BIOMARKERS, 2000, VOL. 5, NO. 3, 182–191

DNA adducts in human placenta as biomarkers for environmental pollution, analysed by the ^{32}P-HPLC method

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Received 21 June 1999, revised form accepted 29 November 1999

During pregnancy, mothers are exposed to complex chemical mixtures, such as air pollution and smoke from incomplete combustion. In this study DNA adducts were measured in human placentas from 29 mothers. Environmental exposure and several possible biomarkers in relation to levels of DNA adducts were measured. Placental aromatic and bulky DNA adducts were measured with the ^{32}P-HPLC method. Mothers living in an urban area in Estonia had significantly higher levels of DNA adducts compared with mothers living in a rural area of Estonia and in Switzerland (19.38 ± 6.91 per 10^8 normal nucleotides [NN] vs 10.35 ± 6.87 10^8 NN and 8.09 ± 6.59 10^8 NN, respectively; $P < 0.01$). No significant correlation was found between smoking and DNA adduct levels for any of the subjects. Among the potential biomarkers tested, a possible correlation was found between DNA adducts and placental copper in the environmentally polluted area, and a correlation was found between DNA adducts and glutathione S-transferase (GST) activity for Swiss mothers abusing drugs. The levels of DNA adducts in the drug abusers were not higher than those of the controls; in fact they were lower. These data suggest that the presence of DNA adducts in human placenta is an environmental biomarker for polluted areas. A further conclusion is that air pollutants to which pregnant women are exposed can induce DNA damage in the placenta and consequently risk exposing the foetus to genotoxins.

Keywords: biomarker, DNA adducts, human placenta, ^{32}P-HPLC.

Abbreviations: [^{32}P]ATP, adenosine 5′-[gamma-^{32}P]triphosphate; GST, glutathione S-transferase; HPLC, high performance liquid chromatography; MN, micrococcal nuclease; NN, normal nucleotides; PAH, polycyclic aromatic hydrocarbons; SDS, sodium dodecyl sulphate; SPD, spleen phosphodiesterase; Tris, tris[hydroxymethyl] aminoethane.

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Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online © 2000 Taylo
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Introduction

In addition to expressing genetic variability, humans are chronically exposed to low doses of complex chemical mixtures, many of which are mutagens and carcinogens. The origins of these chemical mixtures are mostly from incomplete combustion, that is, smoke, air pollutants, and pyrolysis products (Warren and Shields 1997). A number of these substances bind covalently to DNA and form DNA adducts (Lutz 1979, Zeisig and Möller 1995), which are the net effect of exogenous carcinogen exposure and inherited traits for absorption, metabolism, and DNA repair (Warren and Shields 1997). DNA adducts are known biomarkers in epidemiological investigations of exposed populations (Perera *et al.* 1982), and they are biologically linked to chemical exposure, tumour formation (Cui *et al.* 1995), and clinically-observed cancer (Warren and Shields 1997). Analysis of biomarkers is done to improve the evaluation of the risk factors and mechanisms responsible for cancer (Perera 1996).

Placenta is a potentially important source of tissue for molecular studies in humans because it is large, readily available, and responsive to maternal exposure to environmental pollutants (Manchester *et al.* 1988). Studies have shown that rats exposed to carcinogens had already formed high levels of DNA adducts in certain tissues within the first days of exposure, and after up to 2 years the same rats had a high incidence of tumours in these tissues. Low levels of DNA adducts in response to exposure also resulted in a low incidence or absence of tumours. This means that early exposure may be crucial for determining what happens later in life (Cui *et al.* 1995). It has been suggested that the presence of DNA adducts in foetal tissue may predispose the individual to develop a serious disease later in life (Hansen *et al.* 1993). Hansen *et al.* (1993) also showed maternal transfer of carcinogens present in cigarette smoke to foetal tissue and that placenta can metabolize the carcinogens into DNA-binding metabolites. A correlation between smoking and DNA adducts has been found in placenta (Everson *et al.* 1988, Topinka *et al.* 1997) and different compounds found in cigarette smoke have been found to form DNA adducts, for example, benzo(*a*)pyrene (Manchester *et al.* 1988, Zeisig and Möller 1995, Arnould *et al.* 1997). 4-Aminobiphenyl, also present in smoke, has been shown to form haemoglobin-adducts in maternal and foetal blood samples (Myers *et al.* 1996). Other studies show no difference between smokers and non-smokers in forming DNA adducts (Daube *et al* 1997, Ichiba *et al.* 1998, Whyatt *et al.* 1998a).

A correlation has been shown between the presence of DNA adducts and people living in polluted areas in Poland, as measured in placenta and lymphocytes (Perera *et al.* 1992, Möller *et al.* 1996, Whyatt *et al.* 1998b). The conclusion Whyatt *et al.* drew was that genetic damage in new-borns was associated with maternal environmental exposure. Correlations were also found between a GSTM1-negative genotype and the formation of DNA adducts in polluted areas by Topinka *et al.* (1997), but not by Whyatt *et al.* (1998b) or Nielsen *et al.* (1996).

The aim of this study was to analyse levels of placental DNA adducts, activities of placental xenobiotic-metabolizing enzymes, and placental concentrations of heavy metals in mothers at term living in urban and rural areas in order to determine whether any of these factors were related and could serve as environmental biomarkers.

Material and methods

Placental samples

Human placental samples were collected from three distinct populations: (1) residents of a rural area in Puru, Estonia; (2) residents of a polluted area in Narva, Estonia, with industry and oil shale; and (3) a population of mothers in Zurich, Switzerland. The study plan was congruent with the Helsinki Declaration and was accepted by the local Ethics Committee. The personal questionnaire included detailed clinical data such as location of residence and duration of the residence, occupation, lifestyle and anamnestic data of general health status including the use of any herbal or medicinal drugs.

The placentas were collected immediately after delivery. Connective tissue and coagulated blood were removed, and the placental tissue was rinsed in cold 0.9% NaCl solution and dried gently between paper towels. Small pieces of tissue (about 10–50 g) were taken from the central parts of the placentas and immersed in liquid nitrogen. All samples were stored at –70 °C until subcellular fractions were prepared (within 2 weeks) by a standard ultracentrifugation technique. Microsomes were isolated in a buffer containing 150 mM KCl and 10 mM EDTA (pH 7.4) and suspended in a buffer containing 100 mM potassium phosphate, 1 mM EDTA and 20% glycerol (pH 7.4) (Honkakoski and Lang 1989). After the suspension was centrifuged at 100 000 × *g*, the supernatant was used for glutathione S-transferase (GST) and NADPH:quinone oxidoreductase (NQO) determinations.

Enzyme assays

Microsomal protein concentrations were determined according to Bradford (Bradford 1976). Among CYP mediated analysis the following enzymatic assays were performed; 7-ethoxycoumarin O-deethylase activity (ECOD) – a general marker for polycyclic aromatic hydrocarbon inducible forms in placenta (Pasanen 1999) – was measured by the method of Greenlee and Poland (1978) using 0.5 mM 7-ethoxycoumarin as a substrate. The activity of 7-ethoxyresorufin O-deethylase (EROD; 0.1 mM substrate), which detects mainly CYP1A1 in human placenta (Pasanen 1999), was determined according to Burke *et al*. (1985). Aromatase activity (CYP19) was measured according to the method of Pasanen (1985). NQO activity was recorded at 600 nm as the dicoumarol-sensitive reduction of 2,6-dichlorophenolindophenol (final conc. 0.04 mM) with NADPH as the electron donor. The assay mixture (1.2 ml of buffer containing 40 mM Tris–HCl and 0.8% Triton X-100, pH 7.5) which contained 0.3–1.0 mg cytosolic (100 000 × *g* supernatant) protein from placental samples was preincubated (30 °C for 5 min) before addition of NADPH (final conc. 0.17 mM) followed by measurement in a Shimadzu double-beam spectrophotometer (Elovaara *et al*. 1977). GST activity for 1-chloro-2,4-dinitrobenzene (final conc. 1.0 mM) – a general marker substrate for GST activity – was recorded spectrophotometrically at room temperature (20 °C) according to Habig *et al*. (1974) using 0.04–0.12 mg cytosolic (100 000 × *g* supernatant) protein from placental samples in a 2.5-ml assay mixture. All enzyme assays were performed as duplicates in a double-blind manner.

Chemical analysis

For the heavy metal and selenium analysis, the placental samples were cut into pieces, and the outer surface was removed using titanium tools. To remove the blood, the 20-g samples from the full-term placentas were rinsed twice with ultrapure water (Millipore Milli-Q RO15, Millipore, Molsheim, France). The samples were lyophilized, and homogenization was performed in agate vessels. The homogenized material (50 mg) was used for the digestion, which was performed in pressurized, closed Teflon vessels with 0.5 ml nitric acid, using a microwave oven (MDS-18D, CEM Corp., Mathews, VA, USA). The digested samples were diluted with 0.01% surfactant. Copper and zinc were analysed by flame AAS, for cadmium and selenium analyses a graphite furnace AAS was used (Perkin-Elmer Zeeman 5000, Bodenseewerk Perkin Elmer GmbH, Norwalk, CT, USA). The results from certified reference samples of bovine liver (Bovine liver, NBS 1577a) included in analytical runs were 153.5±4.7 [$\mu g\ g^{-1}$] ($n = 7$; recommended 158 ± 7).

Serum cotinine was determined by the method of Kolonen and Puhakainen (1991) with minor modifications. Analyses were carried out on a programmable HPLC (Waters 600 E, Waters, Milford, MA, USA) with UV- detection (Schoeffel Instrument Group, Labtronic Ltd, Vantaa, Finland) and equipped with a Rheodyne 7125 (Rheodyne, Cotati, CA, USA) injector. Separation was carried out with a silica column (Spherisorb S5 ODS2, 25 cm × 4.6 mm; Phase Separation Ltd, Deeside Industrial Park, UK) using buffer containing 40% methanol and 60% 0.1 M phosphate (pH 4.5) and a flow rate of 0.5 ml min^{-1}. Benzimidazole was used as an internal standard. All samples were stored at –80 °C until they were analysed.

DNA preparation

Tissue was homogenized in 10 ml g^{-1} tissue of SDS–EDTA buffer (1% SDS, 1 mM ethylendiaminetetraacetic acid disodium salt) and 240 µl g^{-1} tissue of Tris–HCl buffer (1 M Tris–HCl, sodium hydroxide to pH 7.4) at 0 °C. DNA was isolated by digestion with 240 µl (120 units) g^{-1} tissue of RNase A (Boehringer Mannheim GmbH, Mannheim, Germany) (10 mg ml^{-1} ribonuclease from bovine pancreas, heat-inactivated by a 3-min incubation at 100 °C, in 50 mM Tris–HCl buffer, pH 7.4) and 80 µl g^{-1} tissue of RNase T1 (Sigma Chemical, Cleveland, OH, USA) (5 U μl^{-1} ribonuclease T1 from *Aspergillus oryzae* in 50 mM Tris–HCl buffer, pH 7.4) for 2 h at 37 °C. Then, 600 µl g^{-1} tissue of protease (10 mg ml^{-1} protease from *Streptomyces griseus*, heat-inactivated by a 2 h incubation at 37 °C, in 50 mM Tris–HCl buffer, pH 7.4) was added, and the mixture was incubated for 30 min at 37 °C. The mixture was then extracted with 10 ml g^{-1} tissue of phenol (saturated with 50 mM Tris–HCl buffer pH 7.4) and centrifuged at 4000 × *g* for 10 min. The aqueous phase was then extracted with 10 ml g^{-1} tissue of phenol-sevag (50% phenol, 48% chloroform, 2% isoamyl alcohol) and centrifuged at 4000 × *g* for 10 min. Following this, the aqueous phase was extracted with 10 ml g^{-1} tissue of sevag (96% chloroform, 4% isoamyl alcohol) and centrifuged at 4000 × *g* for 10 min. The DNA was precipitated with 0.1 volume 5 M NaCl and 1 volume ethanol. After centrifugation and a wash with 70% ethanol, the DNA was redissolved in water (1 ml g^{-1} tissue). Finally, the DNA concentration and purity were measured by ultraviolet spectrometry. The DNA was dried through evaporation in a Speedvac (VLP120) (Savant Instruments, Holbrook, NY, USA) and stored in a non-hydrolysed form at –80 °C until it was analysed.

Aliquots of 10 µg DNA dissolved in water (to 0.5 µg μl^{-1}) were hydrolysed by the addition of 80 mU μg^{-1} DNA micrococcal nuclease (0.2 U μl^{-1} from *Staphylococcus aureus* Foggis strain) (Sigma Chemical, Cleveland, OH, USA), 5 µl of 3 mM bicine (pH 9.0), and 5 µl of 0.5 mM $CaCl_2$ and incubated at 37 °C for 2 h. Then, 1.6 mU μg^{-1} DNA of SPD (Boehringer Mannheim GmbH, Mannheim, Germany) (1 mU μl^{-1} dialysed phosphodiesterase from calf spleen in 10 mM ammonium acetate, pH 5.0) was added, and the mixture was incubated for 2 h at 37 °C. The DNA and nucleotide samples were adduct-enriched by butanol extraction and then enzymatically ^{32}P-postlabelled (Möller and Zeisig 1993, Möller *et al.* 1993). The 5′-[γ-^{32}P]triphosphate [^{32}P]ATP, with an activity of 3000 Ci $mmol^{-1}$ was obtained from Amersham International, Little Chalfont, UK.

^{32}P-HPLC

^{32}P-HPLC analyses were performed by injecting the buthanol-extracted, unrefined ^{32}P-postlabelled mixture (40 µg of DNA) into the HPLC column and eluting with 2 M ammonium formate, 0.4 M formic acid (pH 4.5) at a rate of 0.5 ml min^{-1} on a linear gradient of 0 to 35% acetonitrile (0 to 70 min). The HPLC system consisted of a DeltaPak™ 5 µ C18-100A, 150 × 3.9 mm i.d. (Waters, Millipore Co., Milford, MA, USA) as the main column and an on-line *A*280 radioactivity detector (Radiomatic Instruments & Chemical Co., Tampa, FL, USA). The ^{32}P-HPLC method is described in detail elsewhere (Möller and Zeisig 1993, Möller *et al.* 1993).

Results

The total level of aromatic DNA adducts in the placental tissue ($n = 9$) from Estonian mothers living in Narva, a polluted area, was 19.38±6.91 per 10^8 normal nucleotides [NN]. The placental tissue from mothers living in Puru ($n = 13$), a rural area in Estonia, had a mean DNA adduct level of 10.35±6.87 10^8 NN, and the placental tissue from mothers living in Zurich, Switzerland ($n = 7$) had a level of 8.09±6.59 10^8 NN (figure 1). The levels of DNA adducts in placentas from the polluted area in Estonia were significantly higher than in those from the rural area of Estonia and from Switzerland ($P < 0.01$).

The ^{32}P-HPLC chromatograms from both areas in Estonia (figure 2) showed that the different samples had the same DNA adduct pattern, with retention times between 40 and 80 min, which is the range in which aromatic and bulky DNA adducts are found.

The correlation between DNA adduct levels and the presence of other potential biomarkers was investigated. In placental tissue from Estonia, the following factors were compared with DNA adduct levels: age, number of years living in the area, placental cadmium (µg g^{-1}), placental copper (mg g^{-1}), placental zinc (mg g^{-1}), placental selenium (µg g^{-1}) and self-reported smoking. When comparing the levels

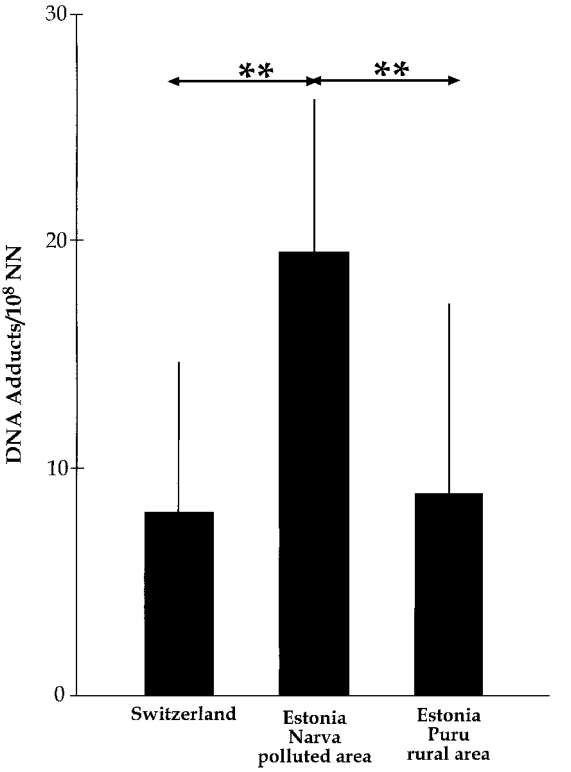

Figure 1. Total DNA adduct levels in human placental samples. Levels are mean ± SD and are expressed as DNA adducts per 10^8 normal nucleotides. Bar 1 corresponds to mothers from Switzerland ($n = 7$); bar 2 corresponds to mothers from Narva, Estonia (polluted area) ($n = 9$); and bar 3 corresponds to mothers from Puru, Estonia (rural area) ($n = 13$). DNA adducts were analysed by ^{32}P-HPLC. The levels of DNA adducts for mothers from Narva were statistically significantly higher ($P < 0.01$) than for mothers from Puru (control area) and Switzerland.

of DNA adducts in relation to smoking status, the smokers in the rural and polluted areas of Estonia had a higher number of DNA adducts than did the non-smokers (polluted area: smokers 22.4 ± 7.5 vs non-smokers 17.0 ± 6.1 DNA adducts per 10^8 NN; rural area: smokers 12.2 ± 7.4 vs 9.4 ± 0 DNA adducts per 10^8 NN) but the differences were not statistically significant. Among the factors analysed, a possible correlation was found between DNA adduct levels and placental copper concentration (figure 3), but none of the potential biomarkers showed a significant correlation with the level of DNA adducts in the placenta.

In placentas from Swiss mothers, correlations between DNA adduct levels and self-reported smoking, cotinine, 7-ethoxyresorufin O-deethylase (ERDE), 7-ethoxycoumarin O-deethylase (ECDE), aromatase, GST enzyme activity, NADPH:quinone oxidoreductase (NQO) enzyme activity and drug abuse were studied. A correlation was found between the presence of DNA adducts and GST activity in mothers who abused drugs ($P < 0.05$) (figure 3). None of the other potential biomarkers had a significant correlation with the level of DNA adducts in the placenta.

Four of the women from Switzerland were drug abusers and three were not. The drug abusers had no additional peaks on the chromatograms compared with

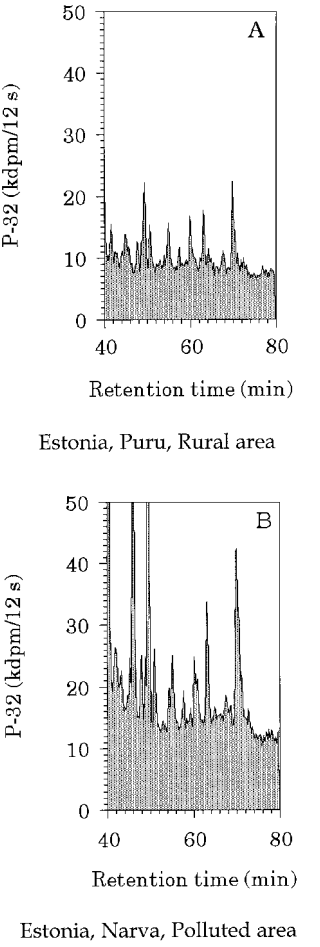

Figure 2. Representative ^{32}P-HPLC chromatograms from human placental tissue. The retention time interval of 40–80 min represents aromatic and bulky DNA adducts. (A) DNA adducts from placental tissue from mothers living in Puru, a rural area in Estonia. (B) ^{32}P-chromatogram showing DNA adducts for placental tissue from mothers living in Narva, a polluted area in Estonia.

the controls, and the total DNA adduct levels were in fact not higher than those of the controls (figure 4) they were lower (non-significantly).

Discussion

It is known that human placental metabolism is sensitive to environmental effects (Manchester and Jacoby 1981, Conney 1982, Hincal 1986). This study showed that placentas from mothers who lived in a polluted area in Estonia had a statistically significantly ($P < 0.01$) higher number of DNA adducts (19.38 ± 6.91 10^8 NN) than did placenta from mothers living in a rural area of Estonia (10.35 ± 6.87 10^8 NN). These results are in agreement with those of Topinka *et al.* (1997), who reported that placental levels of DNA adduct were higher for mothers in a polluted area in the Czech Republic. In addition, Whyatt *et al.* (1998b) found higher numbers of DNA adducts in white blood cells for mothers and new-borns

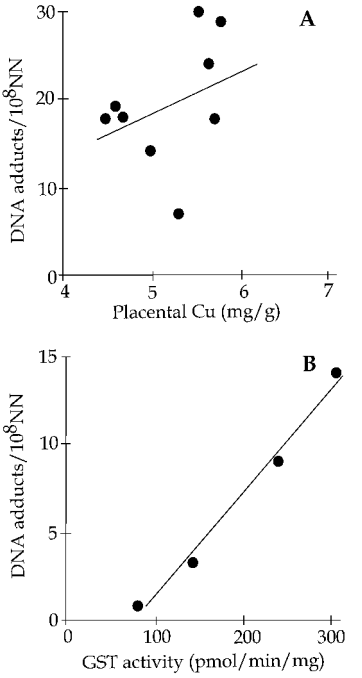

Figure 3. (A) Correlation between DNA adducts and placental copper in mothers from Narva, a polluted area in Estonia ($r = 0.36$, $P < 0.01$). DNA adducts were measured per 10^8 normal nucleotides and the placental copper concentration was measured in mg g^{-1} of placental tissue. (B) Correlation between DNA adducts and placental GST activity in Swiss mothers abusing drugs ($r = 0.98$, $P < 0.05$). GST activity was measured per pmol min^{-1} mg^{-1}.

who lived in a polluted area in Poland. In another study on white blood cells, DNA adduct levels were higher for people living in Silesia, a polluted industrial region in Poland, than for people living in a rural area in Poland (Möller *et al*. 1996).

Smokers from Estonia had higher numbers of DNA adducts than did non-smokers, but the difference was not statistically significant. These findings are in agreement with earlier results from Whyatt *et al*. (1998a) and Daube *et al*. (1997). A correlation between smoking (self-reported) and cotinine levels and levels of DNA adducts was not found for the mothers from Switzerland. These results suggest that the higher DNA adduct levels found in women in the polluted area of Estonia are probably due to air pollution and not to smoking. The lower levels of DNA adducts in drug abusers from Zurich could possibly be related to induction of detoxification enzymes in these persons.

Carmichael *et al*. (1995) studied patients with Wilson's disease and primary haemochromatosis. These patients had a dysfunction in the intracellular deposition of copper and iron that led to higher concentrations of these metals in the tissues, and the livers of these patients had statistically significantly higher levels of DNA adducts than did those of the controls. In this study, a possible correlation between levels of placental DNA adducts and concentrations of placental copper in mothers living in a polluted area in Estonia was observed.

A significant correlation between levels of DNA adducts and GST activity was observed in placentas from Swiss mothers who abused drugs. Others have reported (Soni *et al*. 1998) that GST activity and levels of DNA adducts are inversely

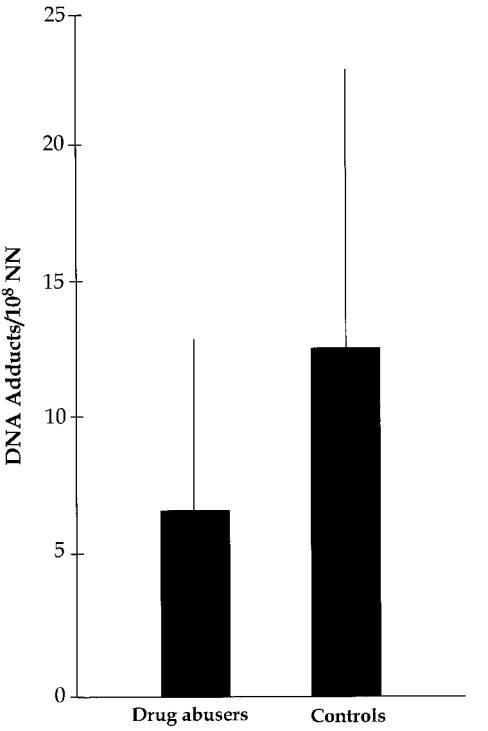

Figure 4. Total DNA adduct levels in human placental samples from Swiss mothers. Levels are mean ± SD and are expressed as DNA adducts per 10^8 normal nucleotides. The left bar represents drug abusers ($n = 4$) and the right bar represents controls ($n = 3$). DNA adducts were analysed by ^{32}P-HPLC. The differences seen were not statistically significant.

correlated, and it has also been suggested that cigarette smoke does not up-regulate human placental GST activity (Pasanen and Pelkonen 1990). The correlation between levels of DNA adducts and GST activity for drug abusers in this study ($r = 0.98$, $P < 0.05$) (figure 3), suggests that the GST enzyme was highly active because it was involved in detoxifying drugs. The reason why the induced GST enzyme in drug abusers was correlated with the number of DNA adducts is not known and this observation although statistically significant, is based on a small number of individuals.

The drug abusers had lower levels of DNA adducts than controls did (figure 4). These results were not statistically significant, but they suggest that women who abuse drugs probably induce certain enzymes, including enzymes that detoxify and repair DNA damage, such as DNA adducts. The main target of the drugs used by mothers in this study is the central nervous system, which was not addressed in this paper.

In conclusion, mothers who lived in an urban area in Estonia had significantly higher levels of placental DNA adducts than did those who lived in a rural area of Estonia. A significant correlation between levels of DNA adducts and GST enzyme activity in mothers who abused drugs was also observed. Other parameters studied, namely, age, number of years living in the area, placental cadmium (μg g^{-1}), placental copper (mg g^{-1}), placental zinc (mg g^{-1}), placental selenium (μg g^{-1}), self-reported smoking, cotinine, 7-ethoxyresorufin O-deethylase (ERDE), 7-ethoxycoumarin

O-deethylase (ECDE), aromatase, NADPH:quinone oxidoreductase (NQO) enzyme activity and drug abuse, did not show significant correlations with levels of DNA adducts.

These data suggest that the level of DNA adducts in human placenta is an environmental biomarker for polluted areas. A further conclusion is that air pollutants to which pregnant women are exposed can induce DNA damage in the placenta and consequently risk exposing the foetus to genotoxins.

Acknowledgements

The expert technical assistance of Riitta Heikkinen and Mary-Ann Zetterqvist is gratefully acknowledged. This work was supported by The Swedish Environmental Protection Agency, MISTRA foundation of Sweden and Academy of Finland grants No. 34555, 29456, European Council, Biomed 2 Program (EUROCYP).

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