Short-term Exposure to JUUL Electronic Cigarettes Can Worsen Ischemic Stroke Outcome by Disrupting the Blood-Brain Barrier

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Abstract

Background

The short and long-term health effects of JUUL electronic cigarette (e-Cig) are largely unknown and warrant extensive research. We hypothesized that chronic exposure to JUUL vapor could promote cerebrovascular toxicities impacting the progression and outcome of ischemic stroke to an extent comparable to that resulting from tobacco smoke (TS) exposure.

Methods

We exposed male C57 mice to TS/ JUUL vapor for 14 days. LCMS/MS was used to measure brain and plasma nicotine and cotinine level. Transient middle cerebral artery occlusion (tMCAO) followed by reperfusion was used to mimic ischemic stroke. Plasma levels of IL-6 and thrombomodulin were assessed by enzyme-linked immunosorbent assay. At the same time, western blotting was used to study blood-brain barrier (BBB) tight junction (TJ) proteins expression and that of key inflammatory and oxidative stress markers.

Results

tMCAO upregulated IL-6 and decreased plasma thrombomodulin levels. Post-ischemic brain injury following tMCAO was significantly worsened by JUUL/TS pre-exposure. TJ proteins expression was also downregulated by JUUL/TS pre-exposure after tMCAO. Similar to TS, exposure to JUUL downregulated the expression of the antioxidant Nrf2. The inflammatory marker ICAM-1 was also significantly upregulated by TS pre-exposure following tMCAO and by JUUL but to a lesser extent.

Conclusions

Our results suggest that JUUL exposure could negatively impact the cerebrovascular system as much as TS exposure.

Introduction

Tobacco smoking (TS) causes more than 480,000 deaths in the United States (US) by contributing to many diseases, including cancer, lung diseases, cardiovascular and cerebrovascular disorders (1). Although there has been a steady decline in cigarette smoking among adults in the US from 42–14% between 1964 and 2019 (1, 2), the increasing use of alternative tobacco products like electronic cigarettes (e-Cigs) poses a new threat to the public health. E-Cigs were first introduced in the US market in 2007, and their popularity has been rising ever since (3). Vaping (the common term associated with smoking e-Cigs) has increased significantly in adult and adolescent populations (4, 5). Nicotine is delivered in aerosol...
form by e-Cig devices from vaporized e-liquid. JUUL is a recently developed portable e-Cig device that physically resembles a universal serial bus (USB) flash drive (6, 7), which currently is one of the most popular e-Cig brands in the US. Although JUUL e-Cig was introduced to help adult heavy smokers quit smoking or as a less harmful alternative to TS, it is also very popular in adolescents. JUUL e-Cig consists of a liquid & heating coil-containing pod and a rechargeable battery. The nicotine in the JUUL-pod is claimed to be salt-based instead of the free base (7, 8), which could facilitate the vapor inhalation process and generate higher nicotine concentrations (9). This could make JUUL more harmful to its users. Rigorous research is required to elucidate the health effects of JUUL e-Cigs.

Stroke is another major cause of morbidity and mortality in the US, causing death every 4 minutes (10). Stroke is primarily of 2 types: ischemic and hemorrhagic. Ischemic stroke comprises 87% of all strokes and is characterized by interrupting blood flow to the brain (10). Smoking is one of the most common comorbid conditions that can increase the risk and worsen the outcome of an ischemic stroke event (11). Our lab has previously shown that exposure to nicotine and smoking can worsen brain injury and neurological outcomes (12, 13) and decrease brain glucose transport (14) & utilization (15) in ischemic stroke. The blood-brain barrier (BBB) is an integral part of the brain's neurovascular unit and plays a vital role in maintaining normal brain physiology and ionic and nutrient balance. BBB disruption, alongside inflammation and oxidative stress, are major pathological hallmarks of ischemic stroke (16). The deleterious role of TS on BBB function, inflammation, and oxidative stress has been depicted in preclinical studies (12, 17–19). Exposure to nicotine-containing JUUL e-Cigs can adversely affect the ischemic brain, leading to a poor clinical prognosis.

Some studies have investigated the cerebrovascular effects of e-Cigs (12, 15, 20), but very few (21) have specifically addressed the toxic effects of JUUL e-Cigs on the brain. Ramirez et al. has shown that short-term JUUL e-Cig exposure can increase the risk of thrombotic events (22). But to our knowledge, no study has yet addressed the effects of JUUL e-Cigs on the cerebrovascular system. In this study, we have investigated the impact of short-term JUUL e-Cig exposure on brain injury, BBB tight junction (TJ) proteins, and inflammatory and oxidative stress markers in ischemic stroke; in direct comparison with TS.

**Material & Methods**

**Animals and surgical procedures**

All studies were approved by the IACUC of Texas Tech University Health Sciences Center, Lubbock, Texas (IACUC protocol# 20026). All experiments were performed in accordance with relevant guidelines and regulations. This study was not pre-registered, and no randomization/blinding was performed. Male C57BL/6 mice (Charles River Laboratories, Inc., Wilmington, MA) were kept under standardized light and dark conditions (12 h), humidity (70%), and temperature (22°C). They were given ad libitum access to food and water.

The behavior of the animals was monitored every day to minimize animal suffering. We applied the following exclusion criteria: severe weight loss, infections, or significant behavioral deficits (decreased
mobility, seizures, lethargy). No animal was excluded from this study. The study is reported in accordance with ARRIVE guidelines. The research design is depicted as a flow diagram in Fig 8.

**In vivo TS/e-Cig vaping**

Mice were exposed (via direct inhalation) to JUUL e-Cig vapor (30 mg/ml nicotine) or 3R4F standardized research cigarettes (9.4 mg tar and 0.726 mg nicotine/cigarette equivalent to full flavor commercial products) mixed with oxygenated air or oxygenated air alone, 6 cycles/day for 14 days. A modified CORESTA (Cooperation Centre for Scientific Research Relative to Tobacco) standard smoking protocol adapted to study JUUL exposure (27.5 ml puff depth volume, 3 s puff duration, 2 puffs per 60 s, 32 puffs/cycle) and a modified CIR (Canadian Intense Regimen) standard smoking protocol (27.5 ml puff depth volume, 2 s puff duration, 2 puffs per 60s, 32 puffs/cycle) to study TS exposure were followed in the laboratory. E-Cig vapor/TS was generated using a Single Cigarette Smoking Machines (SCSM, CH Technologies Inc., Westwood, NJ, USA) following a previously published method (12, 15). These methods were followed to mimic the smoking behavior of a human chronic and heavy smoker/vaper and yield plasma levels of cotinine (43 & 195 ng/ml for JUUL and TS, respectively) which is in the range of blood cotinine levels found in other preclinical models of chronic TS/e-Cig exposure (23-25). The smoking exposure was done between 9 am to 2 pm.

**Plasma nicotine and cotinine level measurement by LCMS/MS**

Plasma concentration of nicotine and its principal metabolite cotinine were measured from the 14 days of JUUL/TS-exposed mice by LCMS/MS analysis using Cotinine-d3 (MilliporeSigma, St. Louis, MO, USA) as internal standard (IS) following a previously published method (25). In brief, samples were prepared by protein precipitation of 25 µL mouse plasma using acetonitrile at 1:8 ratio. Using the electrospray ionization technique, the mass spectrometer was operated in positive polarity under the multiple reaction monitoring mode. The transitions of m/z 163.2 → 132.1, 177.2 → 98.0, and 180.2 → 101.2 were used to measure the nicotine, cotinine, and IS, respectively. The elution of nicotine (MilliporeSigma), cotinine (MilliporeSigma), and IS were at 1.89, 1.77, and 1.76 min, respectively. This was achieved with a mobile gradient phase consisting of 5 mM ammonium bicarbonate, acetonitrile, and methanol (3:1, v/v) at a 0.3 mL/min flow rate on a Kinetex EVO C18 column (Phenomenex, Torrance, CA, USA).

**Open field test**

Open field test was performed according to our previously published study (20, 26). This test evaluated the locomotor activity of the JUUL/TS-exposed or control mice with or without stroke. We used Versamax software (Accuscan Instruments., Columbus, OH) to automatically calculate the activity of the animals (total distance traveled). Briefly, mice were introduced to a 16” x 16” unobstructed glass chamber. They were monitored and recorded for 1 h. The first 10 min of 1 h was excluded as the acclimatization period. All experiments were performed between 8 am, and 11 am.

**Transient Middle cerebral artery occlusion with reperfusion**
Transient Middle cerebral artery occlusion (tMCAO) surgery was performed in mice (24–28 g) as previously reported (26, 27), using a Zeiss OP pico I surgical microscope (Carl Zeiss GmbH, Jena, Germany). Mice were anesthetized with 4% and maintained at 1.5% isoflurane in N2O/O2 mixture (70/30) using a SurgiVet Vaporizer (Smith Medical North America, Waukesha, WI, USA). Continuous blood flow was measured with a Laser Doppler probe (Moor Instruments, Wilmington, DE, USA). The probe was placed on the skull directly above the left MCA region (1 mm posterior and 3 mm lateral to the Bregma). Body temperature was maintained at 37 °C and controlled by a thermostatic blanket (TC-1000 Temperature Controller, CWE, USA). After aseptic preparation with betadine, a 1.5 cm long incision was made on the neck midline. The left common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were carefully isolated from surrounding tissue. After CCA was occluded, a micro clip was placed on the ICA, and the ECA was ligated and coagulated. A small incision on the ECA was made to introduce a 6–0 nylon microfilament with a round tip (0.20–0.25 mm), and it was gradually inserted until it blocked the MCA bifurcation. A decrease in blood flow of 80% from baseline was considered a successful occlusion. After 30 min of occlusion, the nylon filament was carefully removed to restore blood flow, leading to reperfusion. An increase of 70% or more of the blood flow during occlusion was considered successful reperfusion. Animals that did not meet the above criteria were excluded from the experimental group.

2,3,5-tripheyltetrazolium chloride staining

2,3,5-tripheyltetrazolium chloride (TTC) staining was used to demarcate viable brain tissue after MCAO (26, 27). Brain tissue with viable mitochondrial was stained dark red while the infarcted brain region remained white. After 24 h of reperfusion following MCAO, animals were euthanized by isoflurane anesthesia followed by cervical dislocation. The brain was quickly extracted and sectioned into 1 mm thick slices using McIlwain Tissue Chopper. Brain slices were then incubated in a 2% solution of TTC in phosphate-buffered saline (PBS) for 5 min at 37 °C. Images of brain slices were scanned as previously described (26) and analyzed for infarct and edema using image analysis software (Image J1.50i, National Institutes of Health, Bethesda, Md, downloadable from (http://rsb.info.nih.gov/ij/download.html)). We measured three areas of each brain slice: infarct area (X) (mm²), area of the infarcted (ipsilateral) hemisphere (Y) (mm²), and area of the noninfarcted (contralateral) hemisphere slices (Z) (mm²). % infarct area in the ipsilateral brain hemisphere and % brain edema for the brain sections were calculated by the following equations, respectively: (X/Y) *100 and ((Y-Z)/Z) *100, and later averaged for each brain.

Enzyme-linked immunosorbent assay

Blood samples collected from JUUL/TS/control mice with or without MCAO were analyzed by Quantikine ELISA kits (R & D systems, Minneapolis, MN, USA) for the quantitative determination of thrombomodulin and IL-6 according to the procedure per the manufacturer's protocol.

Western blot
JUUL/TS/control mice brain without MCAO (normoxia) or contralateral and ipsilateral brain hemispheres 24 h after MCAO were lysed using RIPA buffer to isolate protein lysate. Protein concentrations of isolated protein lysates were determined using bicinchoninic acid (BCA) assay. Exactly 30 μg of protein from each sample was loaded and separated using a 10% Tris-glycine polyacrylamide precast gel (Bio-Rad Laboratories, Hercules, CA; Cat# 4568034). This method has been used previously to analyze Western blot immunoreactivity (20, 28). Protein samples were then transferred to a polyvinylidene difluoride membrane (Thermo Fisher; Cat# IPVH00010), and then membranes were incubated in blocking buffer (1% Tween-20 containing Tris-buffered saline (TBST) with 5% bovine serum albumin) to block the nonspecific protein bands for 2 h at room temperature. Membranes were incubated with rabbit polyclonal anti-ZO-1 antibody (1:2000, Thermo Fisher; Cat# 40-2200), rabbit polyclonal anti-claudin-5 antibody (1:2000, Thermo Fisher; Cat# 34-1600), rabbit polyclonal anti-occludin antibody (1:1000, Thermo Fisher; Cat# 40-4700), rabbit polyclonal anti-MMP-9 (N-terminal) antibody (1:1000, Proteintech; Cat# 10375-2-AP), mouse monoclonal anti-ICAM-1 antibody (1:500, Thermo Fisher; Cat# MA5407), rabbit polyclonal anti-Nrf2 antibody (1:2000, Thermo Fisher; Cat# PA5-88084), mouse monoclonal anti-NQO-1 antibody (1:10000 MilliporeSigma; Cat# A5441) in TBST with 5% bovine serum albumin at 4°C overnight. After 4 times washing with TBST for 15 min each, membranes were incubated with anti-rabbit (Sigma Aldrich; Cat# GENA934-1ML, RRID: AB_2722659) or anti-mouse (Sigma Aldrich; Cat# GENX931-1ML, RRID: AB_772209) IgG-horseradish peroxidase secondary antibody (1:10000) in TBST with 5% bovine serum albumin for 2 h at room temperature. After 4 times of 15 min wash with TBST, the protein signals were detected by enhanced chemiluminescence-detecting reagents (Thermo Fisher; Cat# 34577) and visualized in X-ray films in the dark. The protein bands were quantified relative to beta-actin in Image J software.

Immunofluorescence

Immunofluorescence staining was performed as previously described with modifications (29). Mice were euthanized by isoflurane overdose 24 h after MCAO. The brains were sectioned at 30 μM of thickness, fixed with 4% paraformaldehyde (Thermo Fisher) for 15 minutes, then permeabilized with 0.1% Triton X-100 for 10 minutes. After washing with the phosphate-buffered saline (PBS), the sections were blocked for 1 hour and incubated overnight with primary antibodies for ZO-1 (1:100, Thermo Fisher) and Nrf-2 (1:100, Thermo Fisher), respectively. Fluorescent secondary antibodies (Thermo Fisher) were used at 1:200 dilutions for 1 hour. 4’,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) was included for nuclear staining. The images (20X magnitude) were captured with a Nikon A1R multi-photon confocal microscope (Nikon Instrument). Mean total fluorescence intensity was calculated for each color channel using NIS elements AR software, and the intensity of the green color (ZO-1/Nrf2) was expressed relative to the blue color (DAPI). Three microscopic fields out of each ipsilateral and contralateral section were used to evaluate the expression levels of ZO-1 and Nrf2.

Statistical Analysis
The sample size for the animal study was estimated based on our previously published literature (12, 15). No sample size calculation was performed, and there were no sample size differences between the beginning and end of the experiments. No test for normality was performed. All data are expressed as the mean ± SD except for Figure 2 and Figure 3 (A, C), which are presented as box and whisker plots. The values were analyzed one-way analysis of variance with Tukey's post hoc multiple comparisons (Prism, version 7.0; GraphPad Software Inc., San Diego, CA). P values less than 0.05 were considered statistically significant.

Results

**TS-exposed mice had higher plasma nicotine and cotinine level than JUUL-exposed mice**

Average plasma nicotine concentrations in JUUL and TS-exposed mice were 22.5 ± 6.68 ng/ml and 74.76 ± 5.95 ng/ml, respectively, whereas those of cotinine were 42.58 ± 5.2 ng/ml and 194.7 ± 24.42 ng/ml, respectively. Plasma concentration of nicotine was significantly higher in TS-exposed mice (P<0.0001) compared to JUUL-exposed mice (Fig 1A). Similarly, TS-exposed mice had a higher plasma level of cotinine (P<0.0001) than JUUL-exposed mice (Fig 1A). Further, when we measured the ratio of plasma cotinine to nicotine, we found that the ratio was significantly decreased in JUUL-exposed mice (P<0.05) than in TS-exposed mice (Fig 1B).

**JUUL and TS exposure caused weight reduction in mice, and TS exposure induced hyperactivity**

We measured the weight of the mice after 14 days of JUUL or TS exposure and 24 h after MCAO (Fig 2A, 2B). We found that JUUL (P<0.0001) or TS (P<0.0001) exposure for 14 days drastically reduced the weight of the mice compared to control (Fig 2A). TS-exposed mice also had significant weight reduction (P<0.001) compared to JUUL-exposed mice. MCAO caused weight reduction in all groups, but no significant weight difference was observed among control, JUUL, and TS-exposed mice 24 h after MCAO. Locomotor activity of the mice was measured by open field test after 14 days of JUUL or TS exposure and 24 h after MCAO (Fig 2C, 2D). JUUL-exposed mice had a non-significant increase in percent baseline activity change compared to control, while TS-exposed mice showed significant hyperactivity (P<0.05) compared to control mice (Fig 2C). There was no significant difference observed in the total distance traveled by the mice 24 h after MCAO among the three groups (Fig 2D).

**JUUL and TS-exposed mice had a worsening brain injury 24 h after MCAO**

We did TTC staining 24 h after MCAO to evaluate brain injury in the mice (Fig 3A-C). JUUL (P<0.01) and TS-exposed (P<0.05) mice had significantly increased brain infarct area compared to control mice (Fig 3A, 3B). There was also an increase in brain edema ratio in TS-exposed mice (P<0.05) compared to control (Fig 3A, 3C). Further, the neurological score was significantly worsened in TS-exposed mice (P<0.01 vs. control) 24 h after MCAO (Fig 3A, 3D).

**Plasma IL-6 level was increased, and thrombomodulin level was decreased 24 h after MCAO**
Plasma IL-6 and thrombomodulin levels were measured in mice by ELISA after 14 days of JUUL or TS exposure and 24 h after MCAO (Fig 4A, 4B). IL-6 level was higher in control MCAO (P<0.01 vs. control, P<0.05 vs. JUUL, and P<0.05 vs. TS) and TS MCAO (P<0.01 vs. control, P<0.05 vs. JUUL, and P<0.01 vs. TS) groups (Fig 4A). Contrastingly, plasma thrombomodulin level was lower in control MCAO (P<0.05 vs. control), JUUL MCAO (P<0.0001 vs. control, P<0.001 vs. JUUL, and P<0.01 vs. TS), and TS MCAO (P<0.05 vs. control) groups (Fig 4B).

**JUUL and TS exposure reduces TJ protein expression at the BBB 24 h after MCAO**

We measured the expression of BBB TJ proteins (claudin-5 and occludin) and TJ-associated protein ZO-1 by western blot in the normoxic and contralateral & ipsilateral brain hemispheres 24 h after MCAO (Fig 5A-L). JUUL (P<0.01) and TS (P<0.01)-exposed mice had a significant reduction of ZO-1 expression after MCAO in the contralateral brain hemisphere compared to control (Fig 5A, 5C). There was no substantial change in the groups’ normoxic and ipsilateral brain ZO-1 levels (Fig 5A, 5B, 5D). This data is supported by immunofluorescence studies which showed JUUL (P<0.05) and TS (P<0.05)- exposure reduces ZO-1 expression in the contralateral brain hemisphere (Fig 7A, 7B) compared to control. At the same time, there was a non-significant downregulation of ZO-1 in the ipsilateral hemisphere (Fig 7A, 7C). Claudin-5 expression in the contralateral brain hemisphere of TS-exposed mice was reduced (P<0.05) compared to control (Fig 5E, 5G). No significant change in claudin-5 expression in the normoxic and ipsilateral brain regions was observed among the groups (5E, 5F, 5H). Occludin expression was also significantly reduced in the contralateral brain regions of JUUL (P<0.05 vs. control) and TS (P<0.01 vs. control)-exposed mice (Fig 5I, 5K). Further, JUUL-exposed mice had decreased occludin expression (P<0.05) in the ipsilateral brain region compared to control (Fig 5I, 5L). No change in occludin expression in the normoxic brains was observed (Fig 5I, 5J).

**JUUL and TS exposure decreases Nrf2, and TS exposure increases ICAM-1 expression in the brain 24 h after MCAO**

We then measured brain expression of the inflammatory marker, ICAM-1, and the antioxidant marker, Nrf2, by western blot in the normoxic and contralateral & ipsilateral brain hemispheres 24 h after MCAO (Fig 6A-H). JUUL (P<0.05) and TS (P<0.05)-exposed mice had a significant reduction of Nrf2 expression after MCAO in the contralateral brain hemisphere compared to control (Fig 6A, 6C). No significant change was observed in normoxic and ipsilateral brain Nrf2 expression among the groups (Fig 6A, 6B, 6D). Interestingly, immunofluorescence studies supported the western blot findings in the contralateral hemisphere, showing a significant reduction of Nrf2 expression by JUUL (P<0.05) and TS (P<0.001) exposure (Fig 7D, 7E). Further, immunofluorescent studies showed a significant reduction of Nrf2 by TS exposure (P<0.0001 vs. control and P<0.001 vs. JUUL) in the ipsilateral hemisphere as well (Fig 7D, 7F). Western blot results showed that ICAM-1 expression was significantly increased in the ipsilateral brain regions of TS (P<0.05 vs. control)-exposed mice (Fig 6E, 6H). There was no significant change in ICAM-1 expression in the normoxic and contralateral brain regions among the groups (Fig 6E-G).
Discussion

JUUL e-Cig has become extremely popular recently, and studies are needed to elucidate its possible toxic effects on the cerebrovascular system. This study has investigated the impact of short-term JUUL exposure on ischemic brain injury, BBB TJ proteins, and inflammatory and antioxidative markers compared to TS in mice. To our knowledge, this is the first study that evaluated cerebrovascular toxicities of JUUL with a side-by-side comparison with TS using a preclinical model of ischemic stroke.

We have used a well-established smoking/vaping exposure model for this study (12, 20, 25, 30). Plasma nicotine & cotinine level in mice after two weeks of TS & JUUL exposure were comparable to previously published in vivo studies (23, 25, 31, 32). Importantly, these concentrations also reflected human cigarette smokers (25, 33). We found higher plasma nicotine and cotinine concentrations in TS-exposed mice than JUUL-exposed mice, consistent with published studies in our laboratory and others involving TS and e-Cigs (23, 25, 31, 32). Further, nicotine to cotinine metabolism was also reduced in plasma of JUUL-exposed mice than in TS. One possible explanation of this could be the formation of nicotine by the gradual oxidation of e-liquids exposed to air. Nicotine inhibits CYP2A enzymes in the lungs and liver, thus could inhibit nicotine metabolism to cotinine by CYP2A6 (34).

The weights of the mice were drastically reduced after two weeks of TS exposure. Significant weight reduction was also observed with JUUL exposure. It has been widely reported that nicotine and TS can reduce body weight in preclinical (35, 36) and clinical studies (37, 38). Vaping was also shown to decrease body weight (24, 39). In our study, TS-exposed mice showed hyperactivity in the open field test. It is consistent with other studies which showed that short-term TS exposure increases physical activity in rodents compared to control (40). Interestingly, mice exposed to long-term (10 months) TS (40) or heavy human smokers (41, 42) displayed reduced physical activity, suggesting a differential effect induced by acute vs. chronic nicotine exposure.

Our study found that JUUL and TS exposure can increase brain injury after ischemic stroke. TS exposure also worsened brain edema and neurological functions. This result is consistent with our group's previous study, which showed that TS and e-Cig (Blu) exposure (12) could worsen ischemic brain injury. We also showed that acute administration of nicotine and nicotine-containing TS extract increases brain edema and infarct ratio after MCAO (13). Other researchers have demonstrated that exposure to nicotine or TS can worsen ischemic brain damage in rodents (19, 43, 44).

In our study, JUUL or TS exposure did not cause any significant change in plasma concentration of the inflammatory marker, IL-6. However, we found that ischemic stroke increases the plasma level of IL-6. Plasma IL-6 concentration 24 h after ischemic stroke was the highest in TS pre-exposed mice, although no significant difference was found between the test groups. Per our findings, IL-6 has been identified as a prognostic marker for ischemic stroke, as it was correlated with worsened ischemic brain injury and outcome in clinical (45–48) and preclinical (49, 50) studies. Thrombomodulin is a natural anticoagulant (51), which exerts a protective effect in acute ischemic stroke by inhibiting coagulation, fibrinolysis, and inflammation, stabilizing barrier function, and increasing blood flow (52). We found decreased plasma
thrombomodulin concentrations after ischemic stroke, but no significant JUUL or TS- pre-exposure effects were observed after MCAO. A clinical study showed that the serum concentration of soluble thrombomodulin decreased at the acute stage and increased after six months of ischemic stroke onset (53). In contrast, plasma thrombomodulin was higher in a clinical study by Zhang et al., which could be due to the small sample size (54). In another study, expression of endothelial thrombomodulin was decreased in the ischemic core region but increased in the peri-infarct area, compared to the contralateral side (55).

Disruption of the BBB is one of the key pathophysiological features of ischemic stroke, contributing to ischemic brain injury and neurological disturbances (56). Ischemic stroke causes disruptions in the TJ proteins at the BBB (57). Claudin-5 is a crucial BBB TJ protein responsible for increased paracellular permeability in experimental stroke settings if disrupted (56, 58). Occludin regulates functional integrity and paracellular permeability of the BBB (59, 60), while ZO-1 connects transmembrane TJ proteins to the actin cytoskeleton (61). TS and e-Cig exposure both decreased the expression of ZO-1 in an in vitro model of BBB (12). Prasad et al. found no significant change in ZO-1 and occludin expression after two weeks of TS exposure. However, four weeks of TS exposure decreased the expression of those TJ proteins (62). In our study, we also did not observe any significant change in the expression of the TJ proteins (ZO-1, claudin-5, and occludin) after two weeks of JUUL or TS exposure. This could be due to the inherent difference between in vitro and in vivo systems and the amount & duration of exposure. In western blot studies, we found reduced expression of ZO-1 and occludin in the contralateral brain hemisphere in JUUL or TS pre-exposed mice.

In contrast, contralateral claudin-5 expression was decreased only with TS pre-exposure after MCAO. Immunofluorescent studies supported these changes in BBB TJ proteins, where we have found a similar reduction of TJ protein expression only in the contralateral hemisphere. We observed the harmful effects of JUUL or TS on BBB TJ protein expression only after ischemic stroke, which implies that adding another insult accentuates the harmful effects caused by JUUL or TS exposure on the BBB. Studies investigating the impact of TS and/or e-Cig on BBB and TJ proteins in acute ischemic stroke have been scarce. Acute exposure to TS extract worsened BBB disruption after the ischemia-like condition in an in vitro study (63). Sladojevic et al. showed that claudin-5 expression in the ipsilateral brain hemisphere was decreased after MCAO, but no change of this protein in the contralateral hemisphere was observed (64).

Similarly, in a photothrombotic stroke model, ZO-1 and occludin expression in the ischemic cortex were significantly decreased; however, their expression was unchanged in the contralateral hemisphere (57). Contrastingly, researchers found BBB damage in the contralateral brain hemisphere in an in vivo model of sub-acute ischemic stroke (65). This change in the contralateral brain was associated with reactive astrocytes and microglia in that hemisphere, indicating an inflammatory response (65). Interestingly, significant changes in brain activity and functional connectivity in the contralateral brain hemisphere in acute ischemic stroke have been reported, linked with functional recovery (66). The reduction of TJ proteins’ expression in the contralateral hemisphere by TS or JUUL pre-exposure, as observed in our study, could be due to an enhanced release of inflammatory mediators (cytokines, chemokines, MMPs, VEGF) in
the bloodstream from the ischemic hemisphere, which may create a profound effect on the non-ischemic hemisphere. The mechanisms of these observed changes will be the subject of future investigation, with focused experiments measuring astroglia and microglia markers in both hemispheres. Overall, these findings bear significance as by reducing the otherwise unchanged TJ proteins in the contralateral hemisphere, the whole ischemic brain could be indirectly affected, thus leading to worsened brain damage after acute ischemic stroke.

One limitation of the study is that we have measured TJ protein expression at the BBB with western blot using total brain tissue instead of isolated brain microvessels. Therefore, the reported values may slightly differ from TJs protein expression levels directly measured from purified brain microvessels. However, this procedure has been previously used for similar studies (12, 30, 62) to assess the cerebrovascular impact of smoking on BBB TJs expression under diseased conditions (e.g., TBI) and the protective effect of potential countermeasures.

Oxidative stress and inflammation play a vital role in the pathobiology of ischemic stroke. Nrf2 is a nuclear transcription factor regulating the cellular antioxidative response system. Nrf2 has also been shown to play an essential role in TS-mediated BBB toxicity and ischemic stroke. Nrf2 was previously shown to be downregulated by chronic TS and/or e-Cig exposure in vitro and in vivo (12)(62). However, we did not observe any significant change after two weeks of JUUL or TS exposure. Nrf2 was shown to be upregulated in tMCAO studies (67), and it also exerted protective effects against ischemic brain damage (67, 68). In our western blot studies, Nrf2 expression was significantly decreased in the contralateral brain after MCAO with JUUL or TS pre-exposure. In immunofluorescence studies, we found a similar reduction of Nrf2 in the contralateral brain by JUUL or TS exposure. These studies also showed a reduction of Nrf2 in the ipsilateral brain only with TS exposure. Kaisar et al. also observed that e-Cig or TS exposure decreases brain Nrf2 expression after ischemic stroke (12). By reducing the antioxidative and cytoprotective actions of Nrf2, chronic exposure to JUUL or TS could worsen the ischemic brain damage and the neurological outcome.

ICAM-1 is an inflammatory marker that helps in leukocyte infiltration in response to an ischemic event (69). We did not observe any significant change in ICAM-1 expression after JUUL or TS exposure. Prasad et al. also observed no significant change in ICAM-1 expression after two weeks of TS exposure (62). Contrastingly, TS extract increased the expression of ICAM-1 in hCMEC/D3 BBB endothelial cells (70). In another study, two weeks of TS and e-Cig exposure increased brain ICAM-1 expression (12). Higher endothelial ICAM-1 expression was observed in the brain after acute ischemic stroke in clinical (69, 71, 72) and preclinical studies (72–74). In our study, ICAM-1 was significantly increased in the ischemic brain hemisphere of TS pre-exposed mice. This increase in inflammation could be one of the mechanisms of TS-mediated exacerbated ischemic brain injury and neurological damage. The unchanged ICAM-1 expression after only JUUL or TS exposure could be due to differences between in vitro and in vivo systems and duration of exposure, as explained earlier. It is also important to mention that changes in the expression levels of mRNAs related to TJ proteins, oxidative stress, and inflammatory markers do not necessarily parallel the expression of the corresponding proteins. The increase of ICAM-1 by TS exposure
in the ipsilateral brain can also be explained by the observed decrease of Nrf2 in the same region in immunofluorescent studies. Nrf2 and its downstream pathway protect against inflammation by regulating anti-inflammatory gene expression and inhibiting inflammation (75). Overexpression of Nrf2 has been shown to inhibit TNF-α-induced ICAM-1 expression in human retinal pigment epithelial cells treated with lycopene (76). On the other hand, knockdown of Nrf2 enhanced brain ICAM-1 expression in a mouse model of traumatic brain injury (77). This inhibitory role of Nrf2 on ICAM-1 can explain the overexpression of the latter in the ischemic brain.

TS exerted more cerebrovascular toxicity than JUUL, as observed in some of our abovementioned findings, which could be due to the higher nicotine concentration in TS-exposed mice. Further, TS has thousands of toxic chemicals, which could also be responsible for the enhanced toxicities. In the future, we would like to investigate the cerebrovascular effects of 4 weeks of JUUL e-Cig exposure with higher nicotine (5%) concentration and compare it to TS exposure.

**Conclusion**

This study found that short-term JUUL e-Cig exposure can cause a comparable level of harmful effects on ischemic brain injury and TJ protein expression at the BBB compared to TS exposure. In our study, only TS exposure caused hyperactivity in mice and altered the expression of brain inflammatory and oxidative stress markers after ischemic stroke. In summary, short-term JUUL e-Cig or TS exposure can enhance the sensitivity to ischemic stroke injury by disrupting the expression of TJ proteins at the BBB.

**Abbreviations**

- Electronic cigarette (e-Cig)
- Tobacco smoke (TS)
- Transient middle cerebral artery occlusion (tMCAO)
- Blood-brain barrier (BBB)
- Tight junction (TJ)
- Universal serial bus (USB)
- Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA)
- Single Cigarette Smoking Machines (SCSM)
- Common carotid artery (CCA)
- External carotid artery (ECA)
• Internal carotid artery (ICA)
• 2,3,5-tripheyltetrazolium chloride (TTC)
• Tris-buffered saline with 0.1% Tween® 20 Detergent (TBST)
• 4',6-Diamidino-2-Phenyindole, Dihydrochloride (DAPI)

Declarations

Ethics approval and consent to participate

• Not applicable

Consent for publication

• Not applicable

Availability of data and materials

• All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

Competing interests

• The authors declare that they have no competing interests

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Authors’ contributions

• Participated in research design: TJA, LC, BV, AES
• Conducted experiments: AES, SN, HV, SRA, YG, SS, YZ
• Performed data analysis: AES, SN
• Wrote or contributed to the writing of the manuscript: AES, TJA, LC

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• Not applicable

References


Figures

Figure 1
Plasma concentration and the ratio of nicotine and cotinine in mice after 14 days of JUUL and TS exposure. (A) Plasma concentration of nicotine and cotinine after 14 days of JUUL and TS exposure. (B) Plasma ratio of cotinine to nicotine after 14 days of JUUL and TS exposure. **** P<0.000; n=23-31 animals for each group.

Figure 2
Weights and locomotor activity of mice after 14 days of JUUL and TS exposure. (A) Percent weight change in mice after 14 days of JUUL and TS exposure. (B) Percent weight change in JUUL/TS-exposed mice 24 h after MCAO. (C) Percent change in baseline locomotor activity in mice after 14 days of JUUL and TS exposure. (D) Total distance traveled (cm) by JUUL/TS-exposed mice 24 h after MCAO. * P<0.05, *** P<0.001, **** P<0.0001; n=21-38 animals for each group (A) and n=9-12 animals for each group (B).

Figure 3
Brain injury, edema ratio, and neurological score in mice 24 h after MCAO. (A) TTC staining of brain slices exposed to JUUL/TS. (B) Percent infarct area in the ischemic brain hemisphere of JUUL/TS-exposed mice. (C) Brain edema ratio of JUUL/TS-exposed mice. (D) The neurological score of JUUL/TS-exposed mice. * P<0.05, ** P<0.01; n= 7-12 animals for each group.

Figure 4

Plasma IL-6 and thrombomodulin level in mice after 14 days of JUUL/TS exposure. (A) Plasma IL-6 concentration in mice after 14 days of JUUL and TS exposure with or without MCAO. (B) Plasma thrombomodulin concentration in mice after 14 days of JUUL and TS exposure with or without MCAO. For (A), α P<0.01, β P<0.05, and X P<0.05 refer to control vs. control MCAO, JUUL vs. control MCAO, and TS vs. control MCAO, respectively, and δ P<0.01, ε P<0.05, and φ P<0.01 refers to control vs. TS MCAO, JUUL vs. TS MCAO, and TS vs. TS MCAO, respectively. For (B), α P<0.05 refers to control vs. control MCAO, β P<0.0001, X P<0.001, and δ P<0.01 refers to control vs. JUUL MCAO, JUUL vs. JUUL MCAO, and TS vs. JUUL MCAO, respectively, and ε P<0.05 refers to control vs. TS MCAO; n= 8-15 animals for without MCAO groups and n= 5-11 animals for MCAO groups.

Figure 5

Brain ZO-1, claudin-5, and occludin expression in mice after 14 days of JUUL/TS exposure with or without MCAO. (A) western blot images of ZO-1 expression in normoxic brain and contralateral & ipsilateral brain hemispheres 24 h after MCAO. (B-D) Quantification of brain ZO-1 expression normalized to beta-actin and expressed as relative to control (1.0) in normoxic, contralateral, and ipsilateral brain regions. (E) western blot images of claudin-5 expression in normoxic brain and contralateral & ipsilateral brain hemispheres 24 h after MCAO. (F-H) Quantification of brain claudin-5 expression normalized to beta-actin and expressed as relative to control (1.0) in normoxic, contralateral, and ipsilateral brain regions. (I) western blot images of occludin expression in normoxic brain and contralateral & ipsilateral brain hemispheres 24 h after MCAO. (J-L) Quantification of brain occludin expression normalized to beta-actin and expressed as relative to control (1.0) in normoxic, contralateral, and ipsilateral brain regions. * P<0.05, *** P<0.001; n= 5-9 animals for each group. Cropped images from blots have been used in some cases to improve the clarity and conciseness of the presentation. The cropped images are delineated with white space; full-length blots developed by X-ray films are presented in Supplementary Figure S1.
Figure 6

Brain Nrf2 and ICAM-1 expression in mice after 14 days of JUUL/TS exposure with or without MCAO. (A) western blot images of Nrf2 expression in normoxic brain and contralateral & ipsilateral brain hemispheres 24 h after MCAO. (B-D) Quantification of brain Nrf2 expression normalized to beta-actin and expressed as relative to control (1.0) in normoxic, contralateral, and ipsilateral brain regions. (E) western blot images of ICAM-1 expression in normoxic brain and contralateral & ipsilateral brain hemispheres 24 h after MCAO. (F-H) Quantification of brain ICAM-1 expression normalized to beta-actin and expressed as relative to control (1.0) in normoxic, contralateral, and ipsilateral brain regions. * P<0.05; n= 5-9 animals for each group. Cropped images from blots have been used in some cases to improve the clarity and conciseness of the presentation. The cropped images are delineated with white space; full-length blots developed by X-ray films are presented in Supplementary Figure S1.

Figure 7

Brain ZO-1 and Nrf2 expression in mice after 14 days of JUUL/TS exposure with MCAO by immunofluorescence studies. (A) Immunofluorescence images of brain slices where green color represents ZO-1 while blue color represents DAPI (nuclear marker). Quantification of contralateral (B) and ipsilateral (C) ZO-1 expression relative to DAPI, expressed as a percentage. (D) Immunofluorescence images of brain slices where green color represents Nrf2 while blue color represents DAPI (nuclear marker). Quantification of contralateral (E) and ipsilateral (F) Nrf2 expression relative to DAPI, expressed as a percentage. Contralateral, non-injured hemisphere of the stroke brain; ipsilateral, injured hemisphere of the stroke brain. *P<0.05, ***P<0.001, ****P<0.0001; n= 4 mice for each group.

Figure 8

Flow diagram of the study design. Male C57 mice were exposed to JUUL or TS for 14 days. At the end of the exposure, plasma nicotine and cotinine levels were measured by LCMS/MS. An open field test was done to investigate the locomotor activity of the mice. Ischemic stroke was induced in mice by middle cerebral artery occlusion (MCAO) followed by reperfusion. 24 h after reperfusion, an enzyme-linked immunosorbent assay (ELISA) was performed to measure plasma IL-6 and thrombomodulin. Western blot and immunofluorescence were done to measure the expression of BBB tight junction proteins as well as inflammatory and antioxidant markers in the brain.

Supplementary Files
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- Supplementaryfile.pdf