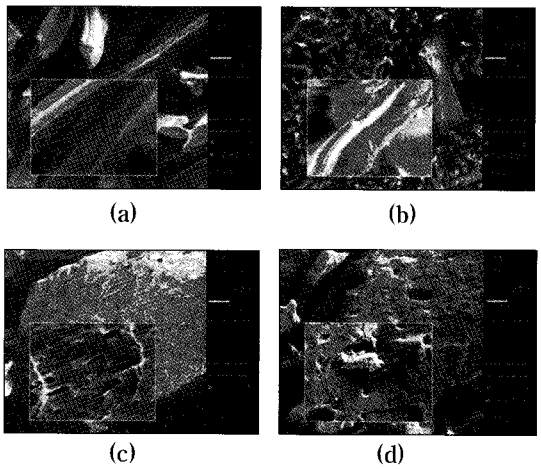
Cytotoxicity analysis

For cell cultures, Chinese hamster ovary (strain K1) cells were purchased from the Korean Cell Line Bank (KCLB), and were maintained on Ham's F-12 medium supplemented with 10 % FBS, 100 units/mL of penicillin, and 100 ㎍/mL of streptomycin. All cells were grown at 37 ℃ in a humidified incubator maintained at an atmosphere of 95 % air and 5 % CO_2. Chinese hamster ovary (CHO) cells were maintained for 3-4 days of passages, and cells obtained between passages 5 and 20 were used for the neutral red assays. Mainstream smoke constituents were collected under the conditions of a 35-mL puff volume, 2 sec puff duration, and 60 sec puff interval according to ISO machine-smoking conditions using a RM20/CS smoking machine (Babich and Borenfreund, 1992). TPMs from the cigarettes were collected on Cambridge filter pad. The pads were extracted with DMSO at a concentration of 10 mg TPM/mL of DMSO. The TPM fractions were stored at -70 ℃ until assayed. The cytotoxicity of the TPM samples was evaluated by a neutral red assay using CHO cells. The neutral red cytotoxicity assay was adapted from the procedure developed by Borenfreund *et al.* and is briefly described as follows. Three batches of TPM were assayed for each tested cigarette type. For each batch, four replicate 96-well culture plates were employed, each at eight different concentrations. Cells were plated in 96-well culture plates at a density of 104 cells per well. Tissue culture plates were placed for 24 hours at 37 ℃ in an atmosphere of 95 % air and 5 % CO_2. The culture medium was aspirated from the wells and replaced with media containing the test samples to be evaluated. At the end of the exposure period (about 22 ± 2 hours), the treatment/control medium was replaced with 200 ㎕ neutral red solution (50 ㎍/mL in culture medium without FBS). After a 3 hour incubation period, the neutral red solution was aspirated and 200 ㎕ of wash/fix solution (1% formalin) was added to each well for 1 minute at room temperature. The wash/fix solution was then immediately aspirated. Quickly, 150 ㎕ of the solvent solution (1 % acetic acid in 50 % ethanol) was added to each well, and the 96-well culture plates were placed on a microplate shaker for 10 minutes. The absorbance of each well was then

measured at 540 nm on a microplate reader (Bio-Tek, USA). The concentration-response curve was constructed via comparison of the concentrations of the smoke fractions with cytotoxicity, which was expressed as a percentage of the control value (absorbance of neutral red obtained from treated cells divided by absorbance of neutral red from control cells). In addition, the regression lines were used to calculate the EC_{50} values (*i.e.* the concentration of cigarette smoke required to elicit 50 % decrease in the neutral red response). Data obtained from the toxicological experiments were expressed as means ± SD. One-way analysis of variance was employed in order to compare the results obtained between each of the cigarette. In cases in which this overall comparison revealed a significant statistical difference between the cigarettes, the Duncan test for pairwise comparison was applied.

RESULTS AND DISCUSSION

Fig. 1 shows the change of surface morphology by treatment of ascorbic acid.

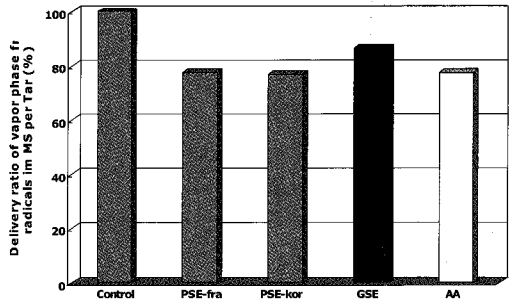


(a) (b)

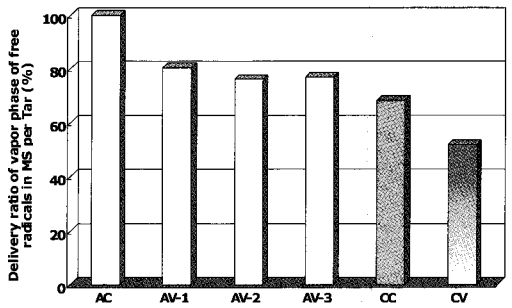
(c) (d)

Fig. 1. Surface morphology of acetate tow and activated carbon according to the treatment of ascorbic acid : (a) Acetate : before (b) Acetate : after (c) Activated carbon : before (d) Activated carbon : after.

Compared to the acetate fiber before and after treatment of ascorbic acid, regarding the surface, we observed that ascorbicacid was coated with or attached to acetate fiber surface. On the other hand, regarding the activated carbon surface, we observed that the pore of activated carbon surface was covered with a ascorbic acid, somewhat reducing the surface porosity.



(a)



(b)

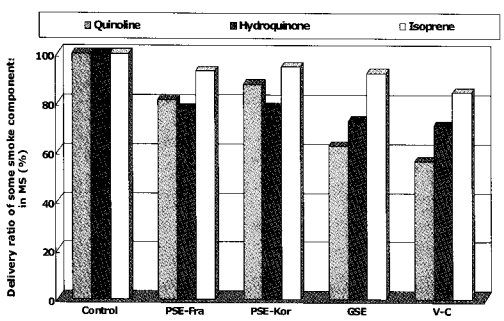
Fig. 2. Delivery ratio of vapor phase free radicals in MS through the cigarette filter loaded with activated carbon and treated with various concentrations of ascorbic acid: (a) effect of various antioxidants and (b) effect of activated carbon and ascorbic acid concentration.

Fig. 2 (a) shows the delivery ratio of vapor phase free radicals in mainstream smoke through the cigarette filter treated with various antioxidants. Delivery ratio was used to express the percentage of free radicals delivery of sample

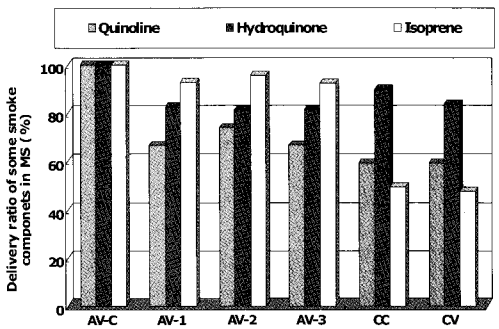
compared with that of control, and it was defined as the percentageof the spin number of free radicals for sample divided by spin number of free radicals for control. According to the introduction of antioxidants, delivery ratio of free radicals for all samples was decreased. Reduction rate of pine bark extracts and ascorbic acid was about 23 % and thatof grape seed extract was about 13 %. Fig. 2 (b) shows the delivery ratio of vapor phase free radicals in mainstream smoke through the cigarette filter loaded with activated carbon, treated with various concentrations of ascorbic acid and loaded with activated carbon. According to the introduction of ascorbic acid, delivery ratio of free radicals for all samples was decreased. Especially, AV-2 as 0.38 mg/tip of concentration was superior to the others for removal of free radicals in mainstream smoke given that up to 0.38 mg/tip of ascorbic acid does not contribute to reduction of free radical in mainstream smoke. And scavenger effect of activated carbon was about 32 %. On the other hands, Scavenger effect of AV-1 cigarette filter only treated with 0.28 mg of vitamin-C was about 19 % and that of CC cigarette filter only loaded with activated carbon was about 32 %. So, ideal scavenger effect of CV cigarette filter loaded with activated carbon and treated with 0.28 mg of ascorbic acid was about 51 %. But, real scavenger effect of CV cigarette filter was about 48 %. We consider that this result was caused by pore filling of activated carbon as introduced ascorbic acid. Quinoline, it is a semi-volatile compound and isoprene, it is a volatile compound very related to vapor phase free radicals. Those constituents were decreased by introduction of ascorbic acid. Hydroquinone, it is a particulate compound very related to particulate phase free radicals.

Fig. 3 (a) shows the change of some Hoffmann's compounds such as quinoline, hydroquinone and isoprene in mainstream smoke through the cigarette filter treated with various antioxidants.

As shown in this figure, cigarette filter treated with ascorbic acid showed the lower amount of the deliveries of quinoline, hydroquinone and isoprene in MS than that treated with the other antioxidants. Fig. 3 (b) shows the change of quinoline, hydroquinone and isoprene in mainstream smoke through the cigarette filter treated with ascorbic acid as change concentration and loaded with activated carbon. Those constituents were decreased by introduction of ascorbic acid and activated carbon.

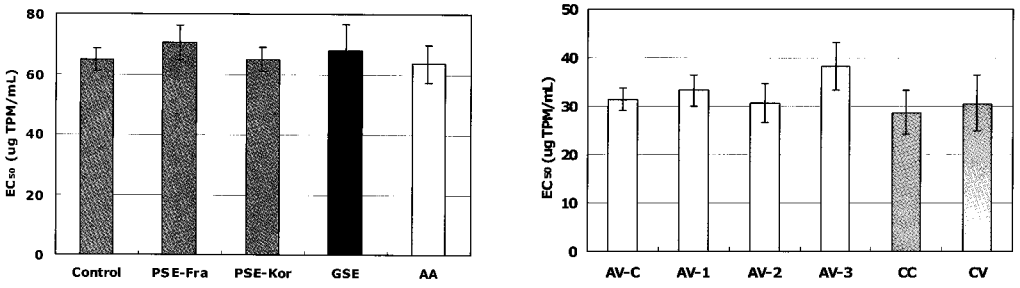


(a)



(b)

Fig. 3. Change of some compounds of MS through cigarette filter treated with various antioxidants and loaded with activated carbon.



(a) (b)

Fig. 4. The results of cytotoxicity of MS throughthe cigarette filter treated with various antioxidants and activated carbon: (a) as changes various antioxidants and (b) as changes ascorbic acid concentration and loads with activated carbon.

Fig. 4 (a) shows the *in-vitro* cytotoxicity of MS through the cigarette filter treated with various antioxidants. In the significant t-test on the difference for the cytotoxicity among the various antioxidantstreated-cigarette filters, there are no significant differences at the 95 % confidence level. Also, fig. 4 (b) shows the *in vitro* cytotoxicity of MS through the cigarette filter treated with ascorbic acid as changes concentration and loaded with activated carbon. In the significant-test on the difference for the cytotoxicity among the various antioxidants treated-cigarette filters, there are no significant differences at the 95 % confidence level.

CONCLUSIONS

This study was conducted to evaluate the effect of antioxidants for free radicals reduction in cigarette filters treated with various antioxidants and concentrations of ascorbic acid and loaded with activated carbon on the delivery of free radicals of MS by ESR. Reduction ratio of free radicals with PSE-Fra, PSE-Kor and AA is above equal and there are higher than it of GSE. In case of reduction ratio of free radicals with change of ascorbic acid concentration, it of AV-2 and AV-3 is higher than it of AV-1. Effect of activated carbon for reduction of free radicals in MS is 32 % and effect of activated carbon and ascorbic acid for reduction of free radicals in MS is 48 %. Those results indicated that antioxidants in cigarette filters react with free radicals in MS and that reaction takes only a very short time during the smoking. Analysis of Hoffmann's analytes in Smoke showed that the contests of hydroquinone, quinoline and isoprene were decreased by the introducing of antioxidant and activated carbon. The result of *in-vitro* cytotoxicity as change of antioxidants, ascorbic acid concentration and loaded activated carbon, there are no significant differences at the 95% confidence level.

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