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Extra Virgin Olive Oil phenols induce autophagy and apoptosis in human bladder cancer cell lines depending on tumor progression

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Abstract: Epidemiological evidences indicate that there is an inverse association between olive oil intake and bladder cancer risk and several data suggest that a key role to support these beneficial effects is played by its phenolic fraction. Bladder cancer is one of the most common cancers in Western countries. In particular, the transitional cell carcinoma histotype shows an aggressive behavior and the current therapies are ineffective. The anti-proliferative effects of an extra virgin olive oil phenolic extract (EVOOE) it has been investigated on RT112 and J82, two human bladder cancer cell lines employed as models of superficial and invasive bladder cancer, respectively. EVOOE reduces cell viability in both cell lines triggering different processes. In RT112 cells, EVOOE triggers an autophagic response, causing a delay in cell growth (132 μg/ml induces 30% reduction). Instead, in J82, the invasive transitional cell carcinoma, EVOOE treatment induces a rapid and remarkable decrease of cell viability (33 μg/ml for 24 h induces 40% reduction) triggering an apoptotic process. EVOOE exerts an antioxidant activity in both cell lines reducing ROS (30% in J82 and 15% in RT112). However, in J82, comparing the effects of EVOOE with those of other well-known antioxidants, the absence of correlation between antioxidant effects and reduced cell viability was evidenced. Data presented show that EVOOE possesses pleiotropic activities that intercept different pathways resulting in anti-proliferative effects independently of its antioxidant property.

Keywords: Extra Virgin Olive Oil phenols; bladder cancer; apoptosis; autophagy; antioxidant;

1. Introduction

Bladder cancer is the 10th most common cancer worldwide [1]. It is a heterogeneous disease with a variable course. Pathologic stage is an important prognostic factors that results critical for patient management The low-grade tumors have a low progression rate and, generally, only require endoscopic treatment and surveillance. The high-grade tumors have a very relevant malignant potential with significant progression and hight cancer death rates [2]. According to the European Prospective Investigation into Cancer and Nutrition (EPIC) study there is an inverse association between the dietary intakes of flavonols and lignans and risk of bladder cancer, particularly aggressive urothelial cell carcinoma [3]. Moreover, epidemiological evidences indicate that a regular consumption of olive oil has a protective effect on bladder cancer risk [4].

Extra virgin olive oil is obtained from the first and second pressings of the olive fruit by the coldpressing method and is composed of a glycerol fraction (about 95–99%) and a nonglycerol or unsaponificable fraction (about 0.4–5%) that contains phenolic compounds (phenols, phenolic acids,

flavonoids, lignans, secoiridoids). These phenolic components strongly contributes to the health effects attributed to extra virgin olive oil, possessing, among others, anti-inflammatory, anti-oxidants and anti-microbial properties [5,6]. In particular, these molecules appear extremely interesting as they are able to interact with different molecular pathways underlying various pathogenesis, including cancer [7]. The aim of present work is to study the effects of an Extra Virgin Olive Oil Phenolic Extract (EVOOE) in bladder cancer cell lines characterized by different tumor progression.

2. Experiments

2.1 Phenolic compounds extraction from extra virgin olive oil

The extra virgin olive oil, an Italian Blend: Sud Italia 637, was provided by Basso Fedele & Figli s.r.l., San Michele di Serino -Avellino- Italy. Phenolic compounds from extra virgin olive oil were isolated following the analytical procedure [8]. Briefly, 10 g of oil were homogenized for 3 min in a solution of 80% methanol-water (10 ml), then were centrifugated 4000 rpm 15 min, and repeated three times. A de-fatting with n-hexane was performed to completely remove the lipid fraction. The extract was re-suspended in DMSO at a stock concentration of 40 mg/mL.

2.2 Cell culture and viability assay

RT112 (low-grade) and J82 (high-grade) human bladder carcinoma cells were cultured respectively in RPMI and MEM supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin, at 37°C in a humidified atmosphere containing 5% CO2. Cell viability was assayed using crystal violet staining. Cells were cultured at density of $8x10^4$/ml in 48 well plates and stimulated as indicated in the text. After stimulation cells were fixed with 10% formalin for 10 min and washed before addition of crystal violet (0.1% w/v) for 30 min. Finally, cells were washed and lysed with 10% acetic acid. Absorbance was spectrophotometrically measured at 590 nm. The quantity of adsorbed dye was proportional to number of living cells.

2.3 Apoptotic bodies staining

To verify the presence of apoptotic bodies, after treatments cells were incubated with Hoechst nucleic acid stain, a cell-permeant nuclear counterstain that emits blue fluorescence when bound to dsDNA, allowing to distinguish condensed pycnotic nuclei in apoptotic cells. After staining cells were photographed using a fluorescence microscopy (Zeiss Axiovert 200, Milan, Italy).

2.4 Annexin V/Propidium Iodide detection

Phosphatidylserine exposure was measured using the binding of fluorescein-isothiocyanate-labelled (FITC) Annexin V to phosphatidylserine (PS), as indicated in the manufacturer's protocol (Miltenyi Biotec, Bologna, Italy). Briefly, after 15 h of treatment J82 cells ($0.15x10^6$/ml) were washed and then suspended in binding buffer. The cells were incubated with Annexin V FITC and propidium iodide in the dark at room temperature prior to analysis by flow cytometry (FACS-Calibur; Becton Dickinson, Mountain View, CA, USA) equipped with argon laser (488nm) and filtered at 530 nm. Data were analyzed using CellQuest software (Becton Dickinson).

2.5 Autophagy determination.

Autophagy was monitored by using the CytoID Autophagy Detection Kit (ENZO Life Science, Milan, Italy) as indicated in the manufacturer's protocol. RT112 cells were incubated for 24 h with EVOOE. After incubation, cells were washed and incubated with the autophagy detection marker (Cyto-ID). Then, cells were rinsed with assay buffer and photographed using a fluorescence microscopy. Finally, autophagosomes were analysed by flow cytometry and quantified using CellQuest software.

2.6 Intracellular ROS measurement

J82 an RT112 cells, 0.1x10^6/ml in 96-well dark plates, were stimulated as indicated in the text and then incubated for 30 min with 10 mM of 2′-7′-dichlorofluorescein diacetate (DCFH-DA), a non-fluorescent compound that freely permeates cells. When DCFH-DA penetrates cell membrane the diacetate group is hydrolyzed by cellular esterase and then DCFH is oxidized by intracellular peroxides to a fluorescent molecule 2′-7′-dichlorofluorescin (DCF). Fluorescence was spectrofluorimetrically determined with an excitation and emission setting respectively of 485 ± 20 nm and 530 ± 20 nm.

2.7 Statistical analysis

The data were obtained from at least three separate experiments and presented as mean ± standard deviation (SD) or, to takes into account also the sample size, mean± standard error (s.e.). The Student's t-test was used to analyze the statistical significance between the EVOOE treatments and control groups.

3. Results

3.1. Extra virgin olive oil phenolic extract reduces cell viability in RT112 and J82 bladder cell lines

To assess the antiproliferative effect of the EVOOE on RT112 and J82 cell lines, representing respectively low- and high-grade tumors, cells were treated for 24 h within a range of concentrations corresponding to 2-132 μg/ml (w/v) of the extract (Figure 1a-b). EVOOE slightly reduced amount of viable RT112 cells, the higher concentration induced 30% of reduction. Instead, J82 cells showed a rapid and extensive response to EVOOE, with 40% decrease of cell viability with 33 μg/ml. It has been calculated an IC50 of 240 μg/ml for RT112 cells and of 65.8 μg/ml for J82 cells.

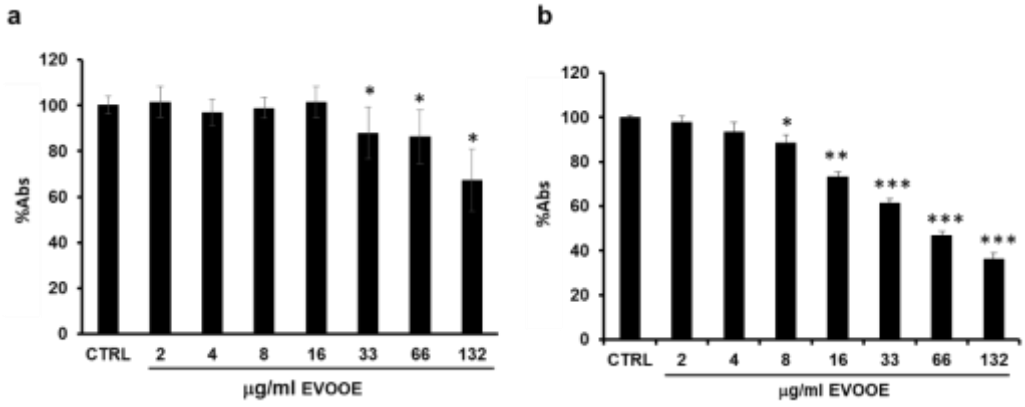

Figure 1. EVOOE reduces cell viability in RT112 and J82 bladder cell lines. RT112 (**a**) and J82 (**b**) cells were treated for 24 h at indicated concentration (w/v) of EVOOE. Cell viability was assessed by crystal violet assay and reported as percentage of absorbance. Bar graphs represent the mean of three experiments (± s.e.). Symbols indicate significance: $p<0.05$ (*), $p<0.0005$ (**) and $p<0.0001$ (***) respect to CTRL.

3.2. Extra virgin olive oil phenolic extract induces autophagy in RT112 cell line

In the treated RT112 cells with EVOOE the presence of numerous intracellular vacuoles was evident by the microscopical observation (data not shown). For this reason it has been hypothesized an involvement of the autophagic process in slowing cell proliferation. After treating RT112 cells with

EVOOE, the cells were specifically stained with Cyto-ID Green autophagy dye to visualize and quantify the autophagosomes. Figure 2 (a-b) reports the activation of autophagy by EVOOE, clearly visible by fluorescence microscopy (Figure 2a), with an increase of autophagic vacuoles of about 30% compared to untreated cells, quantified by flow cytometry.

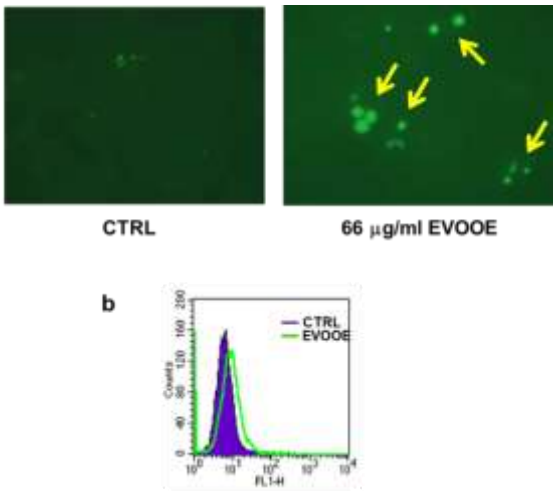

Figure 2. EVOOE induces autophagy in RT112 cells. (a) Representative images of cells untreated (left) and treated EVOOE (right) and stained with CytoID. Cells were visualized using a fluorescent microscopy and photographed in FITC filter with 200× magnification. The arrows indicate the presence of autophagic vacuoles. (b) Representative histogram of CytoID flow cytometry experiments.

3.3. Pro-Apoptotic effects of extra virgin olive oil phenolic extract in J82 cell line

To verify, in J82 cell line, if the reduction in cell viability induced by EVOOE was due to the induction of apoptotic cell death the presence of apoptotic bodies and the exposure of phosphatidylserine (PS) on the cell surface were evaluated. As showed in Figure 3a the phenolic exctract strongly induced apoptosis, as emerge from the presence of numerous apoptotic bodies evidenced by nuclear staining. This observation has been also confirmed by Annexin V/PI fluorimetric assay, where EVOOE at 66 µg/ml significantly induced apoptosis increasing Annexin V positivity of about 20% comparing to untreated cells (Figure 3b).

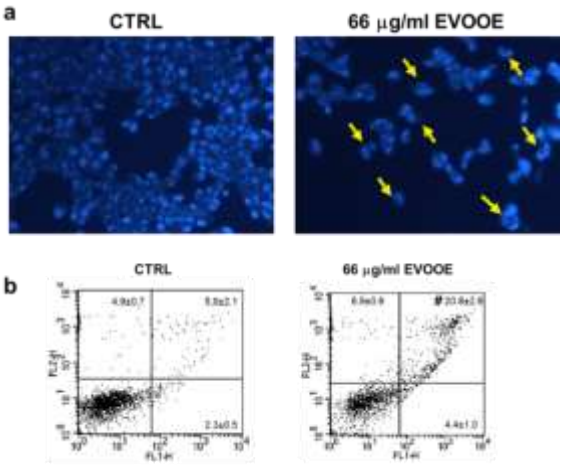

Figure 3. Apoptotic induction by EVOOE in J82 cells. (a) Representative images of cells untreated (left) and treated EVOOE (right) and stained with Hoechst nuclear stain. Cells were visualized using a fluorescent microscopy and photographed in DAPI filter with 200× magnification. The arrows indicate the presence of apoptotic bodies. (b) Representative plots of Annexin V-FITC flow cytometry and PI staining experiments. Numbers in quadrant indicate the percentages of counted positive cells (means ± SD) from three independent experiments. Symbol indicate significance: $p<0.05$ (#)

3.4. Antioxidant activity of extra virgin olive oil phenolic extract in bladder cancer cell lines and absence of correlation with the antiproliferative effect

Treating RT112 and J82 cells for 30 min with EVOOE it has been measured a significant reduction of intracellular ROS, stronger in J82 cell line (Figure 4a-b). To verify the possible correlation between the antioxidant and the antiproliferative effect induced by EVOOE treatment, we compared, in J82 cells, the effects obtained treating cells with the extra virgin olive oil extract to that induced by several well known antioxidant. In Figure 4b we reported results obtained treating J82 cells with 5 μg/ml (w/v) of a phenolic extract obtained from green tea (highly rich of polyphenols) and with 30 μM of pure molecules belonging to the polyphenols family, quercetin, gallic acid, myricetin, kaempferol and rutin (concentrations have been chosen in the same range of EVOOE concentration). All the extracts and the molecules strongly reduced intracellular ROS level, but only rutin, kaempferol (slightly) and quercetin (strongly) reduced cell viability. These data suggest an absence of correlation between antioxidant and antiproliferative effect induced by EVOOE in this cell model.

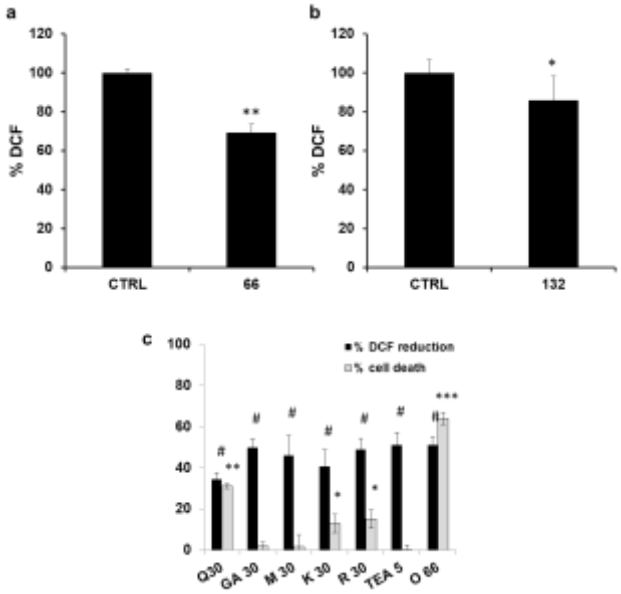

Figure 4. Antioxidant effect of EVOOE and absence of correlation with the antiproliferative effect. Intracellular ROS were monitored as DCF fluorescence; J82 (a) and RT112 (b) cells were treated with 66 μg/ml (w/v) of the extract for 30 min. Bar graphs represent means of three separate experiments ± s.e. Symbols indicate significance: $p < 0.05$ (*) and $p<0.005$ (**) respect to CTRL. (c) Reduction of intracellular ROS (black bars) and cell death (grey bars) induced by EVOOE. Cells were treated with 30μM of Q (quercetin), GA (Gallic Acid), M (Myricetin), K (Kaempferol), R (rutin), 5 μg/ml green tea extract and O (EVOOE) 66 μg/ml, for 30 min, to measure ROS level, and 24 h to assess cell viability by crystal violet assay (reported as percentage of cell death). Bar graphs represent the mean of two

experiments (± SD). Symbols indicate significance: $p<0.05$ (#), $p<0.05$ (*), $p<0.005$ (**) and $p<0.0001$ (***) respect to CTRL.

4. Discussion

The health benefits of extra virgin olive oil can be attributed besides the richest source of the mono-unsaturated fatty acid (MUFA) oleic acid (OA; 18:1n-9) also to the minor components, with particular reference to hydrophilic phenolic compounds, such as secoiridoids (that include aglycone derivatives of oleuropein, dimethyloleuropein and ligstroside) and lignans [such as (+)-pinoresinol and 1-(+)-acetoxypinoresinol] [5]. Considering the epidemiological evidences showing a preventive effect of phenols [3] and olive oil [4] against bladder cancer risk, in the present work we investigated the role of an EVOOE on different stages of bladder cancer progression.

Data obtained showed as the EVOOE induced different response depending on the staging of tumors. In RT112 cell line, representing a low-grade bladder cancer, the phenolic extract induce the autophagic process which leads to a slowdown in cell growth. In J82 cell line, representing a high-grade bladder cancer, EVOOE induced, through the apoptotic pathway, an incisive and rapid reduction of cell viability. Moreover, it is generally assumed that phenols provide health benefits mainly because of their antioxidant activity. In literature, several observations suggest that ROS may have a role in the regulation of programmed cell death [9] and also that antioxidant effects of olive oil polyphenols are correlated to the antiproliferative potential [8]. To clarify the presence of a possible correlation between the antioxidant and the antiproliferative effect induced by EVOOE treatment, we compared the effects obtained treating J82 cells with this specific phenolic extract to that induced by several well known antioxidant. The results obtained suggested that in the case of EVOOE there is no correlation between the antioxidant and the antiproliferative effect induced in this cell model.

Data presented show that EVOOE possesses pleiotropic activities that intercept different pathways resulting in anti-proliferative effects.

The observed antitumoral potential of EVOOE in bladder cancer cells is extremely relevant considering that the hydrophilic fraction of olive oil is physiologically excreted through urines, thus the problem of bioavailability could be considered partially overcome. Therefore in the light of these considerations and the results obtained in the present work it will be interesting to continue to deepen in the future the investigation of the exact mechanisms underlying anti-proliferative effect and the different response depending on bladder tumor staging induced by the mixture of phenolic compounds in extra virgin olive oil.

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Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used C.S. conceived, designed, performed the experiments and wrote the manuscript; S.M. and I.T. performed the experiments; M.R. and C.C. review; G.L.R. provided ideas and financial support. All authors have read and agreed to the published version of the manuscript

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

EVOOE: extra virgin olive oil phenolic extract
DCF: dichlorofluorescin
S.D.: standard deviation
s.e.: standard error
ROS: reactive oxygen species
IC50: half maximal inhibitory concentration

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