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ORIGINAL ARTICLE

The effect of smoking on the urinary excretion of 8-oxodG and 8-oxoGuo in patients with type 2 diabetes

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ABSTRACT

Over the past decades, attention has been paid to understanding the impact of oxidative stress and related modifications of DNA and RNA on various human health risks. A recent meta-analysis comprising 1915 smokers and 3462 non-smokers found a significantly higher level of DNA oxidation measured as urinary 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-oxodG) excretion in smokers compared with non-smokers in a healthy population. We aimed to investigate if an increased urinary excretion of 8-oxodG in smokers versus never smokers and former smokers could be verified in a population with type 2 diabetes. Additionally, we measured RNA oxidation levels through urinary excretion of 8-oxo-7, 8-dihydroguanosine (8-oxoGuo). Our study included urinary samples from 2721 type 2 diabetic patients, analyzed using ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). Logistic regression was used to examine the relationship between daily smokers ($n = 462$) versus former ($n = 1341$) and never smokers ($n = 918$) regarding the RNA and DNA oxidation, respectively. We did not find any significant effect of smoking on urinary excretion of 8-oxodG or 8-oxoGuo in our study. Due to a sparse study area, it is still too early to draw any conclusions on smoking and RNA-oxidation. Regarding DNA oxidation, our study suggests that the effect of smoking seen in healthy populations might be attenuated in patients with type 2 diabetes.

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Introduction

Over the past decades, attention has been paid to oxidative stress and modifications of DNA and RNA. Oxidative stress results from an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense mechanisms in the cell [1]. ROS are products of cellular metabolism, e.g. mitochondrial respiration and activated phagocytes [2] but also external sources such as tobacco smoking and ionizing radiation contribute to the ROS production [3]. ROS can react with any structure or molecule. The most studied alteration in DNA and RNA is the C-8 hydroxylation of guanine. The repair mechanisms of DNA-oxidation are well studied and include glycosylase and endonuclease activity followed by replacement of single bases or nucleotides [4]. On the other hand, little is known about the repair mechanisms of oxidized RNA. Even though such repair mechanisms are thought to exist [5], the major way of dealing with damaged RNA in the cell seems to be degradation and elimination [6]. As a result of repair and/or degradation of DNA and RNA, the urinary excretion of 8-oxo-7,8-dihydroguanosine (8-oxoGuo) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) is interpreted as a measure of oxidative stress and damage to DNA and RNA *in vivo* [7].

The exact mechanism by which tobacco smoking induces oxidation of DNA and RNA is not known, but oxidants in the smoke as well as in the tar may contribute to the process [8]. Furthermore, an increase in the metabolic rate and thereby an enhanced production of ROS is also thought to be part of the mechanism. In 1992, Loft et al. reported an association between tobacco smoking and high excretion of 8-oxodG in a study investigating healthy smokers ($n = 30$) versus non-smokers ($n = 53$) [9]. A recent meta-analysis [10] has found that there is a significantly higher level of 8-oxodG excretion in smokers compared with non-smokers.

To date, only a few studies have been reported concerning RNA oxidation in smokers vs. non-smokers, using the measure of urinary excretion of 8-oxoGuo [11]. Regarding the association between diabetes and oxidative stress, there seems to be a link between the pathogenesis of diabetes and

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its complications. Redox unbalance is thought to be associated with both beta-cell-dysfunction and insulin resistance. Furthermore, oxidative stress has been linked to the development of vascular complications in diabetes [12].

The effect of smoking on the pathogenesis of diabetes and its complications has been examined in many studies. A recent meta-analysis comprising 51 studies concluded that cigarette smoking is a cause of type 2 diabetes and that active smokers have 30–40% higher risk of developing diabetes than non-smokers. Furthermore, the number of cigarettes smoked seems to be related to the risk of developing diabetes in a positive dose-response relationship [13].

As indicated by Ellegaard and Poulsen [10], the effect of smoking on oxidative stress has mainly been investigated in healthy groups and little is known [14] about how the urinary excretion of 8-oxoGuo and 8-oxodG behave in a non-healthy population. In a cohort of type 2 diabetes patients we aimed to investigate if daily smokers had higher levels of nucleic acid oxidation compared to never smoking type 2 diabetes patients and former smokers with type 2 diabetes.

Materials and methods

Study population

Patients included in the study were identified using the Danish Personal Identification Number and the Danish Civil Registration System, which holds information on every citizen in Denmark. The study population was identified using an algorithm based on high HbA1c, frequency of HbA1c measurements, prescription of anti-diabetics and ICD-10 Diabetes diagnosis and self-reported type 2 diabetes (2%) [15].

Urine samples

Urine samples were collected as spot urine and analyzed using ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) at Laboratory of Clinical Pharmacology at Bispebjerg Hospital. Detailed information of the assay is described in the Appendix under the heading 'Materials and methods for analysis of 8-oxoGuo and 8-oxodG'. Random spot urine results were available for 2727 type 2 diabetes patients. Due to unknown smoking status of six patients, 2721 type 2 diabetes patients were included in the analyses. The values of 8-oxodG and 8-oxoGuo are reported as 8-oxodG/creatinine ratio and 8-oxoGuo/creatinine ratio to correct for variable spot urine concentrations. Additional biochemical measures were analyzed as previous described by ERB Petersen et al. [16].

Ethics considerations

The local ethics committee approved measurement of oxidative modified nucleic acids in urine on 3 April 2013 (S-20080097, amendment protocol 37831). After 15 May 2012 register-based research projects which demanded ethical approval no longer needed approval from the Danish Data Protection Agency; nonetheless, they were informed.

Statistics

Data was grouped related to smoking status in three categories: 'never smokers', 'former smokers' and 'daily smokers' based on answers from a questionnaire filled in by the participants. Former smokers included 100 occasional smokers. Means and standard deviations were calculated for continuous traits. Binary outcomes were stated as percent of the group total. Differences between the three groups were calculated using ANOVA-tests (analysis of variance) and Chi-squared tests.

The relationship between the 8-oxodG- and 8-oxoGuo-levels and smoking status was further investigated by logistic regression. We made a new group of 'non-smokers' including never smokers and former smokers and performed logistic regressions for both types of nucleic acids comparing the new group of 'non-smokers' with the daily smokers. The study population was divided into low and high oxidizers corresponding to above or below the median of 8-oxodG levels (1.679 nmol/mmol creatinine) and 8-oxoGuo levels (2.739 nmol/mmol creatinine). Outcome was set to a binominal factor where levels of 8-oxodG or 8-oxoGuo above the median were 1 and levels below the median were 0. The logistic regressions in the study were adjusted for age, sex and body mass index (BMI). Due to eight missing values for BMI, 2713 type 2 diabetes patients were included in the adjusted analyses. The significance level in all analyses was set to $p < .05$. All primary data was registered in Microsoft excel 2010, and converted to a csv for import to R [17] and analyzed with R version 3.2.2.

Results

General characteristics

Table 1 shows the general characteristics of the study populations according to smoking status. The three groups were significantly different from each other in terms of gender, age, BMI, CRP, fasting p-HDL and urinary 8-oxodG.

Nucleic acid oxidation

The crude analysis showed a slightly significant difference between the three groups regarding the level of 8-oxodG excretions. In contrast, no differences were found for 8-oxoGuo urinary levels. Table 2 shows the results of the logistic regression as the OR of being in the group of high oxidizers with levels of 8-oxodG and 8-oxoGuo above the median. The influence of smoking was analyzed in daily smokers using former and never smokers as reference group. The logistic regression did not show any significant effect of smoking status on the level of 8-oxodG or 8-oxoGuo. However, it showed that sex, age and BMI had a significant effect on 8-oxodG and 8-oxoGuo levels (Table 2).

Discussion

We aimed to investigate if daily smokers with type 2 diabetes have higher levels of nucleic acid oxidation compared

Table 1. General characteristics of the populations.

	Missing values	Never smokers	Daily smokers	Former smokers	*p*-value
Sex (*n*)					
men	0	413 (45.0%)	300 (64.9%)	950 (70.8%)	<.001
women		505 (55.0%)	162 (35.1%)	391 (29.2%)	
Age (year)					
mean		62	61	64	
min	0	28	30	28	<.001
max		78	78	79	
BMI (kg/m^2)					
mean		30.96	29.88	31.06	
min	8	16.30	15.5	18.25	<.001
max		60.0	59.0	66.0	
CRP (mg/L)					
mean		3.23	4.09	3.91	
SD	9	5.11	6.77	7.44	.024
HbA1c (mmol/mol)					
mean	11	7.00	7.11	6.98	.070
SD		1.05	1.15	1.04	
Fasting p-HDL mmol/L					
mean	9	1.34	1.22	1.26	<.001
SD		0.35	0.37	0.35	
Urinary 8-oxodG (nmol/mmol creatinine)					
mean		1.92	1.82	1.79	
SD	0	0.83	0.78	0.82	.001
Urinary 8-oxoGuo (nmol/mmol creatinine)					
mean	0	2.99	2.92	2.96	.654
SD		0.99	1.18	1.38	

P-values were derived from ANOVA (continuous variables) and Chi-squared test (categorical variables) comparing all three groups. SD: standard deviation.

to never smokers and former smokers with type 2 diabetes. Studies have shown that in relation to smoking cessation, the effect on the excretion of oxidized DNA is obtained within 4 weeks [18]. Based on these results we concluded that the effect on excretion of 8-oxodG and 8-oxoGuo had reached steady state in the group of former smokers (minimum 6 months of smoking cessation). Therefore, we made a new group of 'non-smokers' including never smokers and former smokers.

The results of the logistic regression did not show any significant effect of smoking status on the level of 8-oxodG or 8-oxoGuo. However, a possible difference in urinary 8-oxodG or 8-oxoGuo between the daily smokers versus never smokers and former smokers could be blurred by the rather wide categories of smoking status. Therefore, more detailed information about smoking habits including detailed information regarding amount and duration of smoking could have contributed to a more accurate analysis of the effect of smoking. Another limitation of the study is the lack of matched controls without diabetes. This would have made it possible to investigate the effect of diabetes on the biomarkers and it would have given an opportunity to investigate whether or not the effect of smoking is attenuated due to the effect of diabetes on the oxidative stress.

Looking at Table 1, the groups differ in sex, age, BMI, CRP and p-HDL, which mean that these parameters are potential confounders. On the basis of biological considerations and the fact that former studies have shown influence of sex [19], age and BMI on the excretion of 8-oxodG [9,20] we *a priori* decided to adjust the logistic regression for these potential confounders. Different studies have shown various results concerning the effect of the potential confounders [4,9,20,21] and the effect of these parameters on the urinary excretion of 8-oxodG and especially 8-oxoGuo remains to be clarified. Further studies are needed to elaborate the knowledge about the interaction between biological parameters and the excretion of 8-oxodG and 8-oxoGuo.

Table 2. Odds ratios (ORs) with 95% CIs for being in the group of high oxidizers, investigating the influence of smoking. The adjusted analyses are adjusted for sex, age and BMI.

Logistic regression comparing daily smokers versus former and never smokers	Odds ratio (95% CI)	*p*-value
8-oxoGuo, unadjusted		
smoking	0.86 (0.70–1.05)	.132
8-oxoGuo, adjusted*		
smoking	1.00 (0.81–1.23)	.967
sex	0.45 (0.38–0.53)	<.001
age	1.05 (1.04–1.06)	<.001
BMI	1.02 (1.01–1.04)	<.001
8-oxodG, unadjusted		
smoking	1.02 (0.84–1.25)	.818
8-oxodG, adjusted*		
smoking	1.05 (0.85–1.28)	.672
sex	0.73 (0.62–0.85)	<.001
age	1.02 (1.01–1.03)	<.001
BMI	0.97 (0.96–0.98)	<.001

*8 missing values from BMI in the adjusted analyses, thus 2713 type 2 diabetes patients in the adjusted analyses.

Looking at the logistic regression in Table 2, there is a difference between the influence of BMI on 8-oxodG and 8-oxoGuo. For 8-oxodG, it indicates that the risk of being in the group of high oxidizers decreases with higher BMI. This is in line with the findings by Loft et al. [9] and Broedbaek et al., who found that people with type 2 diabetes having the highest excretion of 8-oxodG had lower BMI than the other groups with lower excretion of 8-oxodG [22]. However, our results suggest that the influence of BMI on 8-oxoGuo is opposite. Table 2 shows that it is more likely to be in the

group of high oxidizers if you have higher BMI which falls in line with a study of Cejvanovic et al., who found that obesity in men is associated with increased urinary excretion of 8-oxoGuo [23]. Further investigation in a general population of this potential difference between the urinary markers is needed.

Other studies have investigated the effect of smoking in diseased populations. Regarding DNA oxidation, Broedbaek et al. did not detect any effect of smoking status on the excretion of 8-oxodG divided into quartiles in a population of type 2 diabetic patients ($n = 1381$) [11]. Loft et al. only found a significant effect of smoking status on the excretion of 8-oxodG in the healthy control group in a study regarding breast cancer. That is, no significant effect of smoking status on 8-oxodG in the subjects suffering from breast cancer [24]. In addition, Loft et al. found no relationship between 8-hydroxyguanine excretion (the oxidized base which is also used as a marker of oxidative stress [25]) and smoking in a study of 481 lung cancer patients [26]. These findings, including our results, suggest that the effect of smoking on DNA oxidation in diseased populations might be attenuated by the effect of the disease, e.g. in cancer or diabetes.

Regarding RNA oxidation, Broedbaek et al. found that people with type 2 diabetes with the highest urinary excretion of 8-oxoGuo were more likely to be non-smokers than smokers [11,22]. In contrast to this, we were not able to find any significant difference in RNA oxidation between daily smokers and never and former-smokers. Further investigation of the relationship between 8-oxoGuo in daily smokers and never and former-smokers in a healthy population would be of relevance.

To expand the study area and thereby making it possible to compare our result to others, it would be relevant to investigate the effect of smoking on oxidative stress in populations suffering from other diseases than the ones mentioned above. This would give the opportunity to see if it is possible to demonstrate the effect of smoking seen in healthy populations or if this effect is attenuated by the disease as suggested by the present study.

Conclusion

Our study did not show any significant differences in urinary excretion of 8-oxodG or 8-oxoGuo in type 2 diabetes patients who were daily smokers compared with never smokers and former smokers. Since previous studies do find that smoking affects urinary excretion of 8-oxodG in healthy populations, we speculate that our findings might be a result of an attenuated effect of the oxidative damage to DNA in individuals with chronic conditions such as type 2 diabetes.

Due to sparse data, it is still too early to draw any final conclusions on the general effect of smoking on DNA and RNA-oxidation. Studies including both healthy participants and patients with chronic conditions should be performed in order to investigate the consequences of smoking on DNA and RNA oxidation.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Appendix

Materials and methods for analysis of 8-oxoGuo and 8-oxodG

Chemicals 8-oxoGuo was purchased from BioLog (Bremen, Germany) and 8-oxodG from Berry & Associates (Dexter, MI, USA). Internal standards, $^{15}N_5$-8-oxodG and $^{15}N_5$-8-oxoGuo, were produced in the lab by electrochemical oxidation [27], of $^{15}N_5$-dG and $^{15}N_5$-Guanosine purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

Acetonitrile (isocratic grade) were from Merck KgaA (Darmstadt, Germany). Methanol (HPLC-grade), lithium acetate dihydrate, acetic acid, and aqueous ammonia (25%) were all from Sigma-Aldrich (Steinheim, Germany). LC-MS Ultra Chromasolv water from Sigma-Aldrich was used for preparation of Litiumacetate-buffer and mobile phase. QC samples were prepared from a pool of urine samples and stored at −20 °C.

Sample preparation Urine samples were stored at −20 °C prior to analysis. The frozen urine was thawed, mixed, and heated to 37 °C for 5 min to re-dissolve possible precipitate and trapped analytes [28]. The heated samples were centrifuged at 10,000 *g* for 5 min. Calibration standards in the range 1.0–60.0 nM, and internal standard solution were prepared in 0.1 M Lithiumacetate, pH 6.4. The final sample preparation in 96-well plates was carried out fully automated at a Biomek 3000 (Beckman Coulter, CA, USA) by the mixing of (1) 90 µL 100 mM lithium acetate buffer, (2) 110-µL of urine/calibration standard/QC sample, and (3) 90 µL of internal standard solution.

Chromatography The chromatographic separation was performed on an Acquity UPLC I-class system (Waters, Milford, MA, USA). The column was an Acquity UPLC BEH Shield RP18 column (1.7 µm, 2.1 × 100 mm) protected with a VanGuard precolumn (BEH Shield RP18, 1,7 µm, 2,1 × 5 mm) both from Waters. The column was operated at 4 °C to focus the analytes at the front end during the injection of 50 µL sample volume. The mobile phase and gradient are shown in Table A1.

Mass spectrometry The MS detection was performed by a Xevo TQ-S triple quadrupole mass spectrometer from Waters, working in the negative ionization electrospray mode. MS-settings were optimized using the Intellistart function (MassLynx 4.1). A desolvation gas flow of 1000 L/h was applied, heated to 500 °C, to ensure a stable spray of the UPLC mobile phase containing less than 5% organic. To reduce contamination of the ion source, a switching valve diverted the mobile phase flow to waste, except the fraction at the time for elution of the target peaks (9–14 min). Detections were performed in the

Table A1. UPLC gradient program.

Time (min)	Flow (μL/min)	%A	%B
0.0	200	100	0
0.5	200	100	0
12.0	200	95	5
14.6	200	10	90
15.0	300	10	90
16.0	300	10	90
17.0	200	100	0
20.0	200	100	0

Eluent A: 5.0 mM ammoniumacetate, pH 5.
Eluent B: Acetonitrile

Table A2. Analytical parameters and mass transitions of the MS/MS detection.

			MRM mass transitions (collision energy)		
Analyte	Rt. (min.)	Ionization	Quantifier	Qualifier	Internal standard
8-oxoGuo	9.27	ESI (−)	298→208 (20 V)	298→165 (24 V)	303→213 (20 V)
8-oxodG	11.87	ESI (+)	284→168 (14 V)	284→140 (34 V)	289→173 (14 V)

multireaction monitoring mode (MRM). The MS/MS transitions and collision energies are reported in Table A2.

Quantification was based on the signal peak area from the transitions 298/208 (8-oxoGuo) and 284/168 (8-oxodG) relative to the signal peak area of the respective internal standards. The transitions 298/165 (8-oxoGuo) and 284/140 (8-oxodG) were applied as qualifier ions to confirm the presence of the analyte and absence of false contributions from co-elution of similar components in the urine sample. The signal ratio of the two fragment-ions (qualifier/quantifier) was calculated for each sample. According the tolerance guidelines [29], results were rejected if the fragment ratio in the urine samples deviated more than ±30% from the mean ratio of the actual standards.

Validation procedures The analytical method was validated in accordance with the FDA guidelines [30], including selectivity, accuracy, precision, linearity, ion suppression and LLOQ. Validation in respect to stability of samples and standards was performed in a previous study [31].

Method performance

Limit of quantification The lower limit of quantification (LLOQ) was 1.0 nM for both 8-oxoGuo and 8-oxodG, based on the quality requirements of CV <20%.

Linearity, precision, and accuracy Linear relationships were obtained for both analytes in the concentration range 1.0–200 nM, using a weighting factor of $1/x^2$. However, as the urine concentration of 8-oxoGuo and 8-oxodG rarely exceeds 60 nM, the applied calibration range is limited to 1–60 nM.

The *within-day* and *between-day* variations were estimated from three series of five human urine samples, all prepared in triplicate, covering the concentration range 1.1–43.5 nM. Variation at repeated analyses of the same sample was in average 0.9% for 8-oxoGuo and 8-oxodG ($n = 4$).

The average within-day precision was 2.3% for 8-oxoGuo and 3.8% for 8-oxodG, and the between-day precision 9.0% and 7.4%, respectively. As a measure of accuracy, the average recovery in fortified human urine samples was to 103.7% for 8-oxoGuo and 104.8% for 8-oxodG.