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Paradoxical Pro-Oxidative Effect of Pomegranate Polyphenol Antioxidants in Cultured Hepatic Cells

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Objectives: In recent years, the number of scientific papers concerning pomegranate (Punica granatum L.) and its health properties has increased greatly, and there is great potential for the use of bioactive-rich pomegranate extracts as ingredients in functional foods and nutraceuticals. To translate this potential into effective strategies it is essential to further elucidate the mechanisms of the reported bioactivity and the effective dose to be supplemented. The purpose of our study was to investigate the effects of a pomegranate fruit extract (POM) or of its specific bioactive compound, as punicalagin (PUN), on antioxidative liver biomarkers under an exogenous oxidative stress.

Methods: HepG2 cells, a human hepatoma cell line, were used as a model system. This cell line has been found to express a wide variety of liver-specific metabolic functions, including those related to the expression on protein with antioxidant properties. First, not cytotoxic effects of whole pomegranate extract or pure punicalagin were verified in our model system. Then the expression of the gene encoding for the main antioxidant enzymes (catalase – CAT; and glutathione peroxidase – GPX; superoxide dismutase – SOD) and their activity were evaluated. Total antioxidant capacity and lipid peroxidation were also verified.

Results: The POM and PUN supplementation did not preserved cells from the reduction in vitality, membrane damage, and the increase in lipid peroxidation induced by stressor exposure. However the supplementations increased cellular TAC. No differences in gene expression were observed, but a decrease in SOD1 transcription in PUN supplemented cells. A significant decrease in SOD1 and 2 activities was clearly detected in supplemented cells.

Conclusions: Overall, upon the oxidative stress the antioxidant enzyme defences appeared reduced in supplemented cells, which appeared more prone to the detrimental effects than unsupplemented ones. These results underline the need to carefully consider the amount of bioactives to be supplemented in order to avoid a paradoxic effect.

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Cytotoxic Effects of Selected Bioactives in HepG2 Cells

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Objectives: Bioactive compounds are natural components of foods that possess biological activity in addition to their nutritional value. *In vitro* studies are often performed to establish their effectiveness, but the scientific uncertainties of the extrapolation of *in vitro* data to humans explains EFSA's requirement that human intervention studies are mandatory for health claim approval. Notwithstanding, the cell culture model represents an important tool to unravel the mechanism of action of bioactives. The possible *in vitro* cytotoxicity of bioactives should be carefully considered even within the physiological range of concentration, since cell cultures represent a close system, and cell exposure to *in vivo* physiological concentrations could anyway induce cytotoxicity and alter the cell response to bioactive compounds. The establishment of the highest *in vitro* not cytotoxic concentration within the *in vivo* physiological range is fundamental to avoid misleading results while using cell cultures as model system for the study of bioactive effectiveness. The aim of this study was to assess the concentration- and time-related cytotoxicity of four different bioactives: i. docosahexaenoic acid, a long-chain omega-3 fatty acid; ii. propioniate, a short chain fatty acid deriving from the colonic microbiota fermentation of beta-glucans; iii. cyanidin-3-glucoside, one of the most representative phenols in anthocyanin rich foods; iv. protocatechuic acid, the main *in vivo* metabolite of anthocyanins.

Methods: The cytotoxicity of scalar concentrations of the above mentioned compounds, alone and in combinations, was assessed in HepG2 cells by cell count, MTT and Trypan Blue assay after 24 and 48 h exposure.

Results: Cell viability and vitality appeared clearly dependent on the type and concentration of the test compound, and on the exposure time. Furthermore, an additive effect was evidenced using some bioactive combinations.

Conclusions: Identifying bioactives, establishing their mechanisms of actions and health effects are all active areas of scientific inquiry and, through industrial exploitation, potential societal benefit. *In vitro* studies represent the first step for evaluating bioactive effectiveness and mechanism of action, but results obtained in cell cultures could be misleading if possible cytotoxicity of test compounds is not carefully considered.

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Development of Spontaneous Neoplasms in a Model of Maternal Protein Restriction (MPR)

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The majority of developmental programming studies are conducted 60 to 120 d of maternal postnatal life. At this stage, the mother, although very fertile, is still growing. We, therefore, hypothesized that the conflict between maternal and offspring metabolic needs would be resolved in different ways.

Objectives: To determine if maternal age (MA) and maternal protein restriction (MPR) during pregnancy and/or lactation influence the frequency of spontaneous neoplasms (SN) in the offspring.

Methods: Pregnant Wistar rats at 2 different MA (70 d and 150 d) were fed ad libitum with normal control (C) 20% casein diet or restricted (R) 10% casein diet during pregnancy. Rats were maintained under controlled room conditions (lighting, 0700 to 1900 h; temperature, 22 to 23 °C), and all procedures were approved by the IACUC. Mothers received the C or R diet during pregnancy or lactation to provide 4 different offspring groups: CC, RR, CR, and RC (combination of diets: first letter = diet during pregnancy, second letter = diet during lactation). At birth, litters were adjusted to 10 pups per litter. After weaning, offspring were fed ad libitum with standard lab animal chow. Eight rats of both sexes from different litters (no siblings), that is, from CC, RR, CR, and RC groups, and different MA, were maintained without dietary intervention for up to 500 postnatal days.

Results: Seventeen of a total of 128 animals developed spontaneous neoplasms. According to anatomic site, the neoplasms were surgically removed or animals were euthanatized. By MA 70 d, the female offspring developed more SN (females: 9/32, males: 2/32) than MA 150 d (females: 5/32, males: 1/32). Mammary fibroadenoma (one case in a male) was the most common tumor with an incidence of 12 of 17. Two pituitary adenomas in females from MA 70 d were also found. Other findings were sarcomas, vaginal fibroma, and epidermal carcinoma.

Conclusions: These findings suggest that the frequency of SN is dependent of MA at conception, but independent of subsequent MPR. These data demonstrate the need to control the MA in models of developmental programming.

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Investigating Adipocytes Differentiation: DNA Methylation Status, Proadipogenic Gene Expression, and Inhibition by Food Bioactives

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Objectives: Adipocytes differentiation is a complex process involving the coordinated interplay of numerous transcriptional regulators and genes; recent findings reveal that epigenetics might also provide an important insight into adipogenesis (1). The aim of this study was to investigate possible changes in both DNA methylation and expression of adipogenic markers during the differentiation process of murine pre-adipocytes. In addition, since discovering natural compounds able to regulate size, number and function of adipocytes could contribute to obesity prevention and treatment, the anti-adipogenic ability of three bioactives, docosahexaenoic acid (DHA), genistein (GEN), and sulforaphane (SFN) was evaluated.

Methods: Using murine 3T3-L1 pre-adipocytes, the DNA methylation status at selected genomic loci reported to be altered during adipogenesis (2) was quantified at growing, postconfluent, differentiating and mature adipocyte cell stages using mass spectrometry-based MassARRAY technology. Changes in the expression of adipocyte marker genes *C/EBPα*, *PPARγ* variant1 and 2, and *GLUT 4* at the four stages of the differentiation process were evaluated by RT-qPCR. Finally, the ability of SFN, GEN and DHA to inhibit 3T3-L1 differentiation was assessed by both lipid accumulation and modulation of *C/EBPα*, *PPARγ*, and *GLUT 4* mRNA expression in mature adipocytes.

Results: Quantitative methylation analyses did not confirm the reported changes in DNA methylation levels (2) at the considered genomic loci at all stages of adipocytes differentiation. Expression of the four marker genes was low and similar at the early stages of pre-adipocytes development; a prominent increase was observed in mature adipocytes. The bioactive compounds were shown to suppress adipocytes differentiation and to decrease the lipid accumulation and the expression of the adipogenic markers to the levels of pre-adipocytes.

Conclusions: Data herein reported can be considered as a first step in the way to find natural anti-obesity strategies. However, further studies are needed to evaluate genome-wide DNA methylation changes, other epigenetic alterations potentially involved in adipogenesis and the influence of bioactives on these mechanisms.

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