

Effect of smoking cessation on oxidative DNA modification estimated by 8-oxo-7,8-dihydro-2'-deoxyguanosine excretion

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Background: Reactive oxygen species from, e.g. tobacco smoke are suggested to be involved in carcinogenesis by oxidative modification of DNA. The urinary excretion rate of the oxidized nucleoside 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) has been validated as a biomarker of the rate of oxidative DNA modification with mechanistic relation to carcinogenesis. In cross-sectional studies, the urinary excretion rate of 8-oxodG has been shown to be elevated in smokers compared with non-smokers. **Purpose:** In this randomised, controlled smoking cessation study, we investigated whether cigarette smoking per se causes oxidative DNA modification. **Methods:** Of the 182 healthy smokers included, 100 were randomized to quit smoking after baseline samples had been taken, and 82 were randomized to continue usual smoking. Before the start of the study and after 4 weeks, the subjects collected 24-h urine samples that were analysed for 8-oxodG content by high-pressure liquid chromatography with electrochemical detection. The subjects randomized to smoking cessation were followed up after 26 weeks. **Results:** Four weeks of smoking cessation resulted in a 21% decrease in 8-oxodG excretion rate (from mean \pm SD, 30.5 ± 13.9 to 24.1 ± 10.5 nmol/24 h, $P < 0.001$) in 58 quitters included in per-protocol data analysis. Sixty-five continued smokers included in per-protocol analysis showed a 9% decrease in 8-oxodG excretion rate (from 31.6 ± 13.2 to 28.7 ± 12.6 nmol/24 h, $P = 0.026$). After 4 weeks, the 8-oxodG excretion rate was 16% (95% confidence interval 4 to 28%) higher in the continued smokers than in the quitters ($P = 0.0085$, ANCOVA), demonstrating the effect of smoking per se. A 23% ($P < 0.005$) decrease in 8-oxodG excretion rate was sustained for 26 weeks in 27 quitters who completed the study. **Conclusion:** Smoking cessation significantly reduces the urinary excretion rate of 8-oxodG, giving direct and controlled evidence that cigarette smoking causes an increased rate of oxidative DNA modification. This could represent a mechanism by which tobacco smoke is carcinogenic.

Introduction

Cigarette smoking is a well-known risk factor for several cancer forms, including lung cancer (1), however, the mechanism of

*Abbreviations: 8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine; CI, confidence interval.

carcinogenesis by cigarette smoking is still not fully understood. Cigarette smoke contains large amounts of reactive oxygen species (2,3) and the production of reactive oxygen species from leukocytes has been found to be higher in smokers than in non-smokers (4). In addition, the endogenous mitochondrial production of reactive oxygen species might be elevated in smokers because of an increased rate of cellular metabolism (5). Reactive oxygen species have been linked to the initiation and promotion of cancer (6,7) and could thus represent important carcinogens in cigarette smoke. *In vitro* studies have demonstrated that cigarette smoke can generate hydrogen peroxide and hydroxylate deoxyguanosine residues in isolated DNA (8) as well as in cultured human lung cells (9). *In vivo*, oxidative damage to leukocyte DNA (10,11) and to sperm DNA (12) has been found to be elevated in smokers compared with non-smokers.

Oxidative modification of DNA includes a variety of base oxidations (13). C-8 hydroxylation of the guanine base in DNA is rapidly and almost completely repaired (14–16), mainly by an excision repair mechanism (17). The excised oxidized nucleoside 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG*) is water-soluble and readily excreted in the urine without further metabolism and its rate of urinary excretion has been validated as a biomarker of the rate of oxidative DNA modification (18–20). The rate of DNA oxidation is probably critical for the risk of failing repair of damaged bases and thereby for the risk of mutations. In population-based studies, it has been demonstrated that smokers excrete ~35% to 50% more 8-oxodG in urine than non-smokers (21,22). However, other differences in life style between smokers and non-smokers than the smoking habit, e.g. diet (23) and exercise (24), could explain such differences in 8-oxodG excretion.

To test if cigarette smoking per se causes oxidative modification of DNA in humans, we conducted a randomized, controlled smoking cessation study measuring the urinary excretion rate of 8-oxodG at baseline and after 4 weeks. Subjects randomized to smoking cessation were followed up after 26 weeks.

Subjects and methods

Study subjects, randomization and smoking cessation

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the local ethics committee. Signed informed consent was obtained from all subjects. Two hundred volunteers were recruited through advertisement in a local newspaper. The study population was a homogenous Danish population. Eligible subjects of both sexes (aged 35 to 65 years) had smoked at least 15 cigarettes per day for >1 year and declared motivation to stop. Exclusion criteria were presence of known disease; daily intake of drugs, including hormonal contraceptives; antioxidant supplements within the last month; and pregnancy or breast-feeding.

After collection of baseline samples, 182 subjects were assigned by a computer generated random number list either to stop smoking the next morning (smoking cessation group, $n = 100$) or to continue usual smoking for another 4 weeks (control group, $n = 82$). After the first 4 weeks, the subjects in the control group left the study and were offered a smoking cessation programme.

Subjects in the smoking cessation group received support by seven visits to the clinic during the 26 weeks of the study. Nicotine patches releasing a

mean of 15 mg nicotine/16 h (Nicorette®, Pharmacia AB, Helsingborg, Sweden) were supplied for 12 weeks (25), after which dosage was tapered to zero during a 4-week period. At the 26-week visit, only four quitters reported use of nicotine patches (two subjects) or nicotine chewing gum (two subjects). Only subjects from the smoking cessation group who stated to be completely abstinent and who expired 10 p.p.m. of carbon monoxide or less were included in per-protocol data analysis: Of the 100 subjects, 85 were non-smokers after the first 4 weeks, and 41 were non-smokers and did show up at the 26-week visit.

After 4 weeks, 58 subjects from the smoking cessation group and 65 subjects from the control group were included in per-protocol data analysis. Twenty-seven subjects from the smoking cessation group were excluded for the following reasons other than smoking: various infections (nine subjects), intercurrent antioxidant supplementation (nine subjects), missing urine sample (four subjects), not giving blood sample (two subjects), treatment with non-steroid anti-inflammatory drugs (two subjects) or other (one subject). Seventeen subjects from the control group were excluded from per-protocol data analysis at 4 weeks because of: missing urine sample (seven subjects), various infections (three subjects), withdrawal from the study (three subjects), intercurrent antioxidant supplementation (two subjects), not giving a blood sample (one subject) or other (one subject). At the 26-week follow-up, 27 subjects from the smoking cessation group were still non-smokers and had fulfilled all criteria throughout the study, including collection of 24 h urine samples (Figure 1).

Assessments

At the visits at baseline, after 4 weeks (both groups) and after 26 weeks (only the smoking cessation group), the subjects brought urine collected during the previous 24 h. The subjects' smoking history, height and body wt were recorded. The smoking status of the subjects was confirmed at each visit by measuring the carbon monoxide level in end-expiratory air with a carbon monoxide analyser (Bedfont Monitor, Sittingbourne, UK), with levels of carbon monoxide exceeding 10 p.p.m. indicating a recent smoker (25). Plasma cotinine concentrations were measured by capillary gas chromatography at the Bioanalytical Laboratory, Pharmacia AB, Helsingborg, Sweden (26).

The urinary concentration of 8-oxodG in 24 h urine samples was determined by high-performance liquid chromatography with electrochemical detection (HPLC-EC) as described previously (21). The samples from each subject were analysed together in at least two different batches. The inter-batch coefficient of variation was 11.5%.

Statistical analysis

Pre-study sample size calculations indicated a group size of 43 persons for comparison of two parallel groups and 22 persons for a paired design for detection of a difference in 8-oxodG excretion rate of a minimum of 20%. Assumptions were a risk of Type I error of 5%, a risk of Type II error of 20%, and a standard deviation of 33% in the 8-oxodG excretion rate measurements assumed from the standard deviation measured in smokers (21).

Continuous variables were tested for normal distribution by the Kolmogorov–Smirnov test. For the normally distributed variables, baseline values for the two groups were compared using the two sample Student's *t*-test. Values are given as means \pm SD. For variables not normally distributed the baseline values were compared for the two groups by the Mann–Whitney *U*-test. These values (no. of cigarettes smoked daily) are given as medians and ranges. Differences between values before and after 4 or 26 weeks in each group were also tested for normal distribution by the Kolmogorov–Smirnov test. When the differences were normally distributed, the changes were tested using the paired Student's *t*-test. The Wilcoxon matched pairs test was used to test the changes after 4 and 26 weeks when the distributions of the differences were not normal (no. of cigarettes smoked daily).

The 8-oxodG excretion rates at 4 weeks were tested for the smoking cessation group and the control group together using ANCOVA, adjusting with the baseline level as a covariate. The 95% confidence interval (CI) for the adjusted difference between the two groups at 4 weeks was calculated. Values were found to fulfil the criteria of homogeneity of variances according to the Hartley *F*-max, Cochran *C* and Bartlett chi-square tests. A two-sided *P*-value of <0.05 was considered statistically significant. Both per-protocol analysis and intention-to-treat analysis were performed. The computer program Statistica® (version 5.0; Statsoft, Inc., Tulsa, USA) was used for the statistical analyses.

Results

Subjects included in per-protocol data analysis in the smoking cessation group and the control group were similar at study start in respect to gender, age, pack years, daily cigarette consumption, carbon monoxide in expired air, *p*-cotinine, body

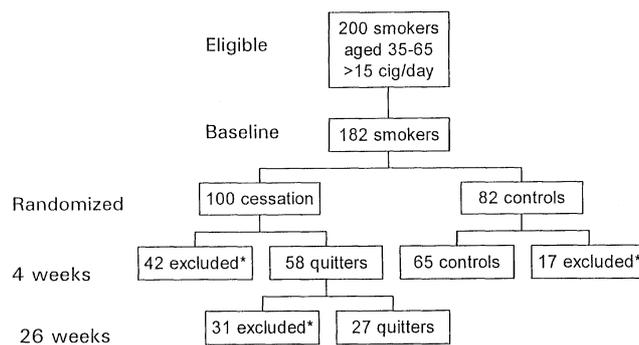


Fig. 1. Flow chart showing the study design. *Excluded because of acute disease, intake of any medicine or antioxidant supplements, missing urine sample, or for the cessation group because of resumption of smoking.

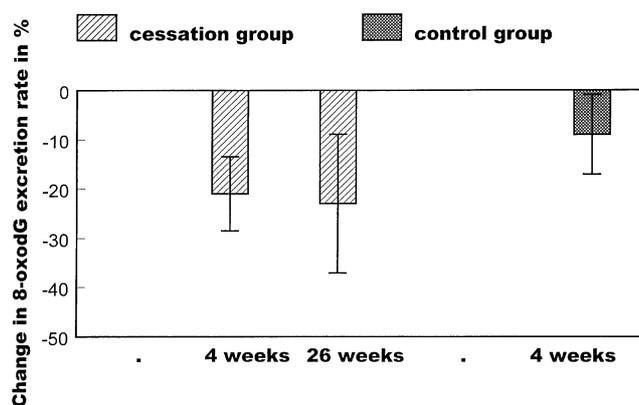


Fig. 2. Relative changes with 95% CI in 8-oxodG excretion rate in the two groups during the study.

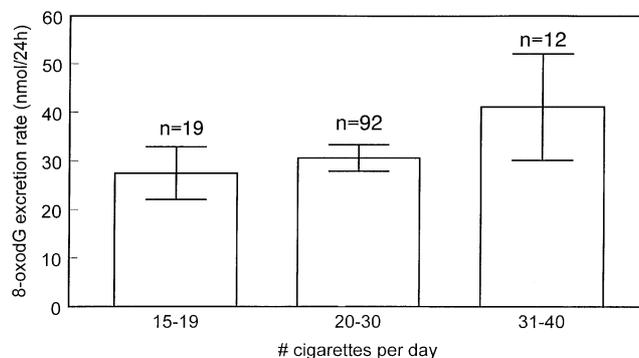


Fig. 3. Dose–response relationship between the daily cigarette consumption and the excretion rate of 8-oxodG (with 95% CI) at baseline for all subjects in per-protocol analysis.

wt, body mass index and the urinary excretion rate of 8-oxodG (Table I). Four weeks of smoking cessation resulted in a significant decrease of 21% ($P < 0.001$) in 8-oxodG excretion rate in the smoking cessation group, however, during these 4 weeks the 8-oxodG excretion rate also decreased by 9% ($P = 0.026$) in the control group (Tables I and II). Tested by ANCOVA with baseline adjustment, the 8-oxodG excretion rates at 4 weeks were significantly different in the two groups, i.e. the control group had 16% (95% CI 4–28%) higher 8-oxodG excretion rate than the smoking cessation group ($P = 0.0085$) (Table I).

Intention-to-treat analysis after 4 weeks showed similar results as the per-protocol analysis: 8-oxodG excretion rates decreased from mean \pm SD, 31.4 ± 14.2 to 26.7 ± 12.6

Table I. Characteristics and values at baseline and after 4 weeks for subjects in per-protocol data analysis

	Baseline		Four weeks	
	Smoking cessation (<i>n</i> = 58)	Controls (<i>n</i> = 65)	Smoking cessation (<i>n</i> = 58)	Controls (<i>n</i> = 65)
Gender (M/F)	33/25	36/29	–	–
Age (years)	44.8 ± 7.7	44.5 ± 7.6	–	–
Pack years of cigarettes	24.6 ± 8.4	26.8 ± 11.2	–	–
No. of cigarettes smoked daily ^a	20 (15–40)	22 (15–40)	0 (0–0) ^b	20 (4–40)
Carbon monoxide in expired air (p.p.m.)	27 ± 10	29 ± 11	4 ± 2 ^b	28 ± 12
<i>p</i> -Cotinine (ng/ml)	284 ± 124	277 ± 97	116 ± 73 ^b	283 ± 101
Body wt (kg)	75.2 ± 13.9	74.8 ± 12.3	76.5 ± 13.9 ^b	75.0 ± 12.3
Body mass index (kg/m ²)	24.8 ± 3.6	24.3 ± 3.0	25.2 ± 3.6 ^b	24.3 ± 3.1
8-oxodG excretion rate (nmol/24 h)	30.5 ± 13.9	31.6 ± 13.2	24.1 ± 10.5 ^b	28.7 ± 12.6 ^c
Mean difference between groups in 8-oxodG excretion at 4 weeks, baseline adjusted (nmol/24 h)	3.9 (95% CI 1.0 to 6.8) ^d			

Data are mean ± SD or ^amedian (range).

^bThe difference between the actual level and the baseline level was significant at the level *P* < 0.001.

^cThe difference between the actual level and the baseline level was significant at the level *P* = 0.026.

^dThe difference was significant at the level *P* = 0.0085 tested by ANCOVA adjusting with the baseline levels as a covariate.

Table II. Values at baseline, after 4 weeks and 26 weeks for 27 subjects from the smoking cessation group who fulfilled the study

	Baseline	Four weeks	26 weeks
No. of cigarettes smoked daily ^a	20 (15–40)	0 (0–0) ^b	0 (0–0) ^b
Carbon monoxide in expired air (p.p.m.)	26 ± 9	4 ± 2 ^b	1 ± 2 ^b
<i>p</i> -Cotinine (ng/ml)	261 ± 119	97 ± 56 ^b	35 ± 53 ^b
Body wt (kg)	76.0 ± 13.0	77.3 ± 13.1 ^b	80.0 ± 13.0 ^b
Body mass index (kg/m ²)	25.5 ± 3.9	26.0 ± 4.0 ^b	26.9 ± 4.0 ^b
8-oxodG excretion rate (nmol/24 h)	26.7 ± 15.3	20.5 ± 11.6 ^b	20.6 ± 12.5 ^b

Data are mean ± SD or ^amedian (range).

^bThe difference between the actual level and the baseline level was significant at the level *P* < 0.005.

Table III. Results from studies where levels of 8-oxodG or 8-oxoguanine (8-oxoGua) measured by HPLC-EC in urine from smokers and non-smokers have been compared

Reference	No. of subjects	Biomarker	Levels	Difference between smokers and non-smokers ^a
Loft <i>et al.</i> (21)	53 non-smokers versus 30 smokers	8-oxodG	213 ± 84 pmol/kg body wt/24 h versus 320 ± 99	50% ^b
Tagesson <i>et al.</i> (27)	24 non-smokers versus 10 smokers	8-oxodG	1.02 ± 0.35 nmol/mmol creatinine versus 1.30 ± 0.38	27% ^b
Loft <i>et al.</i> (22)	21 non-smokers versus 12 smokers	8-oxodG	318 ± 130 pmol/kg body wt/24 h versus 431 ± 168	36% ^b
Lagorio <i>et al.</i> (28)	22 non-smokers versus 10 ex-smokers versus 33 smokers	8-oxodG	1.32 ± 0.50 nmol/mmol creatinine versus 1.29 ± 0.62 versus 1.41 ± 0.46	8% Not statistically significant
Suzuki <i>et al.</i> (29)	6 non-smokers versus 7 smokers	8-oxoGua	3.81 ± 1.93 ng/mg creatinine versus 7.41 ± 1.47	94% ^b

Data are mean ± SD.

^aDifference between the level in smokers and non-smokers in % of the level in non-smokers.

^b*P* < 0.05.

nmol/24 h (*P* < 0.001) in 85 subjects randomized to smoking cessation and from 32.3 ± 15.6 to 29.1 ± 13.8 nmol/24 h (*P* = 0.015) in 76 subjects randomized to continued smoking from whom samples were available. For the 27 subjects from the smoking cessation group who completed the whole study there were significant differences between the 8-oxodG excretion rates at baseline and at 4 weeks (–23%, *P* < 0.001) and at 26 weeks (–23%, *P* = 0.003) (Table II). For these 27 subjects, the mean 8-oxodG excretion rates at 4 and 26 weeks

were similar. Intention-to-treat analysis after 26 weeks included 43 subjects randomized to smoking cessation from whom samples were available both at baseline, after 4 and after 26 weeks and showed a decrease in 8-oxodG excretion rate from 28.3 ± 14.2 at baseline to 22.8 ± 12.5 nmol/24 h after 26 weeks (*P* = 0.001).

At 26 weeks, seven out of the 27 subjects had plasma cotinine concentrations of 15 ng/ml or more suggesting continued nicotine substitution of some kind. Statistical analysis

for the 20 subjects with plasma cotinine levels <15 ng/ml gave similar results as analysis for all 27 subjects and the excretion rate of 8-oxodG at 26 weeks was similar in the seven subjects with plasma cotinine concentrations of 15 ng/ml or more and the 20 subjects with plasma cotinine concentrations of <15 ng/ml ($P = 0.64$).

At baseline there was a significant dose-response relationship between the daily cigarette consumption and the excretion rate of 8-oxodG ($r = 0.25$, $P = 0.006$, $n = 123$). When the subjects were grouped according to their daily cigarette consumption at baseline (<20, 20–30 or >30 cigarettes per day), the baseline 8-oxodG excretion rate differed significantly between these three groups tested by ANOVA ($P = 0.016$). The intra-individual coefficient of variation in the 8-oxodG excretion rate was 32%, calculated as the standard deviation of the change after 4 weeks divided by the mean baseline 8-oxodG excretion rate for the control group.

Discussion

We found a statistically significant effect of smoking cessation for 4 and 26 weeks on the urinary excretion rate of the DNA repair product 8-oxodG. The 16% difference in 8-oxodG excretion rate at 4 weeks between the control group and the smoking cessation group gives direct and controlled evidence that smoking induces oxidative DNA modification. This difference was less than the effect of smoking found in four out of five cross-sectional studies presented in Table III (21,22,27–29). However, cross-sectional studies may overestimate the difference between smokers and non-smokers because of the influence of confounders related to life style such as diet (23), exercise (24) and body wt. The design of the present study allows control for the unexplained 9% change in the group of continued smokers and provides a more realistic and direct estimate of the effect of smoking.

After 4 weeks, plasma cotinine concentrations, carbon monoxide levels in expired air and the number of cigarettes smoked daily were without significant changes in the control group, indicating unchanged cigarette smoke exposure. Other published data from the present study cohort (30) showed significant increases in plasma ascorbic acid concentrations of 23% in the smoking cessation group and of 10% in the control group after 4 weeks, which was sustained for 26 weeks in the smoking cessation group. The moderate decrease in 8-oxodG excretion rate in the control group, which was mirrored by an increase in plasma ascorbate, was most likely caused by unidentified changes in life style in this group during the first 4 weeks of the study. However, the changes in plasma ascorbic acid concentrations did not correlate significantly with the changes in 8-oxodG excretion rates on an individual basis ($r = 0.13$, $P = 0.17$, $n = 123$; data not shown). The lack of correlation may be caused by inter-individual variation in the relative effect of smoking cessation on the plasma compartment antioxidant level (ascorbic acid) and on the rate of oxidative modification in the intracellular compartment (8-oxodG).

Nicotine has been shown to potentiate the production of reactive oxygen species from leukocytes (31,32). Thus, at 4 weeks the 8-oxodG excretion rate in the smoking cessation group could have been influenced by the use of nicotine patches. However, this is not compatible with the finding of similar 8-oxodG excretion rates at 4 and 26 weeks in the subjects from the smoking cessation group who fulfilled the whole study, independently of the plasma cotinine concentra-

tions. Our finding of no further decrease in the 8-oxodG excretion rate in the smoking cessation group from 4 to 26 weeks suggests that the beneficial effect of smoking cessation on the level of oxidative DNA modification is obtained within 4 weeks after smoking cessation.

The relevance of 8-oxodG as a biomarker of oxidative DNA modification is supported by the observations that the guanine base is a major target of oxidative DNA damage (19,33,34) and that the oxidation of guanine residues in DNA is mutagenic (35). The relation of oxidative DNA modification to cancer is founded on *in vitro* experiments showing that oxidation of guanine bases in replicating DNA leads to G→T transversions (36), as found in the activated *K-ras* oncogene (37) and in the *p53* tumour suppressor gene of human lung cancers (38). Recently, it has been shown that benzo[*a*]pyrene, a carcinogenic constituent of tobacco smoke, causes strong and selective adduct formation at guanine positions, including codon 248 of the *p53* gene, which is a major mutational hotspot in human lung cancers (39). Interestingly, treatment with oxidants has been shown to cause similar mutations in the same codons, 248–250, of *p53* (40).

We conclude that cigarette smoking is an important factor causing an increased rate of oxidative DNA modification. This could represent a novel mechanism by which cigarette smoke is carcinogenic.

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