Molecular biomarkers of oxidative stress and role of dietary factors in gasoline station attendants

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A B S T R A C T

Exposure to benzene promotes oxidative stress through the production of ROS, which can damage biological structures with the formation of new metabolites which can be used as markers of oxidant/antioxidant imbalance.

This study aims to assess modifications in circulating levels of advanced oxidation protein products (AOPP), advanced glycation end-products (AGE) and serum reactive oxygen metabolites (ROMs) in a group of gasoline station attendants exposed to low-dose benzene and to evaluate the influence of antioxidant food intake on these biomarkers of oxidative stress. The diet adopted by the population examined consisted of compounds belonging to the classes of terpenoids, stilbenes and flavonoids, notably resveratrol, lycopene and apigenin.

Ninety one gasoline station attendants occupationally exposed to benzene and 63 unexposed male office workers were recruited for this study. Urinary trans, trans-muconic acid (t,t-MA) concentration, determined to assess individual exposure level, resulted significantly higher in exposed workers.

In subjects exposed to benzene, we observed a significant increase (p < 0.001) in ROMs and AOPP levels, which were also negatively correlated with fruit and vegetables consumption. By contrast, AGE did not show a significant increase and consequently any relation with antioxidant food intake. Only ROMs, representing a global biomarker of oxidative status, resulted correlated to t,t-MA levels (p < 0.01), probably due to low-dose exposure.

Increase of ROS induced by reactive benzene metabolites may promote specific biochemical pathways with a major production of AOPP, which seem to represent a more sensitive biochemical marker of oxidative stress in workers exposed to benzene compared to AGE. Furthermore, this is the first study demonstrating ROMs increment in subject exposed to benzene. These biomarkers may be useful for screening purposes in gasoline station workers and other subjects exposed to low-dose benzene. Moreover, a diet rich in fruits and vegetables demonstrated an inverse association with the levels of oxidative stress markers, suggesting a protective role of antioxidant food intake in workers exposed to oxidant agents.

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Abbreviations: ACGIH, American Conference of Governmental Industrial Hygienists; AGE, advanced glycation end-products; ALE, advanced lipoperoxidation end-products; AOPP, advanced oxidation protein products; ARE, antioxidant response element; IARC, International Agency for Research on Cancer; NIOSH, National Institute for Occupational Safety and Health; Nrf2, nuclear factor 2; ROMs, serum reactive oxygen metabolites; ROS, reactive oxygen species; t,t-MA, t,t-muconic acid.

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1. Introduction

Benzene, an important environmental pollutant widely used in industrial settings, is classified in EU as Carc 1A and as a group 1 human carcinogen by the International Agency for Research on Cancer (IARC).

The American Conference of Governmental Industrial Hygienists (ACGIH) and the National Institute for Occupational Safety and Health (NIOSH) have set an exposure limit value for benzene at 1.6 mg/m³ (0.5 ppm) and 0.32 mg/m³ (0.1 ppm) respectively.

Recent studies demonstrated that subjects exposed to benzene air concentrations <0.1 ppm metabolize benzene about nine times more efficiently than heavily exposed workers. This suggests that the health risks associated with low and very low benzene exposures can be considerably greater than those predicted by linear extrapolation from epidemiological studies involving workers exposed to air concentrations of tens to hundreds of ppm (Kim et al., 2006).

The chronic exposure of humans to benzene in work places has been associated with hematotoxicity, leukemia and aplastic anemia, after long-term exposure to high concentrations (Snyder, 2012; Lagorio et al., 2013; Bassig et al., 2015). Moreover, recent studies demonstrated hematological and genotoxic disorders in subjects occupationally exposed to low levels of benzene (Glass et al., 2003; Lan et al., 2004; Smith et al., 2007; Rappaport et al., 2013). However, although it is well established that benzene requires metabolism to induce its effect (McHale et al., 2012), the exact mechanism responsible for its toxicity still remains unclear.

The enzymatic bioactivation of benzene leads to the formation of reactive metabolites, such as phenol, catechol and idroquinone, which play a key role in the production of reactive oxygen species (ROS) (Barreto et al., 2009). Several studies have demonstrated increased ROS levels after benzene exposure in vivo and in vitro (Ho and Witz, 1997; Winn, 2003). The imbalance between the production of ROS and antioxidants in favor of free radicals causes a state of oxidative stress (El Batsch et al., 2015). Toxic effects are caused through the production of peroxides and free radicals that damage important cellular components - proteins, carbohydrates, lipids and nucleic acids - and may enhance inflammatory response. New compounds and modified structures (which can serve as markers of these mechanisms) are formed, as advanced oxidation protein products (AOPP), advanced glycation end-products (AGE) and advanced lipoperoxidation end-products (ALE) (Kalousova et al., 2005). Most of these products have been extensively investigated in various human tissues and blood and they have been proposed as biomarkers for risk evaluation in workers exposed to benzene (De Palma and Manno, 2014). Risk assessment of residual compounds in food and environmental pollutants has emerged as a significant need for the evaluation of toxicity and hazard index in human populations (Tsakiris et al., 2015).

Furthermore, recently serum reactive oxygen metabolites (ROMs) have been reported to be a reliable biomarker to assess oxidative stress (Chen and Kotani, 2015).

The aim of this study is to assess modifications of AOPP, AGE and ROMs circulating levels, as early markers of oxidative stress, in a group of gasoline station attendants exposed to low dose of benzene.

Additionally, t,t-muconic acid (t,t-MA) in urine, as biomarker of benzene exposure, and the influence of vegetables and fruits consumption, a rich source of exogenous antioxidants, on these biomarkers have also been evaluated.

2. Material and methods

2.1. Study population

A group of 91 men, employed in gasoline stations located in Eastern Sicily, was enrolled for the study and compared with a control group (n = 63) of male office employees with no occupational exposure to benzene. Workers were enrolled in a health surveillance program for the prevention of occupational diseases. Written consent was obtained for participation in this study. A custom-made questionnaire was used to collect information on socio-demographic characteristics (age and Body Mass Index), lifestyle (smoking habit, alcohol consumption, fruits and vegetables intake) and occupational features (lifetime exposure to benzene, use of personal protective equipment) and to exclude known disorders or diseases (job-related diseases, infection or other pathology involving oxidative stress) in the three months preceding the survey.

2.2. Assessment of benzene exposure

Environmental monitoring data, provided by gasoline station managers, indicated benzene levels <0.1 ppm as measured at pump site.

Urine samples of both exposed and control group were collected at the end of the work shift, after three consecutive days of exposure, and stored at −80 °C until analysis. Urinary t,t-MA concentration, as a biomarker of benzene exposure, was determined by solid phase extraction followed by high performance liquid chromatography with diode array detection with an Agilent 1200 series HPLC, using a kit produced by Eureka Lab Division (Ancona, Italy); t,t-MA levels were expressed as µg/ml.

2.3. Evaluation of molecular biomarkers of oxidative stress

AGE and AOPP levels were determined using the methods employed by Kocak et al. (2009) with some modifications described in a previous study (Costa et al., 2015).

Briefly, for AGE measurement serum samples were diluted 1:50 with phosphate buffered saline (PBS, pH 7.4) and pipetted in a black microtiter plate. Fluorescence intensity with λ_exc 350 nm and λ_em 440 nm was measured with a Sinergy HT microplate absorbance reader (Biotek, Winooski, USA) and expressed as arbitrary units (AU) per ml.

To measure AOPP serum concentrations, diluted serum samples (200 µl, 1:5 in PBS) were pipetted in a microtiter plate with 10 µl of 1.16M KI and 20 µl acetic acid. Absorbance was measured at 340 nm with a Sinergy HT reader, using a calibration curve with 0–128 μM chloramine T for AOPP quantification.

In order to assess reactive oxygen metabolites, d-ROMS test was used (Diacon International). Absorbance at 505 nm was recorded and measurements were expressed as Carr Units (U CARR) (Hirose et al., 2009).

The coefficient of variation for replicate measurements was <5% for all assays.

2.4. Statistical analysis

Data were analyzed by Prism version 5.01 (GraphPad software, La Jolla, CA, USA) using Student's t test to compare benzene-exposed subjects to control group, while correlation analysis was performed by Spearman test. A p < 0.05 was adopted as a limit of significance.
3. Results

Table 1 reports main data regarding socio-demographic characteristics and lifestyle of the study population are presented in. None of the participants showed signs or symptoms of occupational diseases, infection or other pathology. Considering normal the consumption of 1 or 2 glasses of wine or beer per day or up to 3 serving of liquor per week, most subjects had an adequate daily intake of alcohol, and also of food rich in antioxidants. Fruits and vegetables commonly consumed in the geographical area involved in the study are those typical of Mediterranean diet, i.e. mainly Citrus fruits, tomatoes, broccoli and other cruciferous vegetables, bell peppers and aubergines. Study subjects also consumed olive oil and red wine which is a good source of resveratrol and anthocyanins.

A small percentage of subjects declared to be smokers, while all workers reported to use adequate personal protective equipment.

<table>
<thead>
<tr>
<th></th>
<th>Benzene-exposed workers</th>
<th>Controls</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>91</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>Age (years, mean ± SD)</td>
<td>38.13 ± 9.46</td>
<td>40.70 ± 11.39</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (mean ± SD)</td>
<td>24.47 ± 2.28</td>
<td>25.22 ± 0.31</td>
<td>NS</td>
</tr>
<tr>
<td>Lifetime exposure to benzene (years, mean ± SD)</td>
<td>14.06 ± 6.23</td>
<td>8 (12.7%)</td>
<td>NS</td>
</tr>
<tr>
<td>Smokers</td>
<td>9 (9.9%)</td>
<td>8 (12.7%)</td>
<td>NS</td>
</tr>
<tr>
<td>Alcohol abuse</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Fruits and vegetables intake (servings/day)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1–2</td>
<td>11 (12.1%)</td>
<td>7 (11.1%)</td>
<td></td>
</tr>
<tr>
<td>2–3</td>
<td>35 (38.4%)</td>
<td>26 (41.3%)</td>
<td></td>
</tr>
<tr>
<td>3–4</td>
<td>36 (39.6%)</td>
<td>22 (34.9%)</td>
<td></td>
</tr>
<tr>
<td>≥4</td>
<td>9 (9.9%)</td>
<td>8 (12.7%)</td>
<td></td>
</tr>
</tbody>
</table>

No correlation was found between oxidative stress biomarkers and age, BMI or tobacco smoking.

4. Discussion

In the present study a set of oxidative stress biomarkers in a group of gasoline station attendants exposed to low benzene doses considered “safe” by international guidelines, has been investigated. In fact both environmental and biological exposure assessment data indicated levels lower than current exposure limit values.

Benzene toxicity has been linked to the ability of its reactive intermediates to produce reactive oxygen species (ROS) and to bind cellular macromolecules to induce damage (Moro et al., 2013). Elevated levels of oxidants promote the oxidation of lipids, proteins and glucose, resulting in the accelerated formation of AGE and AOPP (Ott et al., 2014) which have been successfully utilized as biological markers of oxidative stress (Prasad et al., 2012; Gubandru et al., 2013; Morimoto et al., 2015), suggesting a positive correlation between smoking habit and AGE, AOPP or ROMs, probably because only approximately 10% of study population declared to be smokers.

This study showed a significant correlation between benzene exposure (in terms of urinary metabolite t.t.-MA) and oxidative stress expressed as ROMS levels, but unexpectedly not with AOPP concentration. A study population with a wider range of benzene exposure levels would probably disclose such correlation. Another possible explanation is that ROMS, representing a global biomarker of oxidative status, showed a three-fold increase in the exposed group, while AOPP presented a sensibly smaller increment.

As regards the antioxidant capacity of fruits and vegetables, our results confirmed previous studies (Shewita and Sheikh, 2011; Di Renzo et al., 2015; Morimoto et al., 2015), suggesting a positive effect of the intake of food rich in antioxidants on oxidative status. Evaluation of AOPP and ROMS in subjects stratified according to fruits and vegetables consumption (0.25 = 1–2 servings/day; found a significant increase in AOPP but not in AGE levels in subjects exposed to benzene. The different behavior observed for AOPP and AGE is very likely due to reactive benzene metabolites with a consequent increase of ROS that promote specific biochemical pathways (e.g., the myeloperoxidase pathway), with a major production of AOPP. Conversely, AGE are produced mainly from auto-oxidation of sugars and other glycation intermediates; this origin can support their increase in diabetes and in dialyzed patients, but is not substantiated in healthy benzene exposed subjects (Sick et al., 2010; Gradinaru et al., 2013; Saeidnia and Abdollahi, 2013; Xie et al., 2014). Consequently, oxidized proteins in benzene exposed subjects may be converted preferentially in direct oxidation byproducts instead of glyco-oxidation byproducts. Thus, AOPP could represent a more sensitive biochemical marker of oxidative stress in workers exposed to benzene.

The ROMS assay measures the derivatives of reactive oxygen metabolites, allowing to evaluate the equilibrium between free radical production and antioxidant defense (Cornelli et al., 2001). Interestingly, in our sample of subjects exposed to benzene we found an increase in ROMS levels which is not supported by the presence of any disease involving oxidative stress. In fact, measuring the levels of hydroperoxides derived from biological macromolecules (lipids, proteins, nucleic acids, etc.) has been recently suggested as a simple clinical marker of oxidative stress, which may be useful for screening purposes in workers exposed to oxidant chemicals (Kotani and Sakane, 2012; Wakabayashi et al., 2014). To our knowledge, this is the first study demonstrating that ROMS are increased in subjects exposed to benzene.

Smoking is a major source of benzene intake and it is considered the most important confounding factor in the biological monitoring of exposure to low levels of benzene (Fustinoni et al., 2005). However, in our study we didn’t highlight any correlation between smoking habit and AGE, AOPP or ROMS, probably because only approximately 10% of study population declared to be smokers.

Table 1
Sociodemographic characteristics and lifestyle of study population. Student’s t test did not indicate significant differences (NS) between benzene-exposed and control subjects. All data are mean ± SD.
day) showed a significant inverse correlation between these oxidative stress biomarkers and antioxidant active principles intake. Therefore, the protective role demonstrated by diets low in saturated fat and rich in fruits and vegetables, as well as a moderate wine consumption, against the development and progression of chronic degenerative diseases may prevent the damage due to occupational exposure to oxidant chemicals (Di Renzo et al., 2015).

The putative association for the protective role of fruit and vegetable consumption on the oxidative-stress status caused by benzene exposure can be explained by the concentrations of various phytochemicals present in dietary food and beverages (Fig. 3). For example red wine is a very rich source of the stilbene resveratrol that has demonstrated antioxidant activity in animal models and humans. Compounds such as curcumin found in cruciferous vegetables (e.g. cauliflower, cabbage and broccoli) have been associated with the reduction of various diseases related to oxidative stress, notably cardiovascular disease and cancer (Pagliaro et al., 2015). More specifically the polyphenolic subclass of flavonoids has attracted the attention of the scientific community during the past years due to the beneficial effect of flavones and flavonols in the prevention of oxidative-stress-related diseases and to their particular abundance in the diet.

The mechanism sustaining the antioxidant action of flavonoids was initially based on scavenging of free radicals and delocalization of the negative charge via the conjugated bonds of the B and C rings of the polyphenol moiety (Brown et al., 1998). Several additions to the existing hypothesis have been proposed lately including modulation of the antioxidant response element and the transcriptional regulation of genes involved in the antioxidative response. It is well accepted that certain phytochemicals activate the antioxidant response element (ARE)/nuclear factor 2 (Nrf2) pathways and thus modulate expression of genes involved in oxidative protection against neurological diseases and cancer. Both naringenin, a flavonoid found in citrus fruits, and curcumin increase the expression of Nrf-2 and activate the ARE downstream signaling (Lou et al., 2014). Curcumin specifically activates the expression of thioredoxin that protects neurons from death caused by oxygen-glucose deprivation (Wu et al., 2015). In addition resveratrol was shown to reduce the expression levels of pro-inflammatory cytokines by increasing phosphorylation of cAMP binding protein (p-CREB) and thereby exerting antidepressant activity (Ge et al., 2015), whereas combinations of several carotenoids (lycopene, phytoene, phytofluene and vitamin E) demonstrated synergistic inhibition of the androgen receptor and activation of the ARE system (Linnewiel-Hermoni et al., 2015).

In the current study, human workers exposed to benzene whose lifestyle included a diet rich in fruits and vegetables demonstrated an inverse association with the levels of oxidative stress markers, notably ROMs and AOPP, as opposed to comparison with benzene exposure alone. Although the study did not address the exact nature and quantities of fruit and vegetable intake, common compounds contained in the basic dietary components of the population exposed are: resveratrol, naringenin, curcumin, vitamin E, citric acid, carotenoids, lycopene and polyunsaturated fatty acids.

A huge number of peer-reviewed publications as well as some open access electronic databases rank foods and beverages on the

Table 2

<table>
<thead>
<tr>
<th></th>
<th>ROMs (U CARR)</th>
<th>AOPP (µM)</th>
<th>AGE (AU/ml)</th>
<th>t.t-MA (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n = 63)</td>
<td>112.08 ± 20.27</td>
<td>30.33 ± 14.91</td>
<td>41.8 ± 0.19</td>
<td>0.67 ± 0.45</td>
</tr>
<tr>
<td>Benzene-exposed (n = 91)</td>
<td>342.57 ± 123.73</td>
<td>50.4 ± 24.4</td>
<td>42.38 ± 0.78</td>
<td>0.89 ± 0.57</td>
</tr>
<tr>
<td>T test Controls vs Benzene-exposed</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>NS</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Spearman test vs t.t-MA</td>
<td>r = 0.312</td>
<td>0.126</td>
<td>–0.073</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Fig. 1. Spearman correlation analysis between consumption of food rich in antioxidant active principles1 and serum levels of Reactive Oxigen Metabolites (A) or Advanced oxidation Protein Products (B).1 0.25 = 1–2 servings/day; 0.5 = 2–3 servings/day; 0.75 = 3–4 servings/day; 1 =4 servings/day.

Fig. 2. Spearman correlation analysis between urinary t.t-MA concentration and serum levels of Reactive Oxigen Metabolites.

0.5 = 2–3 servings/day; 0.75 = 3–4 servings/day; 1 ≥4 servings/day) showed a significant inverse correlation between these oxidative stress biomarkers and antioxidant active principles intake. Therefore, the protective role demonstrated by diets low in...
basis of the content of individual polyphenols and/or classes or subclasses of polyphenols. Pure citrus juice contains 40–60 mg/100 ml flavanones, 5–20 mg/100 ml flavones and 1–5 mg/100 ml anthocyanins; olives and artichokes contain 40–60 mg/100 g flavones and >100 mg/100 mg hydroxycinnamic acids; tomatoes have 1–5 mg/100 g hydroxycinnamic acids (Manach et al., 2004; Perez-Jimenez et al., 2010). However, though individual effect estimate of single food components can be weak, when healthy foods are combined in a dietary pattern outcomes estimates are usually stronger, suggesting a synergism to obtain more potent antioxidant and anticancer activities. The beneficial effects of a diet rich in fruits and vegetables are attributed to the complex mixture of phytochemicals present in foods, therefore no single antioxidant can replace the combination of natural phytochemicals contained in fruits and vegetables (Liu, 2004; Melchini et al., 2009, 2013; Stagos et al., 2012; Apostolou et al., 2013; Matthaiou et al., 2014; Margina et al., 2015a).

Further studies in the future may assess the antioxidant protection conferred in humans by individual compounds such as those mentioned above, or combinations instead of dietary habits, as well as the transcription of genes involved in the ARE pathway. Furthermore it is important to stress that the interactions between xenobiotics or pharmaceutical drugs and dietary compounds can significantly alter the pharmacokinetic parameters, absorption and distribution patterns of the above mentioned compounds (Margina et al., 2015b).

In conclusion, this study provides evidence that AOPP are a more sensitive biomarker of oxidative stress in workers exposed to low doses of benzene than AGE. Further studies are required in order to evaluate the use of ROMs as biomarkers of oxidative stress in workers exposed to benzene.

Conflict of interest

The authors declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fct.2016.01.017.

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