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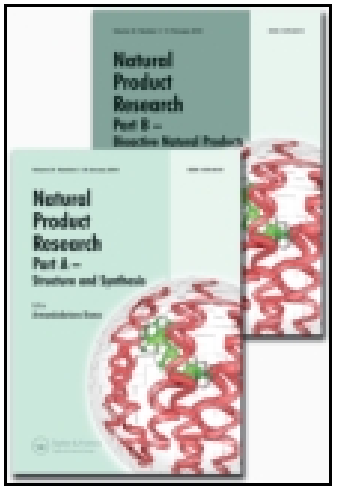

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Protective effects of a standardised red orange extract on air pollution-induced oxidative damage in traffic police officers

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Several pathological conditions have all been associated with a higher release of atmospheric pollutants. There is growing evidence that oxidative stress may represent one of the agents involved in the initiation and/or progression of many of these pathologies. The aim of the present study was to evaluate the effects of short-term dietary supplementation with a standardised red orange extract (ROC) on a group of traffic police officers exposed to traffic exhaust pollution and cigarette smoking, by measuring some noninvasive biomarkers of oxidative stress. At the beginning of the study, all the groups showed similar serum lipid hydroperoxide levels, but traffic officers showed lower serum concentrations of thiol (SH) groups; furthermore, the frequency of spontaneous sister chromatide exchanges (SCEs) in peripheral lymphocytes was increased by smoking (but not by pollution exposure alone) at a higher degree in subjects exposed to traffic pollution. After 1 month of ROC administration, serum lipid hydroperoxide levels decreased only in all non-smoking subjects; furthermore, SH group levels measured in traffic officers appeared restored to normal values observed in the respective controls. Finally, the increase in SCE frequency induced by smoking was reduced by treatment with ROC especially in traffic officers. Our study suggests that ROC supplementation could be useful to minimise the detrimental effects caused by exposure to air pollution and smoking.

Keywords: air pollution; oxidative stress; red oranges; lipid hydroperoxides; thiol groups; sister chromatide exchanges

1. Introduction

In recent years, concern has grown about the adverse effects of air pollution on human health. Pathological conditions, such as pulmonary and extra-pulmonary cancer, reduced lung function, aggravation of asthma, increased susceptibility to respiratory infections, and more frequent cardiovascular and respiratory mortality have all been associated with an higher release of atmospheric pollutants (Brunekreef et al., 1997; Hoek, Brunekreef, Fisher, & Van Wijnen, 2001; van Vliet et al., 1997). Moreover, some of these health effects

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have been associated with proximity to vehicle traffic (Brunekreef et al., 1997; Brunekreef, & Holgate, 2002), suggesting increased hazards associated with exposure to fresh emissions. In particular, a recent work (Motta, Federico, Saccone, Librando, & Mosesso, 2004) showed cytogenic damage associated with air pollution in the city of Catania (Italy).

There is a strong body of evidence indicating oxidative stress as the major mechanism that mediates the toxic effects of most forms of air pollution (Kelly, 2003). Ambient air contains a mixture of compounds including free radicals (for example nitrogen dioxide), ozone, and tiny particles (particulate matter $<10\,\mu$ in aerodynamic diameter) able to drive free radical reactions. Exposure to air pollutants can lead to the activation of inflammatory cells and generation of large amounts of free radicals and reactive oxygen species (ROS) (Kelly, Mudway, & Blomberg, 1999). Although healthy individuals have a range of antioxidant defenses, including enzymatic and low molecular weight nonenzymatic molecules, in some conditions endogenous antioxidants are deficient and/or their efficiency is limited, so that they may be unable to counteract air pollution-induced oxidative stress and to protect against the consequent cell damage. The sensitivity of individuals to air pollution has been demonstrated to vary according to the concentration of their antioxidant defenses; in particular, susceptible subjects, such as asthmatic patients, have been shown to have lower levels of ascorbic acid in the lung lining fluid, resulting in a lower level of antioxidant defenses and higher sensitivity to air pollution (Kelly, 2003). Finally, dietary supplementation with antioxidants, such as vitamin E, proved to be a successful strategy to provide protection against the damage caused by air pollution (Elsayed & Mustafa, 1982; Elsayed, 1987).

Red Orange Complex® (ROC, Bionap, Italy) is a standardised red orange extract, obtained from three red orange varieties (*Citrus sinensis* var. Moro, Tarocco and Sanguinello), and has recently been proposed as a new antioxidant food supplement. The main active principles of ROC are phenolic compounds (anthocyanins, flavanones and hydroxycinnamic acids) and ascorbic acid, the antioxidant activity of which is well recognised (Chen & Ho, 1997; Galvano et al., 2004; Kahkonen & Heinonen, 2003; Kong, Chia, Goh, Chia, & Brouillard, 2003; Noda, Kaneyuki, Mori, & Packer, 2002; Rice-Evans, Miller, & Paganga, 1996; Saija et al., 1999; Tusda et al., 1994; Wang, Cao, & Prior, 1997). We have previously demonstrated that ROC possesses strong *in vitro* free-radical scavenging/antioxidant activity and *in vivo* photoprotective effects against UVB induced skin erythema (Bonina et al., 1998). *In vitro* studies have shown that ROC is able to protect human skin-derived cells (NTTC 2544 keratinocytes and HFFF2 fibroblasts) against iron-induced lipid peroxidation (Morini, Dusatti, Bonina, Saija, & Ferro, 2000). Russo et al., (Russo et al., 2000; Russo et al., 2002) and Sorrenti et al., (2004) have demonstrated the antioxidant capability of ROC to protect DNA, scavenge free radicals, inhibit xanthine oxidase activity, and prevent LDL oxidation. Dietary supplementation with ROC was also shown to reduce oxidative stress in healthy smoker volunteers (Cornelli, Bonina, Valsasina, & Cornelli, 2000), in type-2 diabetic patients (Bonina et al., 2002), and in handball players (Bonina et al., 2005).

In the present study, we have investigated the effects of a short-term dietary supplementation with ROC on a group of road traffic policemen, by measuring some noninvasive biomarkers of oxidative stress such as serum levels of thiol (SH) groups (an indirect measurement of glutathione activity in serum) and lipid hydroperoxides and the frequency of spontaneous sister chromatide exchanges (SCEs; a biomarker of early cytogenetic damage) in peripheral lymphocytes.

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2. Materials and methods

Twenty traffic police officers (males, 38–52), including 7 smokers and 13 non-smokers, all working in a metropolitan area of a busy city centre (Catania, Italy), and therefore exposed to traffic exhausts, were enrolled in this study. A sex and age-matched control group of healthy indoor office workers, including four smokers and eight non-smokers from the same area underwent the same experimental procedures. The study was conducted in accordance with the Declaration of Helsinki, and a written informed consent was signed by all volunteers. None of the subjects were taking any kind of medication, including vitamins or antioxidant supplements. The volunteers were requested not to modify their dietary habits during the study.

All subjects were recommended to take two capsules of 50 mg each of ROC a day. The ROC capsules were a gift from Bionap (Rome, Italy); compliance of the volunteers was checked by counting the remaining capsules. The ROC extract was obtained by an original process from oranges of three pigmented *C. sinensis* varieties (More, Tarocco, Sanguinello) and was composed of 3.1% anthocyanins (cyanidin-3-glucoside), 2.07% hydroxycinnamic acids (caffeic, cumaric, ferulic and sinapic acids), 8.1% flavanone glycosides (narirutin and hesperedin), and 7% ascorbic acid.

Blood samples were collected from fasting patients on day 0 and after 1 month from the beginning of the study. Blood biochemistry parameters were measured by routine laboratory kits.

The serum levels of SH groups and lipid hydroperoxides were measured according to the methods described by Elman (1959) and Cornelli et al. (2001) respectively, using commercial kits purchased from Diacron (Grosseto, Italy). Briefly, the SH group determination system employs 5,5'-dithiobis-2-nitrobenzoic acid, which reacts specifically with thiol groups to give a highly-coloured yellow anion measured at 405 nm. The lipid hydroperoxide determination system is based on the ability of transition metals to catalyse in the presence of peroxides with formation of radicals which are trapped by an alkylamine giving a colored radical detectable at 505 nm; the results are expressed as Carratelli units (U. Carr). To determine spontaneous SCE frequency, 0.8 mL of whole heparinized blood were cultured in 10 mL RPMI-1640 medium supplemented with 20% fetal calf serum, 2% phytohemagglutinin, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (all Biochrom, Berlin, Germany). The cultures were incubated at 37°C for 70 h in an atmosphere of 5% $C0_2$ in air. 5-Bromo-2-deoxyuridine (Fluka, Milano, Italy) was used to identify the first and subsequent metaphases, and was added at a concentration of 10 μg mL^{-1} following 24 h incubation. Colchicine (0.2 μg mL^{-1}) was added 2 h before harvesting to arrest cells at metaphase. After hypotonic treatment (0.75 M KCl at 37°C for 15 min) and fixation in Camoy (3 : 1 methanol–acetic acid), the cells were resuspended and dropped onto clean slides. The slides were stained with fluorescence dye Hoechst 33258 (Fluka, Milan, Italy) and then by the Giemsa staining solution (Sigma, Milan, Italy) (Perry & Wolff, 1974). All slides were blindly coded and scored by one investigator to minimise observer bias. The second division metaphase with 46 chromosomes and good differential staining was selected for the scoring of SCEs. The metaphases with less than 46 chromosomes were not taken in consideration. About 20 second division metaphases were randomly selected and scored as SCEs/cell.

Data are expressed as mean ± standard deviation and were analysed using the two-way Student's *t*-test. A level of probability of < 0.05 was taken as indicating statistical significance.

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3. Results and discussion

In the present study, we have evaluated the effects of a short-term dietary supplementation with ROC on a group of police officers exposed to traffic exhaust pollution and cigarette smoking, by measuring serum lipid hydroperoxide and SH group levels and the frequency of SCEs in peripheral lymphocytes. In particular, one has to note that cellular thiols are critical in maintaining the cellular antioxidant defense mechanisms of the organism, and play a key role in regulating redox-sensitive signal transduction process. Furthermore, an increased frequency of SCEs is considered a sensitive indicator of exposure to agents or conditions capable of producing DNA damage. Studies conducted on populations exposed to environmental pollution have shown increased levels of several markers of cytogenetic damage, including chromosome aberrations, SCEs, and ras oncogene overexpression (Perera et al., 1992; Perera, Jedrychowski, Rauch, & Whyatt, 1999). Furthermore, Piperakis, Petrakou, and Tsilimigaki (2000) observed that the damage induced by air pollution on lymphocyte DNA is higher in smoking subjects.

At the beginning of the study, all groups showed similar serum lipid hydroperoxide levels (Table 1), but non-smoking and especially smoking traffic officers showed lower serum concentrations of SH groups (Table 2); furthermore, SCE frequency in peripheral lymphocytes was increased by smoking (but not by pollution exposure alone) at a higher level in traffic officers (Table 3).

After 1 month of ROC administration, serum lipid hydroperoxide levels decreased only in non-smoking subjects (both traffic officers and controls), but not in smoking subjects (Table 1), and SH group levels measured in traffic officers appeared restored to normal values observed in the respective controls (Table 2). Finally, treatment with ROC reduced SCE frequency induced by smoking especially in traffic officers (Table 3).

Interestingly, our results are consistent with other observations where an anthocyanin-rich extract was shown to be able to protect against oxidative DNA damage induced by vitamin E-deficiency in the rat (Ramirez-Tortosa et al., 2001), and flavonoids to protect diabetic human lymphocytes against oxidative DNA damage (Lean et al., 1999). Finally, cyanidin and cyanidin-3-glucoside have been proved to be able to prevent DNA cleavage *in vitro* (Acquaviva et al., 2003).

Blood orange juice was proven to be a bioavailable source of several antioxidants, such as vitamin C, cyanidin-3-glucoside, β-cryptoxanthin, and zeaxanthin (Riso et al., 2005). However, only relatively high amounts of blood orange juice are able to affect *in vivo* antioxidant status. For example, Riso and coworkers (Riso et al., 2005) have recently demonstrated that in human volunteers the consumption of 600 mL per day of blood

Table 1. Serum levels of lipid hydroperoxides measured in traffic police officers and healthy volunteers, determined using the D-ROMs test at day 0 and after 1 month of supplementation with ROC. Data are expressed as mean ± SD.

	Lipid hydroperoxide (U. Carr.)	
Subjects	Day 0	1 Month
Controls	343.88 ± 36.44	279.38 ± 48.00
Smoking controls	358.25 ± 19.97	336.75 ± 23.71
Traffic officers	350.54 ± 35.59	296.77 ± 42.38
Smoking traffic officers	319.43 ± 51.07	301.57 ± 36.08

Table 2. Serum levels of SH groups measured in traffic police officers and healthy volunteers at day 0 and after 1 month of supplementation with the ROC. Data are expressed as mean ± SD.

	SH Groups (μmol L^{-1})	
Subjects	Day 0	1 Month
Controls	381.00 ± 18.83	422.13 ± 39.27
Smoking controls	381.75 ± 36.10	409.19 ± 42.38
Traffic officers	339.69 ± 33.08*	528.46 ± 71.32**
Smoking traffic officers	272.71 ± 27.81***	459.46 ± 53.25***

*$p < 0.05$ *vs*. the respective controls at the same time.
**$p < 0.05$ *vs*. the respective day 0.
***$p < 0.05$ *vs*. the respective non-smoking controls.

Table 3. Frequency of spontaneous SCEs in peripheral lymphocytes of traffic police officers and healthy volunteers at day 0 and after 1 month of supplementation with the ROC. Data are expressed as mean ± SD.

	SCE frequency (mean SCEs/cell)	
Subjects	Day 0	1 Month
Controls	7.37 ± 0.67	7.41 ± 0.59
Smoking controls	8.84 ± 0.71**	8.35 ± 0.67**
Traffic officers	7.39 ± 0.52	7.25 ± 0.50
Smoking traffic officers	9.66 ± 0.75**	8.61 ± 0.57***

*$p < 0.05$ *vs*. the respective controls at the same time.
**$p < 0.05$ *vs*. the respective non-smoking group.
***$p < 0.05$ *vs*. the respective day 0.

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orange juice for 21 days determines a significant increase in plasma antioxidants and increases lymphocyte resistance to DNA oxidative damage without significantly affecting other markers of lipid oxidation. On the other hand, orange juice contains several nutrients, including 100–130 g L^{-1} of total sugars (Albertini et al., 2006; Moufida, & Marzouk, 2003), thus its introduction in the daily diet has to be regarded as a significant contribution to the total nutritional and caloric intake.

Furthermore, the protective effect of blood orange juice intake against oxidative DNA damage in human leucocytes appeared related not only to its vitamin C content, but to that of the other phytochemicals contained in it (Guarnieri, Riso, & Porrini, 2007). In the past, the biological properties of phytocomplexes, which are constituted by several and different ingredients, have been justified by the presence of a particular molecule having that specific biological activity. Nowadays, many scientific works underline that the pharmacological effectiveness of a phytocomplex is due not to one or a few of its active principles, but rather that it is determined by a combined effect of some, or all, the components of the phytocomplex. Thus, the dietary supplementation with ROC may be preferred to and considered more efficacious than that with the whole blood orange juice or with its isolated antioxidant components.

In conclusion, our study suggests that dietary supplementation with ROC may be able to decrease oxidative damage occurring in populations exposed to air pollution and smoking, by augmenting the endogenous antioxidant defenses. In fact, maintenance of vitamin C and E levels has been demonstrated to protect thiol groups in proteins (such as the enzyme glutathione peroxidase), which are especially vulnerable to oxidative damage and inactivation by the formation of sulphur-seleno bridges (Li, Cowan, Mickle, Weisel, & Burton, 1996), and the phenolic antioxidants in orange juice have been proved to protect vitamin C against oxidative decomposition (Miller, 1998). In addition, besides being inexpensive, supplementation with ROC has the additional advantage of being well tolerated, since no subject enrolled in the present trial reported unpleasant side effects. Therefore, supplementation with ROC might have a beneficial effect, particularly on subjects exposed to high levels of oxidative insults, such as air pollution and smoking.

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