GRAPE SEED EXTRACT COMPONENTS INDUCE STRESS SIGNALS IN A2780 HUMAN OVARIAN CELLS

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Abstract: This study attempted to identify possible regulatory sites and mechanisms of antiapoptotic flavan-3-ols, focusing on reactive oxygen species mediated signaling in A2780 ovarian cancer cells. The cells were treated different concentrations of some rich flava-3-ols extracts. The results obtained for grape seed extract were compared to those of a standardized green tea extract. A concentration of 5 \(\mu\)g/ml grape seed extract induced apoptosis, producing intracellular high levels of reactive oxygen species through mitochondrial pathway. We noticed that 2.5 \(\mu\)g/ml grape seed extract reduce the expression of TNF\(\alpha\) and the cellular proliferation. The overall data indicate that grape seed extract posses a potent protective effect against ovarian cancer cells, with dose-dependent characteristics.

INTRODUCTION

Historically plants have provided a source of inspiration for novel drug components and have shown great promise in the treatment of diseases. The great varieties of secondary metabolites from plants have been sources of commercially important pharmaceutical compounds. Some 41% of newly approved drugs between 1983-1994 had a natural product origin and this increased to 60% when considering anti-infective and anticancer compounds. [3]

The daily consumption of green tea and grapes, foods rich in phenolic phytochemicals, is believed to have many beneficial effects on health, especially in preventing mutagenesis and carcinogenesis that are associated with free radicals. Epidemiological studies link tea beverage consumption to a lower incidence of certain types of cancer [5, 7].

Green tea is produced from the unfermented leaves of *Camelia sinensis*, and polyphenols, known as catechins, constitute its principal chemical components. Epicatechin, epicatechingallate, epigallocatechin, and epigallocatechin gallate are the major catechins contained in green tea. Animal *in vivo* studies and human epidemiological observations indicated that green tea possesses inhibitory effects on the growth of tumor cell lines. [9]

Grape skins and seeds are also rich sources of health-promoting polyphenols, including flavan-3-ols. Grape seeds contain lipid, protein, carbohydrates, and 5-8% polyphenols depending on the variety. Polyphenols in grape seeds are mainly flavonoids: gallic acid, the monomeric flavan-3-ols: catechin, epicatechin, gallocatechin, epigallocatechin, epigallocatechin gallate,
dimers, trimers, and more highly polymerized procyanidins. The most abundant phenolic compounds isolated from grape seed are catechins, epicatechin, procyanidin, and some dimers and trimers. [6]

Grape seed extract may involve a mitochondrial oxidative pathway to induce apoptosis. The induction of apoptosis has been associated with opening of the mitochondrial permeability transition pores and mitochondrial membrane potential alterations characteristic of apoptosis potential loss can lead to release of cytochrome c from mitochondria into the cytosol. Interestingly, a low dose of grape seed extract affects cells, but has no toxicity towards normal cells. [4]

TNFα concentrations are significantly increased in ovarian cancer patients, and the levels of TNFα expression are positively correlated with tumor grade. TNFα has selective cytolytic activity against some but not all tumor cells. The resistance of human epithelial tumor cells to TNFα appears to be associated with the expression of this cytokine and controlled by a protein synthesis-dependent mechanism. [1]

This study aimed to test whether grape seed extract and green tea extract induce apoptosis in ovarian cancer cell lines.

**MATERIALS AND METHODS**

**Cells and cell line.** Human malignant ovarian cell line A2780 were cultured in RPMI 1640 medium supplemented with fetal bovine serum, penicillin, streptomycin in a humidified atmosphere with 5% CO2. This cell line was obtained from the Oncologic Institute “Ion Chiricuta” (Cluj-Napoca, Romania).

In the experiments, $10^4$ cells/200 µl were used in 96 well plates, and were allowed to attach for a 24 hour period. Cells were treated afterwards with different concentrations of extracts.

**Reagents.** 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide, 2’7’-dichlorofluorescein diacetate (DCF-DA), DMSO was purchased from Sigma Chemical Co (St. Louis, MO). The grape seed powder (GSE) was obtained from ECOM. The green tea extract (GTE) it is a standardized extract product from China. These agents were dissolved in hot water obtaining a final concentration of 1 mg/ml.

**MTT assay.** The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by microplate reader Biotek Synergy HT.

Plate cells at $10^4$ cell/well were incubated for 24 hours, after the treatment with GSE and GTE. 80µl MTT reagent was added to each well and incubated 2 hours until the purple precipitate is visible. Washing the cells with 100 µl DMSO and recording the absorbance at 570 nm.

**ROS assay.** Intracellular reactive oxygen species (ROS) level were measured using the fluorescent probe 2’7’-dichlorofluorescein diacetate on $10^4$ cells/well. DCF (dichrolofluorescein) fluorescence intensity is demonstrated to be parallel to the amount of reactive oxygen species formed intracellular.
Various concentrations of GSE and GTE were added to the cells simultaneously with DCF-DA, with final concentration of 2 µM and incubated at 37° up to 4 hours. DCF fluorescence intensity was detected at different time intervals using a microplate reader Biotek Synergy HT, with excitation at 485 nm and emission at 530 nm. [2]

**TNFα Elissa assay.** This assay employs the quantitative sandwich enzyme immunoassay technique using Quantikine human TNFα kit. A monoclonal antibody for TNFα has been pre-coated onto a microplate. Standards and samples were pipetted into wells and any TNFα present is bound by the immobilized antibody. After washing an enzyme-linked polyclonal antibody is added to the wells. Following a wash, a substrate solution is added to the wells and color develops in proportion to the amount of TNFα bound in the initial step. The color development is stopped and the intensity of the color is measured.

### RESULTS AND DISCUSSION

1. **Cell proliferation using MTT assay**

Unspecific MTT reduction to the formazan product already occurred at GSE concentrations of 2.5 µg/ml and for GTE 25 µg/ml. Ovarian cancer cells proliferation is reduced by low quantities of GSE after 24 hours of treatment.

After 48 hours from the treatment there are small differences comparable to the control at the small concentrations of GSE and GTE, were observed the proliferative effect, while at higher concentrations of GSE and GTE 25 µg/ml was less intense.

In conclusion the best effect appears, were observed for GTE and GSE to be at low concentrations in a short time.

![Fig. 1 Effect of grape seed extract on A2780 cell viability](image)
2. Antioxidant activity by ROS assay

Fig. 2 shows the effect of GSE on reactive oxygen species formation in A2780 ovarian cancer cell. Higher concentrations of GSE inhibit the intracellular reactive oxygen species formation. After 4 hours incubation, the DCF fluorescence intensity dropped significantly for the ST4 probe, with concentration of 2.5 µl/ml. The experimental probe were noted: ST1, ST2, ST3, ST4. ST1 is the more concentrated probe 50 µl/ml, then ST2 with 25 µl/ml, ST3 5 µl/ml and ST4 2.5 µl/ml.

The 5 µg/ml of GSE induce a significant increase of DCF fluorescence, the quantity of free radicals is higher in the cells, and the apoptosis is induced. For the others concentrations of GSE the apoptotic effect decrease in cancer cells.

Fig 2. GSE induce intracellular reactive oxygen species formation in A2780 cells

It is also observed that in the control ovarian cancer cells, untreated cells, the fluorescence intensity increased up to 4 hours, suggesting that a considerable amount of reactive oxygen species is formed in A2780 cells.

3. The expression of TNFα by Elissa assay

TNFα is a pleiotropic cytokine that can induce differentiation, proliferation, and apoptosis in many cell types and has been suggested to play an important role in the biology of ovarian cancer and tumorigenesis. Ovarian tumor cells produce a macrophage colony-stimulating factor, a potent chemoattractant for monocytes that secretes TNFα.

Fig 3. Expression of TNFα in A2780 cells
A dose of 2.5 µg/ml of GSE decrease the quantity of TNFα in ovarian cancer cells from 127.5 pg/ml in untreated cells to 110 pg/ml in the cells treated with different concentrations of GSE. For a 10µg/ml and a 25ug/ml concentration of GSE the quantity of TNFα increase the antioxidant effect in cancer cells in obviously.

We have obtained different results for GTE, the higher concentration of the extract can reduce the TNFα level in ovarian cancer cells. The lowest concentration of GTE 2.5 µg/ml has no effect on the expression of this cytokine.

From our experiments we obtained results which demonstrate that the GSE has the strongest prooxidant effect in ovarian cancer cells compared to GTE. So, the reactive oxygen species are produced in higher quantities in the tumoral cells facilitates their suicide.

CONCLUSION

Our studies aimed to test whether the grape seed extract reduce cellular proliferation through MTT assay and the impact on prooxidant capacity and TNFα expression.

Through MTT assay we obtained that the cellular proliferation is reduced by the lowest concentration of grape seed extract (2.5 µg/ml) after 24 hours of treatment. Comparatively the cell proliferation was inhibited by green tea extract by a higher dose of extract (25 µg/ml), at the same time.

A concentration of 5 µg/ml of grape seed extract caused a significant increase of dichlorofluorescein fluorescence, as an indication of higher release of reactive oxygen species which induce apoptosis, through mitocondrial pathway. The grape seed extract has the strongest prooxidant effect in ovarian cancer cells compared to green tea extract.

The TNFα assay reveals that grape seed extract and green tea extract may exert pro-apoptotic effect in ovarian tumor cells in small doses, not only by intrinsic apoptotic pathway (mitochondrial pathway), but also through the extrinsic one (at the plasma membrane level). A dose of 2.5 µg/ml of grape seed extract decrease the quantity of TNFα, in the same cells, more intense than green tea extract control.

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