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Potential anti-tumor effects of *Mugil cephalus* processed roe extracts on colon cancer cells

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ABSTRACT

The salted-semidried mullet ovary product, bottarga, is a Mediterranean food rich in n-3 PUFA EPA and DHA. We studied and compared the effects on cell viability, sensitivity to the anti-tumor drug 5-fluorouracil, and lipid composition, in colon cancer Caco-2 cells after 24 h incubation with oils and hydrophilic extracts obtained from two bottarga samples stored at different conditions. The cellular absorption of bottarga lipids was assessed in cancer cells by the evaluation of lipid accumulation in cytoplasmic lipid droplets by fluorescence microscopy. Bottarga oil showed a significant *in vitro* inhibitory effect on the growth of cancer Caco-2 cells and the ability to potentiate, at non-toxic concentration, the growth inhibitory effect of 5-fluorouracil. Moreover, bottarga oil induced in cancer Caco-2 cells marked changes in fatty acid composition, with a significant accumulation of the n-3 PUFA EPA and DHA, and cytoplasmic lipid droplet formation. Also bottarga hydrophilic extract, characterized by means of ^{1}H NMR spectroscopy, exhibited a reduction in cancer cell viability, without affecting cell lipid profile. Cell cholesterol levels were unmodified by all treatments. The results showed interesting anti-tumor properties of bottarga lipids, and qualify this fish product as a food with nutraceutical properties and potential benefits in colon cancer prevention.

1. Introduction

Colon cancer is one of the most common forms of cancer in the more developed countries (Sala-Vila et al., 2010; Theodoratou et al., 2007; Oraldi et al., 2009). The etiology of this type of cancer is complex and involves both genetic and environmental factors, the most important environmental factor probably being the diet (Roynette et al., 2004; Sala-Vila et al., 2010; Theodoratou et al., 2007; Oraldi et al., 2009). Epidemiological studies suggest an association between quantity and quality of dietary fat and colon cancer risk (Sala-Vila et al., 2010). One major dietary component that has been associated with prevention of colon cancer is fish oil (Turk and Chapkin, 2013). A number of epidemiological, clinical, and experimental studies indicate a role for fish oil and n-3 polyunsaturated fatty acids (n-3 PUFA) derived from fish oil, in particular eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), in colon cancer prevention (Jordan and Stein, 2003; Turk and Chapkin, 2013; Theodoratou et al., 2007; Roynette et al., 2004).

The exact mechanism of action of n-3 PUFA in colon cancer is not yet fully elucidated and is likely to be very complex (Jordan and Stein, 2003; Turk and Chapkin, 2013). Two of the most abundant bioactive lipids enriched in fish oil, EPA and DHA, have been shown to have pleiotropic effects (Turk and Chapkin, 2013; Chapkin et al., 2008). Several mechanisms have been proposed as being implicated in the action of n-3 PUFA in colon carcinogenesis. These include oxidative stress, modification in the cell membrane composition and structure, the ability to affect numerous cellular functions like membrane fluidity, receptor binding, signal transduction processes, activity of membrane-associated enzymes, eicosanoid production, and regulation of gene expression (Roynette et al., 2004; Oraldi et al., 2009; Riediger et al., 2009; Biondo et al., 2008; Bathen et al., 2008). In addition, numerous studies on the impact of modifying tumor lipid profiles by increasing the exposure to n-3 PUFA have reported a beneficial impact on colon tumor cell response to chemotherapy *in vitro* (Sala-Vila et al., 2010; Jordan and Stein, 2003; Biondo et al., 2008).

The main dietary sources of n-3 PUFA are fish and fish oil, but also fish roe products contain significant amount of lipids having high levels of long chain n-3 PUFA (30–50% of total fatty acids) (Bledsoe et al., 2003; Rossawan et al., 2011). The salted and semidried mullet (Mugil genus) ovary product is regarded as a food delicacy and is produced in several countries in the world: in Italy as "bottarga", in Greece as "avgotaracho", and in Japan as "karasumi" (Bledsoe et al., 2003; Scano et al., 2008; Kalogeropoulos

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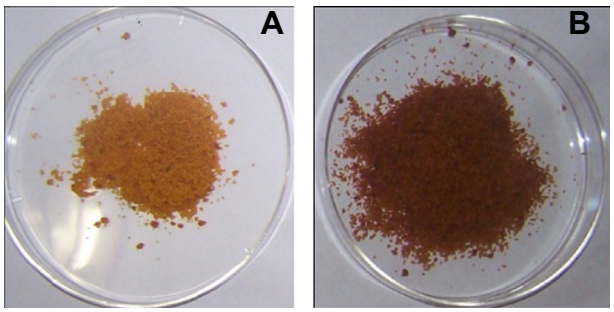

Fig. 1. Digital images of bottarga samples B1 (A) and B2 (B).

et al., 2008). In recent works we have studied the lipid composition, oxidative stability, browning processes, and water-soluble low molecular weight metabolite profile of commercial products of whole and grated bottarga samples manufactured in Sardinia, Italy (Scano et al., 2008, 2009, 2012; Rosa et al., 2009, 2011, 2012). Mullet bottarga emerged as an important natural, stable source of n-3 PUFA, in particular EPA and DHA, that amounted to 10–13 mg/g and 20–33.5 mg/g of edible portion, respectively (Scano et al., 2008; Rosa et al., 2009, 2011, 2012). Moreover, we previously studied the effect of the bottarga oil on cell viability, lipid composition, and lipid peroxidation in differentiated Caco-2 cell monolayers as an intestinal epithelial cell model, in relation to the food oxidative status and browning process (Rosa et al., 2011). The treatment of intestinal epithelial cells with bottarga lipids resulted in a significant modification of the cellular fatty acid composition, with a selective increase in n-3 PUFA levels, indicating a process of absorption of these important bioactive components (Rosa et al., 2011). Preliminary results also showed that mullet bottarga oil is able to affect viability in colon adenocarcinoma cells (Rosa et al., 2011).

The main objective of this work is to demonstrate the potential health role of this food, naturally rich in n-3 PUFA, commonly consumed in the Mediterranean region, in colon cancer prevention. To this goal, we studied and compared the cytotoxicity, and the modulatory effect on lipid composition of oils obtained from mullet bottarga samples in a cell line of human adenocarcinoma (undifferentiated Caco-2 cells). The lipid extracts were obtained from two aliquots of a commercial grated bottarga sample, stored, immediately after manufacturing, at two different conditions, i.e. 35 days at −20 °C in the dark (yellow sample[1], B1, Fig. 1A) and 7 months at room temperature under light exposure (browned sample, B2, Fig. 1B). The cellular absorption of bottarga lipids was assessed in Caco-2 cancer cells by the evaluation of the accumulation of lipids in cytoplasmic lipid droplets. Bottarga oil was also tested in Caco-2 cells for its ability to potentiate the growth inhibitory effect of 5-fluorouracil (5-FO), a commonly used anticancer drug (Jordan and Stein, 2003).

Furthermore, we also tested the impact on the same cellular lines (cytotoxicity and lipid profile modulation) of the hydrophilic molecular pools extracted from the same bottarga samples. The hydrophilic low molecular weight metabolites were characterized by ^{1}H NMR spectroscopy.

2. Materials and methods

2.1. Chemicals and reagents

Cholesterol, triolein, trilinolein, standards of fatty acids and fatty acid methyl esters, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5-fluorouracil (5-FU), and Desferal (deferoxamine mesylate salt), were purchased from Sigma–Aldrich (Milan, Italy). All solvents used, of the highest available purity, were also from Sigma–Aldrich. The methanolic HCl (3 N) was purchased from Supelco (Bellefonte, PA). *cis,trans*-13-Hydroperoxyoctadecadienoic acid (*c,t*-13-HPODE) and *cis,trans*-9-hydroperoxyoctadecadienoic acid (*c,t*-9-HPODE) were obtained from Cascade (Cascade Biochem. Ltd., London, UK). Cell culture materials were purchased from Invitrogen (Milan, Italy). All of the other chemicals used in this study were of analytical grade.

2.2. Preparation of bottarga extracts

Grated bottarga sample of mullet was kindly supplied by the company "Stefano Rocca s.r.l." located in Sardinia (Italy). Ingredients reported in the label were mullet roe and salt. The aqueous and lipophilic bottarga extracts used for cancer Caco-2 cell line study were obtained from two portions of bottarga sample stored at two different time and temperature conditions, 35 days at −20 °C in the dark (yellow sample B1, Fig. 1A), and 7 months at room temperature under light exposure (browned sample B2, Fig. 1B), respectively, as previously reported (Rosa et al., 2011). The extraction was performed by the method described by Folch et al. (1957) by addition of 12 mL of $CHCl_3$/MeOH (2/1, v/v) solution. After addition of 4 mL H_2O and centrifugation at 900 g for 1 h, the $CHCl_3$ fraction (lipophilic extracts, BL 1 and BL 2) was separated from the MeOH/H_2O mixture (hydrophilic extracts, BHE 1 and BHE 2). Total lipids in $CHCl_3$ fraction were quantified by the method of Chiang (Chiang et al., 1957). For experiment in cell cultures, aliquots of the lipophilic and aqueous extracts were dried under vacuum and the residues were dissolved in EtOH and water, respectively. Aliquot of the $CHCl_3$ fraction was subjected to lipid component analysis. The MeOH/H_2O fraction was dried by rotor vacuum and dissolved in 0.7 ml of D_2O, ready for the NMR experiments.

2.3. NMR experiments

^{1}H NMR experiments were performed with a Varian Unity Inova 400 spectrometer operating at 399.94 MHz for proton. The spectra were recorded at 25 °C with a spectral width of 5624 Hz, a 90° pulse, an acquisition time of 3 s, a delay of 20 s, 64 scans. The residual water signal was suppressed by applying a presaturation technique with low-power radiofrequency for 1.5 s. The chemical shifts are referred to the resonance of TSP at 0.00 ppm. The assignment of signals was based on the data reported in the literature (Locci et al., 2011; Scano et al., 2012). Quantitative data are reported as mol% and calculated by integrating the area of diagnostic peak for each metabolite and scaling it to the number of resonating protons.

2.4. Cell cultures

The Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC) (Salisbury, Wiltshire, UK). Caco-2 cells have been obtained from a human colon adenocarcinoma (Travelin et al., 2002). Subcultures of the Caco-2 cells were grown in T-75 culture flasks and passaged with a trypsin–EDTA solution. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and penicillin (100 units/mL)–streptomycin (100 μg/mL), at 37 °C in 5% CO_2.

2.5. Cytotoxic activity of aqueous and lipophilic bottarga extracts in cancer Caco-2 cells: MTT assay

The cytotoxic effect of aqueous and lipophilic mullet bottarga extracts was evaluated in cancer Caco-2 by the MTT assay (Schiller et al., 1992; Rosa et al., 2011). Cells were used in the experiments at a passage number of 50–57 and 53–55 for lipophilic and aqueous mullet bottarga extracts, respectively. Caco-2 cells were seeded in 96-well plates at a density of 5×10^4 cells/mL in 100 μL of medium and cultured overnight. Prior to the addition of bottarga extracts, the cell culture medium was removed, Caco-2 cells were washed with PBS containing Ca^{2+} and Mg^{2+}, and then fresh medium was added. Cells were subsequently exposed to various concentrations of the aqueous (corresponding to 0.25–5 mL MeOH/H_2O fraction obtained from 60 mg of edible portion) and lipophilic (50–500 μg/mL, in EtOH solution) extracts in complete culture medium and incubated for 24 h. For lipophilic extracts an equivalent volume of EtOH was added to the controls. The cell culture medium was then removed from each well of the 96-well plates; an 8 μL portion of MTT solution (5 mg/mL of H_2O) was added to cells in fresh medium and left for 4 h at 37 °C. The medium was aspirated, 100 μL of DMSO was added to the wells, and color development was measured at 570 nm with an Infinite 200 auto microplate reader (Infinite 200, Tecan, Austria). The absorbance is proportional to the number of viable cells.

2.6. Effect of lipophilic bottarga extract BL 1 on cytotoxic activity of 5-FU in cancer Caco-2 cells: MTT assay

The cytotoxic effect of 5-FU, in the absence or in the presence of the lipophilic bottarga extract BL 1, was evaluated in cancer Caco-2 by the MTT assay. Cells were used in the experiments at a passage number of 65–68. Caco-2 cells were seeded in 96-well plates at a density of 5×10^4 cells/mL in 100 μL of medium and cultured

[1] For interpretation of color in Fig. 1, the reader is referred to the web version of this article.