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8-Hydroxydeoxyguanosine as a biomarker of oxidative DNA damage in workers exposed to low-dose benzene

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ABSTRACT

The present study aims to investigate the relation between exposure to low-dose benzene and the occurrence of oxidative DNA damage in gasoline station workers, as well as the possible role of interfering or confounding factors.

Urine levels of 8-OHdG were evaluated by a competitive immunoassay in a group of 80 men, employed in gasoline stations located in East Sicily and compared with a control group ($n = 63$) of male office employees not occupationally exposed to benzene. Information regarding socio-demographic characteristics, lifestyle and job-related records were provided through a questionnaire.

Significantly higher ($p < 0.05$) urinary t,t,-MA and 8-OHdG levels were observed in gasoline station attendants compared to subjects not exposed to benzene.

Pearson's test demonstrated a strong correlation ($r = 0.377$, $p < 0.001$) between 8-OHdG and benzene exposure level. 8-OHdG significantly correlated also with job seniority, ($r = 0.312$, $p < 0.01$), whereas the relation with age resulted weaker ($r = 0.242$, $p < 0.05$). Multiple linear regression analysis, performed to exclude a role for confounding factors, showed that variables like gender, smoking habit, alcohol consumption and BMI did not have a significant influence on the measured biomarkers. No subject enrolled in the study presented signs or symptoms of work-related disease or other illness linked to oxidative stress.

These results suggest that low-level chronic exposure to benzene among gasoline station attendants can determine oxidative damage on DNA, as indicated by alteration of 8-OHdG which may represent a non-invasive biomarker of early genotoxic damage in exposed subjects.

1. Introduction

Benzene is an aromatic chemical compound which can be found in several working and living environments; thus exposure to this substance represents an important public health issue [1]. Additionally, European Union (EU) classified benzene as a category 1A carcinogen. For this reason, its applications have been limited but due to its high octane number it remains an important constituent of gasoline (approximately 1% v/v), which is one of the main sources of environmental and occupational benzene pollution.

Based on epidemiological studies, human exposed to high levels of benzene show an increased risk to develop several acute and chronic diseases, such as acute myeloid leukemia, acute and chronic lymphocytic leukemia, non-Hodgkin lymphoma, multiple myeloma and aplastic anaemia [2]. Moreover, exposure to low doses of benzene has been linked to increased incidence of myelodisplastic syndrome and decreased resistance to infection [3], which may be explained by its immunotoxic activity targeting mainly T-cells [4].

Experimental studies have found that benzene can be enzymatically bioactivated to reactive intermediates (catechol, hydroquinone,

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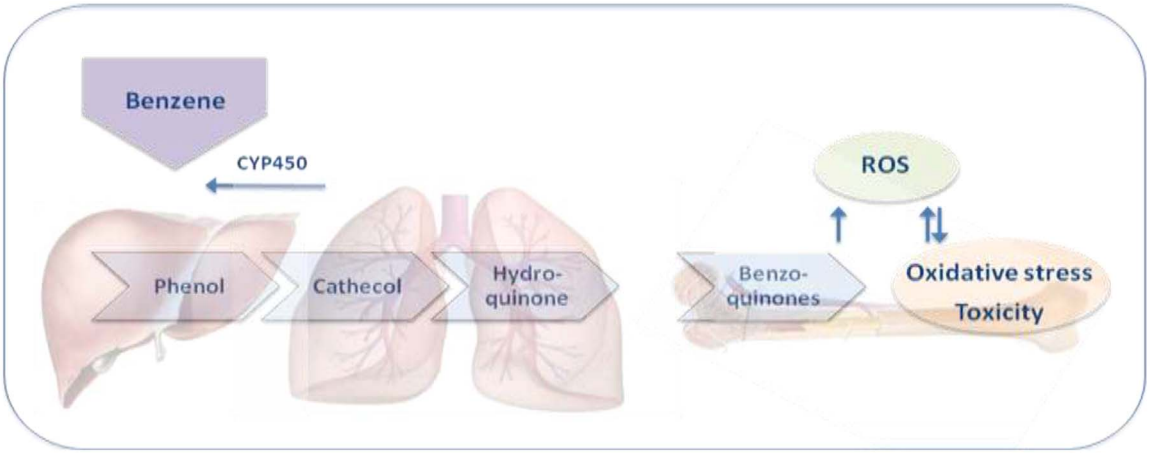

Fig. 1. Benzene metabolism in liver, lung and bone marrow can produce free radicals, which lead to pro-oxidant/antioxidant imbalance and oxidative stress.

phenol) which can produce reactive oxygen species (ROS) through redox cycling as illustrated in Fig. 1 [5,6]. On this basis, serum reactive oxygen metabolites have been recently suggested as biomarkers in oxidative stress assessment [7,8]. ROS are advocated as mediators in key steps leading to benzene toxicity. They also interact with cellular biomolecules originating peroxidised derivatives including advanced oxidation protein products (AOPP), advanced glycation end-products (AGE) and 8-hydroxy-deoxyguanosine (8-OHdG); several studies suggested to adopt these by-products as biomarkers to assess individual risk in workers exposed to benzene [9–12,7].

In particular, 8-OHdG is a ROS-induced DNA base modification due to hydroxyl radical attack of guanine; when left unrepaired, this damage also seems to be involved in mutagenicity and cancer promotion [13]. Consequently, 8-OHdG represents an indicator of endogenous oxidative DNA damage, aimed to risk evaluation of cancer and degenerative diseases [14]. Recently, it has been suggested that 8-OHdG might be a suitable biomarker of DNA damage due to occupational exposure to toxic compounds; in fact, workers exposed to benzene [15,12], Cr VI [16,17], polycyclic aromatic hydrocarbons [18,19] or nanoparticles [20] showed increased urinary 8-OHdG levels.

In the present study we investigated the relation between exposure to benzene and the occurrence of oxidative DNA damage in gasoline station workers, as well as the possible role of interfering or confounding factors. Benzene exposure level has been assessed by determination of its urinary metabolite trans,trans-muconic acid (t,t-MA), a validated biological exposure index.

2. Material and methods

2.1. Study population

We recruited a group of 80 men, employed in gasoline stations located in East Sicily, and matched it with a control group (n = 63) of male office employees not occupationally exposed to benzene. The same population had been investigated in previous studies for different endpoints [7,21]. Subjects provided information *via* custom-made questionnaires regarding socio-demographic characteristics (age and Body Mass Index), lifestyle (use of tobacco smoke and alcoholic beverage) and job-related records (lifetime exposure to benzene, use of personal protective equipment). The existence of occupational diseases, infections or other disorder in which oxidative stress plays a role in the previous trimester was also evaluated.

2.2. Exposure assessment

After three successive days of exposure, urine samples of workers enrolled in both groups were collected at the end of the work shift, divided into aliquots and preserved in polyethylene tubes at −80 °C. Urinary t,t-MA concentration was determined for individual benzene exposure monitoring by solid phase extraction followed by high performance liquid chromatography, using a kit supplied by Eureka Lab Division (Ancona, Italy) with an Agilent 1200 series HPLC equipped with diode array detector; t,t,-MA levels were extrapolated from a calibration curve and expressed as μg/ml urine.

Environmental monitoring records were provided by gasoline station managers and reported median airborne benzene concentrations at pump site well below the Threshold Limit Value-Time Weighted Average suggested by ACGIH (0.5 ppm) [34].

2.3. Evaluation of oxidative DNA damage

Urine levels of 8-OHdG were evaluated by a competitive immunoassay (OxiSelect™ Oxidative DNA Damage ELISA kit, Cell Biolabs, Inc., San Diego, CA, USA), following the manufacturer's instructions. Absorbance of samples was read at $\lambda = 450$ nm against a standard curve with a Synergy HT Microplate Reader (Biotek, Winooski, USA) and 8-OHdG concentrations were expressed as ng/ml. The assay was performed in technical duplicates.

2.4. Statistical analysis

Data were analyzed by Prism version 5.01 (GraphPad software, La Jolla, CA, USA) using Student's *t*-test for the comparison between benzene-exposed and not-exposed workers. Two-tailed Pearson test was used for correlation analysis. Multiple linear regression analysis was performed to determine the contribution of potential confounding factors on the biomarkers of internal dose and effect. Non-parametric tests were chosen when normality check, performed by Kolmogorov-Smirnov test, showed a non-Gaussian distribution of data. A $p < 0.05$ was adopted as a limit of significance.

3. Results

Table 1 reports core data obtained from questionnaires concerning socio-demographic characteristics, lifestyle and duration of occupational exposure to benzene (job seniority) of the study population. No subject enrolled in the study presented signs or symptoms of work-related disease or other illness linked to oxidative stress. No subject reported an excessive daily intake of alcohol, when < 2 glasses/day of wine or beer were considered normal. Only a minor fraction (approximately 10%) of subjects were smokers.

All subjects declared to use personal protections while working.

As shown in Fig. 2, significantly higher urinary t,t,-MA levels ($p < 0.05$) were observed in gasoline station attendants

Table 1
Sociodemographic characteristics, job seniority and lifestyle of study population. Student's *t*-test did not highlight any significant difference (NS) between benzene-exposed and control group.

	Benzene-exposed workers	Controls	P
N	80	63	NS
Gender	80 M, 0 F	63 M, 0 F	–
BMI (mean ± SD)	24.42 ± 2.34	25.22 ± 0.31	NS
Age (years, mean ± SD)	37.44 ± 9.13	40.70 ± 11.39	NS
Occupational exposure to benzene (years, mean ± SD)	13.74 ± 5.47	–	–
Smokers	7 (8.7%)	8 (12.7%)	NS
Alcohol abuse	0	0	NS

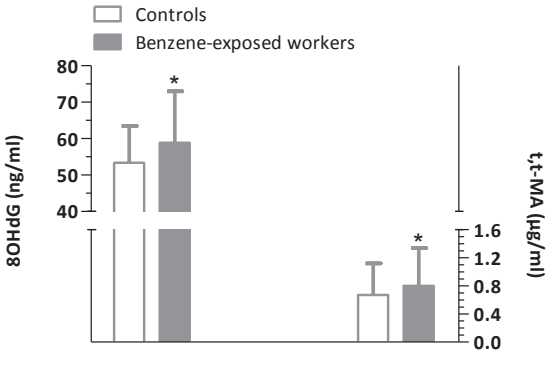

Fig. 2. Urinary concentration of t,t-muconic acid and 8-hydroxydeoxyguanosine in gasoline station attendants and control group.* = $p < 0.05$.

(0.83 ± 0.54 µg/ml, mean ± SD) compared to controls (0.67 ± 0.45 µg/ml, mean ± SD). Moreover, a significant increase in 8-OHdG ($p < 0.05$) levels was also observed in subjects exposed to benzene (57.92 ± 13.26 ng/ml, mean ± SD vs 53.35 ± 10.11 ng/ml in controls).

Two-tailed Pearson's correlation analysis was performed between mean urinary 8-OHdG levels and all other variables to test associations (Table 2). Pearson's test demonstrated a strong correlation ($r = 0.377$, $p < 0.001$) between this biomarker of oxidative stress and benzene exposure level (as urinary t,t-MA concentration, Fig. 3A). As shown in Fig. 3B, 8-OHdG significantly correlated also with job seniority, ($r = 0.312$, $p < 0.01$), whereas the relation with age is still significant but weaker Fig. 3C, ($r = 0.242$, $p < 0.05$).

Multiple linear regression analysis, performed to exclude a role for confounding factors, showed that variables like gender, smoking habit, alcohol consumption and BMI did not have a significant influence on the measured biomarkers.

4. Discussion

This study reports oxidative DNA damage in gasoline station workers exposed to low-dose benzene.

There is general agreement on the hypothesis that the enzymatic bioactivation of benzene plays a significant role in benzene-initiated toxicity through oxidative damage of DNA [22]. Benzene metabolism can induce free radicals production, which leads to pro-oxidant/antioxidant imbalance and oxidative stress [11]. More specifically benzene metabolism through the cytochrome P450 leads to the formation of various metabolites, which are responsible for free radicals production. As a result, antioxidant reserves are exhausted and the excess of free radicals can oxidize various cellular molecules causing the occurrence of oxidative stress. In previous studies [2,21] we suggested that benzene can be responsible of epigenomic damage and that reactive oxygen species (ROS) increase in low-dose benzene-exposed workers can cause toxicity by numerous signaling pathways like NF-κB, p38-MAPK, SAPK/JNK and STAT3, which play a role in the regulation of cell proliferation, differentiation and apoptosis [23]; given their possible involvement also in leukemogenesis [24,25], it is hypothesized that chronic exposure to benzene may cause toxicity by acting on these pathways even at low doses.

Urinary levels of 8-OHdG represent one of the most studied DNA lesions, and are a well-established biomarker suggestive of short-term DNA damage. Therefore, it is a suitable method to assess DNA damage induced by exposure to potential human carcinogens.

In the present study 8-OHdG, consistently with its genesis, was present in all urine samples from both exposed and not-exposed groups, at overall concentrations comprised between 27.8 and 101.4 ng/ml. Statistical relevance of the difference between the two groups was barely higher than the level of significance, presumably due to low benzene exposure level; this hypothesis is supported by the comparable behavior of t,t-MA, representative of internal dose of benzene.

It is well known that oxidative stress determines disturbance of intracellular metabolic processes which cause alterations including oxidation of lipids, proteins and DNA with consequent long-lasting modifications, as suggested by several studies [10]; resultant oxidation products include 8-OHdG, an oxidized nucleoside originated from DNA oxidative damage and repair an excreted with urine [26]. In our results, Pearson correlation analysis of the relationship between of t,t-MA and 8-OHdG revealed that urinary levels of 8-OHdG were significantly increased along with internal dose of benzene.

There is evidence that DNA damage tends to increase with age, as repair functions become less efficient [27]. As shown in Fig. 3, we found this trend in exposed subjects of this population ($p < 0.05$), but correlation was stronger considering years of job seniority instead of age ($p < 0.01$) and even more robust with the biomarker of benzene internal dose ($p < 0.001$). Coherently, years of occupational exposure

Table 2
Pearson's correlation coefficients among urinary 8-OHdG concentrations, benzene exposure level (t,t-MA), smoking habit, alcohol consumption, BMI, age and job seniority.

	t,t-MA	Smoking habit	Alcohol consumption	BMI	Age	Job seniority
8-OHdG	0.377^{***}	-0.131^{NS}	0.085^{NS}	0.132^{NS}	0.242^{*}	0.312^{**}
t,t-MA	1.00	-0.039^{NS}	0.272^{*}	-0.102^{NS}	0.101^{NS}	0.136^{NS}
Smoking habit		1.00	-0.204^{NS}	-0.121^{NS}	0.044^{NS}	0.057^{NS}
Alcohol consumption			1.00	0.002^{NS}	-0.081^{NS}	-0.145^{NS}
BMI				1.00	-0.029^{NS}	-0.115^{NS}
Age					1.00	0.844^{***}

$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

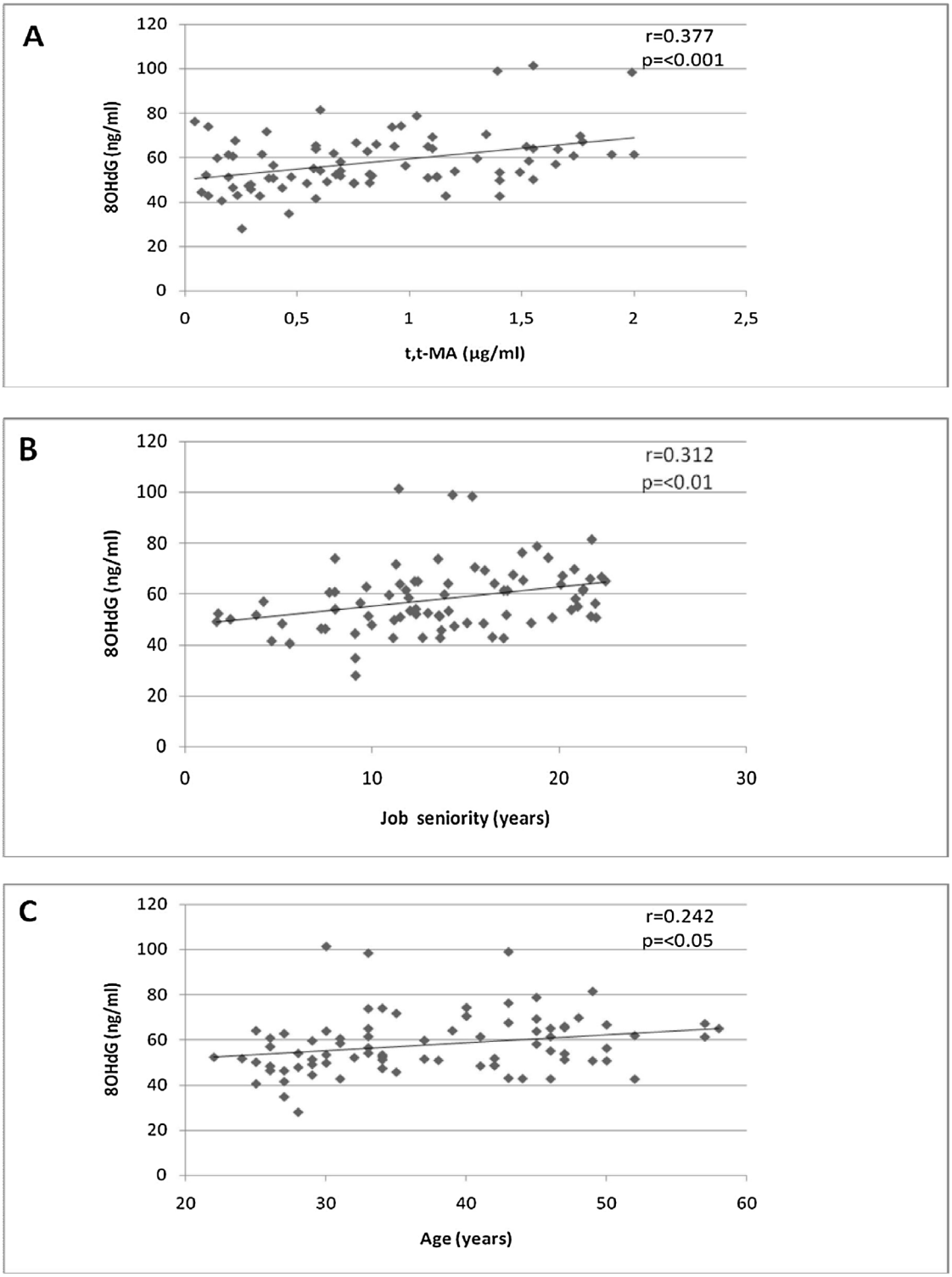

Fig. 3. Pearson's correlation analysis of urinary levels of 8-hydroxydeoxyguanosine with t,t-MA (A), with occupational lifetime exposure to benzene (B) and with age (C) in low-dose benzene exposed workers.

to benzene, i.e. job seniority, resulted associated with age ($p < 0.001$). No other relationship was observed between the variables analyzed in this study.

Multiple linear regression analysis, applied to adjust the influence of possible confounding factors, showed that no covariates had a significant influence on 8-OHdG and t,t-MA levels. This result is particularly relevant for smoke, a well known interfering factor for the biological monitoring of benzene exposure. As already observed in a previous study [7], it can be explained by the low percentage of smokers in this population and by interdiction of smoke near gasoline filling pumps. Though we would have expected a more evident outcome of smoke on 8-OHdG levels, statistical analysis excluded also this confounding effect; this trend has been already observed by other authors, and in our opinion it deserves further study because literature reports very controversial data [28].

None of the workers enrolled in this study showed signs or

symptoms of diseases referable to benzene exposure. Moreover, urinary t,t-MA levels, though allowing to distinguish exposed from not-exposed subjects, confirmed the low environmental concentrations of benzene, which were below the TLV-TWA level proposed by ACGIH. Nonetheless, our results suggest that oxidative damage to DNA occurred in these subjects owing to benzene exposure. Other authors found significantly higher 8-OHdG concentrations in gasoline station attendants [12,29,30] and policemen [31] exposed to low environmental concentrations of benzene, in children co-exposed to benzene toluene and polycyclic aromatic hydrocarbons [32] and in teenagers [27]. However, not all studies report a strong association between 8-OHdG concentrations and environmental exposure to benzene [33].

In conclusion, our results suggest that low-level chronic exposure to benzene among gasoline station attendants can determine oxidative damage on DNA, as indicated by alteration of biomarkers which may represent early signals of damage to biomolecules in exposed subjects. Though statistically relevant, this relationship is weakened by the nature itself of 8-OHdG, which has not yet been validated as a biomarker and does not originate by specific contact of DNA with benzene but by a variety of oxidative stress sources. Moreover, a larger population would improve the robustness of this cross-sectional design study. Nonetheless, 8-OHdG may be a suitable biomarker of effect or effective dose following cumulative exposure to oxidant chemicals or even of susceptibility, indicating the efficacy of various repair systems. Sample availability and non-invasiveness would be further advantages.

Conflict of interest

Authors declare that there are no conflicts of interest.

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