Acta Biochimica Polonica

Vol. 50 No. 1/2003

197–203

www.actabp.pl

Communication

High levels of bulky DNA adducts in human sperm correlate with impaired fertility★✪

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Received: 07 September, 2002; revised: 28 November 2002; accepted: 02 December, 2002

Key words: human, fertility, spermatozoa, DNA damage, adducts, ^{32}P-postlabelling

Progressive decline in fertility and sperm quality has been reported over the last few decades, especially in industrialized nations. It has been proposed that exposure to factors that induce damage in DNA of spermatogenic cells may significantly contribute to impaired fertility. Here, the ^{32}P-postlabelling method was used to analyze the levels of bulky DNA adducts in sperm cells in a group of 179 volunteers, either healthy subjects or patients with an impaired fertility. The levels of DNA adducts were 1.35-fold higher in the infertile group as compared to healthy individuals ($P = 0.012$). Similarly, a significant negative correlation between the levels of DNA adducts and measures of semen quality (sperm concentration and motility) has been observed ($P \leq 0.001$). In addition, the levels of bulky DNA adducts in sperm cells positively correlates with amounts of leukocytes in semen, which were significantly higher in semen of infertile subjects.

Impaired fertility is an increasing health problem, especially in industrialized nations. The male factor is incriminated in about 50% of infertility cases, however the etiology of a

★Paper presented at the 32nd Annual Meeting of European Environmental Mutagen Society, September 2002, Warsaw, Poland.

✪This work was supported in part by the State Committee for Scientific Research (KBN, Poland) grant 4P05A01519.

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Abbreviations: PBS, phosphate-buffered saline; TE, Tris/EDTA buffer; TLC, thin-layer chromatography.

large proportion of them remains obscure. Several studies have reported a progressive decline in sperm quality and fertility over the last few decades, and it has been speculated that this was a combined effect of increased stress and environmental pollution (WHO, 1987; Giwercman & Bonde, 1998). Exposure to some environmental factors, of either a chemical or physical nature, has been reported as a risk factor of male infertility. These factors include smoking, heat, ionizing and non-ionizing electromagnetic radiation, heavy metals, pesticides, organic solvents and aromatic compounds. Several infertility factors are known, or putative mutagens or carcinogens and induction of mutagenic events can be involved in the etiology of some infertility cases (Lahdetie, 1995; Tas *et al.*, 1996; Hruska *et al.*, 2000). It has been shown that active metabolites of chemical carcinogens could be transported in the serum to different organs and tissues (Ginsberg & Atherholt, 1989). Because of the existence of a blood-testis barrier, the majority of developing germ cells are not in direct contact with the circulation. However, detection of nicotine and its metabolites in the semen of smokers (Zenzes *et al.*, 1999) suggests that mutagens might pass through this barrier and react with germ cells as well. Some evidence suggests that paternal exposure to genotoxic agents (e.g. smoking) can be associated with congenital anomalies and childhood cancers in offspring (Vine, 1996).

Covalent modifications of nucleotides termed DNA adducts are widely accepted as biomarkers of exposure to chemical mutagens and carcinogens in molecular epidemiology studies (Harris, 1986). Numerous studies have shown elevated levels of DNA adducts in individuals exposed to behavior-, occupation-, and environment-related genotoxic agents, in either surrogate or target tissues (Beach & Gupta, 1992; Lewtas *et al.*, 1993). However, only limited data concerning DNA adducts in human spermatogenic cells and spermatozoa are available at the moment. Two earlier published papers studied bulky DNA adducts in human spermatozoa. However, both were limited to small groups of subjects and did not refer to correlation between DNA damage and fertility (Zenzes *et al.*, 1999; Gallagher *et al.*, 1993). The ^{32}P-postlabelling is a method of choice for analyzing DNA adducts in humans exposed to complex mixtures like tobacco smoke, air pollutants, engine exhausts or coke oven emission. The aim of the present study was to use the ^{32}P-postlabelling method to analyze bulky DNA adducts in human sperm cells as a measure of DNA lesions, and to relate these findings to possible defects of human fertility.

MATERIALS AND METHODS

Study participants, semen collection and examination. Semen samples were obtained by masturbation from 179 volunteers aged between 22 and 47 years. Twenty subjects were healthy sperm donors with proven fertility. 159 subjects were patients of our infertility clinic. Male-factor infertility was suspected in 94 of them, based on case records and semen analyses. Sixty five patients, who were not suspected of male-factor infertility were joined to sperm donors as the healthy group. These latter patients did not differ significantly from sperm donors in respect to parameters of sperm quality. Information on smoking and drinking habits, occupational and demographic data were obtained through a questionnaire. The "healthy" and "infertile" groups did not differ significantly in age or smoking and drinking habits. Analysis of semen parameters (i.e. volume, sperm concentration, motile sperm percentage, amount of leukocytes in semen) was performed according to standard methods (WHO, 1992).

DNA isolation. Sperm cells washed in phosphate-buffered saline (PBS) were incubated with proteinase K (final concentration 0.2 mg/ml) in the presence of 0.5% SDS and 10 mM EDTA for 12–16 h at 37°C. DNA was then

purified by 2–3 repeated extractions with phenol/chloroform and recovered by ethanol precipitation. Precipitated DNA was dissolved in Tris/EDTA (TE) buffer, incubated with RNaseA to remove traces of RNA, then re-purified by phenol/chloroform extraction and ethanol precipitation. DNA concentration was measured spectrophotometrically and its integrity examined by agarose gel electrophoresis.

Adduct analysis. Bulky DNA adducts were assayed according to the ^{32}P-postlabelling method (Gupta *et al.,* 1982) with some modifications as described in detail elsewhere (Widłak *et al.,* 1996). Briefly, 10 μg of DNA was digested to mononucleotides with micrococcal nuclease and spleen phosphodiesterase and then non-modified mononucleotides were removed by digestion with nuclease P1. Modified nucleotides were radiolabelled with [γ-^{32}P]ATP and T4 polynucleotide kinase, and then applied to pre-washed PEI-cellulose TLC plates (Macherey-Nagel). The chromatography was carried out in 1 M sodium phosphate, pH 6.0, for 6 h to a paper wick (D1). Modified nucleotides were separated by chromatography in 8.5 M urea, 3.5 M lithium formate, pH 3.5 (D2), and then 8.5 M urea, 0.8 M LiCl, 0.5 M Tris, pH 8.0 (D3). D2 and D3 were developed into paper wicks, and the time of chromatography required for optimal and reproducible resolution of adducts was determined experimentally for each batch of TCL plates. The adduct spots were visualized by autoradiography, and then radioactivity of appropriate areas of TLC plates was counted to calculate the level of DNA adducts.

RESULTS AND DISCUSSION

Bulky DNA adducts were analyzed by the ^{32}P-postlabelling method in sperm cells of 179 male volunteers, 85 healthy controls and 94 patients with impaired fertility. Figure 1A shows a few examples of thin-layer chromatograms of DNA samples from sperm cells. All chromatograms contained several discrete radioactive spots that represent bulky adducts, as yet unidentified. We have not detected any adduct pattern that would be common to all sperm samples and, in fact, several types of patterns were detected. However, we have not found any systematic difference in the adduct patterns between groups of healthy and infertile subjects. Adduct levels in each of 179 individuals ranged from 0.34 to 18.5×10^{-2} fmol per μg of DNA, with median 2.47×10^{-2} and mean $3.23 \times 10^{-2} \pm 2.69 \times 10^{-2}$ (S.D.). Adduct levels were compared between groups of healthy and infertile subjects, and both groups differed significantly (P = 0.012). The amounts of adducts were 1.35-fold higher in the "infertile" group, on the average (Table 1 and Fig. 1B).

Semen parameters, including volume, sperm concentration, motility and morphology, are valuable and generally accepted markers reflecting male fertility. Because the "infertile" and "healthy" groups of subjects differed significantly ($P < 0.0001$) in respect to sperm concentration, motility and amounts of leukocytes in semen, the correlation between these sperm parameters and adduct levels were examined in further analyses. Figure 2A presents the relationship between the levels of DNA adducts and sperm motility (percentage of spermatozoa with rapid forward progression). A strong negative correlation between these two parameters was determined ($r_S = -0.2334$, $P = 0.0018$). A significant negative correlation between the levels of DNA adducts and sperm concentration was also found ($r_S = -0.2497$, $P = 0.0008$) (Fig. 2B). The quality of sperm can be also expressed as the concentration of motile spermatozoa (concentration of spermatozoa × percentage of rapidly moving spermatozoa). A strong negative correlation between these two parameters was observed ($r_S = -0.3048$, $P < 0.0001$) (Fig. 2C). Semen volume of the vast majority of samples (98%) was at its normal value (2.0 ml or more, according to WHO standards (1992)) and this parameter was not

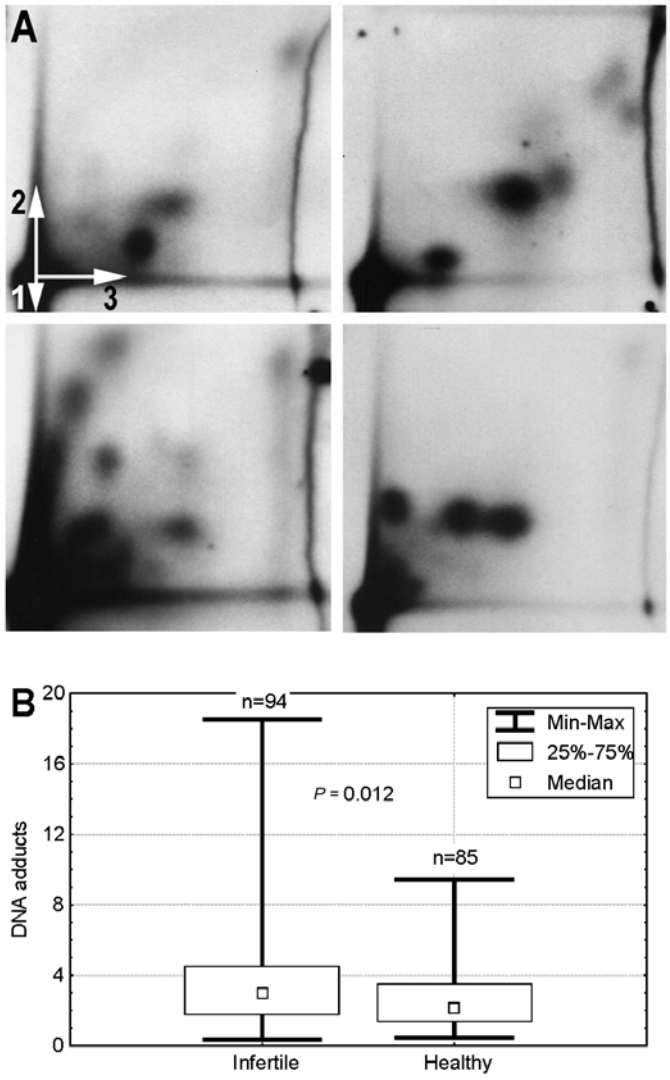

Figure 1. Bulky DNA adducts detected in human sperm.

Panel A. Thin-layer chromatograms of DNA samples analyzed by the ^{32}P-postlabelling method. Shown are directions of chromatography. **Panel B**. The levels of DNA adducts in sperm of healthy individuals and patients with suspected male-factor infertility. The significance level (P) of the difference was determined by the Mann-Whitney U test. DNA adducts levels are shown as fmol per μg of DNA $\times$ 10^2.

studied in further analyses. In the majority of semen samples some leukocytes, mostly granulocytes (about 90%, on the average), could be detected. The amounts of leukocytes in semen were significantly higher ($P < 0.00001$) in individuals with predicted male-factor infertility than in semen of healthy subjects. Medians were 1.1% and 5% for healthy and infertile groups, respectively, when concentrations of leukocytes were given in proportion to concentrations of spermatozoa. We found a strong negative correlation between the amounts of leukocytes in semen and the concentration of spermatozoa ($r_S = -0.6377$, $P < 0.0001$) or their motility ($r_S = -0.516$, $P < 0.0001$). Correlation between the amount of leukocytes in semen and DNA adduct levels in sperm was also determined. A significant positive correlation ($r_S = 0.2088$, $P = 0.0053$) between the concentration of leukocytes and the adduct level in sperm was found (Fig. 2D).

Bulky DNA adducts that can be detected by the ^{32}P-postlabelling method are either induced by exogenous environmental factor or

Table 1. Levels of sperm DNA adducts in groups of healthy donors and patients with male-factor infertility.

Adducts levels are shown as fmol per μg of DNA $\times 10^2$.

Group	n	Median	+95% CI	−95% CI	Lower quartile	Upper quartile	Mean	S.D.
Healthy	85	2.16	2.32	3.13	1.35	3.54	2.73	1.87
Infertile	94	2.94	3.04	4.34	1.76	4.51	3.69	3.19

are endogenous products of metabolism. The majority of subjects studied in this project, both healthy or infertile, were inhabitants of Silesia, a heavily industrialized and polluted area in the south of Poland. It has been reported that the Silesian population showed elevated levels of bulky DNA adducts in lymphocytes from circulating blood (Perera *et al.*, 1992). The adduct levels showed seasonal variations that matched the seasonal variations in the air concentrations of polycyclic aromatic hydrocarbons; adduct levels were 2–3-fold higher in samples collected during winter (Grzybowska *et al.*, 1993; Hemminki *et al.*,

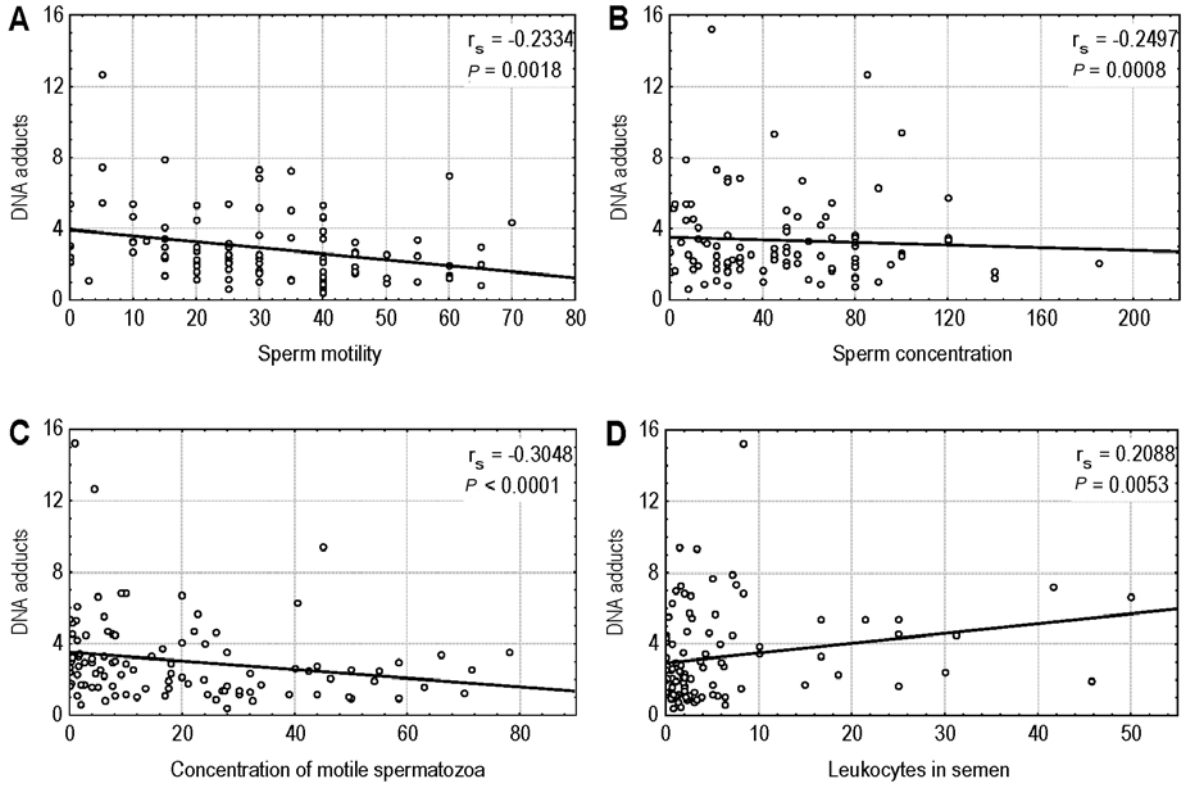

Figure 2. Correlation between the levels of sperm DNA adducts and measures of sperm quality.

Panel A. Correlation between levels of DNA adducts in sperm cells and sperm motility (percentage of rapidly moving spermatozoa). **Panel B**. Correlation between levels of DNA adducts and sperm concentration (amount of spermatozoa $\times 10^6$/ml). **Panel C**. Correlation between levels of DNA adducts in sperm cells and concentration of motile spermatozoa (amount of rapidly moving spermatozoa $\times 10^6$/ml). **Panel D**. Correlation between levels of DNA adducts in sperm cells and amount of leukocytes in semen (shown as the percentage of spermatozoa). DNA adducts levels are shown as fmol per μg of DNA $\times 10^2$. Presented are the values of Spearman R correlation coefficient (r_S) and its significance level (P). N = 176.

1996). Here we have observed that levels of bulky DNA adducts in sperm were significantly lower as compared to lymphocytes; approximately 1–2 adduct per 10^8 nucleotides in sperm and 5–10 adducts per 10^8 nucleotides in lymphocytes, on the average. Noteworthy, the DNA adduct patterns observed in sperm and lymphocytes from the same individual are totally different (not shown). We have detected no seasonal variations in the levels of sperm adducts (not shown). These findings suggest that spermatozoa might be protected against environmental factors and that endogenous tissue-specific factors could play an important role in induction of DNA adducts detected in spermatozoa. It has been reported that steroid estrogens and their metabolites are among the endogenous DNA-reactive molecules and chronic exposure of rodents to estrogens (e.g. estradiol) induces formation of bulky DNA adducts in hormone-responsive tissues that can be detected by ^{32}P-postlabelling (Roy & Liehr, 1999). On the other hand, several bulky DNA modifications termed I-compounds have been detected in rodents not exposed to known genotoxic chemicals and their formation is strain-, age-, gender-, and tissue-dependent (diet, chemicals and hormonal exposure also affect formation of such compound) (Randerath *et al.*, 1999). However, the identities of the DNA adducts detected in human spermatozoa remain to be established.

Epidemiological and experimental studies indicate that DNA adducts induced by chemical carcinogens are involved in initiation and promotion of tumors. However, much less is known about the role of bulky DNA modifications induced by endogenous sources, as well as about the role of bulky DNA damage in etiology of non-malignant diseases. Here we showed that the levels of bulky DNA adducts were higher in sperm of patients with suspected male-factor infertility than in sperm of healthy individuals. Similarly, the levels of covalent modifications in human sperm DNA were negatively correlated with spermatozoa concentration and motility, measures of sperm quality. Several mechanisms could be proposed to explain this correlation. One could suggest an indirect relationship between elevated levels of DNA adducts and decreased sperm quality. For example, the detected bulky DNA modifications may results from action of factors that induce general cytotoxicity, hormonal imbalance or otherwise affect germ-line cells. Increased levels of DNA adducts might be a "side-effect" of pathological processes related to impaired fertility. A direct relationship between both factors (e.g. DNA adducts being a causative factor of low sperm quality) seems much less probable. A functional correlation between bulky modifications of sperm DNA and fertility is not clear at the moment. However, our results imply that DNA adducts could be applied as a potential biomarker in studies of human infertility.

We thank Ms Lucyna Ponge for excellent technical assistance and Dr. Grażyna Motykiewicz for helpful discussions.

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