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Final Report

Title

Assessment of the e-Cigarette Impact on Vascular Health

評估電子香煙對血管健康的影響

Submitted
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Table of Content

List of Abbreviations

AAA	Abdominal Aortic Aneurysm
COX-2	Cyclooxygenase-2
CSE	Cigarette smoke extract
DMEM	Dulbecco's modified eagle medium
EC	E-cigarette
EJ	E-cigarette juice
ELISA	Enzyme-linked immunosorbent assay
IHC	Immunohistochemical staining
miRNA	Micro ribonucleic acid
MMP-2	Matrix metalloproteinase-2
MMP-9	Matrix metalloproteinase-9
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NC	Normal cigarette
q-PCR	Real-time polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TUNEL	Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling
VSMCs	Vascular smooth muscle cells
WB	Western Blot

Executive summary

Abstract of the research

(English):

Since the introduction of e-cigarette in the smoking market in 2007, their usage has increased substantially. Until now, the impact of e-cigarette on human vascular health and whether e-cigarette vapor inhaled has similar effect as a normal cigarette on human aortic cells is still largely unknown. Given their increasing usage, it is crucial to investigate their biological effects on human vascular health. Smoking is a significant risk factor for the fatal Abdominal Aortic Aneurysm (AAA) disease. Thus; in the current study, we examined the effects of cigarette smoke-induced vascular injury by 1) evaluating the impact of e-cigarette smoking in terms of inflammation, matrix degradation, and cell death of human aortic cells; 2) comparing the harmful impact of e-cigarette smoking with that of normal cigarette smoking and 3) delineating the underlying mechanism of its impact on human aortic cells by studying the specific gene expression for e-cigarette smoking.

We found that 6% of e-cigarette smoke extract (CSE) treatment induced more matrix degradation enzymes, MMP-2 expression, higher elastin degradation activity, significant cell apoptosis, and more inflammatory mediators in AAA explant cultures cells. Similar harmful impacts were also observed in CSE-treated normal human vascular smooth muscle cells (VSMCs). For a more comprehensive study, we treated the vascular cells with normal cigarette and e-cigarette juice incubation to delineate the potentially harmful impact of normal smoking and e-cigarette without combustion respectively. Thirteen % of normal cigarette smoke extract and 6% e-cigarette juice induced significant harmful effects on vascular cells. The gene expression study

revealed that there was no differential expression of specific microRNAs among the harmful concentration of e-cigarette, normal cigarette, or e-cigarette juice. These results reveal that the potential vascular injury may not follow the specific microRNA signaling pathway. Those observations provide compelling evidence for apparent e-cigarette smoking-related vascular injury and should not be regarded as a healthier alternative than normal cigarette smoking. This research result supports the new legislation for banning the use and selling of e-cigarette in Hong Kong to safeguard the public health of our citizens.

(Chinese):

自 2007 年起，電子煙進入吸煙市場後，其使用量大幅增加。直到現在，電子煙對人體血管健康的影響很大程度上仍是未知數，此外，亦不知道吸入電子煙釋放的蒸汽是否對人體動脈細胞具有類似普通香煙的影響。鑑於愈來愈多人使用電子煙，評估它們對人體血管健康的影響是非常重要。事實上，我們已知吸煙是致命的腹主動脈瘤疾病的重要危險因素，因此，在目前的研究中，我們通過以下方式檢查其香煙煙霧引起的血管損傷及影響：1）評估吸食電子煙對人類動脈細胞炎症、基質降解和細胞死亡的影響；2) 比較吸食電子煙與普通香煙的有害影響；3) 通過研究特定基因表達，判斷吸食電子煙對人類血管細胞的影響的潛在機制。

我們發現 6% 的電子煙煙霧提取物在腹主動脈瘤外植體培養細胞中誘導了更多的基質降解酶、MMP-2 表達和活性、更高的彈性蛋白降解活性、顯著的細胞凋亡和更多的炎症介質。在經過電子煙煙霧提取物 處理的正常人血管平滑肌細胞中觀察到有類似的有害影響。為了進行更全面研究，我們還用電子煙油浸泡血管細胞，以測試電子煙在沒有燃燒的情況下的潛在有害影響。13% 的普通香煙煙霧提取物 和 6% 的電子煙油均對血管細胞產生顯著的有害影響。基因表達研究表明，在有害濃度的電子煙、普通香煙或電子煙油中，並沒有特定微小核糖核酸 的

表達。這些結果顯示潛在的血管損傷可能不是通過特定微小核糖核酸信號通路。總括來說，這些研究指出吸食電子煙會損害血管，並提供了令人信服的證據，不應將其視為比普通香煙更健康的替代品。這項研究結果支持新法例，禁止在香港使用和銷售電子煙，以保障市民的公共健康。

Layman summary on policy implications and recommendations

(English):

Our research has appropriate and prompt policy implications for assessing the potentially harmful impacts of e-cigarette and the concerning consequences on human vascular health. Our meaningful findings could be used to make important policy interventions to inform the public about the knowledge, understanding, and perceived potential risks of cigarette alternative (electronic cigarette).

The Tobacco Control Legislation executed the banning of 'Alternative Smoking Products' last year. From April 30th, 2022, no person may import, promote, manufacture, sell, or possess alternative smoking products for commercial purposes, including electronic ones; anyone who carries out any of the above acts in relation to an alternative smoking product commits an offense. Hence, our study supports the new legislation with strong pieces of evidence to ban e-cigarette smoking entirely locally, as this is one of the most effective health protection measures for Hong Kong citizens. Moreover, considering that quitting traditional cigarette smoking is already one of the most recommended methods for smokers to prevent atherosclerotic cardiovascular disease in Hong Kong's population reinforces the need for this ban. This recommendation was previously stated in the 2016 Consensus statement on the "Prevention of atherosclerotic cardiovascular disease in the Hong Kong population."

Our findings showed that both e-cigarette and normal cigarette smoking harm vascular cells. These results could be used as a strong recommendation and reference for the government to reinforce the new e-cigarette public policies and, if necessary, can be used to refine the current policies or establish advanced policies to tackle the health risks of both smokers and non-smokers. We hope to contribute to improving the public governance of cigarette regulation in the future.

(Chinese):

我們研究目的是評估電子煙與人體血管關係及其潛在有害的影響，對制定如何提高人們血管健康意識具有適當的政策意義。本研究數據對認知和理解香煙替代(電子香煙）的潛在風險具有重要意義，為制定重要的政策干預提供理論依據。

控煙法例中的另類吸煙產品禁令已於去年執行。由 2022 年 4 月 30 日起，任何人不得進口、推廣、製造、售賣或以商業目的管有另類吸煙產品，包括電子煙、加熱煙產品及草本煙，如作出以上有關行為即屬違法。我們的研究數據為新控煙法例提供了堅實的理論支持，並且提供有力的證據來限制香港民市吸食電子煙，這是保護香港市民健康的措施之一。對香港吸煙者而言，戒菸已經是最有效預防動脈粥樣硬化性心血管疾病發生的方法，這項建議是刊載於 2016 年《預防香港人口動脈粥樣硬化性心血管疾病》的共識聲明裏面。

我們的研究結果表明電子煙和普通香煙都對血管壁細胞有害，可以為政府加強新電子煙公共政策提供建議和參考，並在必要時完善現行政策或提出更好的推進政策建議，降低吸煙者和非吸煙者的健康風險。我們希望在未來能夠為煙草監管的公共治理做出貢獻。

Introduction

Most smoking studies focus on the human respiratory system and showed the potential and specific risk of tobacco use in lung and airway (Brozek et al., 2019; Glantz & Bareham, 2018). The potential harmful impact of smoking is still largely unknown in vascular health. Over the recent decades, industries have introduced an alternative choice known as electronic cigarette (e-cigarette) claiming it is a healthier alternative to tobacco smoking and a help to people who attempt to quit smoking cigarette. Instead, e-cigarette has simply become another class of tobacco product that is maintaining and expanding the tobacco epidemic. However, a growing number of international researchers found that e-cigarette contains chemicals that are harmful to human health.

Some toxic and carcinogenic substances will be released after heating and vaporization of e-cigarette, including nicotine, propylene glycol, glycerol, formaldehyde, acetaldehyde, additives (Cheng, 2014). They could cause addiction, unwell feeling, coughing, damage to body cells and tissues, as well as respiratory and cardiovascular diseases. Severe cases can cause cancer and death (Qasim et al., 2017). There is no international regulation of the use of e-cigarette available (Qasim et al., 2017). A US study of public health policy of e-cigarette pointed out that banning tobacco products has shown to reduce smoking risk in youth and as such, strong e-cigarette regulation measures are needed for prevention (Bhalerao et al., 2019). A newly 2023 review found that there is not enough data obtained from independent studies that could safely indicate that these reduced amounts of toxic chemical entities in the composition of heated tobacco products, including e-cigarette do not induce any harmful effect (Kusonic et al., 2023). Most important, there is still sparse data available to show the direct impact of e-cigarette smoking on human health, particularly for vascular health. Taken together, e-cigarette definitely contains harmful substances that may pose a risk

to public health but needs scientific data to support the statement.

Last year, a longitudinal study is performed to assess the association of e-cigarette use with incident cardiovascular disease (Berlowitz et al., 2022). The researchers analyzed data from a representative cohort study with self-reported data. They did not find a significant difference in the cardiovascular risk of exclusive e-cigarette use compared with nonuse of cigarette and e-cigarette, although analyses were limited by a small number of CVD events in e-cigarette users. Dual use of cigarette and e-cigarette was associated with a significantly increased risk of CVD compared with nonuse. Another systematic review aimed to assess the effect of vaping on disease outcomes in people with existing health conditions (McNeill et al., 2022). However, for cardiovascular diseases, they did not identify any studies on people with existing cardiovascular conditions. And A Netherlands report described a questionnaire-based market survey of 456 daily or weekly e-cigarette. The risks of e-cigarette use were then compared with the risks of tobacco smoking. They concluded that the health risks associated with smoking conventional cigarettes are considerably higher than those associated with using e-cigarette. However, the health risks are strongly dependent on individual vaping and smoking habits (Visser et al., 2015) Actually, tobacco smoking also accounts for up to 30% of heart disease–related deaths in the United States every year (Gattone & Lammert, 2015). Thus, quitting smoking is one of the most convincing recommendations for smokers to prevent the atherosclerotic cardiovascular disease, as stated in the 2016 Consensus statement on prevention of atherosclerotic cardiovascular disease in the Hong Kong population (Cheung et al., 2017).

Recently, there are newly e-cigarette policies adopted by the Hong Kong government with some noise and dispute. Firstly, according to the Pharmacy and Poisons Ordinance

(Cap 138) in Hong Kong, any e-cigarette with nicotine is categorized as pharmaceutical products and must be registered with the Pharmacy and Poisons Board of Hong Kong before sale or distribution. Secondly, according to the Smoking (Public Health) Ordinance, no person shall smoke an e-cigarette in statutory non-smoking area. Under this new law, it will still be legal to use the device, though doing so in a non-smoking area will be subject to a fixed penalty. Thirdly, the Tobacco Control Legislation of banning on Alternative Smoking Products was executed last year. From April 30, 2022, no person may import, promote, manufacture, sell, or possess for commercial purposes alternative smoking products, including electronic smoking products. Anyone who carries out any of the acts in relation to an alternative smoking product commits an offence. So, our study could generate impactful results and support the new local legislation with strong evidence to ban e-cigarette smoking entirely.

Up to now, no comprehensive study of direct harmful impact of e-cigarette on human vascular health is available. Particularly, its health effects on vascular disease patients remain largely unknown due to the novelty of this product. Therefore, our proposed new study on the harmful effects of e-cigarette is urgently needed to inform policy makers in supporting their newly e-cigarette's regulation, which is set to minimize the product's potential health risks to the public.

Objectives of the study

The objective of this study is to provide scientific evidence, which could alert smokers and citizens about the vascular health risk of e-cigarette. It is hoped that the results can provide solid evidence as reference for policy makers to support their determination of the optimal e-cigarette regulation to safeguard the public health by achieving the following three aims:

Aim 1: To evaluate the impact of e-cigarette smoking in terms of inflammation, matrix degradation and cell death of human aortic VSMCs;

Aim 2: To compare the harmful impact of e-cigarette smoking with that of normal cigarette smoking and

Aim 3: To delineate the underlying mechanism of e-cigarette smoking and its impact on human aortic VSMCs

Research methodology

(i) AAA sample collection

AAA tissue specimens were resected from AAA patients undergoing open surgical aneurysmal repair in Queen Mary Hospital. Institutional Review Board approval for the surgical specimens was sought for the study. All surgical aortic specimens collected were thoroughly washed with Dulbecco's modified eagle medium (DMEM) (Life Technologies, Carlsbad, CA) prior to perform explant culture.

(ii) Explant AAA cells culture

Approximately, 2 × 2 mm-sized segment from each AAA specimen was dissected out and placed onto a complete cell culture medium containing DMEM (Cat#11995065) supplemented with 10% HI-FBS (heat-inactivated fetal bovine serum, Cat#10500064), 1% GlutaMax 100X (Cat#35050061), 20mM HEPES (Cat#15630080), 100 unit/mL penicillin and 100 ug/mL streptomycin (Penicillin-Streptomycin, Cat#15140122). All culture medium and reagents were purchased from Gibco (USA). Cells were maintained in a 37℃ incubator with 5% CO2 and 95% humidity with regular replacement of complete cell culture medium in every 3 days. All cells have undergone mycoplasma screening before use. Cells growing out from the aortic tissue

segment were passaged before reaching confluence by trypsinization using 0.025% trypsin/ethylenediaminetetraacetic acid (Life Technologies). The VSMCs cultured between passages 4 and 6 were used for the proposed experiments. Maintenance of VSMC phenotype was confirmed by immunohistochemical staining of smooth muscle α-actin with anti-human smooth muscle α-actin antibody (cat. no.: M0851; 1:200; DakoCytomation, Glostrup, Denmark) in cells cytospined on Polysine microscope slides according to the standard IHC procedure. All cells were exposed to serum-free medium for at least 24 hours before any exposure with cigarette smoking extract (CSE). This special preparation of VSMCs is beneficial to acquire high homogenous cell type for determining the harmful impact of VSMC after exposure with cigarette smoking extract.

(iii) Healthy aortic vascular smooth muscle cells as control

Healthy human aortic vascular smooth cell was purchased from American Type Culture Collection (ATCC, Manassas, USA) as control and grown in a complete culture medium (hVSMCs, ATCC-PCS-100-042) for the subsequent e-cigarette study.

(iv) Cigarette smoking extract (CSE) preparation

Briefly, aqueous CSE was prepared via a smoking apparatus (either for smoking e-cigarette or normal cigarette) to which smoke from the device were slowly bubbled into DMEM (1 cigarette/10 ml serum free medium). The customized apparatus was set up with modification with previous studies (Y. T. Cui et al., 2021; Olmedo et al., 2016). (Fig. 1). The core of the device was a 3-way regulator that allows for unidirectional flow in a controlled manner. To prepare the CSE, smoke of 55 mL per puff volume was first drawn into the syringe and then pushed into the storage container, using the 3-way regulator as flow control. Puff duration and interval was adjusted accordingly for both

normal cigarette and e-cigarette following the guideline from WHO (World Health & Initiative, 2012). The narrow opening of the tubing and pipette tips aided in the condensation of smoke or aerosol as it flew through. CSE was prepared fresh daily for the cell experiments. The pilot study of Wylam research group found that ≥5% CSE concentration produced significant cell death, especially with prolonged exposures (Wylam et al., 2015). Accordingly, we used lower concentrations (1% and 3%) to determine the modulatory effects of CSE on human VSMCs. A time period of 24 hours cell incubation with CSE was selected based on the previous observation of altered protein expression within that time frame (Vassallo et al., 2005). In the study, human aortic VSMCs were treated with 1%, 3%, 6% and 13% of CSE for 24 hours for all subsequent functional experiments. The experimental data was measured with 24 hours of CSE incubation in AAA explant and normal aortic culture respectively. A measurement of all experimental factors of VSMCs incubated with 24 hours of DMEM without any CSE will be performed as sham control.

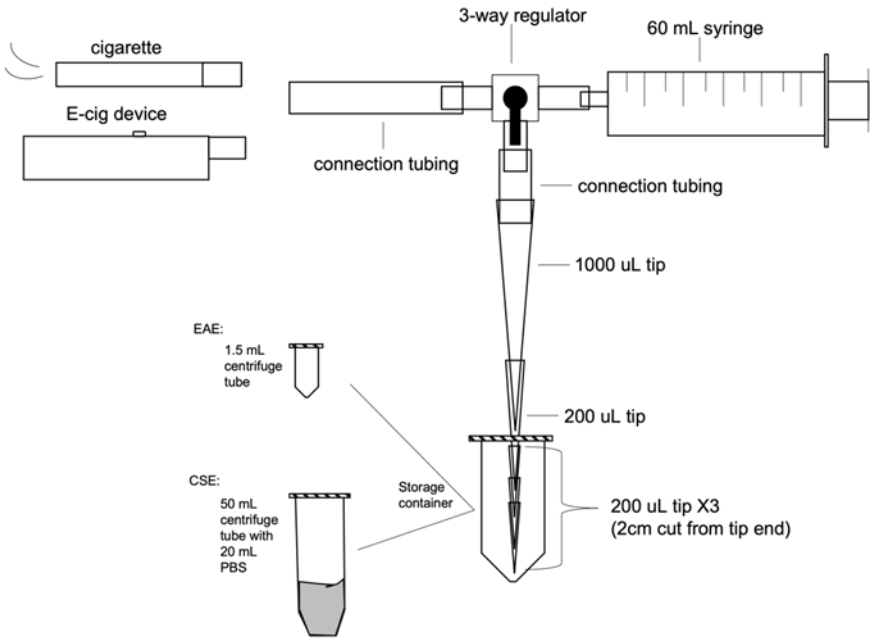


Figure 1. A schematic diagram of the customized CSE extraction system.

Specifically, for collecting CSE from normal cigarette, smoke of 2 commercial cigarette (12 mg of tar. 1.0 mg of nicotine, Marlboro) were bubbled through 20 mL PBS at 2s duration with 30s interval and sterile filtered using 0.22 um membrane filter (Czekala et al., 2019; Vassallo et al., 2005). To standardize the preparation, OD absorbance at 320 nm was being measured using a spectrophotometer CLARIOstar plus (BMG Labtech, Germany)(Wirtz & Schmidt, 1996). Absorbance patterns (OD: ~1.0) showed little variation across different preparations and confirmed the stable concentration of CSE used in all experiments. This CSE solution was regarded as 100% CSE and was stored in aliquots at -80 ˚C and protected from light. Subsequent dilution of different CSE concentrations was prepared using 10% HI-FBS complete medium.

For collecting CSE from e-cigarette, puffing was done at 4s duration with 30s interval using a commercial e-cigarette device purchased from local retail shop before the new banning policy of e-cigarette purchase. Usage survey revealed e-cigarette user to have longer smoking duration per puff as compared to conventional cigarette (Robinson et al., 2016). OD absorbance at 320 nm was not an effective measurement for standardizing CSE of e-cigarette preparation, as shown by other study (Taylor et al., 2016) and our preliminary experiments (data not shown). To allow quantification in treatment concentration, the CSE of e-cigarette was collected directly into a storage container as condensed droplets without the use of PBS and regarded as 100% CSE. The specific CSE stock were stored in aliquots at -80 ◦C until further dilution. CSE of different concentrations were prepared using 10% HI-FBS complete medium as diluent and sterile filtered with 0.22 um membrane filter before application.

(v) CSE treatment

All cultured cells were plated into 6-well plate at ~54,000 cell/well density and allowed to adhere for 24hr. Before each treatment, all cells were starved for 24hr with DMEM medium containing 0.2% HI-FBS, 1% GlutaMax 100X, 20mM HEPES, 100 unit/mL penicillin and 100 ug/mL streptomycin.

CSE of normal cigarette (NC) and e-cigarette (EC) were thawed right before use and diluted using 10% HI-FBS complete medium. PBS was used as vehicle control for NC. For a comprehensive study, we also examined the e-cigarette juice (EJ) content used in EC preparation. Thus, the vaporizing effect of EC could be understood. Cells were incubated with 10% HI-FBS or treatment medium for 24hr before protein and RNA extraction.

(vi) MTT

All cells were seeded at the MTT plate with 2500 cell/well density (determined by preliminary experiment) onto 96-well plate and allowed to adhere for 24hrs for CSE treatment first. Starvation and treatment were carried out as the procedures of CSE treatment that described in previous section. After incubated with treatment medium for 24hr, all wells were washed 2 times with pre-warmed 1x PBS before incubating with 0.5 mg/ml MTT for 3hrs in a humidified 37 ˚C incubator. To solubilize the formazan crystal formed, 100 ul DMSO was added into each well and plate was shaked at 150 rpm for 15 mins in dark. Absorbance was measured at 570 nm and 650 nm (reference) using a Multiskan FC machine (ThermoFisher, USA). Experiment was performed in triplicates. Cell viability was determined based on absorbance after subtracting reference and blank.

(vii) Inflammatory effect of CSC on human aortic VSMCs

a) Immumohistochemical staining (IHC) detection of inflammatory macrophage of treated VSMCs

After performing the above experiments, the cells were harvested from the culture plate by means of collagenase D solution (2 mg/mL) and attached to the glass slide by a cytospin apparatus (Shandon, Inc). After inhibition of endogenous peroxidase activity, the cells were incubated with primary antibodies against CD68 (A tissue macrophage marker) in the recommended diluent overnight at 4 °C. The detection was performed according to the manufacturer's instructions using Signal Stain Boost Detection Reagent (Cell Signaling) and incubated in a humidified chamber for 30 min at room temperature followed by washing. Then Signal Stain DAB were added to each section and subjected to dehydration of the sections by using 95% and 100% ethanol, respectively, before mounting.

b) Western blotting (WB)

In order to confirm the protein expression levels of an interested inflammatory mediator (COX-2), WB was performed for the CSE treated VSMCs at 0 hour and 24 hours. Protein from cultured cells was extracted using RIPA (Cat #89900, ThermoScientific, USA) with protease inhibitor PMF (Cat #93483, Sigma-Aldrich, USA). Ten microgram samples were separated on 8% SDS-PAGE gels and transferred electrophoretically onto nitrocellulose membranes. Membranes were blocked for one hour in 5% non fat milk in TBS-T. Primary antibodies of COX-2 (1:10000, Cat#ab79800, Abcam, USA), MMP-9 (1:2000, Cat#NBP1-40610, Novus Biologicals, USA) GAPDH antibody (1:20000, Cat#2118L, Cell Signaling Technology, USA), internal control) were probed onto the membranes overnight at 4^{o}C, followed by horseradish labelled secondary antibody (anti-rabbit, 1:2500, Cat#31460, ThermoFisher Scientific, USA for COX-2, 1:10000

for MMP-2, MMP-9 and GAPDH) at room temperature for 1 hour. Bands were visualized by enhanced chemiluminescence (Clarity Western ECL substrate, Cat#1705060, Bio-Rad, USA) following manufacturer's protocol. Because COX-2 and MMP-2 detected sizes are both 72kDa, so the membranes were then stripped with 2-mercaptoethanol (Cat#M3148, Sigma-Aldrich, USA, USA), blocked again and probed for MMP-2 (1:20000, Cat# 40994S, Cell Signaling Technology, USA) (2nd Ab 1:10000). Relative protein expression levels for Western blotting were quantified using the Image J program (National Institute of Health, Bethesa, MD).

c) Enzyme-linked immunosorbent assay (ELISA)

In order to measure the secretary levels of the pro-inflammatory cytokines (PGE_2, MCP-1, TNFα, IL-1β), and Matrix degradation (MMP-2 and -9), ELISA was performed for the CSE treated VSMCs at 0 hour and 24 hours. Briefly, the supernatants of the cultured VSMCs plate were collected and analyzed for the secretary levels of inflammatory factors using ELISA kits (BioSource; Camarillo, CA, USA) according to the manufacturer's instructions. After the supernatant were removed for the ELSIA detection, the treated cells were be harvested for the subsequent Immunohistochemical staining (IHC), Western blotting (WB) and TUNEL study.

(viii) Elastin degradation

Level of elastin degradation and elastin degrading enzymes (MMP-2 and MMP-9) will be investigated by (a) elastin zymography and (b) ELISA respectively in CSE treated VSMCs at 0 hour and 24 hours. It is proposed that high MMP enzyme levels will be responsible for more elastin degradation of aortas.

a) Elastin zymography

An elastin zymogram was prepared by incorporating 3mg/ml elastin into a sodium dodecyl sulfate-polyacrylamide gel eletrophoresis (SDS-PAGE). Proteins harvested from cells will be loaded into the zymogram and electrophoresed under non-reducing condition. Purified MMP-2 (Cat#902-MP-010, R&D Systems, USA) was also electrophoresed as positive control. The zymogram was washed 2 times with 2.5% Triton X-100 for 30 mins to remove SDS, followed by incubation into a digestion solution containing 1% Triton X-100, 50mM Tris pH 7.5, 5mM $CaCl_2$ and 1μM $ZnCl_2$ at 37°C for 2 hrs to allow activation of elastinolysis. The zymogram was stained with Coomassie brilliant blue. The clear band against blue background indicated the digested elastin due to the presence of elastin-degrading proteases. Densitometry of the clear band were done by Image J software (National Institute of Health, Bethesda, MD). A regular SDS-PAGE without elastin incorporated were performed in parallel under identical conditions as negative control.

b) ELISA

The supernatants of the cultured VSMCs plate were collected and analyzed for the secreted PGE_2 (Cat#KGE004B), MCP-1 (Cat#DCP00), TNF-α (Cat#DAT00D), IL-1β (Cat#DLB-50), MMP-2 (Cat#MMP200) and MMP-9 (Cat#DMP900) level using ELISA kits (R&D Systems, USA) according to the manufacturer's instructions.

(ix) Loss of VSMCs by apoptosis

Apoptotic analysis was performed by Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL). To prevent cell loss due to detachment caused by treatment, cytospin technique was used to fix cells onto slides for staining. Cells were initially seeded at ~2,600 cell/well density onto a 24-well plate

for CSE treatment. After treatment, cells were collected from supernatant and harvested from plate by TryPLE. Cells were concentrated by centrifugation and resuspended in 200 ul 1x PBS before cytospin onto slide at 13000 cell/mL density using Cytospin 3 (Thermo Shandon, USA) set at 500 rpm for 3min. After the supernatant was removed for ELISA detection, the cells were harvested for TUNEL detection to quantify the apoptotic cells. TUNEL-positive cells were counted with automatic segmentation in a blind fashion using ZEN 3.3 (Zeiss, Germany).

(x) Expression of miRNAs

a) Total RNA isolation

Total RNAs was isolated using Trizol reagent (Invitrogen) and further purified using a RNeasy mini kit (Qiagen, Valencia, Calif). The concentration of all miRNA samples were quantified by NanoDrop 1000 (Thermo Scientific, Wilmington, DE, USA). RNA quality control was further performed using Bioanalyzer 2100 in Genome Research Center. Target MiR expression levels were detected by Real-time polymerase chain reaction (q-PCR) method.

b) q-PCR for altered miR expression

Quantitative real-time PCR Assay

The mRNA expression level of COX-2, MMP-2 and MMP-9 was determined using quantitative real-time PCR assay. Total RNAs were isolated using the miRNeasy mini kit (Cat#217004, Qiagen, USA) and quantified by a Nanodrop 1000 machine (ThermoScientific, USA). Four hundred ng RNA was reverse transcribed to cDNA using PrimeScript RT reagent kit with gDNA eraser (Cat#RR047A, Takara, Japan) following the manufacturer's protocol. Real-time PCR was performed in triplicate using the LightCycler LC480 II (Roche, Germany). The reaction solution was prepared

at a final volume of 20 ul consisted of 1 ul cDNA (10 ng/ul), 10 ul 2X iTaq Universal SYBER Green Supermix (Cat#1725122, Bio-Rad, USA), and 0.5 ul each of forward and reverse primer (10 uM) (See Table 1 for primer sequences). Amplification started with cDNA denaturation and polymerase activation at 95℃ for 2 min, followed by 40 cycles of 95℃ for 15 sec and 60℃ for 1 min. Primer specificity was confirmed by melt curve analysis at 54 ℃ with temperature gradually increased to 95℃ in 0.5℃ increments. Relative expression was measured by the 2−ΔΔCt method using GAPDH as internal control.

Table 1. Primer Pairs used for qPCR Experiments

Gene		Primer Sequence	Amplicon
MMP-9	Forward	TTG ACA GCG ACA AGA AGT GG	179bp
	Reverse	GCC ATT CAC GTC GTC CTT AT	
MMP-2	Forward	TTG ACG GTA AGG ACG GAC TC	153bp
	Reverse	ACT TGC AGT ACT CCC CAT CG	
COX-2	Forward	CCC TTG GGT GTC AAA GGT AA	132bp
	Reverse	GAA GTG CTG GGC AAA GAA TG	
GAPDH	Forward	GGC CTC CAA GGA GTA AGA CC	147bp
	Reverse	AGG GGT CTA CAT GGC AAC TG	

In addition, the expression of the 3 potential gene targets (miR 1250, 516a-5p and 205) that were found to be important miRNAs in our previous AAA surgical samples (Chan, Chan, et al., 2017; Chan, Cheuk, et al., 2017; Cheuk & Cheng, 2014) were also detected for delineating their potential pathological role in aortic cells. The primer sequences of miR 1250, 516a-5p and 205 were prepared as previous studies.

Research results

a) AAA sample collection and explant cultures

During the study period, there were 10 surgical samples resected from AAA patents underwent open surgery and available for the e-cigarette study. Eight of them could be successfully cultured for subsequently biochemical analysis. The demographic data of the AAA was showed in Table 1. Most of them were smokers and male without other disease history.

Table 2. Demographics of the abdominal aortic aneurysm (AAA) patients that have explant cultures for the current study

	Subgroup	AAA (n=8)
Age, year (mean±SEM)		70.4±2.06 year sold
Sex	Male	5 (62.5%)
	Female	3 (37.5%)
Smoking	Smoker	5 (62.5%)
	Non-smoker	3 (37.5%)
Diabetes	Disease history	1 (12.5%)
	Normal	7 (87.5%)
Hypertension	Disease history	1 (12.5%)
	Normal	7 (87.5%)
Cardiac Disease [a]	Disease history	1 (12.5%)
	Normal	7 (87.5%)
Pulmonary Disease [b]	Disease history	2 (25%)
	Normal	6 (75%)
Renal Disease [c]	Disease history	2 (25%)
	Normal	6 (75%)
Dyslipidemia [d]	Disease history	3 (37.5%)
	Normal	5 (62.5%)

AAA, abdominal aortic aneurysm; SD, standard deviation.

[a] Cardiac disease refers to ischemic heart disease, angina or myocardial infarction;

[b] Pulmonary disease refers to documented lung disease, asthma or chronic obstructive pulmonary disease;

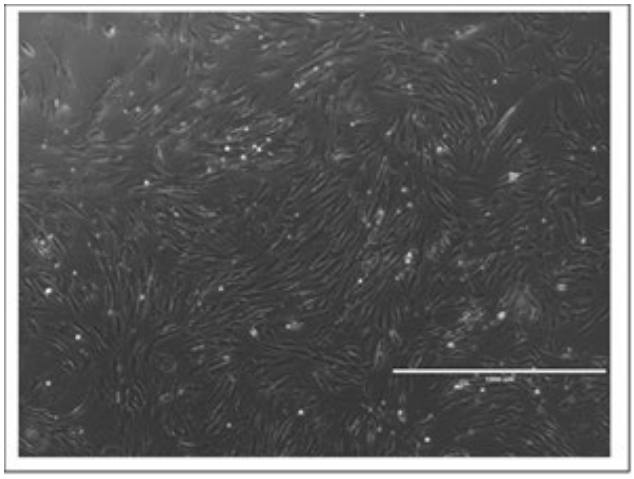
[c] Renal disease refers to renal impairment or end-stage renal failure;

[d] Dyslipidemia refers to raised triglycerides or raised cholesterol levels

b) <u>Vascular cells culture</u>

All available surgical samples were collected and proceeded for explant culture. The cultured cells were confirmed with no mycoplasma contamination and eligible for subsequent biochemical studies with vitality (Picture 1). This checking was important as mycoplasma often out-compete the host cells for essential nutrients resulting in altered growth and protein production, in which will alter almost every cell culture property and characteristic measured.

Picture 1 showed the morphology of the explant Vascular Smooth Muscle Cells (VSMCs) culture of a representative AAA surgical sample at Day 25. It reached 90% confluence and was passaged for enough cells in the e-cigarette study. Confirmation of smooth muscle cell type was done by α-actin staining.



The following experiments result addressed Aim 1: To evaluate the impact of e-cigarette smoking in terms of inflammation, matrix degradation and cell death of human aortic VSMCs and Aim 2: To compare the harmful impact of e-cigarette smoking with that of normal cigarette smoking.

c) <u>Identification of the harmful concentration of the CSE (e-cigarette and normal cigarette) and E-juice without combustion on vascular cells for subsequent experiments.</u>

For a comprehensive study, we also incubated the cells with the e-cigarette juice for delineating the potential harmful impact of e-cigarette without combustion. We first examined CSE incubation induced cell death in human vascular cells by *in vitro* viability MTT assays. MTT-based analyses revealed the treatment with 6% e-cigarette, 13 % normal cigarette and 6% e-juice incubation reduced the number of viable cells significantly (around 50%) compared with the exponential growth of cells without any treatment. Indeed, all CSE was dissolved in PBS, so we would like to check the PBS (as solvent) pose any additional risk in the cultures. We found that there was no significantly cell death with PBS incubation. So, it is safe to use PBS as solvent for CSE in the treatment incubation. Table 2 showed the percentage of number of viable cells vs control (no treatment) of AAA explant cultures with different treatments. The harmful concentration of CSE with pathological effect was then identified for the subsequent experiments.

Table 3 showed the total cell viable (presented with percentage) with different treatment.

	Concentration					
	1%	**3%**	**6%**	**13%**	**25%**	**50%**
E-cigarette (EC)	67%	61%	<u>53.9%</u>	5%	5%	3%
Normal cigarette (NC)	99%	83%	87%	<u>57%</u>	37%	1%
E-juice (EJ)	92%	74%	<u>56%</u>	15%	1%	2%

d) Inflammation and matrix degradation of CSE treated cells by Western Blot (WB)

The WB analysis quantified an important inflammatory mediator, COX-2 and matrix degradation enzymes (MMP-2 and-9) protein expression in the treated aortic cells. The analysis of normal VSMCs was also performed for comparing the pathological impact of cigarette treatment. An incubation without any smoking extract in the cells was used as negative control.

Detection of COX-2 protein level in explant aortic tissues from a representative explant culture of abdominal aortic aneurysm (AAA) surgical samples by Western Blotting was showed in Figure 1.

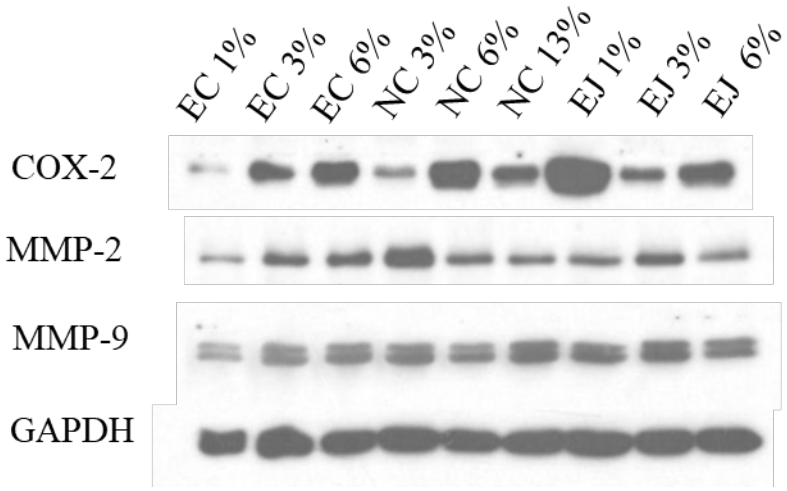
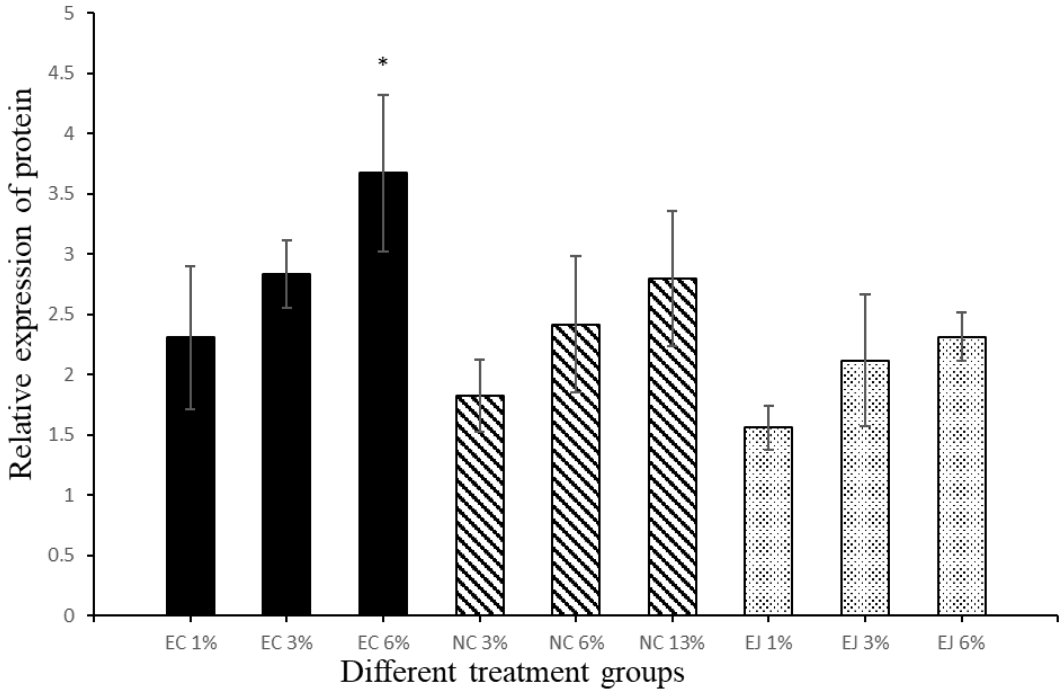


Figure 1 showed the representative western blot gel photos of aneurysmal cells probed with specific COX-2 antibody (72kDa), MMP-2 antibody (72kDa), MMP-9 antibody (92kDa) and GAPDH antibody (35kDa) under different concentration of e-cigarette (EC), normal cigarette (NC) and e-juice (JC) incubation.

(A) COX-2 protein expression



(B) MMP-2 protein expression

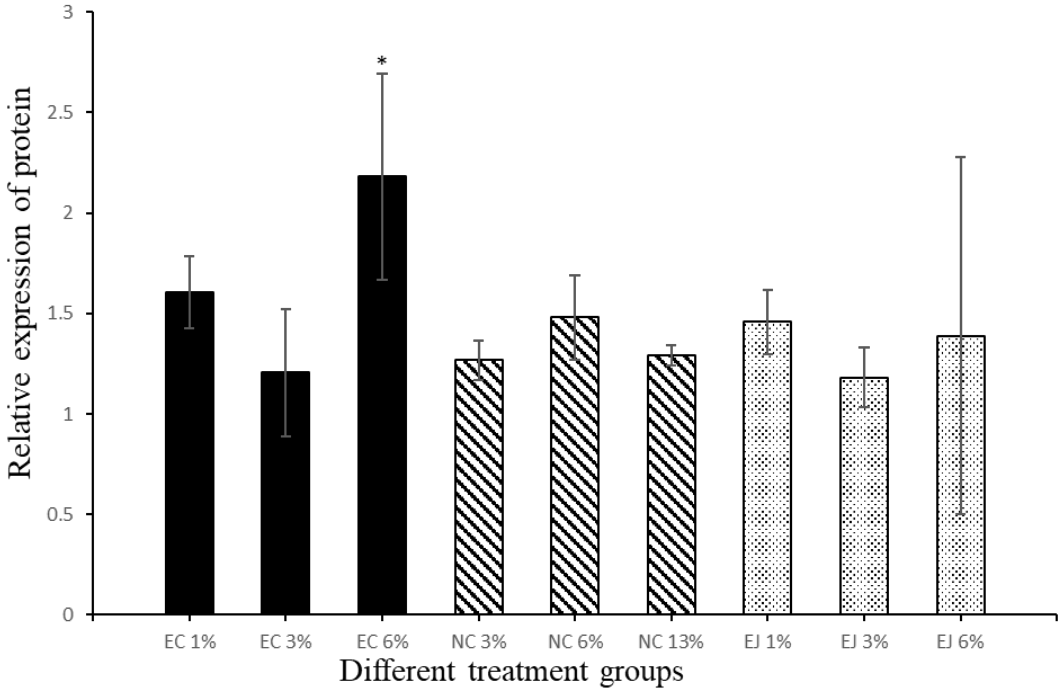


Figure 2 showed the relative COX-2 (A), MMP-2 (B) and MMP-9 (C) in AAA cells treated with different concentration of CSE from e-cigarette (EC) and normal cigarette (NC) and e-juice (EJ). It showed that 6% of CSE of e-cigarette (EC) induced significant COX-2 protein than that without treatment. (3.67±0.65) ($p<0.05$). Same concentration of e-cigarette (EC) induced significant MMP-2 expression too (2.18±0.51, $p<0.05$). The relative MMP-9 expression level in AAA aortic cells (1.17±0.13) showed no statistically difference as compared with that without treatment (1.013±0.24) ($p>0.05$) (Figure not shown).

Healthy VSMCs treated with different concentration of CSE (e-cigarette and normal cigarette) and e-juice. It showed that 6% of e-cigarette induced significant COX-2 protein than that without treatment. (1.92±0.11vs1.03±0.02) (*$p<0.05$*). Same concentration of e-cigarette induced similar upward trend of MMP-2 protein expression, and reached statistically significant. There was no impact of MMP-9 expression in all treatments. Other harmful concentration of normal cigarette and e-juice incubation did not exhaust any impact of the COX-2, MMP-2 and MMP-9 protein expression level.

In brief, CSE induces COX-2 and MMP-2 protein synthesis in aneurysmal cells. Cell extracts, prepared as described in materials and methods, were fractionated on a 10% SDS-PAGE gel and blotted with COX-2-specific antibodies. CSE induces COX-2 in aneurysmal cells treated with 24 hours with increasing concentrations of CSE in a dose-dependent manner. CSE also induced COX-2 protein in healthy VSMCs and no statistical different with that of aneurysmal cells. So, the AAA disease did not pre-dispose any pathological impact in aortic cells under CSE treatment.

e) q-PCR result for COX-2, MMP-2 and MMP-9

CSE increased COX-2 and MMP-2 mRNA levels in human aneurysmal cells and healthy VSMCs. These data revealed that following aneurysmal cells exposure to 6% CSE of e-cigarette with a trend of up-regulation of COX -2 and MMP-2 mRNA levels, but did not reach statistically significant. These observations supported the Western blot results with a significant increase in COX-2 protein after CSE exposure. There was no similar upward trend of MMP-9 mRNA detected levels found in the treated cells.

f) Inflammation status and matrix degradation of CSE treated cells by Enzyme-linked immunosorbent assay (ELISA)

The supernatants of the treated cells were collected for studying the secretory level of pro-inflammatory cytokines PGE_2, MCP-1, TNF-α and IL-1β and matrix degradation enzymes, Matrix Metalloproteinases (MMP-2 and -9) in VSMCs explant cultured from human aortic vascular smooth cell line after incubation of Cigarette Smoke Extract (CSE) (either from e-cigarette or normal cigarette) and e-juice without combustion for 24 hours by Enzyme-linked immunosorbent assay (ELISA).

*Table 4 showed the detected levels of PGE_2, MCP-1, TNF-α, IL-1β, MMP-2 and MMP-9 (mean± S.E.M.) in the VSMCs with different treatments (*p<0.05).*

	e-cigarette		**normal cigarette**		**e-juice**	
	0%	**6%**	**0%**	**13%**	**0%**	**6%**
PGE_2	150.2	*253.2	90.26	*320.5	100.15	*190.3
pg/mL	±20.2	±11.5	±3.2	±16.8	±35.1	±23.5
MCP-1	90.2	100.5	103.5	110.5	89.68	90.5
pg/mL	±23.2	±11.5	+25.5	+30.5	+25.2	+33.1
TNF-α	50.22	62.2	49.81	50.5	66.2	59.23
pg/mL	+11.5	+18.5	+21.5	+31.2	+11.5	+21.5
IL-1β	1.02	0.86	2.62	0.91	0.80	4.33
pg/mL	±0.36	±0.8	±1.44	±0.2	±0.5	±0.25
MMP-2	89.42	*13.74	113.05	*72.45	118.27	*53.54
ng/mL	±28.64	±6.87	±10.37	±1.3	±26.91	±10.15
MMP-9	90.12	68.92	68.36	66.8	77.5	80.5
ng/m\L	±6.25	±35.2	±23.51	±30.5	±15.6	±10.5

Cigarette smoke extract (CSE) of e-cigarette induced inflammatory mediators' and matrix degradation enzymes (MMPs) secretion in the supernatant of explant culture cells. All cells were treated with the harmful concentrations of CSE for 24 hours, and supernatants were harvested and analyzed for detective levels. Results were presented as pg/mL or ng/mL ± S.E.M. Experiments were done in duplicate of 6-8 surgical samples. *$P < 0.05$ indicates statistical significance between cells treated with CSE and

e-juice relative to cells incubated with media alone. There were significantly more PGE_2 secretion and less MMP-2 detected in the cultured cells upon harmful concentration of CSE from e-cigarette and normal cigarette or e-juice treated cells.

g) <u>Expression of inflammatory macrophage was tested in the treated cells using immunohistochemical (IHC) staining</u>.

There was no detected macrophage in the aneurysmal cells treated with 6% e-cigarette, 13% normal cigarette and 6% e-juice without combustion.

h) <u>Matrix degradation by studying their elastin degradation levels with elastin zymography</u>.

The zymography analysis showed that harmful concentration of CSE (from e-cigarette, normal cigarette and e-juice) treated cells exerted high elastic degradation activity and MMP-2 enzyme was used as a positive standard to degrade elastin in the analysis as showed in Figure 3.

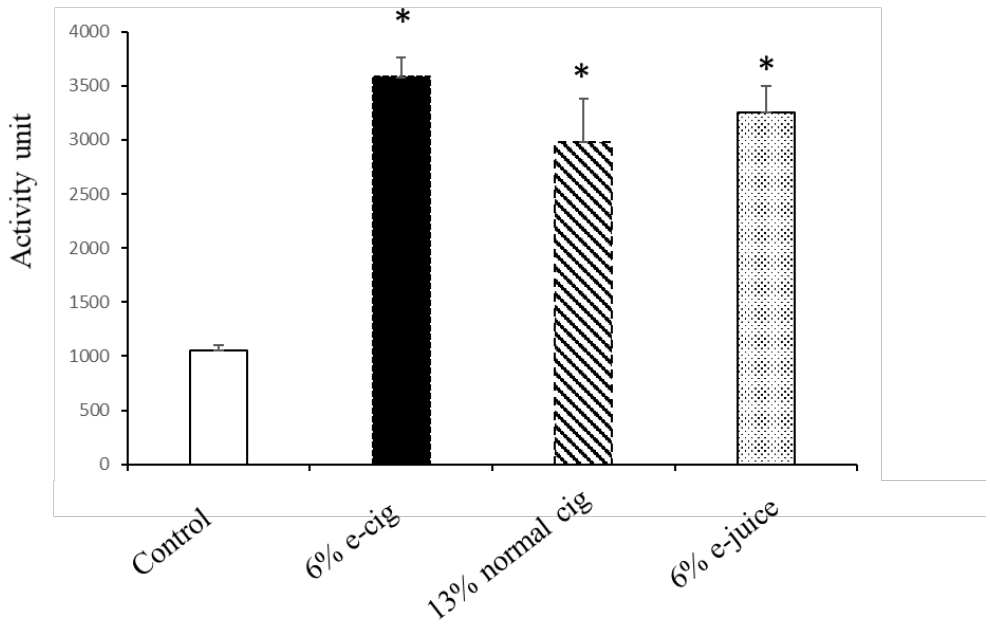
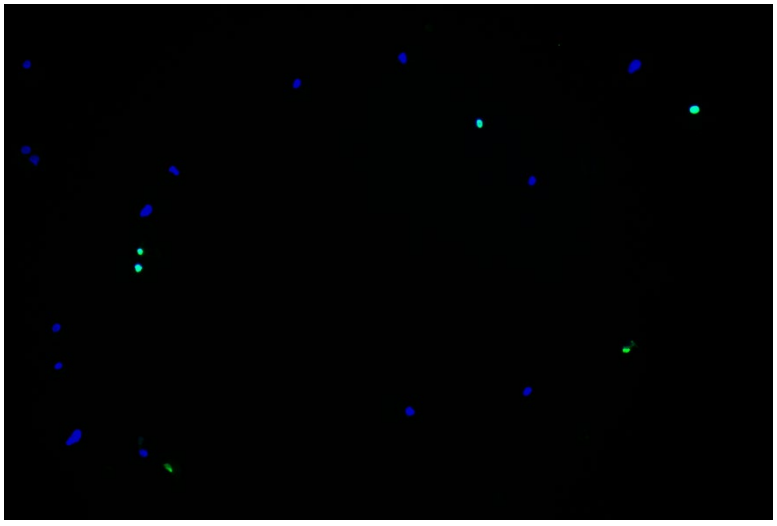


Figure 3 showed the activity unit of elastin degradation of the protein extract of 6% CSE of e-cigarette (e-cig), 13% CSE of normal cigarette (normal cig) and 6% of e-juice

*treated cells as compared with cultured cells without any treatment (negative control), *p<0.05. (n=8).*

i) Apoptotic cell death of AAA explant culture and normal cells with TUNEL method

The apoptotic cells detected in the harmful concentration of concentration of CSE from 6% of e-cigarettein TUNEL analysis was showed in Picture 2 The percentage of green fluorescence positive cells (apoptotic cells) s all cells after CSE (e-cigarette and normal cigarette) and e-juice treatment was shown in Table 3.



Picture 2 showed a representative green fluorescence image of apoptosis taken under microscope after aneurysmal cells treatment with 6 % CSE of e-cigarette after 24 hours.

A) AAA explant cultures

	Concentration			
	1%	**3%**	**6%**	**13%**
e-cigarette (EC)	6.98±1.5%	8.34±3.2%	9.68±1.5%*	NA
Normal cigarette (NC)	N.A.	3.24±1.1%	3.35±2%	5.03%
e-juice (EJ)	2.96±1.5%	3.53±2%	2.71±3%	NA

B) Normal VSMCs

	Concentration			
	1%	**3%**	**6%**	**13%**
e-cigarette (EC)	5.56±2%	4.44±1.5%	4.76±1%	NA
normal cigarette (NC)	NA	0.1±0.05%	0.88±0.1%	2.56±1.2%*
e-juice (EJ)	1.82±1%	1.67±1%	1.66±0.5%	NA

Table 4 showed the quantification of apoptotic cells deaths upon different treatments (percentage of cell death of total number cells) detected by TUNEL method in A) AAA explant culture cells and B) healthy VSMCs. Results represent the mean ± SEM of at least 3 independent experiments. N.A, because all cell died with high CSE concentration treatment.

The following experiments result addressed Aim 3: To delineate the underlying mechanism of e-cigarette smoking and its impact on human aortic VSMCs:

Q-PCR analysis was performed to quantify the 3 target microRNA expression levels in all treatment. However, there were no statistically difference in terms of their expression levels as compared with the cells without any treatment. The harmful impact of e-cigarette, normal e-cigarette smoking and e-juice was found to independent on the specific microRNAs which was previously found to play important role in the pathological pathway in AAA disease.

j) Statistical analysis

Statistical significance was determined by using Student's paired two-tailed t test where $P < 0.05$ indicates statistical significance between tested samples.

Policy implications and recommendations

i) Policy implications

E-cigarette use is popular among youth and adults, despite limited evidence about the long-term risks or benefits. Regulatory policies that strike a balance between the potential benefit of these devices for adult smokers who are trying to quit smoking with reducing the appeal and addictive potential for youth are critically needed.

Our scientific examination of the direct harm of e-cigarette on vascular disease cells and hence vascular health does support the legislation to ban e-cigarette entirely for the cessation of e-cigarette smoking. The use of e-cigarette does not pose benefits to youth and adults, and data our indicated that the use of e-cigarette and its liquid without combustion could lead to the same harmful use of normal cigarette. Thus, the new legislation can ensure on youth access include e-cigarette is intended to protect youth from exposure to e-cigarette, which may lead to addiction and vascular health issues.

Also, our normal cigarette data could provide significance evidence to influence the government to consider further policy decision or refinement in the smoking issue to safeguard the public health.

There is still a heated debate of the drastic change of the new banning e-cigarette policy adopted by the Hong Kong SAR government last year. Our data are supportive to the execution of this new e-cigarette banning policy and further implicate the potential risks of e-cigarette should not be underrated for deciding the relevant smoking policy. The public should not regard e-cigarette as a healthier alternative for normal cigarette smoking. Also, it should not state that it can help for smoking cessation.

Because the use of e-cigarette is recent, there is only one longitudinal data (Berlowitz et al., 2022) available to show the critical issues related to it, including the likelihood of addiction and other health problems for users and those passively exposed. The long-term risks have not yet been identified because this would require monitoring users for years.

Also, some of the flavorings used in e-cigarette liquid are generally recognized as safe for ingestion as food, our data implicated it is not safe as it is claimed. So, the health effects of their inhalation are generally unknown now and cannot be neglected. Further research on this issue is still urgently needed.

ii) Recommendations

World widely, e-cigarette has been proposed by some as a harm-reduction strategy and as a tool for smoking cessation, but our data do not support e-cigarette for harm reduction. These findings provide solid evidences to highlight the urgent need for local and other countries to move toward an accelerated implementation of a set of strong smoking control practices, thus curbing the burden of smoking-attributable diseases and deaths. The policy recommendations are particularly important as they affect the use of e-cigarette by youth and adults. The efforts to prevent their use by young people are needed.

To achieve the goals of minimizing e-cigarette smoking specifically among the youngsters, the research, the stakeholders must work together, which means working with individuals and families, civic and community leaders, public health and health care professionals, e-cigarette manufacturers and retailers, voluntary health agencies and researchers. Our results explained what will happen about e-cigarette on vascular

cells, hence vascular health and has reviewed the recent policy options. However, the gaps in scientific evidence still exist, and as the patterns of its use and relevant policies may be changing quickly. Prompt scientific, policy and preventive measure research to reduce the public health threat posed by e-cigarette use among young people should be performed. In other words, further research to understand the long-term health effects in vascular aspect and to accelerate policies and programs that can reduce e-cigarette use among young people are required. It highlights the need to implement proven strategies that will prevent potentially harmful effects of e-cigarette use among young people.

Based on the direct harmful effect of e-cigarette, its liquid and normal cigarette on AAA and normal vascular cells, we recommend a comprehensive smoking control policy for the government's consideration. Includes:

- Smoking bans;
- Health warnings;
- Advertising bans;
- Revised smoking taxes.

Also, should prohibit the claim that e-cigarette is effective in smoking cessation and includes e-cigarette in public education campaigns, particularly communicating the facts that they are not "harmless water vapor," and do pollute the air. Monitoring study of smoking cessation rate for the efficacy of the new banning e-cigarette should be performed.

In addition, in the context of young people, the precautionary principle should apply. Because smoking activities may lead to significant vascular harm that is scientifically

based and certain, government shall take strong action to avoid or diminish that harm.

The government should also consider the likelihood that former smokers will again become addicted to use combustible products after being reintroduced to them again.

We hope that all the above recommendations are effective in reducing smoking prevalence. Continued research on the health effects and consequences of e-cigarette use will still be essential to inform e-cigarette product standards and smoking regulatory policies as scientific research strives to keep pace with the ever-changing landscape of e-cigarette technology.

Public Dissemination

Actually, we just finished data collection this month and aim to publish our research findings of the study in both international conferences and journals in the fields of vascular health, cigarette studies, and health promotion for the smokers. Hope that a report highlighted the interesting and impactful findings of this e-cigarette study could be conveyed to various stakeholders, including the public.

We had conducted a departmental presentation with the title of " *Assessment of the E-cigarette Impact on Vascular Health*" for students (Postgraduate and medical students) and laboratory staff on 13th OCT 2022. There was fruitful discussion, data update and sharing.

Due to the COVID-19 pandemic, there were less relevant conference available and we were not able to attend them with long quarantine period before 2023.

Now, we are also preparing the manuscript of the PPR project data for peer-reviewed

journal and aim for publication. This can disseminate our output and knowledge with policy impact to other international experts.

Conclusions

Smoking is a major public health threat for both smokers and nonsmokers. Accumulating evidence demonstrated that smoking is associated with many cardiovascular diseases (Y. Cui et al., 2021). In the recent years, the number of e-cigarette users has increased significantly worldwide because of the perception that the device serves as a healthy substitute to tobacco smoking with minimal or no harm and more appealing to young consumers. But recent studies indicated that some toxic substances are present in the e-cigarette (Cheng, 2014). Another study showed that healthy subjects have a significant increase in their heart rate, blood pressure and arterial stiffness after exposure to e-cigarette (Antoniewicz et al., 2019). So, the potential risk of consuming e-cigarette should not be underestimated.

Last year, the Hong Kong SAR government has adopted a new regulation of banning e-cigarette locally. However, there are still many unanswered questions regarding the overall safety and the direct harmful impact of this device on human vascular health. In this funded project, we elucidated the impact of e-cigarette on vascular disease aortic cells thoroughly. In addition, cigarette smoking is one of the most important and preventable risk factors for fatal abdominal aortic aneurysm (AAA) disease. Because of the complex composition of e-cigarette and normal cigarette smoke, the detailed pathophysiological impact in human vascular health are worthy to understand by our current scientific studies. In conclusion, the 3 proposed objectives of the study have been achieved successfully.

This is a first comprehensive report where changes at the cellular level of Abdominal Aortic Aneurysm (AAA patients) were evaluated for associations with functional vascular changes in the context of normal cigarette, e-cigarette (without nicotine), and liquid with e-cigarette (without nicotine) inhalation. Healthy normal aortic cells were also studied for comparison. Firstly, we analyzed the effects of CSE on the viability of patient aortic cells *in vitro* in which the relevant studies are increasing suddenly in recent years. This phenomenon showed its importance in the e-cigarette research world.. During the project period, there is a new study showed that nicotine alters vasoreactivity through endothelium-dependent and/or endothelium-independent mechanisms, leading to clinical manifestations in both cigarette smokers and e-cig users. In addition, nicotine induces vascular remodelling through its effects on proliferation, migration and matrix production of both vascular endothelial and vascular smooth muscle cells (Whitehead et al., 2021). Another newly released report showed that chronic e-cigarette use could impairs endothelial function (Mohammadi et al., 2022). Our e-cigarette study without nicotine could provide additional information on the potential harm of e-cigarette for policy makers, public and users consideration. Thus, our study plus those newly released reports provided evidence that nicotine of normal cigarette and e-cigarette exhaust harmful impact at physiological and cellular levels.

In our study, we examined the potential toxic effects of CSE from both e-cigarette and normal cigarette on aortic cells by viability assays and tested for the induction of cell death by MTT. However, MTT assay cannot differentiate between the inhibition of proliferation and the induction of cell death, so we also directly tested for the induction of cell death by TUNEL method with immunofluorescence staining. The MTT results showed that 6% of CSE obtained from e-cigarette induced significantly half of the human aortic cells died. Higher normal cigarette concentration and 6% e-juice

treatment had around 50% cell death rate.. Generally, cell death could be classified into two forms: apoptosis, also known as ‘programmed cell death' and necrosis described as uncontrolled and accidental form of cell death. Apoptosis is an energy-dependent process characterized by cell shrinkage, nuclear condensation and fragmentation, as well as and the resulting induction of inflammation in the affected tissue caused by the release of intracellular components (Fink & Cookson, 2005). Thus, our immunofluorescence TUNEL analysis showed that some of the death signalling was mediated by apoptotic pathway, and not necrosis. The outcome of CSE-induced apoptotic cell death was also highly concentration dependent. Taking together, our data proved that CSE is lethal to VSMCs.

There was higher concentration of CSE (13%) of normal cigarette exhausted similar lethal impact on aortic cells. Without hindering the taste, the cigarette company is trying to make a cigarette less harmful by reducing the percentage of toxic and carcinogenic compounds in the smoke of the cigarette. The researcher found the concentrations of suspended solids and droplets in the smoke decrease significantly if the cigarette is equipped with filter (Kollati & Mohapatra, 2021). It may explain the phenomenon that normal cigarette with filter is apparently safer for smoker to inhale. Nevertheless, we cannot neglect the fact that the CSE preparation method from e-cigarette and normal cigarette are not exactly the same as other studies.

Secondly, we focused on the impact of CSC-induced inflammation on aortic cells. The western blot and qRT-PCR results showed that similar concentration of CSE of e-cigarette induced significant inflammatory COX-2 protein with a trend of higher COX-2 gene expression in aneurysmal cells. In this case, the transcription level of COX-2 did not reflect the translation product level. Actually, the quantitative relations between

RNA and protein are still not fully understood. A research showed that the protein levels are more conserved than the mRNA levels, and that changes in transcription are associated with translational changes that exert opposite effects on the final protein level. Researchers demonstrated that the protein-to-mRNA ratio in steady state varies in a direction that lessens the change in protein levels as a result of changes in the transcript abundance. They suggested that partial buffering between transcription and translation ensures that proteins can be made rapidly in response to a stimulus. (Perl et al., 2017). This may explain our interesting result of high COX-2 protein with insignificant COX mRNA detected in e-cigarette treatment.

Then, we showed that cigarette smoke extract (CSE) of e-cigarette induced MMP-2 protein (not MMP-9) expression of human aortic cells in a dose-dependent manner, accompanied by increased MMP-2 gelatinase activity of binding activity (meaning more elastin degradation). MMP-2 (gelatinase A) is one of the ECM-degrading proteases and is shown to cleave type I collagen, elastin, and degrades collagen that has been denatured into gelatin at normal body temperature; it has also been especially recognized as a major mediator of the degradation of basement membrane, which consists mainly of collagen type IV, laminin, and proteoglycans (Li et al., 1996) (Ohnishi et al., 1998). MMP-2 and -9 are the important proteinase to weaken the aorta with elastin degradation. Interestingly, there was a significantly higher MMP-2 protein, but not MMP-9 expression in CSE treated aneurysmal cells with more elastin degradation. Longo study suggested that macrophage-derived MMP-9 and mesenchymal cell MMP-2 are both required and work in concert to produce AAA (Longo et al., 2002, Davis et al., 1998). Our explant cells are mainly VSMCs without macrophage, so it may explain the phenomena of up-regulated MMP-2, but no impact on MMP-9 in the CSE treatment. Here, we are the first to show the MMP-2 up

regulation in CSE treated aortic cells. The intracellular signaling mechanisms activated by tobacco smoke that mediating the MMP-2 function and subsequent matrix degradation in the VSMCs are not well understood.

Also, there was significant secretion of PGE_2 of the supernatant of the treated cells. The PGE_2 synthesis (approximately 3-fold) with an increase COX-2 protein levels. The data supported that COX-2 is the key enzyme responsible for the increase of its metabolite - PGE_2 with CSE and e-juice exposure. We proposed that human VSMCs, when exposed to CSE, elicited COX-2 expression with consequent prostaglandin synthesis, thus creating a proinflammatory environment and promotes aortic cells inflammation. Furthermore, there was a dramatic decrease (2-fold) in secretion of the key matrix degradation enzyme (MMP-2) in the treated cells. It may imply that there was up-regulated MMP-2 protein in the cells, and less MMP-2 secreted out from the cells. This has important implication for understanding how cigarette smoking may incite and exacerbate inflammatory process inside the cells that are critical in vascular disease pathology. Further experiments are warranted to support this hypothesis.

With public understanding, the most harmful components of cigarette smoke derive should come from the combustion process, which claims significantly reduced in the electronic cigarette aerosol, thus providing an alternative option in harm reduction strategies. However, there is no study available of the electronic cigarette juice (e-juice) impact on vascular cells. To develop a comprehensive e-cigarette study, it is therefore necessary to screen for the impact of e-juice. We found that e-juice exerts their cytotoxic effect (inflammation, apoptosis cell death and matrix degradation) in aneurysmal cells by the proposed *in vitro* assays, their impact was comparable with that of CSE from e-cigarette and normal cigarette. In addition to the variety of devices on the market, an

issue should be taken into account regards the formulation of the juices used to generate the aerosol and their toxicological effects. The e-juice consists largely of propylene glycol (PG) and vegetable glycerin (VG), and a smaller part of food chemical flavorings and nicotine (which is absent in our study) (Polosa et al., 2019). The e-juices are produced through the use of industrial processes and automated mechanical systems with metal and plastic parts and marketed in plastic containers, typically darkened to protect the nicotine which is photosensitive. This could lead to the presence of heavy metal residues, such as arsenic, lead, aluminum, iron, mercury and cadmium, or nanoplastics (NPs) and microplastics (MPs) in the juices themselves. Heavy metals contamination of e-juices could be responsible to produce oxidative species and subsequent adverse effects on consumers' health (Fu & Xi, 2020). Moreover, tissue accumulation of heavy metals leads to an imbalance and can be used as substitutes for essential elements (e.g., calcium replaced by lead, zinc by cadmium, and most trace elements by aluminum). In conclusion, these results supported the notion that there were toxicity potential of e-juice and comparable to e-cigarette and normal cigarette in an *in vitro* model resembling real life smoke exposure.

The 3 pathological processes (inflammation, matrix degradation and apoptosis) were also detected in the CSE and e-juice treated healthy VSMCs without any smoking history. The harmful impact was insignificant different of both aneurysmal cells (cultured from surgical samples of AAA patients who were mostly smokers) and healthy aortic cells. So, smoking history may not pre-dispose any potential pathological process upon CSE stimulation. And those studied pathological processes are well known to be involved in atherogenesis initiation and may contribute to vascular dysfunction *in vivo* and finally lead to vascular disease and related death.

Despite the fact that our scientific and other epidemiological evidence linking e-cigarette smoking with vascular injury and cardiovascular disease, the precise elements of e-cigarette smoke responsible for this relationship and the mechanisms by which they exert their effect have not yet been elucidated. Also, the chronic impact of e-cigarette on vascular health are still missing in the research world. Both required further investigations.

This study does inform on the promising impact of e-cigarette and normal cigarette driven vascular smooth muscle cells changes on vascular function of available AAA patients' samples. There is still an unavoidable limitation that we need to consider. Because the number of patients' aortic samples are decreasing as less open surgery were performed for this fetal disease. Recently, most AAA patients will undergo minimal invasive procedure called endovascular aortic repair (EVAR). So, less surgical samples could be collected for study purpose. Also, the explant culture is a challenge and rather difficult to perform on human disease samples successfully for further biochemical analysis. So, we could not have more patients' data to do further analysis on the confounding factors or any variate that has impact on the vascular function.

There is only e-juice without nicotine available in Hong Kong last year (before totally ban) for the current study. In earlier time, the public may regard it is a healthier alternative for smoking with normal cigarette. Thus, the purpose of this study is to identify the critical knowledge gaps regarding the effects of smoking e-cigarette without nicotine on the vascular cells and hope our result could stimulate the continued e-cigarette research on human health. Further work will be necessary to consolidate the evidence for the burning of the elements inside e-cigarette and its smoke, which may be responsible for the functional damage to aortic cells that has been observed in our

culture system. We also hope to contribute to improving the public governance of cigarette regulation in the future and aim to liaise with China centers for more aortic samples and can ultimate perform multicenter study to strengthen our results and provide evidence-based support for the policy guideline.

Substantial global effort has been devoted to curtailing the tobacco epidemic over the past two decades. At the same time, e-cigarette companies claim that e-cigarette is a healthier alternative for tobacco smoking and may help for tobacco smoking cessation. Last year, in the recognition of the potential harm resulting from e-cigarette use, a strengthened e-cigarette control was executed as a local development banning policy target. Here, we showed that the comprehensive tobacco control policies and the new e-cigarette banning legislative can be supported by our scientific evidence that e-cigarette smoking definitely harm human vascular cells. The harmful impact is comparable with that of normal cigarette smoking. E-juice without combustion is also harmful to vascular cells. These findings highlight the urgent need for all countries to move toward an accelerated implementation of a set of strong tobacco control practices, thus curbing the burden of smoking-attributable diseases and deaths.

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