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Exocyclic DNA Adducts and Oxidative Stress Parameters: Useful Tools for Biomonitoring Exposure to Aldehydes in Smokers

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Abstract

Exocyclic DNA adducts are proposed as being potential biomarkers of cancer risk related to oxidative stress and exposure to aldehydes in smokers. In fact, aldehydes could potentially arise directly from tobacco combustion and endogenously by lipid peroxidation. The purpose of this study was to investigate the relationship between a profile of nine aldehydes induced DNA adducts and antioxidant activities in order to better characterize the exposure to systemic exposure to aldehydes. Adducts levels were quantified in the blood cells DNA of thirty-four active smokers using our previously published method for simultaneous analysis on UPLC-MS/MS of nine exocyclic DNA adducts deriving from eight exogenous and endogenous aldehydes. The antioxidant vitamins (A, C and E), coenzyme Q10, β-carotene, superoxide dismutase (SOD) and autoantibodies against oxidized low-density lipoprotein were measured. Adducts induced by tobacco smoking-related aldehydes (acrolein, crotonaldehyde (Croto), acetaldehyde (AA), formaldehyde (FA), malondialdehyde, 4-hydroxyl-nonenal, glyoxal and methylglyoxal) were quantified at levels reflecting an oxidative production from lipid peroxidation. We also observed a significant correlation between SOD and Croto-induced adducts ($p = 0.0251$). β-carotene was negatively correlated with adducts of FA and AA ($p = 0.0351$; $p = 0.0413$, respectively). Vitamin C tended to inversely correlate with AA induced adducts ($p = 0.0584$). These first results are promising and the study is now conducted on larger number of smokers with the perspective of evaluating the impact of smoking cessation program on the evolution of adducts profile and antioxidants activities.

Keywords: aldehydes; biomarkers; DNA adducts; tobacco smoke; oxidative stress; antioxidants

Introduction

Tobacco smoking continues to be a major health hazard; it represents an important risk factor for numerous diseases affecting many biological systems. In fact, cigarette smoking is well-known to promote alkylating agents and reactive oxygen species (ROS) that can damage DNA to form mutagenic lesions. Among these reactive species, aldehydes originating from pollutant exposures or endogenous processes are the main compounds implicated in the process of carcinogenesis and various diseases related to stress tobacco-induced, including for example cardiovascular diseases [1-5].

Cigarette smoke is a major supplier of aldehydes directly by exposure to its vapor phase components. High levels of carbonyls in particular acetaldehyde (AA), acrolein (Acro), formaldehyde (FA), propanal, methylglyoxal (MG) and glyoxal (Gx) have been detected in mainstream cigarette smoke [6] where these compounds are formed by nonenzymatic browning reactions [7]. In addition, many studies showed the involvement of cigarette smoke in the oxidative stress mostly generated by an imbalance between the production of ROS and the antioxidant defense systems, causing cell death and serious tissue damages, some of which may participate in the carcinogenesis and/or inflammatory pathologies [8,9]. The increased production of ROS leads to lipid peroxidation (LPO) of polyunsaturated fatty acids and subsequently the generation of a complex mixture of phospholipid products including hydroperoxides which can decompose leading to aldehydes like Acro and crotonaldehyde (Croto) but also malondialdehyde (MDA) and 4-hydroxy-nonenal (HNE), the two major LPO derivatives [10, 12]. In the same way, endogenous sources such as the cell aerobic metabolism, inflammation, or internal exposition to a variety of chemicals are widely involved in the production of aldehydes via LPO [13].

Aldehydes from exogenous and endogenous sources are well known as highly reactive electrophilic compounds which diffuse into the cellular medium and damage not only the proteins but also the nucleosides generating exocyclic DNA adducts with the risk of evolving into mutations [1, 2,14]. Aldehydes could therefore not be measured properly as free compounds in vivo. However, the corresponding exocyclic DNA adducts are more stable and can be measured to quantify internal/exposure dose [15]. Many studies have investigated the application of DNA adducts induced by LPO as potential biomarkers for the assessment of cancer risk related to oxidative stress [16] and tobacco smoke in humans [12]. In fact, DNA exocyclic adducts induced by aldehydes have already been detected at significantly high levels in bronchoalveolar cells, in bronchial and pulmonary epithelia of smokers [17-19] as well as in saliva, buccal cells and sputum of healthy smokers [20, 21].

Also, significant variations between smokers and non-smokers adduct levels were observed in various tissues such as DNA adducts derived from AA, Acro and Croto detected at higher levels in human buccal cells, sputum and lung tissues of smokers comparing to non-smokers samples [21]. Similarly in our previous study, nine adducts derived from eight aldehydes represented in figure 1 were detected and quantified in ten human blood samples with significant differences in adduct mean levels between smokers and non-smokers [22].

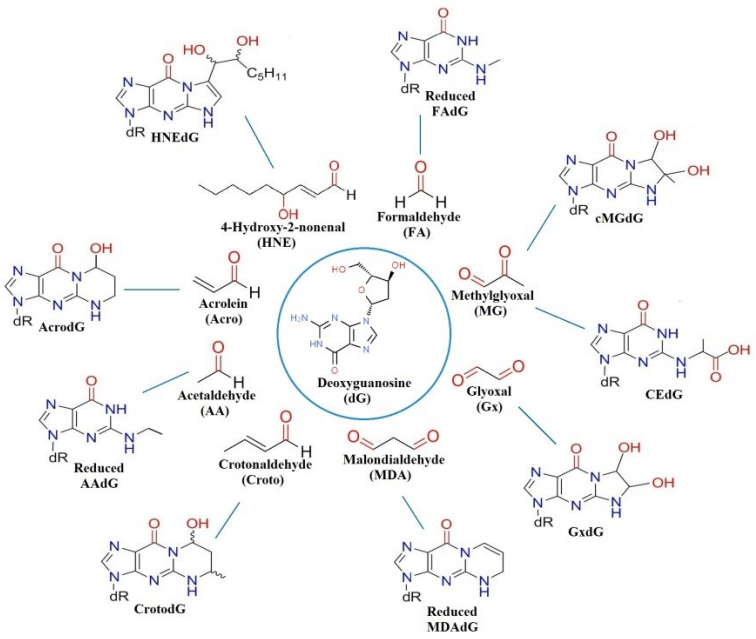

Figure 1: Structures of the 9 exocyclic DNA adducts detected and quantified in human blood by our analytical method [22].

Concerning oxidative stress-induced imbalance, it has been reported that people notably smokers, with low blood levels of antioxidants (superoxide dismutase and vitamins A, C or E) or high levels of lipids or DNA oxidation markers, have a higher risk of developing cardiovascular disease or cancer than people with normal antioxidant balance [23, 24].

Given the often-low concentration of biomarkers in biological samples, a very sensitive analytical method is required, preceded by a suitable sample preparation technique. Over the years, analyzing oxidative stress biomarkers with liquid chromatography coupled to mass spectrometry (LC-MS) methods has become more and more interesting as mass spectrometers become more sensitive [25]. So, in a previous work, we have developed and validated a UPLC-MS/MS method for the simultaneous quantification of nine exocyclic DNA adducts [26]. The method proved to be sensitive and accurate enough for low-level quantification of aldehydes adducts in the blood of two healthy populations of five smokers and five non-smokers in order to evaluate the effect of tobacco exposure on the profile of adducts [22]. Despite the small number of samples severely limiting the previous analysis, correlations were observed between the levels of exocyclic DNA adducts and the main smoking-related markers. Since the epidemiologic data concerning endogenous oxidative stress were unknown in the previous work, we aimed here to evaluate the levels of antioxidants and establish relationships with exocyclic DNA adducts in larger cohorts of smokers.

Materials and Methods

Study population

Thirty-four active smokers were recruited at the Tabacology unit of Namur University Hospital in Belgium after giving their written warranty statement on the use of samples for this research and the confidentiality of personal information. Study subjects are monitored with a regular analysis of the biological parameters during smoking cessation program. The smoking histories of these subjects were based on their files reporting clinical data such as age, sex, height, weight, medication, medical/surgical history, diet, smoking status and tobacco dependencies. The study was approved by the Scientific and Ethics Committee of the Namur University Hospital (NUB: B039201316167).

Smoking status and oxidative stress estimation

Measurements of smoking and oxidative stress markers were realized at baseline before the smoking cessation program starts. The exhaled carbon monoxide (CO) amount was determined by electrochemistry with a Smokerlyzer® monitor (Bedfont Scientific Ltd, Harrietsham, UK). Creatinine in urine was assessed by an enzymatic method (VITROS CREA Slide for VITROS 5600 Integrated System, Ortho Clinical Diagnostics, High Wycombe, UK). Urinary cotinine concentration was measured by an ultra-high performance liquid chromatography (UPLC, Acquity, Waters, Milford, USA) and mass detection (QDa detector, Acquity, Waters) [27]. As for oxidative stress markers, vitamin C in plasma, coenzyme Q10 and β-carotene in serum were quantified by Chromsystems kits (Munich, Germany) and a high-performance liquid chromatography (HPLC) system connected to an ultraviolet (UV) detector (alliance 2695, detector 2489, Waters). The Chromsystems Vitamins A and E kit was used to treat plasma samples. Treated samples were then injected on an UHPLC reverse phase column (Acquity UPLC HSS T3 1.8 µm 2.1 x 50 mm, Waters) with a flow rate of 0.6 ml/min and photodiode array detection (Acquity, Waters). All chromatographic data were acquired and processed by Empower 3 (Waters). Superoxide dismutase (SOD) was analyzed in EDTA-treated blood by the colorimetric activity kit RANSOD (Randox, Crumlin, UK) and the VITROS 5600 System (Ortho Clinical Diagnostics). Enzyme immunoassay was performed for the determination of Ab oxy-LDL, human IgG autoantibodies against oxidized low-density lipoprotein in serum (OLAB IgG kit, Biomedica, Vienna, Austria).

DNA extraction from blood cells and adducts analysis

Five milliliters of venous blood were collected from smokers (n = 34) in a BD Vacutainer® spray-coated K_2EDTA tube and immediately frozen at -80° C. The method for purifying genomic DNA from smokers' blood was the same described in our previous work [22] using Macherey-Nagel kit (Macherey-Nagel, Nucleobond® CB 100).

To detect and quantify exocyclic DNA adducts in smokers blood, the isolated DNA was dissolved in ultrapure water (1 mg/mL) in the presence of $NaBH_3CN$ in order to reduce adducts of AA, FA and MDA to their most stable forms (see Figure 1 for adducts structures). Samples were then enzymatically hydrolyzed prior to analysis on LC-MS/MS according to our recently developed and validated method [26].

The level of each DNA adducts, expressed in adducts per 10^7 normal nucleotides, was calculated using the following formula [20]:

$$RelativeAdductlevel = \frac{(DNAadductConcentration/MWofDNAadduct)}{(DNAConcentration/meanMWofbases)}$$

Statistical Data Analysis

All statistical analyses were performed using Student t-test or Wilcoxon test by SAS software (Version 9.4) and the level of statistical significance was $p < 0.05$. The categorical variables were shown in terms of frequencies, while for continuous variables the means, standard deviations and quartiles were represented. Spearman's correlation was used to define relations between adducts levels (AcrodG, CrotodG, reduced AAdG, reduced FAdG, reduced MDAdG, HNEdG, GxdG, CEdG, and cMGdG), the markers of oxidative stress (SOD, Vitamins A, E and C, coenzyme Q10, β-carotene, and Ab oxy-LDL) and tobacco smoking-related markers (CO in exhaled air and urinary cotinine concentration reported over creatinine).

Results and Discussion

The population study comprised eighteen female and sixteen male smokers aged 25 to 72 years. Simple statistics of the carried-out analysis and measurements are shown in Table 1.

Table 1: Simple statistics of all the analysis and measurements realized among thirty-four current smokers.

Variable (n=34)	Mean	± S.D.	Median	Minimum	Maximum
Age (years)	51.85	± 13.05	54	25	72
Creatinine (mg/dL)	89.87	± 56.72	89.15	19.50	241.20
Cotinine/Creatinine	1939	± 1375	1463	419.60	5924
CO (ppm)	18.53	± 6.50	18	5	34
SOD (U/mL)	219.26	± 28.64	218.20	141.60	304.70
Vitamin A (mg/L)	0.62	± 0.26	0.56	0.23	1.19
Vitamin E (mg/L)	15.14	± 4.67	14.23	8.20	24.67
Vitamin C (mg/L)	9.80	± 4.23	9.62	1.92	20.71
Coenzyme Q10 (µg/L)	869.18	± 398.78	792.81	324.41	1926
β-carotene (ng/mL)	162.32	± 90.79	147.55	20.95	374.41
Ab oxy-LDL (mU/mL)	229.43	± 399.01	112.15	13.90	2237
AcrodG/10^7 normal nucleotides	19.47	± 37.80	1.99	0	140.76
CrotodG/10^7 normal nucleotides	56.94	± 38.80	48.96	0	166.59
Reduced AAdG/10^7 normal nucleotides	2.70	± 4.16	0.91	0	19.75
Reduced FAdG/10^7 normal nucleotides	5.80	± 6.59	2.10	0.10	25.25
Reduced MDAdG/10^7 normal nucleotides	35.62	± 34.55	25.30	4.11	137.22
HNEdG/10^7 normal nucleotides	0.98	± 0.71	0.71	0.34	2.94
GxdG/10^7 normal nucleotides	72.90	± 87.46	31.59	3.41	345.42
CEdG/10^7 normal nucleotides	221.78	± 118.09	214.04	48.04	642.30
cMGdG/10^7 normal nucleotides	176.27	± 163.77	107.67	21.21	649.19

Cigarette smoke contains significant amounts of aldehydes already reported in the literature [6, 28]. In order to better estimate the true smoking status of subjects, biological markers of tobacco exposure have been used, in particular the concentrations of cotinine, major metabolite of nicotine and the level of CO in exhaled air [29, 30]. Cutoff values were reported according to the literature to distinguish current smokers from non-smokers. Knowing that urine is readily obtained and metabolites are generally present in sufficient quantities for reliable quantification, cotinine concentration was reported to creatinine concentration in urine. The latter ranged between 19.50 and 241.20 mg/dL, which is among the acceptable values and under the reference limit of 300 mg/dL according to the WHO. So, urinary cotinine levels - reported over creatinine concentrations - confirmed that all the subjects in the study were active smokers. With an average of 1939, the ratio ranged between 419.60 and 5924, which are way higher than the cutoff value of cotinine previously reported at 100 [31]. As for CO measurements in exhaled air, the average level of 18.53 ±6.50 ppm was consistent with our previously reported level of 18.00 ±8.46 ppm in 5 active smokers [22].

The obtained mean CO level in our present study largely exceeded the cutoff value reported at 6.5 ppm, and was consistent with a previous study conducted on 243 healthy active smokers in which the mean exhaled CO level was 17.13 ±8.5 ppm measured also by a Smokerlyser [30]. Since both smoking-related markers confirmed separately the inclusion of active smokers taking into consideration the cutoff values, statistical test was then conducted to assess the relationship between these two markers. The analysis showed a correlation between exhaled CO levels and urinary cotinine concentrations (Spearman's correlation, $r = 0.40709$, $p = 0.0256$). This result highlighted the intensity and impregnation of smoking in our population and consequently suggests a high level of exposure to aldehydes.

Mechanistically, these reactive compounds released during tobacco combustion cause a set of cellular abnormalities, including DNA damage, inflammation, and oxidative stress. Concerning DNA adduction, we carried on to observe a profile of aldehydes DNA adducts at different levels in a larger number of smokers samples compared to our previous study [22]. The adducts GxdG, CEdG and cMGdG induced by Gx and MG, aldehydes produced by excessive intake of dietary sugars [32] , were the most abundant in all samples. And, it is well-known that nicotine influences the metabolism of glucose by enhancing the production of cortisol that is a hyperglycemic hormone, in blood [33]. So, the high levels of Gx and MG-induced adducts might be indirectly related with the hyperglycemic activity of tobacco nicotine. AcrodG levels were relatively high but CrotodG prevailed mostly over the rest adducts. These observations were consistent with a previous study reporting that adducts induced by Croto are predominant compared to those induced by Acro in normal lung tissues of tobacco smoking lung cancer patients [21]. The authors also demonstrated that AcrodG and CrotodG are the major adducts formed in buccal cells and lung tissues of tobacco smokers. In this respect, it is noteworthy that Acro and Croto are the main aldehydes present in cigarette smoke among other aldehydes. In the same way, the relatively high levels of reduced adducts of AA, FA and MDA could be due to the proportion of the corresponding aldehydes in tobacco smoke. HNEdG remained at low levels in smokers samples, whether in the present or the previous study [22]. Indeed, HNE is extremely reactive mainly towards plasma proteins and cytosolic glutathione. So, there are few HNE molecules that reach the nucleus and cause damage to DNA [14].These aldehydes as well as their DNA adducts are also formed through endogenous lipid peroxidation arising from oxidative stress [2,34]. It is for this reason that we evaluated antioxidant parameters and established correlations with the levels of detected DNA adducts.

Many studies have demonstrated that oxidative status parameters were higher in smokers than in non-smokers while the antioxidant parameters were significantly increased in non-smokers [5]. A previous study on animal models showed that cigarette smoke exposure reduces antioxidant defense by a decreased activity of SOD and an increased LPO [24]. SOD is an important endogenous antioxidant enzyme which acts as the first-line defense system against ROS. Its concentration in our study subjects ranged from 141.60 to 304.70 U/mL which seemed to remain very low in comparison to previous reported concentrations in non-smoking healthy males [35]. Antioxidant vitamins, such as vitamin A, C, and E as well as β-carotene are effective free radical scavengers and can also be useful markers of antioxidant status. Presumably, a higher production of ROS due to severe oxidative stress induced by tobacco smoke, could lead to a higher metabolic consumption of the antioxidant vitamins, and this would be reflected in their lower serum levels [36] like the very low levels of vitamin A obtained in our study (mean 0.62 ±0.26 mg/L). Besides, β-carotene could be converted to vitamin A in the intestine and liver, and smokers are known to have lower plasma levels of β-carotene [37]. Its average concentration in our study was of 162.32 ±90.79 ng/mL that is below previous reported concentration of 257.65 ±128.82 ng/mL in 28 non-smoking healthy males [38]. Regarding vitamin C, it is a powerful antioxidant by acting directly on peroxyl radicals or indirectly by boosting the antioxidant properties of vitamin E [36]. Lower plasma levels of vitamin C were found in smokers [37]. Actually, vitamin C level in non-smokers was previously reported to be 4.3 times more than in smokers [39]. Our obtained levels were relatively low ranging from 1.92 to 20.71 mg/L. Vitamin E is another parameter reflecting altered endothelial function and redox status by reducing the innate antioxidant response. In our study, the vitamin E levels ranged from 8.20 to 24.67 mg/L with a median of 14.23 mg/L. This result was close to a previous study on 72 healthy smokers with median of 16 mg/L and a range of 8 to 128 mg/L [39].

Besides, the authors reported significantly higher levels of vitamin E in the group of 82 non-smokers than in the smokers group (median 67 mg/L, range 12-141 mg/L). In addition, Carnevale et al. [40] reported a decrease in vitamin E levels in 20 smokers, right after smoking cigarettes. Coenzyme Q10 has also an antioxidant role in the plasma membrane and other intracellular membranes, protecting the phospholipids from peroxidation [25] In our study subjects, coenzyme Q10 was found at an average of 869.18 ±398.78 μg/L. Lee and coworkers [41] have found significant lower plasma coenzyme Q10 and higher SOD levels in subjects with coronary artery disease compared to control group. The authors also examined a significantly negative correlation between the plasma coenzyme Q10 concentration and smoking habits.

In smokers, low-density lipoprotein (LDL) is more susceptible to oxidation by excessive ROS presence, thus increasing levels of Ab oxy-LDL [25]. In healthy subjects, no significant difference was ever reported between control and smokers. However, moderately increased levels of Ab oxy-LDL Ab oxy-LDL were noted in smokers or hypercholesterolemic patients [42]. In the present study, Ab oxy-LDL ranged starting 13.90 mU/mL and reached 2237 mU/mL among smokers.

Finally, the statistical analyses performed here showed a significant correlation between CrotodG levels and SOD (Spearman's correlation, $r = 0.38368$, $p = 0.0251$) while AcrodG levels tended to correlate with SOD (Spearman's correlation, $r = 0.32842$, $p = 0.0579$). Moreover, β-carotene levels were inversely correlated to reduced FAdG levels (Spearman's correlation, $r = -0.36257$, $p = 0.0351$) and reduced AAdG levels (Spearman's correlation, $r = -0.35175$, $p = 0.0413$). Similarly, Vitamin C level tended to inversely correlate with reduced AAdG levels (Spearman's correlation, $r = -0.33290$, $p = 0.0584$). However, no significant correlation was obtained between vitamin E, Ab oxy-LDL or coenzyme Q10 and any other specific marker. Also, statistical tests showed no correlation of Sex and age with adducts and antioxidants levels.

Even if the whole profile of exocyclic adducts in DNA extracted from the blood cells of smokers strongly seem to reflect the contribution of genotoxic effects of aldehydes, which are partly released by tobacco and oxidative stress, the interpretation of all obtained results must be done with extreme caution for several reasons: i) the precise effects of antioxidants on mutagenesis and carcinogenesis remain unclear; ii) an antioxidant is essentially a redox agent that provides protection against free radicals, but may promote free radical generation under certain circumstances or may exert pro-oxidant effects [36], iii) the presence of a synergetic effect between vitamins [23], iv) the influence of confounding factors not considered here such as lifestyle and diet rich in antioxidants through vegetables and fruits [43] and v) the relatively small sample size. At this step, the relation between the concentrations of antioxidants and exocyclic DNA adducts in the blood should be carried on larger cohort in the aim to examine if there are representative measures of the situation in the target tissue of the carcinogenesis and a true reflection of overall oxidative cellular DNA damage.

Conclusion

It is admitted that smokers are exposed to a pattern of aldehydes generated by tobacco combustion but also by oxidative stress. In our study, exocyclic DNA adducts induced by aldehydes are proposed as being potential biomarkers of cancer risk related to oxidative stress. We find the profile of 9 adducts evolving towards a preponderance of those certainly generated by the systemic intake of tobacco with predominance for adducts induced by aldehydes found in the vapor phase: CrotodG > AcrodG > FAdG > AAdG. It is noteworthy that adducts induced by LPO major derivatives (HNE and MDA) and by hyperglycemic activity (Gx and MG) were also detected and quantified at levels similar to those reported in our previous work. Although this study is limited by the relatively small population, we hereby provide the first correlative data of smoking impact on genotoxic damage and oxidative stress. As a perspective, further analysis monitoring subjects during smoking cessation are required to assess the use of this profile of exocyclic DNA adducts as a specific biomarker of systemic exposure to aldehydes in oxidative environments.

Author Contributions

Conceptualization, M.L. and M.VDS.; methodology, M.L., H.A. and L.G.; formal analysis, N.H. and M-L.C.; investigation, H.A. and M-L.C.; resources, M.L and L.G.; data curation, M.L. and L.G.; writing—original draft preparation, H.A.; writing—review and editing, M.L.; visualization, H.A.; supervision, M.L. and L.G.; project administration, M.L. and L.G.; funding acquisition, M.L. and H.A. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was approved by the Scientific and Ethics Committee of CHU UCL Namur, Belgium (NUB: B039201316167).

Informed Consent Statement

Written informed consent was obtained from all subjects involved in the study.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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