Cytotoxic effects of selected bioactives in HepG2 cells

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1. Introduction and Aim

Bioactive compounds are natural components of foods that possess biological activity. *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 (DHA), a long-chain omega-3 fatty acid; ii. Propioniate (PRO), a short chain fatty acid deriving from the colonic microbiota fermentation of beta-glucans; iii. cyaniding-3-glucoside (C3G), one of the most representative phenols in anthocyanin rich foods; iv. protocatechuic acid (PCA), the main *in vivo* metabolite of anthocyanins.

2. Methods

The cytotoxicity of scalar concentrations of the mentioned compounds, alone and in combinations, was assessed in a human hepatoma cell line (HepG2) [1]. Cells were seeded in 12-well plates at 6 x 105 cells/ml concentration, and after 24 hours, cells were incubated for 24 or 48 h with the different bioactive compounds. Cytotoxicity was evaluated by cell count, MTT [2] and Trypan Blue assay [3]. Cell viability by MTT and by TB are expressed as percentage of unsupplemented cells (US) (assigned as 100%) and as percentage of total cells, respectively. Cell count is expressed as number of cells/well. Data are mean ± SD of six samples obtained from two independent experiments (n=6).

3. Results: DHA cytotoxicity

DHA cytotoxicity was assessed after 24h exposure at 60, 80, 100, 120, 140, 160, 180 and 200 µM concentration. No significant differences in cell number/well were detected up to 140 µM DHA concentration, while the % of viable cells significantly decreased at 120 µM DHA concentration by the TB method and at 100 µM DHA concentration by MTT assay (Figure 1A). In addition, DHA cytotoxicity was assessed after 48h exposure using the five highest non-toxic concentrations after 24h exposure. No significant modifications in cell number/well were detected at any concentration, while cell vitality evaluated by the MTT method significantly decreased at 60 µM DHA concentration (Figure 1B).

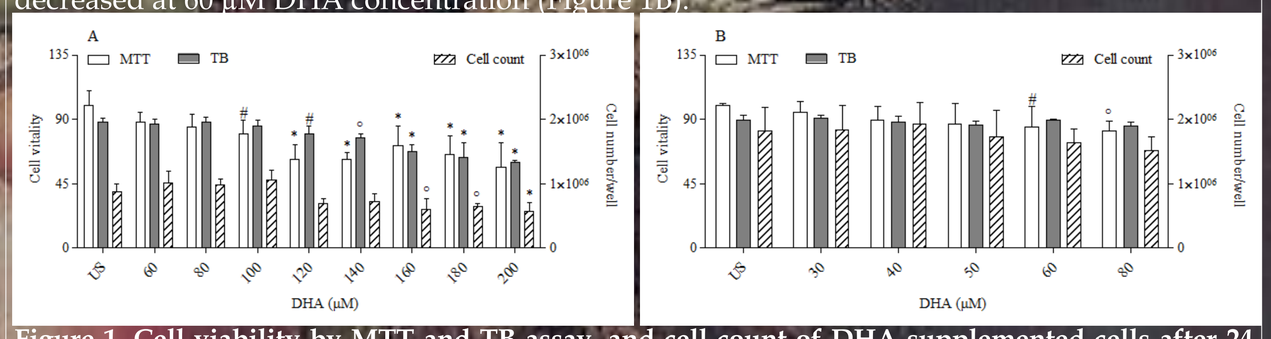

Figure 1. Cell viability by MTT and TB assay, and cell count of DHA supplemented cells after 24 (A) and 48 (B) hours of supplementation.
*Statistical analysis was one way ANOVA (panel A: MTT and cell count p<0.001, TB p<0.05; panel B: MTT and TB p<0.05, cell count n.s.) using Dunnet's as post test to compare supplemented cells to US ones (# p<0.05; ° p<0.01; * p<0.001).*

4. Results: PRO cytotoxicity

Propionate cytotoxicity was assessed after 24h exposure using 6 different concentrations (50, 60, 70, 80, 90 and 100 µM). No significant modifications in cell number/well and % viable cell (TB method) were detected at any concentration, while cell vitality measured by the MTT method slightly but significantly decreased starting at 80 µM propionate concentration (figure 2A). Moreover, propionate cytotoxicity was assessed after 48h exposure using the two highest concentrations that were not toxic after 24h exposure (60 and 70 µM). No significant modifications in cell number, % viable cells (TB method), and cell viability (MTT assay) were detected at any concentration used (data not shown).

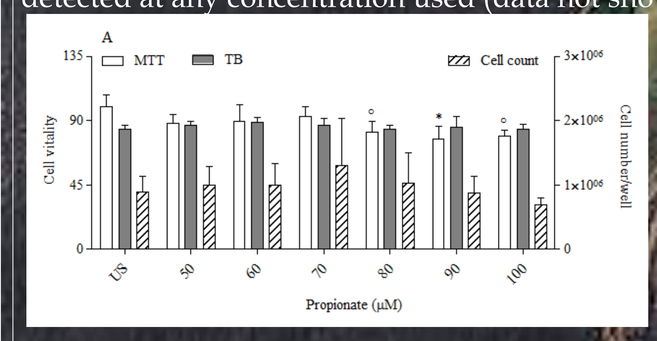

Figure 2. Cell viability by MTT and TB assay, and cell count of propionate supplemented cells after 24 (A).
*Statistical analysis was one way ANOVA (panel A: MTT p<0.001, TB and cell count n.s.) using Dunnet's as post test to compare supplemented cells to US ones (° p<0.01; * p<0.001).*

5. Results: C3G cytotoxicity

C3G cytotoxicity was assessed after 24h exposure using 6 different concentrations (100, 120, 140, 160, 180 and 200 nM). No significant modifications in cell number/well and % viable cell (TB method) were detected at any concentration, while cell vitality measured by the MTT method started decreasing significantly at 160 nM Figure 3A).Furthermore, C3G cytotoxicity was assessed after 48h exposure using the two highest concentrations that were not toxic after 24h supplementation (120 and140 nM). No significant modifications in cell number/well, % viable cell (TB method), and cell vitality measured by the MTT methods were detected at any concentration used (data not shown).

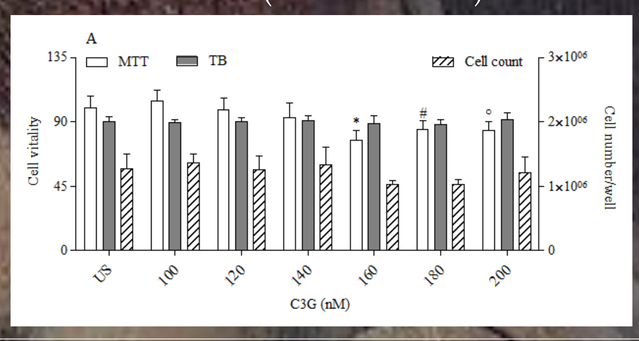

Figure 3. Cell viability by MTT and TB assay, and cell count of C3G supplemented cells after 24 (A). *Statistical analysis was one way ANOVA (panel A: MTT p<0.001, TB and cell count n.s.) using Dunnet's as post test to compare supplemented cells to US ones (° p<0.01; * p<0.001).*

6. Results: PCA cytotoxicity

PCA cytotoxicity was assessed after 24h exposure using 6 different concentrations (10, 12, 14, 16, 18 and 20 µM). Neither modifications in cell number/well, and % viable cell (TB method) nor in cell vitality measured by the MTT methods were detected at any concentration used (data not shown). PCA cytotoxicity was assessed after 48h exposure using the two highest concentrations that were not toxic after 24h supplementation (18 - 20 µM). No significant modifications in cell number/well, % viable cell, and cell vitality (MTT method) were detected at any concentration used (data not shown).

7. Results: C3G:PCA cytotoxicity

Because PCA is one of the major benzoic acid derivatives from C3G degradation (Vitaglione 2007), and in vivo studies of individual anthocyanins reveal that a small amount, generally <1%, of consumed quantities is present in plasma in the native form respect PCA (Milbury et al., 2010), the possible cytotoxicity due to the combined supplementation with C3G and PCA was assessed at 24 and 48h maintaining a C3G:PCA ratio = 1:100. Experiments were performed using the six higher non-toxic C3G concentrations plus the corresponding 100-times PCA concentration. Therefore the combinations were tested for cytotoxicity after 24h exposure from 90nM C3G:9µM PCA to 140nM C3G:14µM PCA. Cell number/well significantly decreased using the 140nM C3G:14µM PCA supplementation, while no significant modifications in % viable cell (TB method) and cell vitality (MTT method) were detected at any concentration (Figure 4A). C3G:PCA cytotoxicity was assessed after 48h exposure using the two highest concentrations that were not toxic after 24h supplementation (120nM C3G:12µM PCA and 130nM C3G:13µM PCA). No significant modifications in cell number/well, % viable cell (TB method), and cell vitality measured by the MTT methods were detected at any concentration used (data not shown).

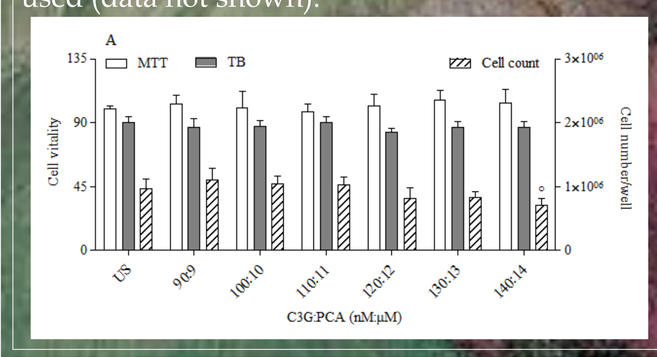

Figure 4. Cell viability by MTT and TB assay, and cell count of C3G:PCA supplemented cells after 24 (A). *Statistical analysis was one way ANOVA (panel A: cell count p<0.01, MTT and TB n.s..) using Dunnet's as post test to compare supplemented cells to US ones (° p<0.01).*

8. Results: DHA:PRO cytotoxicity

The possible cytotoxicity due to the combined supplementation with DHA and propionate was assessed using different concentrations at 24 and 48h. For propionate we considered as high concentration the highest non-toxic concentration at 48h (70 µM), as low concentration an average plasma physiological concentration founded in human after a fibres rich meal (1 µM) and as medium concentration the average of the previous two (35 µM). For DHA we considered as high and low concentration 60 and 50 µM respectively, a typical range of physiological plasma concentration. Medium concentration was the average of the previous two (55 µM). Accordingly, 9 different combinations were tested. At 24h, no significant differences in cell number/well, viable cell (TB method) and cell vitality (MTT method) were detected at any combination used (data not shown) compared to US. After 48h of supplementation, cell vitality (MTT method) decreased in all combination but propionate 70 µM, while no significant modifications in cell number/well and viable cell (TB methods) were detected at any combination used (Figure 5B).

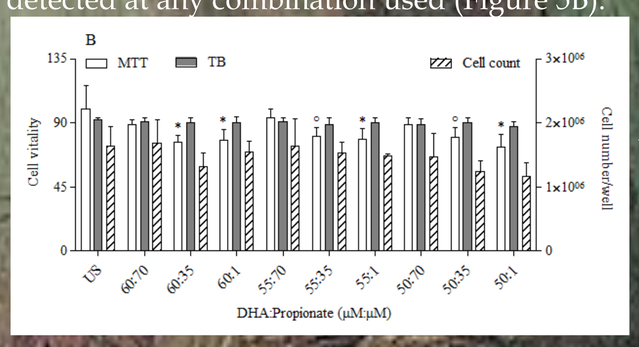

Figure 5. Cell viability by MTT and TB assay, and cell count of DHA:PRO supplemented cells after 48 (B). *Statistical analysis was one way ANOVA (panel B: MTT p<0.001, TB and cell count n.s.) using Dunnet's as post test to compare supplemented cells to US ones (° p<0.01; * p<0.001).*

9. Results: DHA:C3G:PCA cytotoxicity

The cytotoxicity due to the combined supplementation with DHA and C3G:PCA (1:100) was assessed using different concentrations. For C3G:PCA, we considered as high concentration the highest non-toxic concentration at 48h, as low concentration an average plasma physiological concentration [4] (1nM C3G:0.1µM PCA), and as medium concentration the average of the previous two. Accordingly, 9 different combinations were tested. After 24h, no significant differences in cell number/well, viable cell (TB method) and cell vitality (MTT method) were detected at any combination used (data not shown) compared to US. At 48h, cell vitality (MTT method) decreased in all combination, while no significant modifications in cell number/well and viable cell (TB method) were detected at any combination used (Figure 6B).

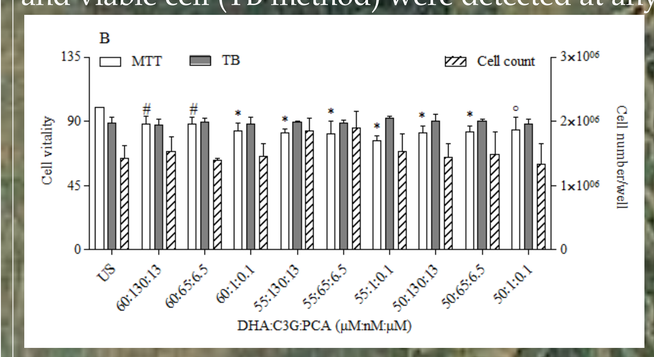

Figure 6. Cell viability by MTT and TB assay, and cell count of DHA:C3G:PCA supplemented cells after 48h (B). *Statistical analysis was one way ANOVA (panel B: MTT p<0.001, TB and cell count n.s.) using Dunnet's as post test to compare supplemented cells to US ones (# p<0.05; ° p<0.01; * p<0.001).*

10. 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.

10. Acknowledgments

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1. Di Nunzio M., et al. Br J Nutr. 2010;103:161-7. **2.** Valli V., et al. J Agric Food Chem. 2012 26;60:12508-15.
3. Di Nunzio M., et al. J Sci Food Agric. 2013;93(14):3565-73. 4. Vitaglione P., et al. J Nutr. 2007;137:2043-8.