

THE CYTOTOXIC POTENTIAL EFFECT OF SOME FLAVORING AGENTS ON A MOUSE FIBROBLAST CELL CULTURE

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Abstract

The study aimed to evaluate the proliferative action and cellular morphology induced by the following food flavoring agents: ($\pm$) - limonene p-methyl-1,8-diene (orange flavoring substance) and ($\pm$) -3,7-dimethyl-6-octenal (lemon flavoring substance) in cell cultures. The tests were performed on normal proliferating adherent cells from the 3T3 cell line (mouse fibroblast) grown in DMEM culture medium (Dulbecco's Modified Eagles Medium) supplemented with 10% fetal bovine serum (SFB), glutamine and antibiotic-antimycotic (complete culture medium). Tests for lactate dehydrogenase (LDH) release and 3- (4,5-dimethylthiazol-2-yl) -5- (3-carboxymethoxy phenyl) -2- (4-sulfophenyl) -2 H – tetrazolium (MTS) reduction were achieved after 18 hours and 24 hours of cells incubation in the presence of the testing substances. For testing, we considered a wide range of dilutions (1:2560000-1:2000) for lemon and orange flavoring substances, compared with the corresponding dilutions of ethanol (used as reference solvent). Orange flavoring substance reduced the number of metabolically active cells in culture at dilution 1/2000 compared to the decreased effect exerted by the scent of lemon. Lemon flavoring substance had a moderate cytostatic effect, but the orange one was strongly cytotoxic at the dilution of 1/2000 (massive release of LDH).

At dilution 1/1000 it was observed a higher cytotoxicity of both flavoring substances, especially the orange flavoring at dilution 1/4000.

Rezumat

Studiul a urmărit evaluarea acțiunii proliferative și a morfologiei celulare induse de următorii aromatizanți alimentari: ($\pm$) - limonen p-metil-1,8-diena (agent aromatizant din portocală) și ($\pm$) -3,7-dimetil-6-octenal (agent aromatizant din lămâie) în culturi de celule. Testarea a fost efectuată pe celule normale proliferative aderente din linia celulară 3T3 (fibroblaste de șoarece), cultivate în mediu de cultură DMEM (*Dulbecco's Modified Eagles Medium*) suplimentat cu 10% ser fetal bovin (SFB), glutamină și antibiotic-antimicotic (mediu de cultură complet). Testele de eliberare a lactat dehidrogenazei (LDH) și reducere a 3- (4,5-dimetiltiazol-2-il) -5- (3-carboximetoxifenil) -2- (4-sulfofenil)-2 H-tetrazoliu (MTS) au fost realizate după 18 și 24 de ore de incubare a celulelor în prezența substanțelor de testat.

Pentru testări s-a luat în lucru un domeniu larg de diluții (1:256000 – 1:2000) pentru substanțele testate, comparativ cu diluțiile corespunzătoare de etanol (utilizat ca solvent de referință). Substanța aromatizantă din portocale a redus numărul de celule

metabolic active în cultură la diluția 1/2000, comparativ cu efectul mult mai slab exercitat de substanța aromatizantă provenită din lămâie. Substanța aromatizantă din lămâie are efect moderat citostatic, iar cea de portocale este pregnant citotoxică la diluția de 1/2000 (eliberare masivă de LDH). La diluția 1/1000 s-a obținut o citotoxicitate pregnantă a ambelor substanțe aromatizante, dar mai ales a substanței aromatizante de portocale la diluția 1/4000.

Keywords: food flavoring substance, cell culture, cell proliferation.

Introduction

Food additives are chemicals added to food in order to improve or maintain their nutritional value, to change the organoleptic characteristics and to increase their quality (flavoring). Given the risk of malignant degeneration using flavoring agents, the global orientation is that their use should be based on the principle of permitted substances demonstrated through toxicological examinations. Depending on the results, acceptable daily intake is established [10].

Compared with other routes of exposure to carcinogenic risks, food is 1000 000 times more often involved in cancer occurrence compared with lung exposure and 1000 times compared with skin exposure. Although food carcinogens are consumed in small amounts over the years, their effect is cumulative, the disease developing after many years. Cancerous disease is established under the combined action of various endogenous factors (age, sex, genetic factors, medical history etc.) and environmental factors. The latter are in causal relationship with carcinogenesis at a rate of 60-90% [7, 8]. Of all environmental factors, diet seems to have a significant role in the emergence and development of malignant tumors. The importance of nutrition in carcinogenesis is underlined by the appreciable greater amount of carcinogenic factors conveyed by enteral way compared to the amount placed on the other means of penetration of carcinogenic substances (skin and lung). The ratio between the amount of carcinogens introduced into the body through food and the amount introduced through the lungs and skin is 1000000/1000/1 [1].

Considering these premises, the aim of our study was the evaluations of the proliferative action and cellular morphology induced by the following food flavoring agents: ($\pm$) - limonene p-methyl-1,8-diene (orange flavoring substance) and ($\pm$) -3,7-dimethyl-6-octenal (lemon flavoring substance) in cell cultures.

Materials and Methods

We have studied the following food flavoring agents ($\pm$) - limonene p-methyl-1,8-diene (orange flavoring substance) and ($\pm$) -3,7-dimethyl-6-

octenal (lemon flavoring substance) which were diluted 1:5 in absolute ethanol. Afterwards they were further 20-folds diluted in DMEM culture medium, obtaining a stock dilution of 1:100. Binary dilutions were made from these stock solutions, using culture medium, so that the final dilutions tested were: 1:256000, 1:128000, 1:64000, 1:32000, 1:16000, 1:8000, 1:4000, 1:2000, 1:1000 and 1:500. *In vitro* studies were performed on adherent proliferating cells of 3T3 cell line (mouse fibroblasts). Cell viability was determined by the exclusion trypan blue test, while determining the cell concentration. Cell experimental culture was performed in sterile culture plates with 96 wells and flat bottom [5]. We used a suspension of fresh trypsinised 3T3 cells, with viability greater than 95%, resulting in a cell culture which did not reach 100% confluence. There were done in parallel three acellular samples (100 mL culture medium), which represented the background of lactate dehydrogenase (LDH) release and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2 H – tetrazolium (MTS) reduction tests. Membrane integrity was assessed as the release of lactate dehydrogenase (LDH release), measured with non-radioactive cytotoxicity kit Cytotox96 Assay [4]. Viability/cell proliferation was assessed by the tetrazolium compound MTS reduction assay (MTS reduction), which is a colorimetric method for determining the number of living cells in cell proliferation experiments and tests cytotoxicity [3]. MTS is bio-reduced by cells in a derivative of formazan dye, soluble in culture medium. The conversion is likely achieved through NADPH or NADH [2]. The literature shows that tetrazolium reagents can substitute with good approximation tritiate thymidine incorporation [11]. MTS reduction test was conducted with the CellTiter One Solution Cell proliferation 96R AQueous Assay (Promega Corporation) Kit.

The results are presented as individual data obtained as average of the triplicate determinations for each sample. Such results obtained in separate experiments were statistically processed as average $\pm$ standard deviation of the average. Statistical comparison of the effect exerted by the compounds was performed with the t-test for pair samples [6].

Results and Discussion

The experimental data (Figure 1a), show that orange flavoring substance reduced the number of metabolically active cells in culture at dilutions greater than or equal to 1/16000, the dilution effect being obvious at 1/2000. The effect exerted by the lemon flavoring substance on the reduction of MTS is much higher than the one exerted by the substance from oranges. Analyzing in parallel the effect of the food flavorings on

LDH release by 3T3 fibroblasts (Figure 1b), we can state that the lemon flavoring substance has a moderate cytostatic effect, but the orange flavoring substance is highly cytotoxic at the dilution of 1/2000 (massive release of LDH).

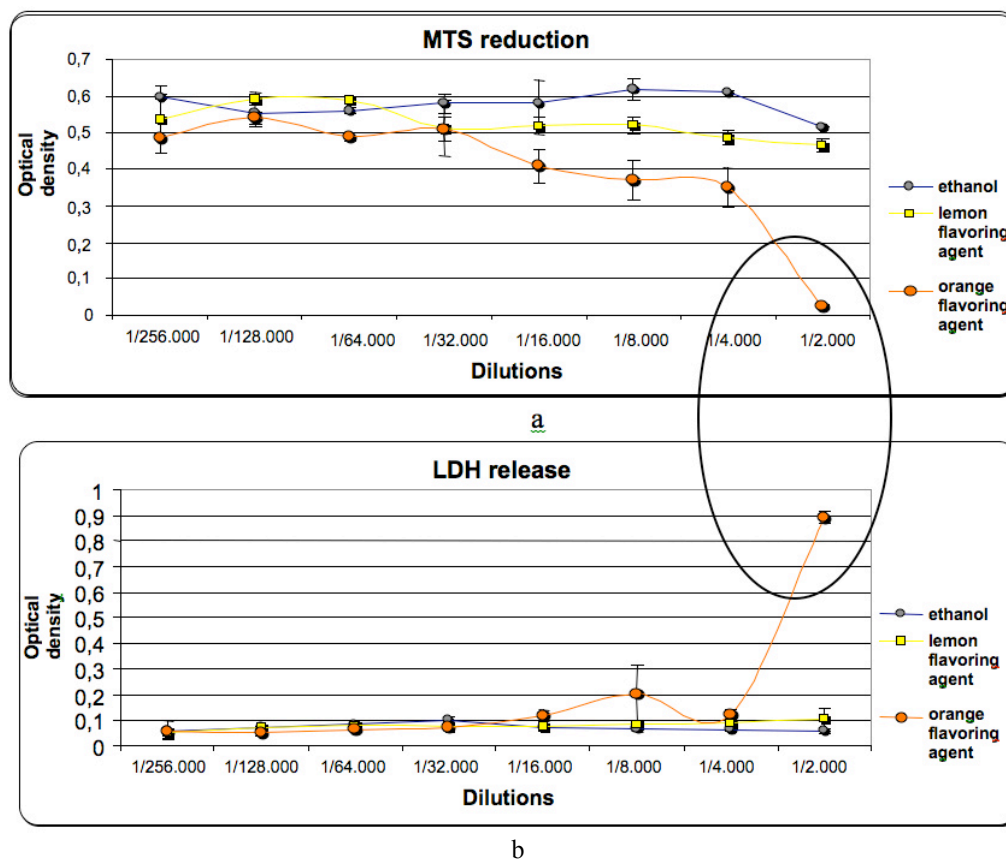


Figure 1

Exerted *in vitro* effect of lemon and orange flavoring substances, diluted in the range of 1:256000 - 1:2000, on the viability and multiplication of 3T3 mouse fibroblasts.

This study showed that flavoring substances can influence cell viability and functionality at dilutions below 1/16000. We also investigated the effect exerted by food flavoring substances in the range of dilutions 1/8000 - 1/1000. The experimental data (Figure 2a, Figure 2b) reveal the differences in cytotoxicity between lemon and orange flavoring. It should be noted the pregnant cytotoxicity of both flavoring agents at the dilution

1/1000, but the orange flavoring substance shows significantly higher cytotoxicity at higher dilutions than the lemon flavoring (1:4000).

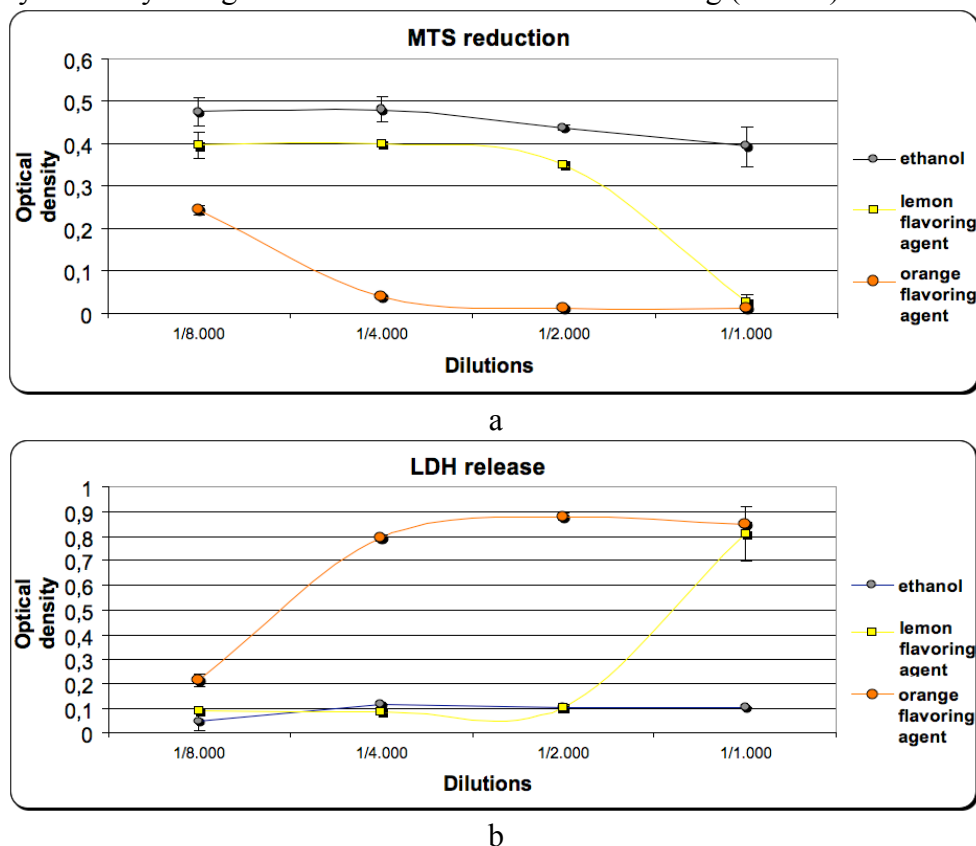


Figure 2

Exerted *in vitro* effect of lemon and orange flavoring substances in the range of dilutions 1:8000 - 1:1000, on the viability and multiplication of mouse 3T3 fibroblasts.

Conclusions

This first experiment shows that the lemon flavoring substance exerts a moderate cytostatic effect at dilutions below 1/2000, but becomes significantly cytotoxic at the dilution 1/1000. At the same time, we observed that the orange flavoring substance is more toxic than the lemon one, showing cytotoxicity at higher dilutions (1/4000 to 1/1000). The study allows us to choose the range of dilutions of flavoring agents at which they show no cytotoxicity, but may influence in a more subtle way the cellular functionality. In future experiments, we aim to investigate the effect exerted

by these flavoring substances at dilutions of 1/32000 and 1/16000 on cells metabolism.

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