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Chemical analysis and *in vitro* toxicological evaluation of aerosol from a novel tobacco vapor product: A comparison with cigarette smoke

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ABSTRACT

The recent rapid increase in the prevalence of emerging tobacco- and nicotine-containing products, such as e-cigarettes, is being driven in part by their reduced-risk potential compared to tobacco smoking. In this study, we examined emission levels for selected cigarette smoke constituents, so-called "Hoffmann analytes", and *in vitro* toxicity of aerosol from a novel tobacco vapor product (NTV). The NTV thermally vaporizes a nicotine-free carrier liquid to form an aerosol which then passes through tobacco, where it absorbs tobacco-derived flavors and nicotine. The NTV results were compared with those for 3R4F cigarette smoke. Chemical analysis of the NTV aerosol demonstrated that Hoffmann analyte levels were substantially lower than in 3R4F smoke and that the most were below quantifiable levels. Results from *in vitro* bacterial reverse mutation, micronucleus and neutral red uptake assays showed that, in contrast with 3R4F smoke, the NTV aerosol failed to demonstrate any measurable genotoxicity or cytotoxicity. The temperature of tobacco during NTV use was measured at approximately 30 °C, which may explain the lower Hoffmann analyte emission and *in vitro* toxicity levels. These results suggest that the aerosol from the NTV has a very different toxicological profile when compared with combustible cigarette smoke.

1. Introduction

Smoking is a known risk factor for smoking-related diseases, such as lung cancer, chronic obstructive pulmonary disease and cardiovascular disease (Forey et al., 2011; Go et al., 2014; Vineis et al., 2004). Cigarette smoke is a complex mixture containing over eight thousands chemicals, many of which have been reported as bioactive substances generated during the processes of tobacco combustion (Rodgman and Perfetti, 2013). The levels of such bioactive substances has been reported to be lower in tobacco products which do not combust tobacco during use, such as smokeless tobacco and heated tobacco products (Hatsukami et al., 2007; Stabbert et al., 2003). This has led to the suggestion that the use of such products could potentially help in reducing the harm associated with continued tobacco use (tobacco harm reduction concept).

To date, there is a wide variety of traditional and non-traditional smokeless tobacco products, such as moist snuff, snus, chewing and dissolvable tobacco available in several countries around the world. More recently, there has been a rise in the use of novel tobacco- and nicotine-containing products, such as e-cigarettes (Adkison et al., 2013) and heated tobacco products. The latter are generally separated into two categories based on the heating mechanisms utilized; namely carbon heat source at the tip of the product or a battery-powered electrical heating device (Borgerding et al., 1998; Sakaguchi et al., 2014; Smith et al., 2016). The health risks associated with the use of traditional non-combusted products have been extensively investigated, in particular for the Swedish smokeless tobacco product snus where there is rich epidemiological data on the health consequences

Abbreviations: CMF-PBS, Calcium- and magnesium-free phosphate buffer saline; CI, Confidence interval; CO, Carbon monoxide; DMSO, Dimethyl sulfoxide; GVP, Gas vapor phase; HCI, Health Canada intense; HCN, Hydrogen cyanide; ISO, International Organization for Standardization; LOD, Limit of detection; LOQ, Limit of quantification; MN, Micronucleus; NAB, Nitrosoanabasine; NAT, Nitrosoanatabine; NNK, 4-(Methylnitrosoamino)-1-(3-pyridyl)-1-butanone; NNN, Nitrosonornicotine; NO, Nitric oxide; NOx, Nitrogen oxides; NRU, Neutral red uptake; NTV, Novel tobacco vapor product; PAH, Polyaromatic hydrocarbon; PQS, Pyridine, quinoline, styrene; TPM, Total particulate matter

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associated with use of the product (Foulds et al., 2003). However, the health risks of more recent product innovations, such as e-cigarettes and electrically-heated tobacco products, are less well understood and are the subject of active scientific and public debate.

Japan Tobacco Inc. has recently developed a novel tobacco vapor product (NTV) as an alternative to traditional tobacco products. The product is composed of a puff activated electrical heating device with battery, a cartridge containing nicotine-free carrier liquid and an atomizer (cartomizer) and a tobacco capsule containing granulated tobacco. The NTV generates a thermally-vaporized aerosol which then passes through the tobacco capsule before being inhaled. In doing so, evaporated tobacco-derived flavors and nicotine are infused into the vapor. This mechanism of tobacco vapor generation differs from most existing heated tobacco products in that it does not heat the tobacco directly during use. Instead, the tobacco is indirectly warmed by the vapor passing through it. The NTV also differs from most e-cigarettes as the carrier liquid does not contain nicotine.

The purpose of this study was to understand the chemical characteristics and *in vitro* mutagenicity, genotoxicity and cytotoxicity of the NTV aerosol. We evaluated the emission levels of over 40 chemicals known to be biologically active tobacco smoke constituents (Hoffmann and Hoffmann, 1998; Hoffmann and Wynder, 1986). These analytes have been specified as priority constituents required, or proposed, for regulatory reporting (Health Canada, 2000; U.S. Food and Drug Administration, 2012; World Health Organization, 2008). In addition to the chemical analysis of the emissions, *in vitro* toxicological activities of the aerosol as a whole, rather than individual chemical constituents, were also investigated using the bacterial reverse mutation test (Ames assay), the *in vitro* micronucleus (MN) assay and the neutral red uptake (NRU) assay. These three *in vitro* toxicological assays form part of the regulatory reporting requirements for tobacco products in Canada (Health Canada, 2000) and are widely used for the safety screening of chemicals and pharmaceuticals. The Kentucky reference cigarette 3R4F, as a representative of combustible cigarettes (Roemer et al., 2004), was used as a positive control in both emission chemistry and *in vitro* toxicological experiments. Finally, in order to correlate the findings with the heating conditions of tobacco, the temperature inside the tobacco capsule of the NTV was also measures during use.

2. Materials and methods

2.1. Test items

The Kentucky reference 3R4F cigarettes were purchased from the University of Kentucky, Kentucky Tobacco Research and Development Center (Lexington, KY, USA). The NTV were obtained from Japan Tobacco Inc. (Tokyo, Japan). The NTV consists of three modules: a puff-activated electrically heating devise with rechargeable-battery, a cartomizer containing a nicotine-free carrier liquid (i.e., propylene glycol, glycerol and triacetin) and a tobacco capsule containing granulated tobacco (Fig. 1). All test items, except for the heating device, were stored at 4 °C ± 3 °C until use.

2.2. Smoke and aerosol generation

All the test items were conditioned for at least 48 h under 22 °C ± 1 °C at 60% relative humidity according to the International Organization for Standardization (ISO) 3402 (ISO, 1999). The battery of the heating device was fully charged prior to aerosol generation. The mainstream 3R4F cigarette smoke and aerosol from NTV were generated by machine smoking using the Health Canada Intense (HCI) puffing conditions (i.e., 55 mL puff volume, 2 s duration, 30 s puff interval and bell-shape puff profile (Health Canada, 2000)). The 3R4F cigarette was smoked until butt length of 35 mm in accordance with ISO 4387 (ISO, 2000). For the NTV, the total puff number for each aerosol collection per tobacco capsule was set as 70 based on the product specification.

Fig. 1. Primary components of the NTV.

2.3. Chemical analysis

The chemical analysis was conducted by Labstat International ULC (Kitchener, ON, Canada) in compliance with principles of Good Laboratory Practice. The yields of major smoke constituents (i.e., TPM, water, nicotine and carbon monoxide (CO)) and the 43 Hoffmann analytes were determined using analytical methods specified in the Supplementary Table 1. Propylene glycol, glycerol and triacetin, which are components of the carrier liquid, were also analyzed using the method specified in the Supplementary Table 1. Room air blank samples for each analyte were also analyzed to identify any contaminants in the ambient air, analytical reagents or equipment. Mean value and 95% confidence interval (95CI) were reported for each analyte from five independent measurements. In case that at least one measurement value was below limit of quantification (LOQ) and median value was higher than LOQ, the median was given but the 95CI was not calculated. When the median was below LOQ or limit of detection (LOD), the LOQ or LOD value was provided as a reference value.

2.4. Temperature measurement inside tobacco capsule

The temperature inside the tobacco capsule of the NTV during aerosol generation was measured. A type K thermocouple (φ 0.15 mm) (CHINO; Tokyo, Japan) was inserted into the center of the tobacco capsule. The aerosol from the NTV was generated as described above in section 2.2. The temperature was recorded over 70 puffs (2100 s) during machine smoking using data logger ZR-RX25 (Omron; Kyoto, Japan). The mean values from three independent measurements were calculated for each measured time point.

2.5. In vitro toxicological testing

The Ames assay and *in vitro* MN assay were performed to evaluate the mutagenicity and genotoxicity of the NTV aerosol and 3R4F cigarette smoke. The NRU assay was applied for cytotoxicity evaluation. All *in vitro* studies, including test sample preparation processes, were conducted in Labstat International ULC (Kitchener, ON, Canada) in compliance with the principles of Good Laboratory Practice.

2.5.1. Sample preparation

The NTV aerosol and 3R4F mainstream smoke were generated using a rotary smoking machine as described in section 2.2. The total particulate matter (TPM) was collected on a 92-mm Cambridge filter pad. The pad was extracted with dimethyl sulfoxide (DMSO) purchased from Sigma Aldrich (St. Louis, MO, USA) and prepared to 10 mg TPM/mL solution for the 3R4F and 50 mg TPM/mL solution for the NTV. The gas-vapor phase (GVP) was collected simultaneously with the TPM collection. The smoke or NTV aerosol fraction which passes through the filter pad was bubbled into 15 mL of ice-cold calcium- and magnesium-free phosphate buffer saline (CMF-PBS). Subsequently, fresh CMF-PBS was added to achieve a final concentration of 10 mg TPM equivalent/mL for the 3R4F and 50 mg TPM equivalent/mL for the NTV. The TPM samples for Ames and MN assays were stored below −70 °C in a cryofreezer until testing. The TPM and GVP samples for NRU assay were prepared within an hour prior to testing.

2.5.2. Ames assay

The Ames assay was performed in general accordance with Health