Genotoxic changes in peripheral lymphocytes after therapeutic exposure to crude coal tar and ultraviolet radiation

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Aims. Goeckerman therapy is based on combined exposure to UV radiation (UVA, UVB) and crude coal tar (PAHs). Some indicators suggest a genotoxic hazard, however, the level of genotoxic risk of the therapy has not yet been investigated sufficiently. This study aims to assesss the genotoxic risk.
Methods. The studied group consisted of patients with chronic stable plaque psoriasis treated by Goeckerman therapy (n = 29). Heparin-treated peripheral blood samples were collected one day before the first treatment and immediately after the last procedure. The lymphocytes were isolated from the blood. The level of genotoxicity was evaluated using an alkaline version of the Comet assay which detects DNA single strand breaks (DNA-SSBs), a neutral version of the Comet assay which detects DNA double strand breaks (DNA-DSBs), and using chromosomal aberrations.
Results. The level of DNA-SSBs increased insignificantly (median; Q1-Q3): 1.4 (0.4; 0.1-1.4) vs. 2.5 (0.6; 0.3-2.7) %tDNA ($P = 0.11$) and the level of DNA-DSBs increased significantly: 7.8 (6.5; 3.4-10.5) vs. 20.7 (19.3; 14.2-24.6) % DNA ($P < 0.001$). The total number of aberrated cells ($P < 0.001$) and structurally aberrated cells ($P < 0.001$) increased significantly.
Conclusion. The elevated levels of the DNA-DSBs and the chromosomal aberrations in the peripheral lymphocytes indicated a genotoxic hazard. However, the elevated level of the chromosomal abnormalities was below the upper level of the reference range for healthy Czech adults. While, the genotoxic risk appears to be low, Goeckerman treatment represents a further contribution to the lifetime load of genotoxic factors.

Key word: crude coal tar, ultraviolet radiation, genotoxicity, comet assay, chromosomal aberrations

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INTRODUCTION

Psoriasis is a chronic, relapsing and remitting immune-mediated inflammatory skin disease with a prevalence of 2-4% in western countries[1,2]. The etiopathogenesis involves individual genetic predisposition as well as environmental factors.

Topical therapy of psoriasis is now applied in approximately 75% of cases which are classified as lightly to moderately severe forms[3,4]. In 1925, William H. Goeckerman reported a successful use of topical crude coal tar (CCT) and broad-spectrum ultraviolet radiation (UVA and UVB) in the treatment of psoriasis. This very effective and low cost medical procedure is currently known as the Goeckerman therapy (GT) (ref.[5]). However, due to presumed genotoxicity, the use of CCT in dermatology has recently decreased[6].

The genotoxicity of CCT is related to large content of polycyclic aromatic hydrocarbons (PAHs). Some of them have proven immunosuppressive, mutagenic, genotoxic and carcinogenic effects[7]. They are also photosensitizers and can cause phototoxic effects by way of oxygen-dependent mechanisms[8]. On the other hand, it has been proven that PAHs induced immunosuppression has significant therapeutic effects without symptoms of systemic toxicity[6].

UVB is a well known factor causing damage to DNA. For a long time, UVA was considered as a weak mutagen because of its lower energy and its limited ability to damage DNA directly. However, the results of recent studies have shown that the UVA can also be a potent inductor of DNA damage and may be associated with skin cancer[9]. Protracted exposure to UVA leads to lysosomal dysfunction in human fibroblasts[10], malignant transformation in human cultured keratinocytes[11] and the formation of skin carcinomas in hairless mice in vivo[12].

Unlike the shorter-wavelength UVB photons, which are almost completely absorbed by the epidermis, the longer-wave length UVA photons can reach deeper dermal layer of skin and its blood vessels. Thus, the peripheral blood cells, including lymphocytes, can be exposed to UVA light even under physiological conditions[13]. The UVA radiation (0.1 J/cm^2) can produce around 80-130 DNA lesions per cell (human lymhocytes) that are detectable by the Comet assay[14]. The combination of UVA and UVB is ranked as carcinogenic to humans according IARC (ref.[7]).

Briefly, the CCT (PAHs) and ultraviolet radiation represents therapeutically efficient, nevertheless toxicologically dangerous factors. Moreover, it was proven that UVA in combination with other common environmental pollutants (including PAHs) can significantly increase skin cancer risk[15]. It therefore seems likely, that DNA damage (induced by UVA during GT) can be further increased by co-exposure to PAHs (ref.[16]).

DNA is a significant target of environmental stress and the loss of its structural integrity may be a reasonable parameter for assessing susceptibility to mutations[17]. The Comet assay is widely used for assessments of DNA integrity in occupationally and environmentally exposed populations[18]. The assay offers a number of advantages over other methods for the detection of DNA damage: (i) it is rapid, simple, and relatively inexpensive to perform; (ii) it allows for the collection of data at the individual cell level; (iii) it requires a relatively small number of cells (<10 000) in each sample; (iv) it shows a high sensitivity for the detection of DNA damage; and (v) almost all types of eukaryotic cells, both in vitro and ex vivo, can be used for analysis[19]. On the other hand, there may be many factors affecting the outcomes of in vitro comet assay, for example, exposure period, sampling period, cells used, S9 components, electrophoresis condition, and cytotoxicity[20]. In the scientific literature, we found no publications that describe the use of Comet assay for genotoxicity evaluation of GT.

It has been proved that chromosomal aberrations in human lymphocytes can predict risk of cancer[21]. The level of chromosomal abnormalities represent well-established biomarker of genotoxicity, probably the only one which has been internationally standardized and validated[22,23]. The declared reference value for the total number of aberrated cells in peripheral lymphocytes of healthy Czech adults ranges from 0.0–2.32% (ref.[22,24]) with no significant correlation to sex or age[25]. Clastogenic activity of PAHs and UV radiation has been clearly demonstrated in human lymphocytes[26,27] and the genotoxic effect of combined exposure to CCT (PAHs) and UVA/UVB was described in our previous work[7].

In summary, some indicators suggesting the presence of genotoxic potential (hazard) of therapeutic exposure to CCT (PAHs) and UV radiation. However, the level of genotoxic risk of the therapy has not yet been satisfactorily quantified[28,29]. This study aims to contribute to quantification of the risk.

MATERIALS AND METHODS

Study group

The group consisted of patients with chronic stable plaque psoriasis treated by GT (Clinic of Dermal and Venereal Diseases, University Hospital, Hradec Kralove, Czech Republic). Within a period of two years, we collected data on 29 randomly selected adult patients. The study group included 14 women (average age 57 years, age range 30–75 years, 7 smokers) and 15 men (average age 43 years, age range 19-69 years, 9 smokers). The patients who had significant prior exposure to PAHs and/or artificial UVR (especially occupational) were excluded. The study was approved by the Ethics Committee of the University Hospital in Hradec Kralove, Czech Republic. Informed written consent was obtained from each patient.

Goeckerman therapy

Dermatological ointment (5% of CCT) was administered daily (overnight) on lesions (16-54% of body surface). Residues of the ointment were removed in the morning (using oil bath) and the patients were whole-body irradiated by ultraviolet radiation (UVA and UVB). The content of selected PAHs in used CCT is described in another article[16]. Duration of the therapy (average duration 11 days; range 3-18 days) was modified according to its effectiveness. The actual status of disease was expressed by using the PASI score (Psoriasis Area and Severity Index) (ref.[30]).

Blood samples

Heparin-treated peripheral blood samples were collected one day before the first treatment and immediately after the last procedure. The samples were placed in the fridge and processed within two hours after collection.

Isolation of peripheral lymphocytes

The 3 mL of Histopaque 1077 (Sigma-Aldrich) was overlaid by approximately 2.5-3 mL of heparinized venous blood. We obtained a white ring which contained lymphocytes by centrifugation (20 °C, 400 g, 30 min). Lymphocytes were counted and diluted to required concentration of 10^5 cells/mL.

Comet assay – alkaline version

The integrity of DNA in peripheral lymphocytes was evaluated using Comet Assay, the common tool for assessment the genotoxicity. A standard version of alkaline comet assay detects DNA single strand breaks (DNA-SSBs), alkali-labile sites and incomplete excision repair sites[31]. In the presented study we used the standard version with modifications according to Buchynska[32] and de Freitas[33]. We analysed the percentage of DNA in the tail (designated as tail DNA or tail intensity; % tDNA) expressed as the ratio between amount of DNA in the head and amount of DNA in the tail[34].

Comet assay – neutral version

The DNA double strand breaks (DNA-DSBs) were detected using a neutral version of the Comet assay. We used the modified method described by Olive[35]. The method of preparation of the cells and slides was identical with the alkaline version, up to the phase of lysis of the cells. We omitted the alkaline unwinding and washed the slides three times in the borate buffer (90 mM Tris, 90 mM boric acid, 2 mM ethylenediaminetetraacetic acid; EDTA) before electrophoresis (borate buffer, 29 V, 6 mA for 40 min at 4 °C). The procedure and evaluation after electrophoresis was analogous to the case of alkaline version.

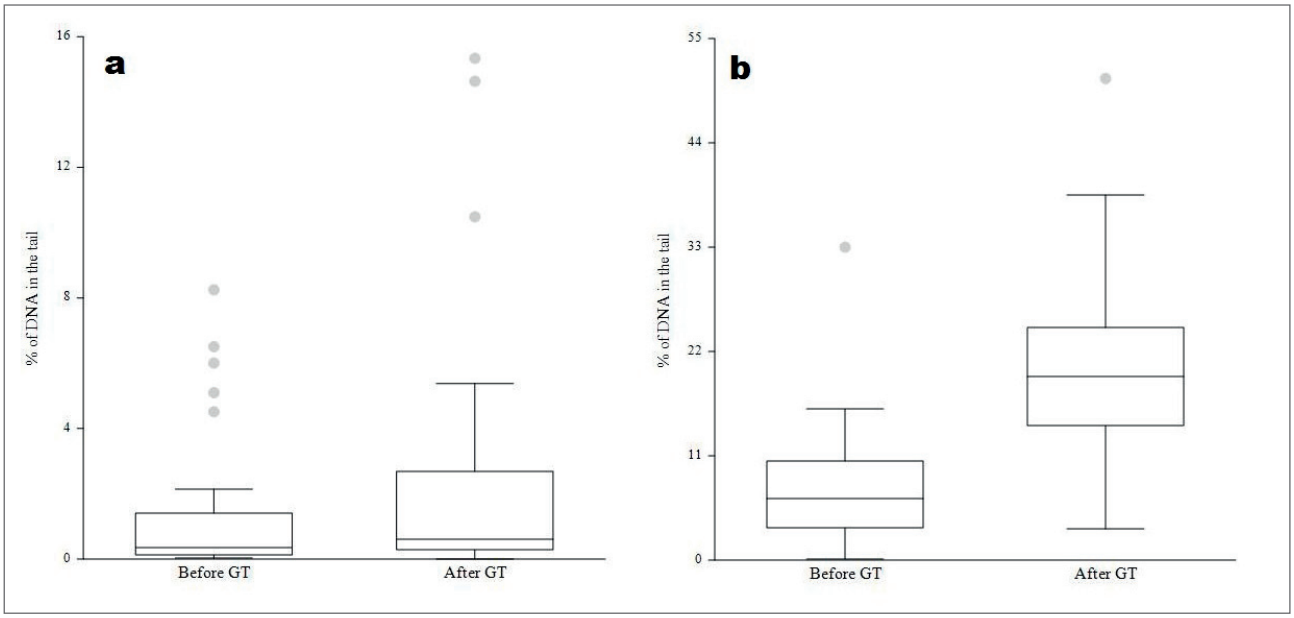

Fig. 1. Comet assay: quantification of DNA breaks (a) DNA-SSBs (alkaline version) and (b) DNA-DSBs (neutral version).

The middle of the box: median
The lower boundary of the box: 1st quartile (Q1)
The upper boundary of the box: 3rd quartile (Q3)
The height of the box: inter-quartile range (IQR)
The lower whisker boundary: the smallest observation that is greater than or equal to Q1 – 1.5 x IQR
The upper whisker boundary: the largest observation that is less than or equal to Q3 + 1.5 x IQR
Points outside the whisker boundaries: outliers

Chromosomal aberrations

The level of chromosomal aberrations in peripheral lymphocytes was evaluated by a standardized method[22] based on microscopic analysis of lymphocytic chromosomes undergoing mitotic metaphase. In each blood sample, 100 of mitotic sets were evaluated. We measured structurally aberrated cells, numerically aberrated cells and total number of aberrated cells.

Statistical analysis

Statistical analysis was performed using the NCSS 2007 software[36]. We used Mann-Whitney test for unpaired comparisons and paired *t*-test, Wilcoxon Signed-Rank test, and Sign test for paired comparisons. Pearson correlation and Spearman rank correlation were used to assess relationships between variables (single and double breaks of DNA, number of aberrated cells, PASI score, body surface covered with CCT ointment, overall time of CCT application and overall time of UVR exposure). To keep the family-wise alpha at 0.05, we used the Bonferroni correction. The resulting significance level for a single test was 0.0021.

RESULTS

Daily irradiation of ultraviolet radiation (UVA and UVB) was individual according to disease status (1-15 min). Density of used radiation was 249.75 μW/cm^2 of UVB and 131.8 μW/cm^2 of UVA (controlled by Sola–Scope 2000 spectrometer; Solatell, United Kingdom). Overall time of exposure to ultraviolet radiation varied from 3 to 122 min (average time 58 min).

Significant decrease in PASI score confirmed the efficacy of GT. Before the therapy, we found (arithmetical mean, SD) 17.5 ± 5.7; after the therapy 5.7 ± 2.4 (P < 0.001).

The percentage of DNA in the tail obtained in the alkaline version of the Comet assay (%tDNA) corresponds with the level of the DNA single strand breaks (DNA-SSBs), the alkali-labile sites and the incomplete excision repair sites. The therapy insignificantly increased the level of DNA-SSBs (Fig. 1a). The arithmetic mean of DNA-SSBs (median, first quartile - third quartile; Q1-Q3) reached levels of 1.4 (0.4, 0.1-1.4) %tDNA before GT and 2.5 (0.6, 0.3-2.7) %tDNA after GT (P = 0.11).

The percentage of DNA in the tail obtained in the neutral version of the Comet assay (%tDNA) corresponded with the double strand breaks (DNA-DSBs). DNA-DSBs level was significantly increased due to the therapy (Fig. 1b). The arithmetic mean of DNA-DSBs (median, Q1-Q3) reached levels of 7.8 (6.5, 3.4-10.5) %tDNA before GT and 20.7 (19.3, 14.2-24.6) %tDNA after GT (P < 0.001).

The therapy significantly increased the total number of aberrated cells (P < 0.001) and structurally aberrated cells (P < 0.001) (Tab. 1). Before GT, we identified 23 aberrations in the total number of 2900 examined cells (0.79%). The aberrations were classified as structural aberrations (19) and numerical aberrations (4). After the therapy, we analyzed 59 aberrations in the total number of 2900 cells (2.03%). Aberrations were classified as structural aberrations (49) and numerical aberrations (10).

DISCUSSION

This study focused on quantification of the risk of genotoxic potential of combined therapeutic exposure to CCT (PAHs) and ultraviolet radiation. CCT (PAHs) may exert their genotoxic, mutagenic and carcinogenic effects in basically two ways. The first pathway includes formation of specific PAHs-DNA adducts (oxygen-independent reaction with DNA, type I reaction) (ref.[37]), the second pathway includes induction of oxidative stress (oxygen-dependent mechanism, type II reaction) (ref.[38]). Both of these pathways induce DNA damage (including strand breaks). If left unrepaired, DNA adducts and strand breaks may lead to permanent mutations and chromosomal aberrations[39] resulting in cell transformation and ultimately to tumour development[40].

We found no comparable study which evaluated the genotoxic effects of GT using Comet assays (both alkaline and neutral version). In sub-population groups, mildly to moderately exposed to PAHs and UVR, the percentage of DNA in the tail obtained in the Comet assay (alkaline version; DNA-SSBs; %tDNA) ranged in the order of units. For example Hininger et al. presented an average level (DNA-SSBs; mean±SD) of ten healthy non-smokers (5 men and 5 women) 4.08±0.96 %tDNA (ref.[41]). Binkova et al.[42] found in a group of healthy non-smoking women, living in an area polluted by PAHs, the average level of 5.35 and 7.12 %tDNA (DNA-SSBs; medians). Other authors studied a group of policemen who were exposed to combination of PAHs and ultraviolet radiation[43]. In summer they found 2.91±1.05 %tDNA (DNA-SSBs; mean±SD), in winter 2.42±1.70 %tDNA. Related control group (non-exposed young healthy males) showed levels 2.62±1.04 %tDNA (summer) and 2.64±1.37 %tDNA (winter). Subsequent study (policemen and bus-drivers exposed to PAHs) found levels of 3.86±1.28 %tDNA (ref.[44]).

The GT in our study insignificantly increased the level of DNA-SSBs. In this respect, we note that DNA-SSBs are repaired more than six times faster (within minutes after their formation) than DNA-DSBs (ref.[45]). We assume, that lower levels of DNA damage, detected by the Comet assay (alkaline version), could be related to this fact.

In the case of DNA-DSBs, the literarature concerning PAHs and UVR is very limited. Baumgartner et al.[46] described elevated levels of DNA-DSBs in lymphocytes (11.08 – 15.23 %tDNA) after in vitro exposure to benzo(a) pyrene (blood from four healthy non-smoking volunteers). In this study we found significantly increased level of DNA-DSBs ($P < 0.001$). Although DNA-DSBs are less frequent type of DNA lesions upon exposure to ionizing radiation (SSBs/DSBs = 25/1), they are potentially lethal for the cell and more problematic than DNA-SSBs. DNA-DSBs represent a worse form of DNA damage because the breaks occur on both strands of DNA. The repair of this damage is difficult and slower than the repair of DNA-SSBs and more prone to error due to a lack of undamaged complementary copy[14,47]. The efficacy of DNA-DSBs repair is crucial for the maintenance of genomic integrity and viability[47]. Failure of that reparation process can lead to cell death or its malignant transformation[48] through the induction of chromosomal aberrations[39].

Table 1. Chromosomal aberrations in peripheral lymphocytes.

Subject (No. of patient)	Chromomal aberration before GT			Chromomal aberration after GT		
	ABC	SAC	NAC	ABC	SAC	NAC
1	1	1	0	5	5	0
2	1	1	0	2	2	0
3	1	1	0	2	2	0
4	0	0	0	1	1	0
5	3	3	0	4	4	0
6	0	0	0	1	0	1
7	1	0	1	3	3	0
8	0	0	0	1	1	0
9	1	1	0	2	2	0
10	0	0	0	1	1	0
11	2	1	1	3	2	1
12	1	1	0	2	1	1
13	0	0	0	3	2	1
14	2	2	0	3	1	2
15	0	0	0	1	1	0
16	0	0	0	2	1	1
17	0	0	0	2	2	0
18	2	2	0	3	2	1
19	1	0	1	3	2	1
20	0	0	0	1	0	1
21	1	1	0	5	5	0
22	1	0	1	0	0	0
23	2	2	0	1	1	0
24	0	0	0	1	1	0
25	1	1	0	3	3	0
26	0	0	0	1	1	0
27	1	1	0	1	1	0
28	1	1	0	1	1	0
29	0	0	0	1	1	0

ABC: total number of aberrated cells
SAC: structurally aberrated cells
NAC: numerically aberrated cells

The literature reports that UVA induces a dose-dependent increase in the level of DNA damage, detected by the alkaline version of the Comet assay, and in oxidative stress, characterized by increased level of 8-oxo-7,8-dihydroguanine[14,49]. It is not known whether UVA can directly cause DNA-DSBs, but DNA-DSBs occur during repair of oxidative damage[49]. In this work, we studied the effects of combined exposure to ultraviolet radiation and PAHs. Under these exposure conditions we found no significant relationship between exposure to ultraviolet radiation and the level of genotoxicity (expressed as DNA-SSBs or DNA-DSBs).

As stated above, DNA-DSBs occur less frequently, are roughly six times more slowly repaired (compared to DNA SSBs) and can be detected with a certain time delay

from exposure. With regard to the nature of evaluated subchronic exposure (GT), we assume that for purposes of biological monitoring, monitoring the levels DNA-DSBs is (in this case) more appropriate.

Apart from our earlier publications[6,16] we found no publication on evaluation of genotoxic effects of GT using chromosomal aberrations in human lymphocytes. Among the other recent works, we can choose e.g. a study aimed at the genotoxic risk of traffic policemen (combined exposure to PAHs and ultraviolet radiation). The mean level of chromosomal aberrations in lymphocytes (observed in different cities and different seasons) ranged from 0.22 to 0.27% ABC (ref.[50]).

It is evident that the therapy significantly increases absolute level of aberrated cells ($P < 0.001$) and thus increases the related genotoxic risk. However, it must be emphasized that the level of abnormalities found after the therapy (2.03%) was still below the upper level of the reference range for healthy Czech adults.

CONCLUSION

While the values of DNA-SSBs ranged within the level of normal non-exposed populations, elevated levels of the DNA-DSBs indicated the genotoxic risk. The risk was also suggested by the elevated levels of chromosomal aberrations; however, the level of chromosomal abnormalities was below the upper level of the reference range for healthy Czech adults. In summary, the level of genotoxic risk appears to be low. On the other hand, it is evident that assessed effective therapy represents a significant contribution to the lifetime load of genotoxic factors.

ABBREVIATIONS

%tDNA, Percentage of DNA in the tail; ABC, Total number of aberrated cells; CCT, Crude coal tar; DNA-DSBs, Double strand breaks; EDTA, Ethylenediaminetetraacetic acid; PAHs, Polycyclic aromatic hydrocarbons; PASI, Psoriasis area and severity index; DNA-SSBs, Single strand breaks.

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Author contributions: LB, ZF: study design; LB, RK, KH: performed the experiments; ISK: data analysis; AM, LB,ZF: manuscript writing.

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