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Research Paper

Offsetting the impact of smoking and e-cigarette vaping on the cerebrovascular system and stroke injury: Is Metformin a viable countermeasure?

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ABSTRACT

Recently published in vitro and in vivo findings strongly suggest that BBB impairment and increased risk for stroke by tobacco smoke (**TS**) closely resemble that of type-2 diabetes (**2DM**) and develop largely in response to common key modulators such oxidative stress (**OS**), inflammation and alterations of the endogenous anti-oxidative response system (**ARE**) regulated by the nuclear factor erythroid 2-related factor (**Nrf2**). Preclinical studies have also shown that nicotine (the principal e-liquid's ingredient used in e-cigarettes) can also cause OS, exacerbation of cerebral ischemia and secondary brain injury. Herein we provide evidence that likewise to TS, chronic e-Cigarette (**e-Cig**) vaping can be prodromal to the loss of blood-brain barrier (BBB) integrity and vascular inflammation as well as act as a promoting factor for the onset of stroke and worsening of post-ischemic brain injury. In addition, recent reports have shown that Metformin (**MF**) treatment before and after ischemic injury reduces stress and inhibits inflammatory responses. Recent published data by our group revealead that MF promotes the activation of counteractive mechanisms mediated by the activation of Nrf2 which drastically reduce TS toxicity at the brain and cerebrovascular levels and protect BBB integrity. In this study we provide additional in vivo evidence showing that MF can effectively reduce the oxidative and inflammatory risk for stroke and attenuate post-ischemic brain injury promoted by TS and e-Cig vaping. Our data also suggest that MF administration could be extended as prophylactic care during the time window required for the renormalization of the risk levels of stroke following smoking cessation thus further studies in that direction are warrated.

1. Introduction

In the past decade a number of alternative vaping products have hit the market, rapidly gaining consumers among adults and, especially, adolescents [1]. Electronic nicotine delivery systems or e-cigarettes (**e-Cigs**) have become the sought-after product partly due to the belief that they are much safer than traditional cigarettes (e-Cig use has surpassed that of conventional cigarettes [2]). Moreover, tobacco smoking (**TS**) has been associated with vascular endothelial dysfunction [3–8] in a causative and dose dependent manner [9] primarily related to the TS content of reactive oxygen species (**ROS**) [4,10], nicotine [11–16], and oxidative stress (**OS**) -driven inflammation [17]. Current scientific opinion considers OS-mediated pathways to play a major role in the pathogenesis of these disorders, especially stroke [18]. Preclinical studies (and data presented herein) have shown that nicotine (the principal ingredient of e-liquid) can cause OS, exacerbation of cerebral ischemia and secondary brain injury [19–21]. Likewise, chronic e-Cig vaping could be prodromal to cerebrovascular impairment and promote cerebrovascular conditions that favor the onset of stroke and post-ischemic brain injury [22]. The health impact of e-Cig vaping is currently

Abbreviations: **ARE**, Anti-Oxidant Response Element; **BBB**, Blood-Brain Barrier; **CS**, Cigarette Smoke; **CSE**, Cigarette Smoke Extract; **2DM**, Type 2 Diabetes Mellitus; **FJC**, Fluoro-Jade C; **FTC**, Federal Trade Control; **ISO**, International Organization for Standardization; **MF**, Metformin; **Nic**, Nicotine; **Cot**, Cotinine; **NQO-1**, NAD(P)H: Quinone reductase I; **Nrf2**, Nuclear factor erythroid 2-related factor; **PECAM-1**, Platelet Endothelial Cell Adhesion Molecule-1; **ROS**, Reactive Oxygen Species; **TJ**, Tight Junction; **tMCAO**, ransient middle carotid artery occlusion; **TS**, Tobacco smoke; **TTC**, Triphenyl tetrazolium chloride; **ZO-1**, Zonulae occludentes-1

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unknown and the limited research and dearth of regulatory guidelines for the content of the vaping solution for e-Cigs (various harmful compounds including aldehydes, nitrosamines etc. have been detected in the e-Cig vapors [23–26]) has become a critical public and regulatory concern. Further, we and others have found that TS promotes glucose intolerance and increases the risk of developing type-2 diabetes mellitus (**2DM**) [27,28] with which it shares other pathogenic traits including the high risk of cerebrovascular and neurological disorders like stroke [29] via ROS generation, inflammation, and blood-brain barrier (**BBB**) impairment [30–32]. Recent findings [33,34] from our group, supports an additive release pattern of angiogenic, oxidative and inflammatory factors by BBB endothelial cells in response to hyperglycemia (**HG**) and/or stroke conditions with comcomitant exposure to cigarette smoke extracts (**CSE**), thus suggesting the involvement of common pathogenic modulators of BBB impairment. To this end, Metformin (**MF**; a widely prescribed, firstline anti-diabetic drug) before and after stroke injury has been shown to reduce stress and inhibit the inflammatory responses [34,35]. These data and recent published work by our group strongly suggests that MF activates counteractive mechanisms mediated by the activation of nuclear factor erythroid 2-related factor (Nrf2) [34,36] which drastically reduce TS toxicity at the level of the BBB. Further, long term health impact and toxicity of e-Cigs are practically unknown.

TS alone is accountable for 434,000 deaths each year in United States (US) [37] and is a prodromal factor for the onset of stroke, Alzheimer's [9,38,39], depression [40] and vascular dementia [41]. OS, inflammation and the resulting BBB impairment [42–44] are the major prodromal factors for ischemic damage. Even upon smoking cessation, former chronic smokers remain at considerable risk for stroke for several years [45]. The only FDA approved drug treatment for ischemic stroke is tissue plasminogen activator (**tPA**) for which the therapeutic efficacy and safety is drastically decreased if not administered within 4.5 h from the onset of stroke [46]. Herein we provide alarming evidences (both in vitro and in vivo) demonstrating that e-Cigs are not safer than conventional tobacco products from a cerebrovascular perspective. Further we also show that MF can indeed provide partial protection against the negative impact of smoking and e-Cig vaping on stroke injury.

2. Methods

2.1. Materials and reagents

Sterile culture ware and molecular biology grade chemicals and reagents were obtained from standard commercial sources (Fisher Scientific, Pittsburgh, PA, USA; Sigma-Aldrich, St. Louis, MO, USA; and Bio-rad laboratories, Hercules, CA, USA). Fluorescein isothiocyanate (FITC; 3000–5000 MW; #FD4) and Rhodamine B isothiocyanate (RITC; 70,000 MW; #R9379) dextrans were purchased from Sigma-Aldrich, while Cascade Blue®-dextran (10,000 MW; #D-1976) was obtained from life technologies (Grand Island, NY, USA). Gel electrophoresis was carried out by using Mini-Protean® TGXTM gels 4–15% (#456–1084) from Bio-rad laboratories (Hercules, CA, USA). The antibodies were obtained from the following sources: Rabbit anti-ZO-1 (# sc-10804), mouse anti-ICAM-1 (#sc-18853), mouse anti-VCAM-1 (#sc-13160), mouse anti-PECAM-1 (#sc-376764), rabbit anti-Nrf2 (#sc-722), mouse anti-NQO1 (#sc-376023). Donkey anti-rabbit (#NA934) and sheep anti-mouse (#NA931) HRP-linked secondary antibodies were obtained from GE Healthcare (Piscataway, NJ, USA); goat anti-rabbit (#A11008, A21428) conjugated to Alexa Fluor® 488 and 555 respectively and anti-mouse (#A11001, A21422) conjugated to Alexa Fluor® 488 and 555 respectively from Invitrogen (Camarillo, CA, USA). Reference full flavor cigarettes (3R4F, 9.4 mg tar and 0.726 mg nicotine per cigarette), were obtained from the Center for Tobacco Reference Products (Kentucky Tobacco Research & Development Center, Lexington, KY) while e- cigarettes (Blu™, 24 mg/ml nicotine) were obtained from commercial sources.

2.2. Experimental design (In Vivo)

The animal protocol for this work was approved by the Institutional Animal Care and Use Committee, TTUHSC, Lubbock, Texas. C57BL/6 J male mice (age range 8–10 weeks old) were purchased from Jackson Laboratories. Mice where divided into 3 major groups including control, TS exposed and e-Cig exposed. Of these the TS and e-Cig exposed groups were divided into 2 subgroups including MF treated and Sham treated (saline). Mice were chronically exposed (via direct inhalation) to cigarette smoke (**CS**) of e-Cig vapor mixed with oxygenated air or oxygenated air alone, 6 times/day; 2 cigarettes/hour, 6–8 h/day, 7 days/week for 2 weeks following International Organization for Standardization/ Federal Trade Commission (ISO/FTC) standard smoking protocol (35 ml draw, 2 s puff duration, 1 puff per 60 s). CS and e-Cig vapor were separately and independently generated using a Single Cigarette Smoking Machines (**SCSM**, CH Technologies Inc., Westwood, NJ, USA) following previously published methods [47]. Mice were sacrificed and samples were collected for further analysis. MF (Sigma, St. Louis, MO, USA) was dissolved in sterile saline at a concentration of 30 mg/ml. MF was administered daily (via intraperitoneal Injections of doses of 200 mg/kg [34,48,49]) to mice either exposed to CS or e-Cig. At the end of the study, mice were sacrificed and samples (plasma and brain) were collected for further analysis. In a parallel study, a similar cohort of mice underwent identical CS or e-Cig exposures and MF treatments (200 mg/kg/day IP) for 10 days followed by tMCAO (see below for details).

2.3. Transient Middle Cerebral Artery Occlusion (tMCAO)

Brain ischemic injury by tMCAO in TS and e-Cig exposed C57BL/6 J male mice was performed as previously reported with slight modifications. Surgery was performed using a Zeiss OPMI pico I surgical microscope (Carl Zeiss GmbH, Jena, Germany). Temperature was maintained at 37 °C, controlled by the thermostatic blanket (TC-1000 Temperature Controller, CWE, USA). Mice were anesthetized with 4% and maintained at 1–1.5% isoflurane with a facemask. The cerebral blood flow was continuously monitored throughout the surgery to confirm the occlusion and reperfusion of the brain by using non-invasive, real-time microcirculation imaging, Pericam PSI system (Perimed Inc., Marble Falls, TX) placed over the exposed skull in the territory of the left middle cerebral artery (**MCA**) perfusion area. A midline incision was made at the neck about 1.5 cm long. The left carotid bifurcation, external carotid artery (**ECA**) and common carotid artery (**CCA**) were isolated from the adjacent tissue. After occlusion of CCA using a micro clip, the left ECA was ligated, coagulated, and cut distally to the cranial thyroid artery. 6–0 nylon monofilament with a silicone coated tip (0.20–0.23 mm; Doccol Corporation) was gently introduced up to ~8.5–9 mm to block the origin of the MCA. IR injury was produced by tMCAO (30 min) according to established procedures in Dr. Abbruscato's laboratory [50]. After 30 min occlusion, the suture was withdrawn up to the left carotid bifurcation to restore blood flow, i.e. reperfusion. Animals that failed to recover at least 80% of baseline within 10 min after reperfusion were excluded from the experimental group. After reperfusion, neurological evaluation using several sensory-motor tests was carried out. These include neurologic deficit score on a four-point scale [50]. Once the brain tissue was resected, coronal sections (10 μm thick) from the contralateral and ipsilateral hemispheres were also analyzed by fluorescent microscopy to assess neuronal degeneration by using fluoro-Jade C (FJC) which is an anionic dye that specifically stains soma and neurites of degenerating neurons [51].

2.4. Behavioral tests

Neurological deficits where assessed using a five-point scale as