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Comparison between electronic cigarette refill liquid and nicotine on metabolic parameters in rats

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ABSTRACT

Aims: Nicotine is known to promote body weight loss and to disturb glucose homeostasis and lipoprotein metabolism. Electronic cigarettes, as a substitute to nicotine, are becoming increasingly popular, although there is no evidence regarding their safety. Considering the dearth of information about e-cigarette toxicity, the present study was designed to compare nicotine alone to e-liquid with or without nicotine on metabolic parameters in Wistar rats.

Main methods: For this purpose, e-liquid with or without nicotine and nicotine alone (0.5 mg/kg of body weight) were administered intra-peritoneally during 28 days.

Key findings: Our results show a significant decrease in food and energy intake after nicotine or e-liquid with nicotine exposure, when compared to control or e-liquid without nicotine. Analysis of lipid status identified a significant decrease in cholesterol and LDL levels in e-cigarette groups, suggesting an improvement in lipid profile. Interestingly, e-liquid without nicotine induced hyperglycemia which is negatively correlated to hepatic glycogen level, acting like nicotine alone. Furthermore, an increase in liver biomarkers was observed in all treated groups. qRT-PCR analysis showed GSK3β up-regulation in e-liquid with nicotine as well as, surprisingly, in e-liquid without nicotine exposure. In contrast, PEPCK genes were only up-regulated in e-liquid with nicotine.

Significance: While some features observed in rats may not be observed in human smokers, most of our data are consistent with, e-liquid *per se i.e.* without nicotine, not being neutral from a metabolic stand point since disrupting glucose homeostasis in rats.

1. Introduction

The World Health Organization (WHO) reported in 2013, 6 million deaths worldwide, due to cigarette smoking. Cigarette smoke contains, in addition to nicotine, carbon monoxide, and a wealth of toxic gaseous and particulate agents [1]. Cigarette smoking has been associated with hypertension, inflammation [2,3], diabetes [4] and abnormal lipoprotein metabolism [5]. Indeed, smokers have elevated levels of inflammatory markers, such as C-reactive protein, white blood cell count, and fibrinogen, whereas they have decreased serum albumin, compared to non-smokers [3]. They are also insulin resistant, exhibiting an increased risk for type 2 diabetes [6,7]. Finally, they have significantly higher serum free fatty acids and triglyceride levels, lower high-density lipoprotein (HDL) cholesterol and a higher proportion of atherogenic small dense low-density lipoprotein (LDL) particles [6,7]. All these features, with increased platelet aggregation and decreased distensibility of vessel walls [8], may lead to cardiovascular diseases.

To break smoking habit, several alternatives exist, one of them having recently emerged: the electronic cigarette. The electronic cigarettes (e-cig) invention, patented by Hon Lik, a Chinese pharmacist, was introduced to the market in 2004, as a substitute to smoking [9]. E-cig contains an e-cig refill (e-fluid or e-liquid), which typically contains humectant (propylene glycol (PG) and/or vegetable glycerin (or VG) and/or polyethylene glycol 400 (PEG400)), concentrated flavor and optionally variable doses of nicotine, which in addition often vary significantly from the concentration on labels [10,11]. Other compounds including tobacco-specific nitrosamines, formaldehyde, acetaldehyde, acrolein, metals, polycyclic aromatic hydrocarbons have also been detected in various amounts [11,12].

These revolutionary products raised a lot of enthusiasm, being regarded as a healthy alternative in the treatment of smoking cessation. At the same time they raised a major public health concern because the risks associated with these new products remain unknown. The WHO recommended that "consumers should be strongly advised not to use

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e-cig until a reputable national regulatory body has found them safe and effective" [13].

Recent *in vitro* studies demonstrated that at least some e-liquids were cytotoxic, with potential for promoting cellular adverse effects, varying from one e-liquid to another. In fact, unless electronic cigarette vapor extract is less cytotoxic than cigarette smoke [14,15,16], e-liquid cytotoxicity was identified in human embryonic stem cells and mouse neural stem cells [17], as well as, in human pulmonary fibroblasts cells [18,19]. Toxicological effects of e-cigarette vapor were found after direct exposure of primary human bronchial epithelial cells [20,21] and human gingival fibroblasts (HGF) [22]. It appears that e-liquid cytotoxicity correlates with the number and concentration of chemicals used for flavoring [17]. For instance, cinnamon flavor contains two chemical compounds (cinnamaldehyde and 2-methoxycinnamaldehyde) that are highly cytotoxic [23]. Moreover, some cell types, like stem cells seem to be more sensitive to e-fluids than others [17].

It was recently shown that e-liquids aerosols, *i.e.* produced after heating exhibited oxidant reactivity by generating Reactive Oxygen Species (ROS) [19,22]. Furthermore, in HGF cells, these ROS were able to induce an oxidative stress leading to an increased in Bax expression and to cell apoptosis [22].

Concerning animal studies, one recent work in mice treated intratracheally with e-cigarette fluid showed increased infiltration of inflammatory cells, airway hyper-responsiveness, and stimulation of cytokines production [21]. Another study, testing exposure of wild type C57BL/6J mice to aerosols produced from e-cigarette, also found increased pro-inflammatory cytokines [19]. But to our knowledge, no study has ever focused on metabolic disorders induced by e-liquids.

In order to bring new insights into e-cigarette toxicity, we analyzed, how e-liquid exposure, in comparison to nicotine, might influence metabolic parameters, in normal healthy rats.

2. Material and methods

2.1. Chemicals

Nicotine bitartrate hydrogen salt was supplied by Sigma St. Louis, Missouri, USA. Electronic cigarette refill bottles certified ISO 9001, with tobacco flavor and with 18 mg/ml of nicotine or without nicotine were used. E-cigarette refill liquid is composed of propylene glycol (50%), vegetal glycerin (40%), distilled water (5–10%), flavorings (1–5%) and nicotine (0–1.8%).

2.1.1. Analysis of e-liquids composition by Gas Chromatography–Mass Spectrometry (GC–MS)

30 μl of e-liquid were diluted in 470 μl of methanol and analyzed on a Trace-GC Ultra gas chromatograph with mass detection performed on an ITQ900® (Thermo Scientific). The injector was set with a split ratio of 1:10 at 250 °C. Compounds were separated with an Agilent Technologies DB5HT column (30 m × 0.250 mm × 0.1 μm) and carrier gas was high-purity helium at 1.1 ml min^{-1} flow. The oven temperature was initially held at 100 °C for 2 min, then raised to 320 °C at a rate of 15 °C min^{-1} and held for 1 min. Compounds were detected by electronic impact ionization, with the source temperature set at 220 °C. Data analysis was performed with Xcalibur™ software using NIST and a homemade database.

2.2. Animals

Male Wistar rats weighing 160 ± 20 g were purchased from SIPHAT (Tunis, Tunisia). Before beginning experiments, all animals were acclimated for 1 week under well-controlled conditions of temperature (22 ± 2 °C), relative humidity (70 ± 4%), and a 12/12 h light–dark cycle with 07:30–19:30 being light phase. Animals were housed as 2 per polypropylene cage. They were fed with standard pellet diet (SISCO, Sfax, Tunisia) and given free access to water *ad libitum* all along the experiment. Procedures involving the animals and their care followed the Guidelines for Ethical Control and Supervision in the Care and Use of Animals. All procedures have been reviewed by the Animal Care and Use Committee of Pasteur Institute.

2.3. Study design

The experimental procedure is depicted in Fig. 1. A total of 32 rats were randomized into 4 groups of 8 animals each as follows: Group 1: Control group, were injected intra-peritoneally with physiological serum (500 μl). Group 2: NICOTINE treated group, received an intraperitoneal injection of 0.5 mg of nicotine/kg of bw/day diluted in physiological serum (500 μl). Group 3: E-CIGARETTE 0% treated group, received an intra-peritoneal injection of electronic cigarette refill liquid without nicotine (less than 10 μl) diluted in physiological serum (500 μl). Group 4: E-CIGARETTE treated group, received an intraperitoneal injection of electronic cigarette refill liquid containing 0.5 mg of nicotine/kg of bw/day (less than 10 μl) diluted in physiological serum (500 μl). Rats were treated for 4 weeks and sacrificed by beheading 24 h after the last treatment.

2.4. Body weight gain and dietary indexes

Food intake and drinking water consumptions were evaluated daily at the same time (09:00–10:00), as food and water provided minus the leftover. The body weights were determined at the beginning and at the last day of the experiment and were used to determine the bw gain (= final body weight − initial body weight). Food intake was used to obtain total energy intake (kcal/day) using the metabolic factor (4 × proprotein, 9 × fat and 4 × carbohydrate).

2.5. Blood and tissue sampling

After decapitation, arteriovenous blood was quickly collected in heparin tubes and centrifuged at 1000 *g* for 10 min at 4 °C. The resulting supernatant (plasma) was immediately transferred into clean polypropylene tubes and plasma aliquots were then stored at −80 °C until use. Liver was carefully dissected out, made free from adherents and stored at −80 °C until biochemical analysis.

2.6. Plasma glucose assay

Glucose was measured by the glucose oxidase and peroxidase using quinoneimine as a chromogen. The amount of plasma glucose is related to amount of quinoneimine which is measured spectrophotometrically at 505 nm [24].

2.7. Hepatic glycogen assay

For determination of glycogen, we followed the gravimetrical technique of Good [25]: 0.5 g of liver was extracted with 3 ml of 30% KOH, incubated for 30 min at 100 °C, then brought to acid pH by addition of 20% trichloroacetic acid. Protein precipitates were removed by centrifugation for 10 min at 3000 *g*. Glycogen was precipitated by ethanol and weighed. The results were expressed as g of glycogen/100 g of liver.

2.8. Lipid profile, cardiovascular and atherogenic indexes

Plasma total cholesterol (TC), low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL) and triglycerides (TG) concentrations were measured using commercially available diagnostic kits supplied by Randox Laboratories (UK). VLDL was calculated as TG/5. Results were presented in g/l.

Cardiovascular risk factors were evaluated using TC/HDL and TG/HDL ratio [26]. Atherogenic index (AI) was calculated as LDL/HDL.