SUPPLEMENTARY MATERIAL

Ex vivo evaluation of the effects of a white grape juice extract on lymphocytic mitochondrial functions

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Abstract

The physio-pathological role of mitochondria in aging and age-related diseases has stimulated the search for compounds able to promote mitochondrial functionality.
Our study was designed to evaluate the effect of a white grape juice extract (WGJe) on mitochondrial activity, in an *ex vivo* experimental model consisting of activated lymphocytes obtained from a younger age group and an older age group of subjects. WGJe steadily decreased the lymphocytic mitochondrial mass in the older subjects, without a relevant effect in their younger counterpart, and significantly enhanced $\Delta\psi m$ in both groups investigated. Finally, WGJe reduced the endogenous mitochondrial production of H_2O_2 in all subjects. The results support the potential use of WGJe to improve mitochondrial functionality, thus maintaining human health and slowing down aging.

Experimental

Chemicals and reagents

All chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise specified.

White grape juice extract (WGJe)

The extract used in this study derived from a mixture of different white grape (*Vitis vinifera*) juices: *Vitis vinifera* var. Catarratto, *Vitis vinifera* var. Grillo and *Vitis vinifera* var. Insolia. In its liquid form, WGJe was provided by the company "Bono & Ditta" (Campobello di Mazzara, Trapani, Italy), and then transformed into dry powder by lyophilisation. It was stored at −20 °C until use. The chemical profile of WGJe was assessed by using a UPLC/QqQ-MS/MS and previously reported (Giacoppo et al., 2015; Andreucci et al., 2015). Thirty-seven different compounds were detected in the phytocomplex. These were 24 flavonoids (i.e. 12 flavonols, 4 flavanols, 2 dihydroflavonols, 2 dihydrochalcones, 1 flavanone and 3 flavones) and 13 non-flavonoids (i.e. 5 hydroxycinnamates, 5 phenolic acids, 2 resveratrols and a single hydroquinone glucoside). In particular, resveratrol molecules, mainly present as *cis* isomers, constituted 32% of non-flavonoids.

Sample collection and lymphocyte cultures

Thirty-one healthy Caucasian individuals (10 males and 21 females), aged 43.5 ± 13.0 (means ± SD) years, voluntarily agreed to participate in the study. To further minimize intragroup variability of lymphocytic mitochondrial function, nine subjects were excluded because of smoking, heavy drinking or consumption of drugs and/or supplements. Considering the importance of the variable age in mitochondrial function, we divided the 22 selected subjects, into two groups. The ~~younger~~ mean of the younger group's age is 31.5 ± 4.1 years, while the mean of the older ones is 55.6 ± 2.6 years. In order to reduce inter-individual variability, a priori selection was made according to a variety of exclusion criteria. These were: exposure to diagnostic X-rays during the last 5 months and the presence of chronic or infectious diseases and/or flogistic conditions during the last 2 weeks. All lymphocytes donors signed informed consent to study participation, according to the principles of the Helsinki Declaration (1964). They completed a questionnaire in order to obtain information about age, gender, smoking, drinking and previous drug intake. In the selected subjects, heparinized whole blood was collected by venepuncture (4 ml) and was immediately added to 1:1 v/v phosphate buffered saline (PBS) for blood lymphocyte separation. The suspensions were carefully layered over 4 ml of Lymphoprep Separation Medium™ (Axis-Shield Oslo, Norway). After centrifugation at 800 × *g* for 20 min, the buffy layer was removed, washed twice with PBS and collected by centrifugation at 250 × *g* for 10 min. The pellets were suspended in RPMI 1640 supplemented with 15% v/v foetal calf serum (FCS), 100 IU

ml^{-1} penicillin and 100 mg ml^{-1} streptomycin. To induce lymphocytic activation 10 µg ml^{-1} phytohemagglutinin (PHA) and 40 IU ml $^{-1}$ of the cytokine interleukin 2 were added to the medium (Di Pietro et al., 2011a). The cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere for 24 h

WGJe treatment of lymphocyte samples

After 24 h of PHA and interleukin 2 stimulation, the lymphocytes were washed in PBS, centrifuged, placed in fresh medium (with 2% FCS) and divided into six aliquots processed in parallel. To assess the effect of WGJe on mitochondrial function, the experimental design involved the use of two different WGJe concentrations with two different treatment times. Therefore, two aliquots were used to evaluate basal mitochondrial function under culture conditions at the set times by adding PBS to cell suspensions. To the others, WGJe solutions at 0.05 and 0.1 µg mL^{-1} (two each, for assessment of 24 and 48 h of treatment) were added. The two concentrations were selected after preliminary experiments, performed to assess the concentration that caused neither cytotoxic nor proliferative effects. Treated and untreated lymphocytic suspensions were incubated at 37 °C.

Detection of mitochondrial parameters

After WGJe or PBS treatment for the established times, samples were centrifuged at 1000 × *g* for 5 min and suspended in PBS (1 x 10^5 cells mL^{-1}) containing 10 mM D-glucose (pH 7.4). To determine the mitochondrial mass, the mitochondrial transmembrane potential ($\Delta\psi_m$) and intracellular H_2O_2 ~~intracellular~~ content, aliquots of lymphocytic suspensions were loaded separately with the respective probes and were assayed by flow cytofluorimetric analyses (FACS), using an argon laser instrument (NovoCyte 2000 flow cytometer; ACEA Bioscences Inc., San Diego, CA, USA). Specifically, to evaluate mitochondrial mass, the cells were incubated with 10-nonyl bromide acridine orange NAO (Invitrogen Molecular Probes), which provides a fluorescent signal proportional to the mitochondrial content. This dye is spontaneously incorporated by mitochondria with a high specificity due to its affinity cardiolipin (CL), the signature acidic phospholipid of the inner mitochondrial membrane (Micale et al., 2013). The incorporation of the fluorescent probe rhodamine 123 (R123; Invitrogen, Milan, Italy) was used to measure $\Delta\psi_m$. The chemical properties of the cationic fluorochrome allow it to cross the mitochondrial membrane and to be stored only in the matrix of functional mitochondria that possess a transmembrane potential, indicative of

an active proton gradient maintained during oxidative phosphorylation (Di Pietro et al., 2011b). The probes NAO and R123 were added to cell suspensions (final concentration 0.2 and 2.0 μM for NAO and R123, respectively) and incubated for 10 min at 37 °C. The emission signals were collected in the FL-1 and FL-2 channels, respectively.
The H_2O_2 content was detected by using the non-fluorescent lipophilic probe dihydrorhodamine (DHR123), which can passively diffuse across membranes and localizes in the mitochondria. Here, it is oxidized primarily by H_2O_2 in a myeloperoxidase-dependent reaction to cationic green fluorescent rhodamine 123. It was used at a final concentration equal to 0.2 μM, and loaded cell suspensions were incubated at 37 °C for 20 min. FACS analyses were performed by collecting signals in the FL-1 channel.
In FACS analyses, the weighted average of emission values for 100 cells was calculated and expressed in arbitrary fluorescence units (AFU).

Statistical analyses

Each FACS detection was conducted in duplicate, and the results were expressed as mean ± standard deviation (SD). Statistical analyses were performed using the STATISTICA program (version 6.0). Bivariate comparisons were performed by using the non-parametric Mann–Whitney *U* test or the Kruskal–Wallis rank-based ANOVA. The relationship between the different variables was assessed by Spearman's rank correlation coefficient.

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Declaration of interest statement

The authors declare that there are no conflicts of interest.

Figure Legends

Table 1. Some mitochondrial parameters ~~lymphocytes~~ in the activated lymphocytes. Data examined after 24 h are expressed as AFU. Compared with the younger group, the older one had higher MM ($P < 0.01$), while $\Delta\psi_m$ values were approximately equal. Considering the $\Delta\psi_m$ values per unit of mass as a marker of mitochondrial function, the values were 1.3-fold lower in the elderly (0.47 *vs* 0.61) than in younger subjects, underlining a physiological decline. The mitochondrial H_2O_2 content was higher in the older group ($P < 0.01$) and, by using H_2O_2 value per unit of $\Delta\psi_m$ as an index of redox imbalance, the age-related difference was confirmed.