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Pycnogenol® in cigarette filters scavenges free radicals and reduces mutagenicity and toxicity of tobacco smoke *in vivo*

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Despite large-scale anti-smoking campaigns throughout the world, the number of smokers remains high and cigarette smoking continues to represent a life-threatening health risk. Until a smoke-free society is achieved, reduction of cigarette smoke toxins may reduce the health burden. Current cigarette filter techniques are limited to the reduction of volatile tar constituents by dilution and by condensation on the filter surface. Vast quantities of harmful constituents, such as polycyclic aromatic hydrocarbons, heterocyclic (aromatic) amines, free radicals and reactive oxygen species, are inefficiently retained in the filter.

We investigated whether neutralisation of free radicals in cigarette filters is feasible and accompanied by a reduction in smoke toxicity. Addition of the bioflavonoid pine bark extract Pycnogenol® to cigarette filters depleted free radicals in a dose dependent manner. This was paralleled by a reduction of toxicity and mutagenicity in rodent test models. In this model system, the acute toxicity of cigarette smoke was markedly reduced by up to 70% in rodents with 0.4 mg Pycnogenol® in filters. Chronic exposure to cigarette smoke for 75 days revealed that Pycnogenol® filters significantly reduced mutagenicity by up to 48% and decreased pathological changes in lung tissue. *Toxicology and Industrial Health* 2002; **18:** 215–224.

Key words: *cigarettes; filters; free radicals; mutagenicity; Pycnogenol®*

Introduction

Since 1950 the health hazards related to smoking of cigarettes, especially its relation to lung cancer, are documented (Levin *et al.*, 1950). Since then, a large number of epidemiological studies have linked cigarette smoking to cardiovascular diseases, stroke, lung carcinoma, chronic bronchitis, chronic obstructive pulmonary disease (COPD) and emphysema (Tønnesen and Vermeire, 2000). Epidemiological data clearly establish that cigarette smoking is the major cause of lung cancer. It has been estimated that 90% of male lung cancer deaths and 75–80% of female lung cancer deaths in the United States are caused by smoking (Shopland, 1995; Hecht, 1999). Long term and heavy smokers have an excess risk for liver and stomach cancer, with significant twofold excess risk among men who smoked 40 or more packs/year (Mizoue *et al.*, 2000). Smoking also increases the incidence of age related macular degeneration, the most common

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cause of irreversible blindness (Chan, 1998). Smoking exacerbates asthma (Althius *et al.*, 1999) and diabetes (Traber *et al.*, 2000). Smoking also increases the risk of cancers of larynx, oral cavity, oesophagus, pancreas and urinary bladder (Nair and Brandt, 2000). Cigarette smoking is associated with significantly higher rates of low birth weight and preterm birth (Moore and Zaccaro, 2000).

The prognosis of lung cancer remains poor, with a five year overall survival rate of $\sim$10% and without much improvement during the last several decades (Peto *et al.*, 2000). Death from smoking exceeds the next several preventable causes of death combined: alcohol, car accidents, AIDS and drugs (Koop, 1991). The US Centers for Disease Control and Prevention (CDC) estimates that smoking kills approximately 419 000 people in the US each year (Nair and Brandt, 2000). There is an estimated one billion cigarette smokers worldwide, one third of whom live in China, where a major epidemic of lung cancer is predicted (Peto *et al.*, 1996; Wald and Hackshaw, 1996). Despite these depressing figures and large scale anti-smoking campaigns, 27% of men and 23% of women in the US smoke cigarettes (Wingo *et al.*, 1999). Cessation of all smokers is most unlikely; instead, many youngsters will begin smoking, among them increasingly more women.

Whereas addiction to cigarette smoking has been attributed to nicotine, which itself is not considered to be carcinogenic, the toxicity and carcinogenicity results from byproducts developing during combustion of tobacco (Tønnesen and Vermeire, 2000). Cigarette smoke is a complex mixture of more than 4700 chemicals, including high concentrations of free radicals and other oxidants (Bluhm *et al.*, 1971; Church and Pryor, 1985). Cigarette smoke contains free radicals in both the gas and the tar phase. The number of free radicals in the non-nitric oxide gas phase has been estimated to 10^{15} per puff, which are primarily of the alkyl, alkoxyl and peroxyl type and nitric oxide (NO) (Pryor and Stone, 1993). NO is present in the gas phase of cigarette smoke at almost 1000 ppm. Condensate (tar) contains more stable free radical species, mainly polycyclic aromatic hydrocarbons and quinones, which are partly removed by cigarette filters (Zhao, 1989).

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 components, one major group being free radicals (Hecht, 1999). Free radicals from cigarette smoke have been shown to cause DNA strand breaks and are implied in human carcinogenesis (Nakayama *et al.*, 1985; Kodama *et al.*, 1997). Components of the lung matrix itself (e.g., collagen, elastin) can be damaged and fragmented by oxidants in cigarette smoke (Cantin and Crystal, 1985). Moreover, free radicals from cigarette smoke play a role in enhancing inflammation in smokers with chronic obstructive pulmonary disease, through the activation of redox sensitive transcription factors regulating gene expression of pro-inflammatory mediators and protective antioxidant gene expression (MacNee, 2000).

The damage of free radicals from cigarettes is not limited to the pulmonary tract. It was found that urine of smokers contains 1.9-fold higher amounts of a typical biomarker of oxidative damage than non-smokers (Kaufmann *et al.*, 2000). The noxious pro-oxidant effects of smoking extend beyond the epicardial arteries to the coronary microcirculation and affect regulation of myocardial blood flow and cause carotid-media thickness (Wingo *et al.*, 1999).

In this investigation, the possibility to inactivate free radicals from cigarette smoke using an antioxidant rich preparation deposited on standard cigarette filters was studied. The antioxidant used should necessarily be effective against a broad range of free radical species, be heat resistant, nonvolatile and display high affinity to the filter surface. The extract from the bark of the French maritime pine, trade name Pycnogenol® (Horphag Research, Geneva, Switzerland), was a promising antioxidant. Pycnogenol® constituents are of phenolic nature, commonly referred to as flavonoids. Pycnogenol® consists of oligomeric procyanidins (catechin oligomers), monomeric catechin, taxifolin and various phenolic acids (e.g., caffeic, vanillic, protocatechuic, hydroxy-benzoic acid).

Pycnogenol® constituents have been shown to potently scavenge a large variety of free radicals species (Packer *et al.*, 1999; Rohdewald, 2002) and more efficiently than other antioxidants neutralise the very energetic species hydroxyl- and superoxide anion radicals (Noda *et al.*, 1997). Furthermore, Pycnogenol® proved to be considerably heat resistant. After ten minutes at 100°C, Pycnogenol® only lost about 3.2% of its superoxide scavenging capacity, whereas other antioxidants like *Ginkgo biloba* extract (EGb761) and green tea extract lost 50% and 59.5% of their activity, respectively (Noda *et al.*, 1997). Furthermore, Pycnogenol® is non-volatile and importantly, has a high binding affinity to various materials (Packer *et al.*, 1999). These properties indicate that Pycnogenol® could be firmly retained within the cigarette filter during smoking.

Materials and methods

Chemicals and reagents

Pycnogenol®, a standardised extract of the bark of the French maritime pine (*Pinus pinaster*), was provided by Horphag Research Inc. (Geneva, Switzerland). Agents for positive control in Ames test, NaN_3, 2-aminofluorene and 9-fluorene, as well as dimethyl sulfoxide (DMSO) and spin trap *N*-tertbutyl-2-phenyl nitrone (PBN) and spin probe TEMPO (2,2,5,5-tetramethyl-1-piperidin-1-oxyl radical) were obtained from Sigma (St. Louis, MO, USA).

Animals

Kunmin strain mice (18–22 g, female) and Wistar rats (230–300 g, female) were obtained from the Animal Breed Center of Chinese Academy of Medical Science (SCXK1100-0006) (Beijing, China). All of the protocol in this experiment were approved by the Animal Experimentation Committee of China Academy of Traditional Chinese Medicine.

Preparation of cigarette filters

Cigarette filters consisting of acetate cellulose were obtained from Hongta Co. (P.R. China). 200 μL of Pycnogenol® solutions in ethanol (95%) were added to cigarette filters equivalent to 0.12 mg, 0.24 mg, 0.4 mg and 0.8 mg Pycnogenol®, evenly distributed in the filter. Normal filters were treated with 200 μL ethanol only.

Generation of cigarette smoke

'Chuncheng' cigarettes from the Kunming Cigarette Factory (Yunnan, China) were used. Cigarette filters equipped with Pycnogenol® or normal filters were attached to cigarettes during this procedure. Cigarette smoke was generated using a smoking-device to imitate human's smoking with a smoke flow of 400 mL/min, inhaling once every two seconds (35 mL/two seconds) at one-minute interval as shown in Figure 1. The cigarette tar was filtered through three layers of Cambridge glass fibre filters. It was whole cigarette smoke when not using a Cambridge filter, and gas phase when the Cambridge filter was used. Thereafter, the gas phase smoke was used for determination of free radicals or chronic and acute toxicity tests as well as for preparation of condensate. The condensate was obtained by passing the gas phase of cigarette smoke through cold acetone. Following evaporation of the acetone, the residual condensate was dissolved in DMSO in defined concentrations for subsequent mutagenicity assays.

Detection of free radicals in gas phase of cigarette smoke

Free radicals present in the gas phase of cigarette smoke were detected by an electron spin resonance

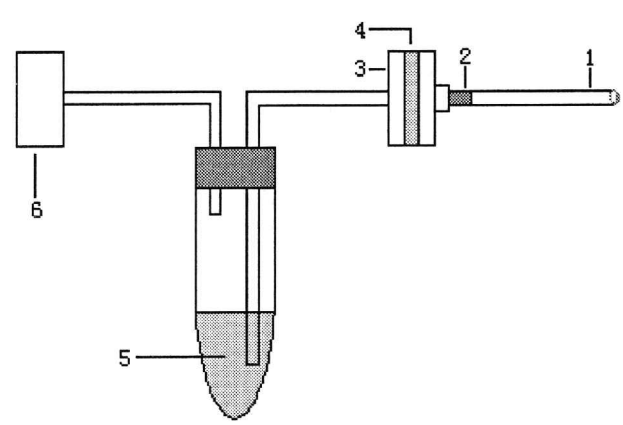

Figure 1. Scheme of the smoke machine. 1, cigarette; 2, filter; 3, hold; 4, carmbridge filter; 5, spin trapping solution; 6, pump.

(ESR) spin trapping technique (Zhao *et al.*, 1991). Cigarette smoke generated, as described above, passed through 2 mL of 0.1 mol/L *N*-tertbutyl-2-phenyl nitrone (PBN) spin trapping solution. The flow of gas phase was controlled at 400 mL/min in all spin trapping experiments. ESR spectra of the spin adducts were measured using a Brucker E 200 spectrometer at 23°C. ESR conditions: X-band, microwave power 20 mW, 100 kHz modulation frequency and 1G modulation amplitude. ESR signal intensity was measured as the relative concentration of free radicals as generated by cigarette smoking. The ESR instrument was adjusted to the same condition by a standard marker using the spin probe, TEMPO. Free radical scavenging rate was calculated by the following formula:

$$E = (HO - HX)/HO \times 100\%$$

where HO represents the peak intensity of the reference system and HX represents the peak intensity of samples containing the free radical scavenger.

Mutagenicity assay

The mutagenicity of cigarette smoke was evaluated by the Ames test with minor modifications (Maron and Ames, 1983). A bacterial strain *Salmonella typhimurium* mutant TA98 (hisD3052, rfa, ΔμυγB, pKM101) was applied. S9 activated system of the metabolic enzymes of Sprague-Dawley male rat liver microsomes (1.8 mg of S9 protein/plate) were prepared (Maron and Ames, 1983). One culture dish was used for solvent control, three dishes were prepared for positive controls: 2.5 μg NaN_3, 10.0 μg 2-aminofluorene and 0.2 μg 9-fluorene. The other dishes contained 50 μg, 150 μg or 500 μg of condensates resulting from the gas phase of cigarettes with different filters. Each condensate dose was applied in triplicates and all tests were repeated twice.

Exposure of rodents to cigarette smoke

To study acute and chronic toxicity rodents were exposed to cigarette smoke in a polyacryl glass chamber ($35.6 \times 35 \times 20$ cm) with two 1.5 cm^2 holes, one located on top of the chamber for ventilation and another at the bottom as entrance for the gas phase. The gas phase of smoke was delivered with a special pump to the chamber containing one group of ten rodents. The concentrations of O_2, NO_x, CO_2 and CO in the chambers were recorded during the experiments with a PG-250 portable gas analyser. Forty rodents were randomly divided into four groups. Rodents in group 1 were treated with the gas phase of smoke from cigarettes with normal filters. Rodents in groups 2 and 3 were treated with the gas phase of smoke from cigarettes with filters containing 0.12 mg and 0.4 mg Pycnogenol®, respectively. Rodents in group 4 served as control and were not treated with cigarette smoke.

Acute toxicity of cigarette smoke in mice

Four groups of ten mice each were exposed to the gas phase of cigarette smoke as described above, recording the time and number of cigarettes until the lethal endpoint was reached. The deceased mice were examined for histopathological changes.

Chronic toxicity of cigarette smoke in rats

Four groups of rats were exposed to the gas phase of cigarette smoke as described above. Each group of ten rats was exposed to the gas phase of 14 cigarettes during 30 minutes. This procedure was carried out twice a day, with an intermission of four hours, over a total time period of 75 days.

Mutagenicity of chronic smoking was established by micronucleus assay. Bone marrow of sacrificed rats was flushed out of femurs, homogeneously mixed with an equal volume of fetal bovine serum, centrifuged, resuspended and spread on a slide. The smear was air-dried and then stained with May-Grünwald/Giemsa. One thousand polychromatic erythrocytes were analysed per animal for micronuclei. To describe a cytotoxic effect the ratio between polychromatic and normochromatic erythrocytes was determined in the same sample and expressed in normochromatic erythrocytes per 1000 polychromatic erythrocytes.

Specimens from lung, heart, liver, spleen, kidney and adrenal gland were taken from the same positions of the organs, fixed with 10% formalin, embedded and sectioned in paraffin and stained with HE. Pathological changes were examined under the light-microscope.

Statistical analysis

The data were expressed as mean ± SEM and analysed by ANOVA. Comparison between the treatment groups was made by analysis using Newman–Keuls test with $P < 0.05$ as the minimum level of significance.

Results

Detection of free radicals in cigarette smoke by ESR

ESR spectra taken of free radicals in the gas phase of cigarette smoke, trapped by PBN in CCl_4 and benzene, had following parameters: $a_N = 13.7G$, $a_H^{\alpha} = 2.1G$; $a_N^{\beta} = 14.1G$, $a_H^{\gamma} = 3.3G$. Mainly identified free radical species were alkoxyl and alkyl radicals (Zhao *et al.*, 1991). The ESR signal intensity is proportional to the relative concentration of free radicals.

Free radical species present in cigarette smoke were not limited to alkoxyl ($RO^{\bullet}$), alkyl ($R^{\bullet}$) and alkylperoxide ($ROO^{\bullet}$) radicals. However, other free radicals were not detected because PBN is not sensitive for detection of these radicals. The generation of free radicals from cigarette smoke is a kinetic process (Church and Pryor, 1985). First, carbohydrates and proteins in the cigarette burn producing alkenes (R) and NO, then NO is oxidised to NO_2 which reacts with R to generate $RO^{\bullet}$, $ROO^{\bullet}$ and $R^{\bullet}$ free radicals.

Free radical scavenging effects of Pycnogenol® in cigarette filters

The relative concentration of free radicals generated in cigarette smoke was significantly ($P < 0.01$) and dose dependently reduced by presence of 0.12–0.4 mg Pycnogenol® in filters (Table 1). However, at the highest concentration of Pycnogenol® (0.8 mg), the scavenging effects did not further increase, but decreased to 20%. This might result from Pycnogenol® blocking channels of the filters at high concentration and thereby decreasing the chance of free radicals in the gas phase to get in contact with Pycnogenol® in filters.

Table 1. Scavenging effects of Pycnogenol® in filters on free radicals in cigarette smoke

(Pycnogenol® per filter)	free radicals (relative concentration)	Scavenging effect	*n*
Normal filter	8.67 ± 1.22	0	100
0.12 mg	6.67 ± 1.22*	22.6%	95
0.24 mg	6.57 ± 1.27*	27.6%	100
0.40 mg	6.11 ± 1.01*	29.1%	98
0.80 mg	6.90 ± 1.02*	20.0%	100

Mean values of repeated measures (*n*) are given.
* Compared to control $P < 0.05$.

In vitro inhibition of mutagenicity of cigarette smoke by Pycnogenol® in cigarette filters

For estimation of the mutagenicity of cigarette smoke and its reduction by Pycnogenol® in filters, preliminary tests were carried out to search for sensitive *S. typhimurium* strains and the strain TA98 was chosen as it gave a dose dependent response in a reproducible manner.

The mutagenicity of cigarette condensates in the absence or presence of Pycnogenol® in filters are given in Table 2. Smoke condensates had reduced mutagenic effects, depending on the amount of Pycnogenol® present in filters.

In vivo reduction of acute toxicity of cigarette smoke by Pycnogenol® in cigarette filters

The efficacy of Pycnogenol® in cigarette filters was accompanied *in vivo* by a significant reduction of acute cigarette smoke toxicity in mice, a precisely defined endpoint. The presence of 0.12 mg Pycnogenol® in cigarette filters significantly increased the survival time and reduced the acute toxicity of cigarette smoke by 47.5% (Table 3). In the absence of Pycnogenol® in cigarette filters, mice died after inhaling the smoke of eight cigarettes. In presence of 0.4 mg Pycnogenol® in filters, mice died only after exposure to the smoke of 14 cigarettes and the acute toxicity of cigarette smoke was reduced by 70.5%. During experiments, the concentrations of O_2, NO_x, CO_2 and CO were monitored at different time points (Table 4). O_2 and CO_2 concentration in the chamber changed little over different time-points after the beginning of experiments. The level of CO increased from 0 to about 3200 ppm, but there was no significant difference between the different groups; NO_x in 0.4 mg/filter group increased from 0 to about

Table 2. Mutagenicity of cigarette smoke condensate and its inhibition by the presence of Pycnogenol® in filters

	Condensate (μg/culture dish)	Reverse mutation (number/culture dish)	Inhibition rate (%)
Cigarette with normal filter	50	29.2±3.76	
	150	38.7±5.47	
	500	67.2±8.11	
Cigarette filter with 0.12 mg Pycnogenol®	50	28.2±4.67	3.5
	150	34.6±9.54 *	11.9
	500	49.6±11.41 **	35.4
Cigarette filter with 0.4 mg Pycnogenol®	50	26.5±2.90 *	10.1
	150	31.8±6.10 **	21.6
	500	48.4±8.60 **	39.5

* Significance as compared to cigarettes with normal filters: $P < 0.05$.
** Significance as compared to cigarettes with normal filters: $P < 0.01$.

50 ppm and the amounts in smoke from normal filter increased little more that of smoke with Pycnogenol®, however the difference with that of 0.4 mg/filter group was significant.

All deceased mice were biopsised and histopathologically examined. In the normal filter group (cigarette filters without Pycnogenol®), an obvious congestion and haemorrhage in lung tissue was observed in 80% of mice. Furthermore, we discovered vasodilation and congestion of small blood vessels in kidneys and a slight vasodilation and congestion of central veins in livers. The presence of 0.12 mg Pycnogenol® in filters reduced the number of mice with these pathological changes to 40%. With 0.4 mg Pycnogenol® in filters, almost none of the pathological changes were observed.

In vivo mutagenicity and toxicity of chronic smoking

The reduction of mutagenicity in the Ames test and of acute toxicity in mice prompted us to demonstrate the prevention of mutagenicity by Pycnogenol® filters *in vivo*. We investigated the incidence of micronuclei in polychromatic erythrocytes as a measure of mutagenicity, and the change of the polychromatic erythrocytes to normochromatic erythrocytes ratio as an indicator of toxicity in rats exposed to cigarette smoke for 75 days. The incidence of micronuclei in polychromatic erythrocytes increased by more than twofold when mice were chronically exposed to cigarette smoke, in accordance with a mutagenic activity of cigarette smoke (Table 5). When cigarette filters contained 0.12 mg Pycnogenol®, the incidence of micronuclei was inhibited by 24% as compared to rats that inhaled smoke from cigarettes with normal filters. The presence of 0.4 mg Pycnogenol® in filters was accompanied by a considerable improvement in micronuclei formation which was inhibited by 48%. The incidence of micronuclei was statistically insignificant in rats not exposed to smoke. Hence, there was a significant reduction in the mutagenicity of cigarette smoke by presence of 0.4 mg Pycnogenol® in cigarette filters.

Additionally to polychromatic erythrocytes, the ratio of polychromatic erythrocytes to normochromatic erythrocytes should normally be about one in healthy animals, while a lower ratio is known to indicate cytotoxicity insults. Accordingly, the polychromatic erythrocytes to normochromatic erythrocytes ratio was decreased in rats that had been exposed to cigarette smoke for 75 days. The presence of 0.12 mg Pycnogenol® in filters does not protect against the toxic effect of cigarette

Table 3. Acute toxicity of cigarette smoke in mice

Group	Body weight (g)	Cigarettes smoked (*n*)	Survival time (min)	Inhibition rate (%)
Normal filters	29.7±1.9	8	12.2±1.0	0
Pycnogenol® (0.12 mg)	27.0±1.5	12	18.0±0.3*	47.5
Pycnogenol® (0.4 mg)	28.5±2.4	14	20.8±2.1*	70.5
Normal (no smoke)	30.0±2.1			

Survival times of mice exposed to smoke from cigarettes with filters containing different amounts of Pycnogenol®. The survival time was recorded for death of all mice of each group.
* The survival time is longer than that of normal filter group $P < 0.01$.

Table 4. The changing of gas components in the cabin in different time points after the beginning of cigarette smoke

Gas	Group	Time			
		0 min	10 min	20 min	30 min
CO_2	Normal filter	0.03%	0.46%	0.7%	0.9%
	0.4 mg Pycnogenol®	0.03%	0.46%	0.7%	0.9%
O_2	Normal filter	21%	20.5%	20%	20%
	0.4 mg Pycnogenol®	21%	20.5%	20%	20%
NO_x	Normal filter	0	19 ppm	46 ppm	60 ppm
	0.4 mg Pycnogenol®	0	17 ppm	39 ppm	50 ppm
CO	Normal filter	0	600 ppm	2500 ppm	3100 ppm
	0.4 mg Pycnogenol®	0	600 ppm	2500 ppm	3200 ppm

smoke, however, 0.4 mg Pycnogenol® in filters brought the polychromatic erythrocytes to normochromatic erythrocytes ratio back to normal (1.01).

The weight of the rodents was followed and it was found that there was no difference between the various groups. Specimens of heart, liver, spleen, kidney, adrenal glands and lung of each animal from all groups were embedded in paraffin, sectioned, stained with hematoxylin-eosin and examined under the optical microscope for pathological changes. In the lungs of animals exposed to cigarette smoke with normal filters, or filters with 0.12 mg Pycnogenol®, a variety of pathological alterations were discovered (Figure 2), while all other organs remained unaffected. We observed an increased thickness of bronchi capillary walls with signs of neutrophil and lymphocyte infiltration. There was a low evidence of mucous epithelial cell proliferation or even exfoliation, an increase of inflammatory exudate and lympho-proliferation in the bronchial lumen. Abscess and low fibre proliferation were found in lung tissue in two rats from the normal filter group and one rat from the group treated by cigarette smoke with 0.12 mg Pycnogenol® in filters. When 0.4 mg Pycnogenol® filters were used, only one rat showed the pathological alterations and five other rats showed minor pathological changes in the lungs (moderate neutrophil and lymphocyte infiltration of alveoli) and in four animals, almost normal lung tissue was observed (Figure 2d).

Discussion

We tested the *in vitro* and biological consequences of equipping standard type cigarette filters with the potent free radical scavenger, Pycnogenol®. Pycnogenol® is a standardised extract from the bark of the French maritime pine, available worldwide as a food supplement. Pycnogenol® is a bioflavonoid rich extract with a high content of oligomeric procyanidins. Of recent reviews (Packer *et al.*, 1999; Rohdewald, 2002), Pycnogenol® exhibits potent free radical scavenging activity and readily binds to surfaces.

As predicted, the presence of Pycnogenol® in cigarette filters reduced the amount of free radicals in the gas phase of cigarette smoke. Free radical scavenging was dose dependent up to an amount of 0.4 mg per filter; higher amounts up to 0.8 mg showed no further increase, and on the contrary had a lower scavenging yield. The reason for this effect is unknown, but likely results from overfilling the filter and thus retarding the interaction of the gas with Pycnogenol®.

Table 5. Chronic toxicity of cigarette smoke in rats

Cigarette filter	PCE/NCE	PCE with micronuclei (%)	Micronuclei inhibition (%)
None (control)	1.02	4.2±1.4	–
Normal filter	0.88	10.0±0.9	0
Filter with 0.12 mg Pycnogenol®	0.88	7.6±2.5*	24*
Filter with 0.4 mg Pycnogenol®	1.01	5.2±2.3*	48*

The number of micronuclei per 1000 polychromatic erythrocytes was determined as measure of *in vivo* mutagenicity. The ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) is given as measure of cytotoxicity. Mean values for ten rats per group are given.
* Significance as compared to control cigarettes with standard filters ($P < 0.01$).

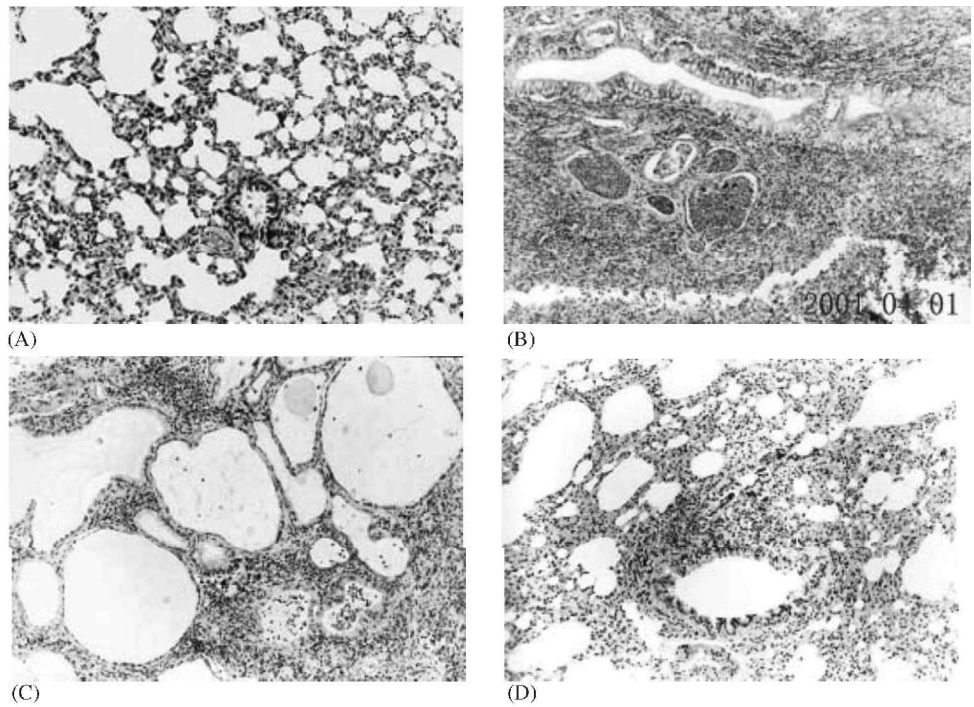

Figure 2. Histopathology of lung tissue: (A) normal rats; (B) rats treated with normal cigarette smoke; and (C) cigarette smoke with 0.12 mg; (D) or 0.40 mg Pycnogenol® in filters.

The reduction of free radicals from cigarette smoke *in vivo* was demonstrated by reduced acute toxicity in mice as an objectively defined end-point. Mice exposed continuously to cigarette smoke, lived up to 70.5% longer when the smoke was filtered through Pycnogenol® containing filters. At this point, it cannot be judged whether the reduction of acute toxicity of cigarette smoke is exclusively a result of radical depletion by Pycnogenol®, or whether reduction of other toxins from cigarette smoke, for example, CO, CO_2, nitrite oxide, acrolein or particles, also have to be taken into consideration. O_2 and CO_2 concentration changed little in the different groups while the level of NO and CO significantly increased. The lethal level for humans is 800 ppm/two hours for CO, and about 100 ppm or more for NO. The level of NO in the chamber was below the lethal level for humans, but the level of CO was higher. Since the susceptibility of animals is usually different from that of humans, it is difficult to deduce that the level is lethal or not. Without doubt, CO and NO must be involved in cigarette smoke toxicity. The concentration of CO in the chamber showed that there were no significant differences between normal filter or the Pycnogenol® group while the concentration of NO_x from the Pycnogenol® group significantly decreased as compared to controls (Table 4). NO and NO_2 are free radicals and are able to cause more free radical generation from reaction with alkenes in gas phase cigarette smoke. The presence of Pycnogenol® could decrease free radicals and the toxicity of cigarette smoke. At the same time, the concentration of other different gases from different groups showed that there were no significant differences between normal filter or the Pycnogenol® group; this suggested that animals in different groups were exposed to the same conditions during the experiments except for minor difference of NO_x. The amounts of acrolein and particles in the chamber from the gas phase of smoke were not measured during the experiment. They must have toxic effects on the animals, but there should not be much difference in their effects in the different groups. What effects Pycnogenol® may have on the unsaturated aldehydes and particles generated from cigarette smoke is not known. Nevertheless, the decrease of toxicity was clearly observed in the group with added Pycnogenol® in filter.

Decreasing the free radical content in cigarette smoke clearly leads to a reduced mutagenicity in

the Ames test of the smoke condensate. The number of reverse mutations in response to cigarette smoke condensate was decreased by up to 39.5% when 0.4 mg Pycnogenol® was present in the cigarette filter. Since mutagenicity is often linked to carcinogenicity, the reduction of lung cancer risk is a potential benefit of Pycnogenol® in the filters.

Chronic exposure of rats to cigarette smoke for 75 consecutive days resulted in the inhibition of micronuclear changes by 48% after smoke was filtered through 0.4 mg Pycnogenol® containing cigarette filters. Furthermore, the polychromatic erythrocytes to normochromatic erythrocytes ratio reached normal values (1.01), suggesting that Pycnogenol® in filters provides protection against chronic cigarette toxicity. Biopsies of the organs of rats clearly demonstrated that the primary target for pathological changes is the lung. The presence of 0.4 mg Pycnogenol® in cigarette filters did not completely abolish but dramatically reduced damage to lung tissue damage.

Comparing with the activated charcoal filter which can physically absorb free radicals and other materials from cigarette smoke, Pycnogenol® not only physically absorbs but also chemically reacts with free reactive radicals and other materials. Hence, Pycnogenol® may have more selective and specific actions on reactive free radicals species.

These experiments point at a considerable, yet largely avoidable health hazard caused by free radicals in the gas phase of cigarette smoke. Though there are other harmful constituents in cigarette smoke besides free radical species, reduction of the latter yields a substantial risk reduction for smokers. As a smoke-free society remains a utopian goal, risk reduction needs to be taken seriously. This study demonstrates that this is feasible and, furthermore, it can be achieved by slight and inexpensive modification of existing filter technologies.

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