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Modulation of the antioxidant/pro-oxidant balance, cytotoxicity and antiviral actions of grape seed extracts

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ABSTRACT

Grape seed extracts (GSEs) were investigated in yeast cells harbouring defects in their antioxidant system (regarding the cellular growth and growth recovery from H_2O_2 insult). GSEs antioxidant activity was detected in wild-type and mutant strains Δ*cta1*, Δ*gsh1* and Δ*oye2glr1*, while pro-oxidant activity in Δ*sod1* cells was seen. Assessment of proliferation of prostate cancer PC3 and HBV-replicating HepG2 2.2.15 cells treated with GSEs has shown higher cytotoxicity of red grape seed extract (RW) than white grape seed extract (WW) subjective to dose and period of administration. No antiviral effect was detected by measuring the secreted virion particles in HepG2 2.2.15 cells treated with GSEs. The GSEs play a dual antioxidant/pro-oxidant role *in vivo* according with the cellular antioxidant system deficiencies and exhibit cytotoxic properties in PC3 and HepG2 2.2.15 cell lines, but no antiviral action against HBV.

1. Introduction

Winemaking by-products are of particular interest because grape is the world's largest fruit crop, with more than 68 million tons produced per year, of which the European Union produce approximately 24.5 million tons per year in 2010 (FAO STAT Database in http://faostat.fao.org (March. 27th, 2012). Grape seeds are waste products of the winery and grape juice industry, containing lipid, protein, carbohydrates, and 5–8% polyphenols, depending on the variety. Grape seed polyphenols contain, aside from phenolic acid precursors (gallic acid), the monomeric flavan-3-ols (catechin, epicatechin, gallocatechin, epigallocatechin and epicatechin 3-O-gallate) and procyanidin dimers, trimers, and highly polymerised procyanidins (Chedea et al., 2011). The pharmacological and health benefits of grape seed extracts (GSEs) include antioxidant (Chedea, Braicu, & Socaciu, 2010), cardioprotective (Schewe, Sadik, Klotz, Yoshimoto, Kühn, & Sies, 2001), hepatoprotective, neuroprotective, anti-inflammatory, antidiabetic, anti-carcinogenic, and anti-ageing effects (Nassiri-Asl & Hosseinzadeh, 2009; Xia, Deng, Guo, & Li, 2010).

Oxidative stress, as a gap between production of reactive oxygen species (ROS) and cellular antioxidant defence, is a key phenomenon in chronic disorders: diabetes mellilitus, cardiovascular diseases, and cancer (Schewe et al., 2001). The harmful effects of oxidative processes in living organisms can be diminished by the dietary intake of polyphenols like flavan-3-ols and procyanidins, present in GSEs (Chedea et al., 2010). Redox cell signalling and the antioxidant potential of various natural compounds have been widely evaluated *in vivo* using yeast, as an eukaryotic model system (Dani, Bonatto, Salvador, Pereira, Henriques, &, Eleutherio, 2008; Zyracka, Zadrag, Koziol, Krzepilko, Bartosz, &, Bilinski, 2005). This takes advantage of the existing elaborate enzymatic

Abbreviations: FRAP, ferric reducing antioxidant power; FBS, fetal bovine serum; GAE, gallic acid equivalents; GSE, grape seed extract; HBeAg, hepatitis B "e" antigen; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HBV, human hepatitis B; MAPK, mitogen-activated protein kinase; OYE, old yellow enzymes; GSSG, oxidised glutathione; PI3K, phosphatidylinositide 3-kinase; PCD, programmed cell death; ROS, reactive oxigen species; RW, red grape seed aqueous extract; GSH, reduced glutathione; TP, total polyphenolic content; WW, white grape seed aqueous extract; Δcta1, Δsod1, and Δgsh1, yeast mutants derived from the wild-type (wt) strain BY474, harbouring deletion of the genes CTA1, SOD1, or GSH1 respectively.

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redox machinery, consisting of catalases, superoxide dismutases, reductases, peroxiredoxins, glutaredoxins, and glutathione transferases (Herrero, Ros, Belli, & Cabiscol, 2007). *Saccharomyces cerevisiae* strains lacking the antioxidant machinery mimic the altered intracellular redox circumstances that are frequently encountered in human pathological conditions, and can be used to assess the ability of antioxidant compounds to protect against oxidative damage (Zyracka et al., 2005). The consumption of polyphenol-rich foods exerts valuable outcomes in oxidative stress-related chronic diseases, including some forms of cancer (Surh, 2003). The anticancer effects of dietary polyphenols occur via a variety of mechanisms, such as the inhibition of cell growth proliferation, the modulation of cancer cell signalling and antioxidant enzymatic machinery, the induction of apoptosis and cell cycle arrest or the elimination of carcinogenic agents (Ramos, 2008). Paradoxically, dietary polyphenols may also perform anti-cancer effects as a result of their pro-oxidant activity (Halliwell, 2008; Surh, 2008). Polyphenols are among the 400 compounds that have been listed as potential chemopreventive agents, 40 of these being currently under clinical evaluation (Cimino et al., 2012).

To address the antioxidant/pro-oxidant capacity of aqueous GSEs to promote/prevent cell survival from oxidative damage we focused on yeast model systems, to simulate common cellular dysfunctions that occur in oxidative stress using strains compromised in important components of their antioxidant machinery. The cytotoxic effect on tumour cells, the antiviral potential of GSEs and the possible link between these two effects was also investigated. This study is ultimately aimed at increasing the use of wine industry waste through inexpensive processing with a view to exploit the material in nutrition to prevent disease, and even to develop novel pharmacological uses.

2. Materials and methods

2.1. Plant material

Varieties of red and white grapes cultivated in Greece were used for all seed samples studied (Chedea, Moussouni, Socaciu, &, Kefalas, 2012), red grape seed aqueous extract (RW) and white grape seed aqueous extract (WW). The grapes were harvested at optimum technological maturity. Grape berries were manually deseeded and seeds were mixed, frozen in liquid nitrogen, and stored in the freezer (−20 °C) until analysed.

2.2. Polyphenol extraction

5 g of seeds were ground with liquid nitrogen, and extracted for 1 h by adding 40 ml of boiled water to the seed powder. After sedimentation of the large solid particles, the supernatant was centrifuged for 10 min at 1500*g*, filtered, and then aliquoted and stored at −20 °C until further analysis. The total polyphenolic (TP) content was measured by the Folin–Ciocalteu method and expressed as mg of gallic acid equivalents (GAE)/g seeds (Chedea et al., 2012).

2.3. Yeast strains

The wild-type (wt) strain BY4741 and the derived mutants Δ*cta1*, Δ*sod1*, and Δ*gsh1*, harbouring deletion of the genes *CTA1*, *SOD1*, or *GSH1*, respectively, were obtained from Research Genetics. The double-knockout strain Δ*oye2glr1* was as previously described (Odat et al., 2007). The yeast strains employed show defects in free radical-scavenging enzymatic system and glutathione metabolism.

2.4. Cell lines

The PC3 cell line derived from prostate metastatic androgen independent tumour was purchased from the Health Protection Agency Culture Collections (http://www.hpacultures.org.uk/collections/ecacc.jsp). PC3 cells were seeded at 25% confluence (i.e. 3×10^{-5} in a 6-well plate), fed with RPMI medium supplemented with L-glutamine, containing 10% foetal bovine serum (FBS), 50 units/ml penicillin, and 50 μg/ml streptomycin.

The hepatocarcinoma–derived HepG2 2.2.15 cells (kind gift from Dr. David Durantel, INSERM U871, Lyon, France), stably transfected with two head-to-tail dimers of the Human Hepatitis B (HBV) genome, were grown in RPMI 1640 medium (Euroclone) containing 10% foetal bovine serum (FBS), 50 units/ml penicillin, 50 μg/ml streptomycin and 2 mM Glutamax (Invitrogen), supplemented with 200 μg/ml of G418 (Gibco).

2.5. Growth recovery curves

Yeast strains were routinely cultivated at 30 °C in YEPD medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose. Single yeast colonies from fresh YEPD plates were used to inoculate starter cultures in 50 ml YEPD broth at A_{600} = 0.1, which were incubated at 30 °C with shaking (160 rpm) for 14–18 h. Aliquots were taken at regular intervals and the absorbance was measured. When A_{600} > 1, aliquots were serially diluted and new measurements were taken. The assay was conducted as previously described (Amari, Fettouche, Samra, Kefalas, Kampranis, &, Makris, 2008).

2.6. Viability colony assay

BY4741 and the mutant Δ*sod1* strains were freshly grown overnight in rich YEPD medium. Viability colony assays were performed as previously described (Amari et al., 2008). All experiments were performed independently in triplicate. The results were statistically analysed by the GraphPad statistical software.

2.7. Cytotoxicity (MTS) assay

PC3 and HepG2.2.2.15 cells were seeded at confluence in 96 wells plates (Greiner) in a final volume of 100 μl/well culture medium. Wells loaded with 0.1 μl milliQ water were used as negative control. GSEs to the final concentration of 1 μM GAE, 5 μM GAE, 10 μM GAE, 25 μM GAE, 50 μM GAE and 100 μM GAE were added to each well. Supplemented cells were left to incubate at 37 °C for 24 and 48 h. The cell proliferation was assayed using WST-1 reagent (Roche Diagnostics Gmbh, Manheim, Germany). Cells were incubated with 10 μl of WST-1 for 30 min. The measurements were done in triplicate for each sample. Colour change was assayed by measuring the absorbance at 420 nm with a microplate reader (optima BMG labtech). The assay quantifies enzymatic activities in metabolically active cells; therefore, dead cells do not contribute to the colour change.

2.8. Quantification of HBV subviral particles secretion by ELISA

Aliquots of supernatants from GSE-treated HepG2 2.2.15 cells were analysed for the secreted HBsAg, using the Monolisa HBsAg Ultra Kit (Bio-Rad). The results were registered as ratios of signal to cutoff and were converted to percentages of HBsAg secretion form control, untreated samples.