IN VITRO COMPARISON OF THE EFFECTS OF "REDUCED RISK" NICOTINE PRODUCTS AND OF CIGARETTE SMOKE ON ADIPOCYTE SURVIVAL AND DIFFERENTIATION

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INTRODUCTION

Cigarette smoking (CS) has been implicated in cardiovascular, metabolic and respiratory diseases and it constitutes a major cause of lung and other cancer in humans. In the adipose tissue, nicotine has been reported to induce lipolysis, leading to body weight loss, while CS has been associated with insulin resistance and hyperinsulinemia. Electronic cigarettes (e-cig) and heated tobacco products have been designed to deliver nicotine in a vaping solution or aerosol, without tobacco combustion. Thus, they are assumed to be safer alternatives to conventional cigarettes.

AIM OF THE STUDY:

To evaluate the cytotoxic effects of 1R6F CS, e-cig vapor and heated tobacco aerosol and the impact of these products on the differentiation of 3T3-L1 pre-adipocytes to "beige" adipocytes.

METHODS

Production of extract-enriched media

By using a commercially available e-cig device, a set of impingers and a syringe pump, e-liquid containing 1.2% w/w nicotine and no flavor was evaporated and extracted into 40 mL of culture medium DMEM. Similarly, extracts of three research cigarettes (1R6F) and four heated tobacco sticks (IQOS, PMI) in DMEM were also produced. The vaping/smoking protocol was: 40 mL puff volume, 2-second puff duration, and 30-second puff interval. LC/MS-MS was applied to determine the levels of nicotine in each extract.

Cell culture and differentiation

3T3-L1 pre-adipocytes were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, kept at 37°C and 5% CO_2 incubator condition. After becoming supra-confluent, 3T3-L1 pre-adipocytes were differentiated to "beige" adipocytes by adding insulin, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), troglitazone, indomethacin and T3, and treated with extracts to reach similar final nicotine concentration (3 ug/mL). After 2 days, the differentiation medium was changed to DMEM containing 10% fetal bovine serum, 1 μg/mL insulin and each of the extracts until day 10.

Cell viability assay

3T3-L1 pre-adipocytes were plated into 96-well plates. Dilutions of the extracts were added and incubated for 24 h and 48 h. Cytotoxicity was determined by the MTT assay and soluble formazan crystals were measured at 492 nm, using a microplate reader.

Oil Red O staining assay

The amount of lipid accumulation of differentiated "beige" adipocytes was determined using Oil-Red O staining at Day 10. In brief, the cells were washed with phosphate-buffered saline and fixed in 10% formaldehyde. Next, the cells were stained with Oil-Red O solution for 1 h, and then washed with distilled water. The monolayers were photographed and the stained lipids were eluted with 100% isopropanol and measured using a microplate reader at 550 nm.

RESULTS

Table 1: Determination of nicotine levels detected in extracts, using LC/MS-MS. Data are presented as the mean ± SD (n=6 determinations in 2 independent repeats).

Types of extracts	Nicotine concentration (ug/mL)
Research cigarette (1R6F) extract	87.35±3.65
Heated tobacco product extract	92.73±3.08
e-cig extract	51.50±1.29
Medium extract	1.04±0.08

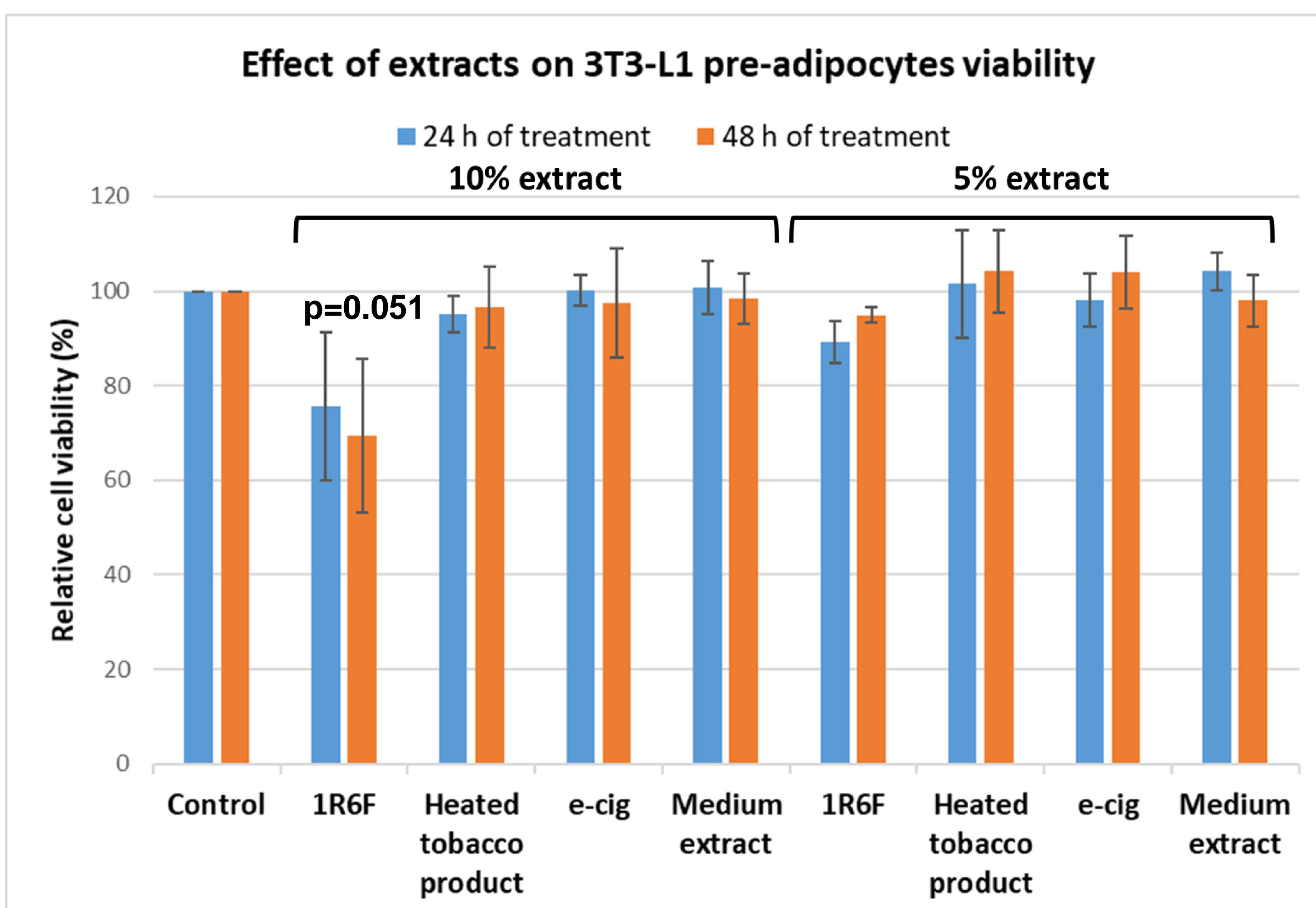

Figure 1: Effects of the four extracts on cell viability of 3T3-L1 pre-adipocytes, after 24 h and 48 h of treatment. The extracts were administered as 5% and 10% final dilutions. No treatment caused statistically significant decrease in viability. Data are presented as the mean ± SD (n=12 determinations in two independent repeats).

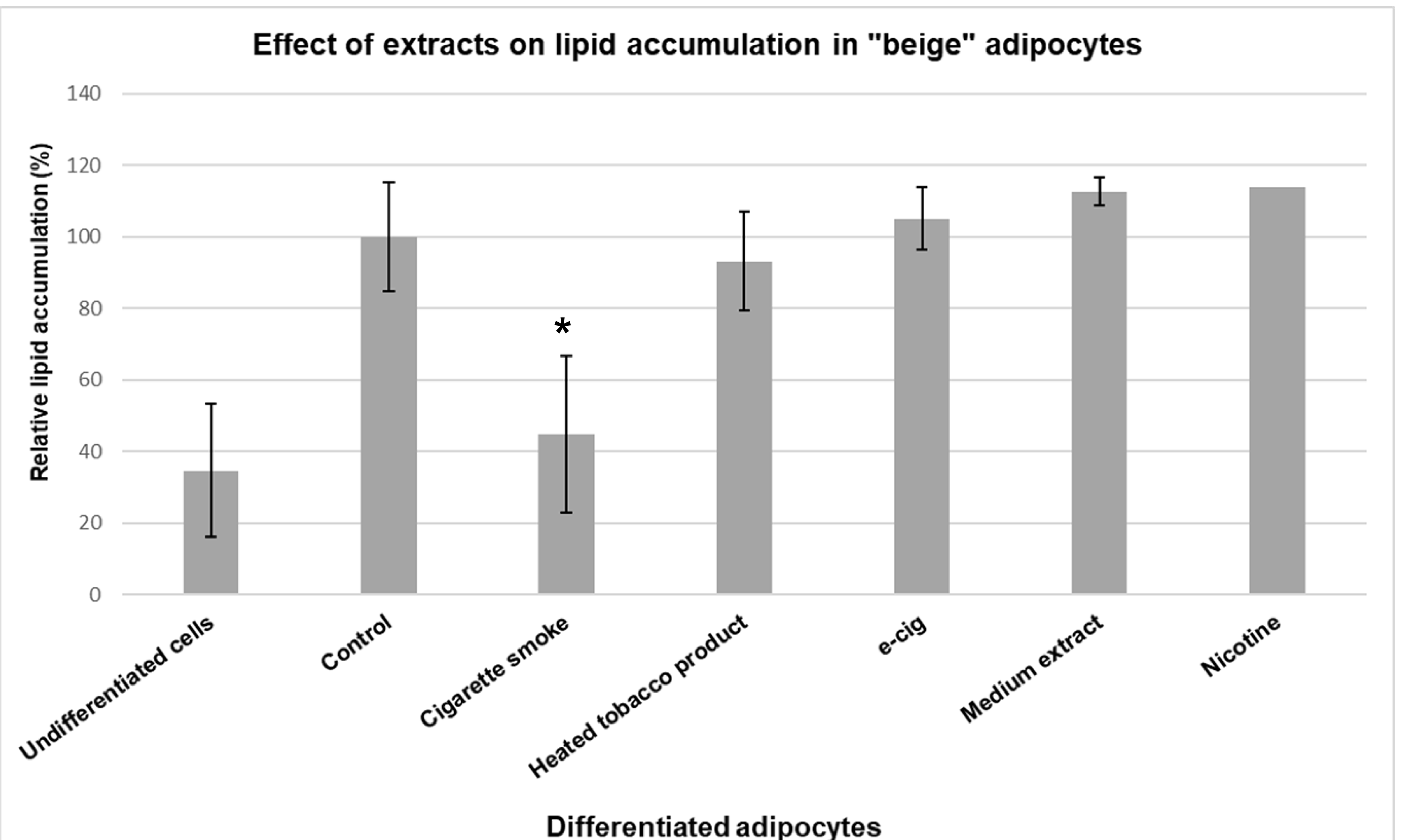

Figure 2: Preliminary results of the effects of the four extracts and pure nicotine (3 ug/mL) on lipid accumulation of "beige" adipocytes. 3T3-L1 pre-adipocytes were differentiated in the presence of each treatment for 10 days. Data are presented as the mean ± SD (n=3 wells per condition). * $P < 0.05$

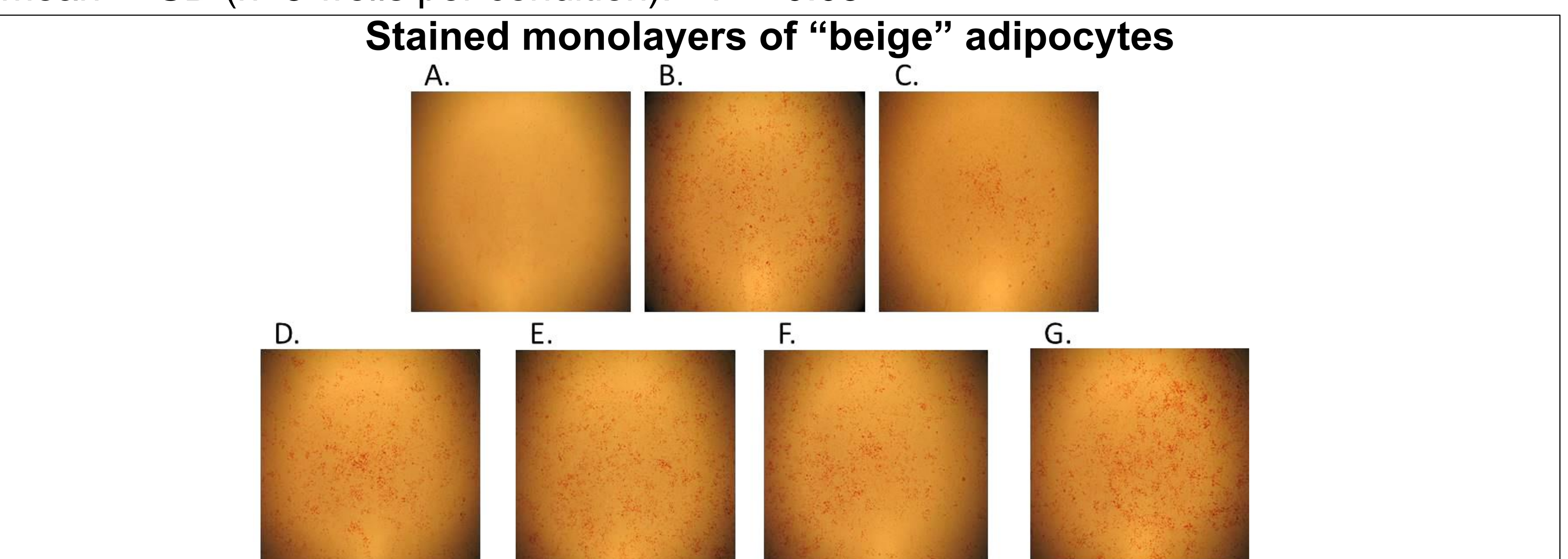

Figure 3: Pictures of the stained cells with Oil Red O were obtained using a dissection microscope. A. Undifferentiated cells, B. Differentiation Control, C. 1R6F extract-treated cells, D. Heated tobacco product-extract treated cells, E. e-cig extract-treated cells, F. Medium extract-treated cells, G. Nicotine-treated cells.

DISCUSSION

- **In preliminary experiments, no statistically significant effects of cytotoxicity were observed in the cases of heated tobacco, e-cig and medium extracts, after 24 h and 48 h of treatment of 3T3-L1 pre-adipocytes, at 5% or 10% dilutions. A borderline significant cytotoxic effect was detected in the administration of 1R6F at 10% dilution after 48 h of treatment.**
- **Administration of the extracts - along with the differentiation factors of «beige» adipocytes - showed that the cells treated with the 1R6F extract were characterized by decreased levels of lipid accumulation. This effect seems to be nicotine-independent.**
- **mRNA obtained from similarly-treated cells will be evaluated for expression of "beige" adipocyte markers.**

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