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ORIGINAL ARTICLE

Third-Hand Smoke Exacerbates H_2O_2-Driven Airway Responses in A549 Cells

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ABSTRACT

Objectives: Third-hand smoke (THS) is residual smoke after extinguishing a cigarette and adhering to surfaces. Re-emission into the air also makes THS a health concern for those who suffer from respiratory diseases. The present study aimed to elucidate the mechanistic pathways involved in THS-induced respiratory toxicity and the accelerative potential of THS in an H_2O_2-induced oxidative stress model of human airway epithelia *in vitro*.

Materials and Methods: THS extracted from terrycloth exposed to 3R4F cigarettes was assessed *via* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay to identify cytotoxicity. The reactive oxygen species (ROS) level was determined *via* 2,7-dichlorofluorescein diacetate (DCFDA) fluorescence intensity in a flow cytometer, and glutathione (GSH), malondialdehyde (MDA), and catalase (CAT) activity were assessed spectrophotometrically. Interleukin-6 (IL-6) level was measured *via* enzyme-linked immunosorbent assay.

Results: THS 50% (*v/v*) with significant cytotoxicity in A549 cells upregulated intracellular ROS levels *via* a right-shifted fluorescence intensity of DCFDA compared with the control ($p < 0.05$), which was also amplified with H_2O_2 co-treatment. MDA levels remarkably increased with THS ($p < 0.05$). Both THS and THS + H_2O_2 led to notable GSH depletion, increased CAT activity, and increased IL-6 levels, which were attenuated by the negative control (N-acetylcysteine, 1 mM) ($p < 0.05$).

Conclusion: The induction of oxidative stress may be an important event in THS-induced airway toxicity that may contribute to the progression of respiratory diseases.

Keywords: Chronic airway diseases, airway inflammation, oxidative stress, third-hand smoke, cigarette

INTRODUCTION

Cigarette smoke (CS) is the main preventable cause of death worldwide, and it poses a significant health risk for both smokers and non-smokers.[1] Recently, a new toxicological concern has arisen due to the residual part of CS, third-hand smoke (THS). THS is referred to as tobacco residue and stale or aged second-hand smoke (SHS). THS is not described as rigid smoke but rather as the by products of smoking and refers to the contamination of surfaces with SHS-emitted compounds.[2] The products of chemical transformations of these constituents, and the off-gassing of volatile substances into the atmosphere, thus represent important public and environmental issues. Apart from traditional tobacco smoke, people may be exposed to THS *via* three routes: ingestion, inhalation, and dermal absorption. In particular, the most important target population is infants and toddlers residing in the homes of smokers who are vulnerable to THS because they spend more time in contact with THS-contaminated surfaces. In addition, they might indirectly be exposed to THS *via* hand-to-mouth transfer *via* contaminated objects.[3] THS has a complex chemical dynamic and consists of nicotine and tobacco-specific nitrosamines (TSNAs), which are highly carcinogenic compounds formed when tobacco burns and can react with other chemicals in the environment to create even more harmful substances, as well as volatile organic compounds (VOCs), such as benzene and formaldehyde.[4,5] Polycyclic aromatic hydrocarbons (PAHs) and heavy metals were also detected in the THS content.[6]

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Due to the inhalation of the re-released SHS-emitted compounds, the other vulnerable population might be individuals with chronic airway diseases such as chronic obstructive pulmonary disease (COPD).[7] The damage of alveolar walls characterizes COPD; in other words, its pathological basis is the injury of alveolar epithelial cells; thus, the ability of alveolar epithelial cells to proliferate is closely linked to the pathological process or prognosis of COPD.[8] COPD is considered a systemic disorder and is more common in individuals with a smoking history.[9] COPD is the most extensively studied inflammatory airway disorder induced by smoking, and its incidence rate at all stages among active smokers was > 35% over 25 years.[10] According to the latest data from the Centers for Disease Control and Prevention, COPD is usually caused by smoking, which accounts for as much as 80% of COPD-related mortality.[11] In addition, COPD remains a socioeconomic burden, especially in countries with a low sociodemographic index between 1990 and 2019.[9] Not only for COPD but also for asthma and bronchitis, THS might represent an important pre-existing factor due to the well-known effects of tobacco on these chronic airway conditions.

It is well-known that CS exposure *via* firsthand smoke or SHS exposure may harm airway epithelial cells through oxidative stress, apoptosis, necrosis, chronic inflammation, and other pathways that are not fully elucidated.[12] According to previous reports, TSNAs and other reactive chemicals can directly damage lung cells, leading to inflammation and scarring. In addition, THS exposure may induce oxidative stress, which directly disrupts lung function and may promote further inflammation in target organs. The amplified inflammatory response may also contribute to the immune response in the respiratory system and trigger chronic inflammation in the airways, leading to thickening and narrowing of the bronchial tubes, which is a hallmark of COPD. Moreover, tobacco smoke can induce epithelial and mucus dysregulation due to damage to the lining of the airways, leading to airway obstruction and difficulty breathing.[5,13-15] The precise contributions of each mechanism are still being studied; thus, THS may worsen symptoms and accelerate the progression of pre-existing respiratory conditions like COPD, asthma, and bronchitis, due to the well-known detrimental effects of tobacco on the respiratory system. Several experimental studies have revealed that THS exposure in mice made the alveolar walls in terminal respiratory bronchioles thicker with increased proinflammatory cytokine levels in lung tissues than in non-exposed animals.[13,15] In addition, THS toxins as a mixture exerted dose-dependent cytotoxicity in A549 human lung epithelial cells, mainly due to the presence of acrolein, phenol, and 2,5-dimethylfuran content.[5] In light of these limited findings, THS exposure may cause a pro-inflammatory and oxidative environment in the lungs, which increases the risk of disease progression in chronic airway diseases. It is known that when free radicals overpower antioxidants, the present oxidative imbalance may lead to stress that damages cells, proteins, and DNA, leading to various pathologies, including chronic airway diseases. This oxidative cascade also contributes to mucus hyperproduction, tissue remodeling, inflammation, airway hyperresponsiveness, and tissue damage such as fibrosis and scarring in the lining of respiratory epithelia.[16-18] The present study aimed to elucidate the mechanistic pathways involved in THS-induced respiratory toxicity and the accelerative potential of THS in an H_2O_2-induced oxidative stress model of human airway epithelia *in vitro*.

MATERIALS AND METHODS

Materials

3R4F research cigarettes were obtained from the University of Kentucky (Lexington, Kentucky, USA). The cell culture chemicals including Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, and penicillin-streptomycin antibiotics were obtained from Gibco (USA). Other chemicals, including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), nicotine standard, DTNB-Ellman's reagent 5,5'-Dithiobis (2-nitrobenzoic acid) (#D8130), sodium bicarbonate (#S6014), cobalt (II) nitrate hexahydrate, and catalase (CAT) enzyme (#C9322), were purchased from Sigma-Aldrich (USA). The kit used for the analysis of cellular reactive oxygen species (ROS) (ab113851) was from Abcam (Germany) and the analysis of interleukin-6 (IL-6) level was assessed *via* the human IL-6 Enzyme-Linked Immunosorbent Assay (ELISA) kit (Elabscience, E-EL-H6156, USA).

Methods

Extraction of THS

THS was extracted from a terrycloth that was manually exposed to 2 of 3R4F research cigarettes (11.0 mg total particulate matter/cigarette; 9.4 mg tar/cigarette; 0.73 mg nicotine/cig, and 12 mg CO/cigarette) in a polystyrene chamber according to the International Organization for Standardization British Standards Institution (ISO BSI 10993-12),[19] with a slight modification in the number of cigarettes used in our previous study.[20] According to the previously mentioned method, once the smoke exposure in the chamber ceased, the mainstream and sidestream smoke was mixed with the minivan in the chamber for 5 minutes, and the exposed terrycloth was extracted in DMEM at 37 °C for 24 hours. The standardization of THS was achieved through the weighing of the tar of different filter papers used for THS batches according to the method of Martins-Green et al.[14]

Nicotine level in THS

The nicotine content of the prepared THS was assessed by liquid chromatography-mass spectrometry (LCMS, Agilent1260 Infinity II, USA) equipped with a solvent pump, manual injection valve, and diode array detector, as described previously in detail.[21] Six dilutions of nicotine standard were used to calculate the equation and the R^2 value (Supplementary Figure S1 and Supplementary Table 1). The area of the standard peaks was calculated according to the mass values.

Cytotoxicity

A549 human lung epithelial-like cells (ATCC, CCL-185™) were used in cell culture studies. For this purpose, the cells were seeded in a 96-well plate and exposed to different concentrations

of THS (12.5-100%, *v/v*) diluted with DMEM for 24 hours. Since previous data reported increased H_2O_2 levels in expired breath condensates of patients with COPD,[22] and oxidative stress contributes to the development and progression of chronşc airway diseases through numerous pathways, such as mucus hyperproduction, tissue remodeling, and inflammation, *in vitro* oxidative stress in the respiratory system was demonstrated by the co-exposure of H_2O_2 (100 µM) in A549 cells. As a negative control, a 2-hour pretreatment with N-acetylcysteine [(NAC), 1 mM] was used in all studies to observe its ameliorative effects against THS itself and under H_2O_2-driven oxidative stress conditions. After the incubation period, cytotoxicity was assessed using the MTT assay, as previously described.[21]

Oxidative stress

Glutathione (GSH) level

GSH levels were measured in cell lysates according to our previous study.[23] The exposed cell lysate prepared in phosphate-buffered saline (PBS) was mixed with DTNB and then mixed with ethylenediaminetetraacetic acid (EDTA) buffer solution (pH 8.2). After incubation at 37 ˚C in the dark for 30 minutes, the absorbance of the yellow chromophore was measured at 412 nm spectrophotometrically (ThermoScientific, Finland). The results are expressed as µmol/g protein GSH, and each measurement was performed in duplicate.

CAT activity

CAT activity was measured using the correlation between the carbonatocobaltate (III) complex and the CAT enzyme. Briefly, the cell lysate was mixed with H_2O_2 as described in our previous study[21] and incubated at 37 ˚C for 2 minutes. Subsequently, a solution containing phosphate buffer (pH 7.4), sodium bicarbonate, and cobalt (II) nitrate hexahydrate was added to the mixture and vortexed. The reaction tubes were kept in the dark for 10 minutes, and absorbances for the kinetic reaction were recorded at 440 nm spectrophotometrically for 2 minutes in duplicate. CAT activity was expressed as U/mg protein.

ROS level

The oxidative effect of THS in H_2O_2-induced A549 cells was assessed using a cellular ROS assay kit *via* flow cytometry. As described previously,[23] under oxidative conditions, 2,7-dichlorofluorescein diacetate (DCFDA), a fluorescence-sensitive dye, is deacetylated by cellular esterases and forms a non-fluorescent compound, which is oxidized into 2,7-dichlorofluorescein by the produced ROS. Briefly, cells pre-treated with NAC/treated with THS/treated with THS + H_2O_2 were collected from 12-well plates and harvested in PBS solution, followed by 20 mM DCFDA addition to each flow cytometer tube in the dark for 30 minutes at 37 ˚C with 5% CO_2. Cells pre-treated with the medium were used as a negative control, whereas the group exposed to 100 µM of *tert*-butyl hydroperoxide (THBP) for 4 hours was used as the positive control (PC). Data were analyzed in triplicate, and intracellular ROS levels were expressed as relative ROS content compared to the PC.

IL-6 level

The inflammatory response induced by THS exposure in A549 cells co-treated with H_2O_2 was determined using the proinflammatory cytokine IL-6 release as determined using a human IL-6 ELISA kit according to the manufacturer's protocol as previously.[24] Cell supernatants were used to detect IL-6 release in A549 cells, and each group was evaluated in duplicate. The results are expressed as pg/mL.

Statistical analysis

The results of each experiment were analyzed with One-Way ANOVA and Tukey's post hoc tests. The statistical significance was accepted as $p < 0.05$ and determined by GraphPad Prism 9.0 Software (LaJolla, California, USA).

RESULTS

Nicotine level in THS

According to the LCMS analysis of the THS extract prepared from 2 of 3R4F cigarettes (100%, v/v), the nicotine concentration of samples was recorded as 0.287 mg/mL (Supplementary Table 2) according to the prepared nicotine standard calibration curve. The mass spectrums and chromatograms of nicotine standards were also given in Supplementary Figure S2-S7, and for THS sample, given as Supplementary Figure S8.

Cytotoxicity

The cytotoxicity of THS and its co-exposure to H_2O_2 *via* the MTT assay showed that THS induced dose-dependent cytotoxicity in A549 cells, remarkably at 50-100% (*v/v*) ($p < 0.05$) (Figure 1A). Based on this finding, THS 50% (*v/v*) (approximate IC_{50} value) was selected for further assessments of the cytotoxicity of THS under respiratory oxidative conditions *in vitro*.

As a step to assess the exacerbative effect of THS under respiratory oxidative conditions, A549 cells were co-exposed to a selected dose of THS (50%, *v/v*) and 100 µM H_2O_2. The results showed that THS significantly reduced cell viability under oxidative conditions compared with the control group ($p < 0.001$). On the other hand, 2 hours of pre-treatment with a potent antioxidant, NAC (1 mM), a powerful antioxidant, significantly reduced the cytotoxicity induced by THS and H_2O_2 alone, as well as their combination (Figure 1B).

Respiratory oxidative damage by THS in H_2O_2-stimulated cells

The oxidative stress conditions of A549 cells exposed to THS, H_2O_2, and THS + H_2O_2 after pre-treatment with NAC indicated the antioxidant capacity of NAC in the present study. According to our findings, THS led to a significant increase in oxidative stress by reducing GSH levels and elevating CAT activity ($p < 0.01$) (Figure 2). In parallel, lipid peroxidation was significantly increased by THS exposure. The oxidative effect of H_2O_2 exposure used in the *in vitro* modeling of COPD notably upregulated CAT activity and MDA levels, which were also elevated with co-exposure. In addition, pre-treatment with NAC significantly ameliorated the oxidative responses of THS alone and under COPD conditions induced by H_2O_2 (Figure 2).

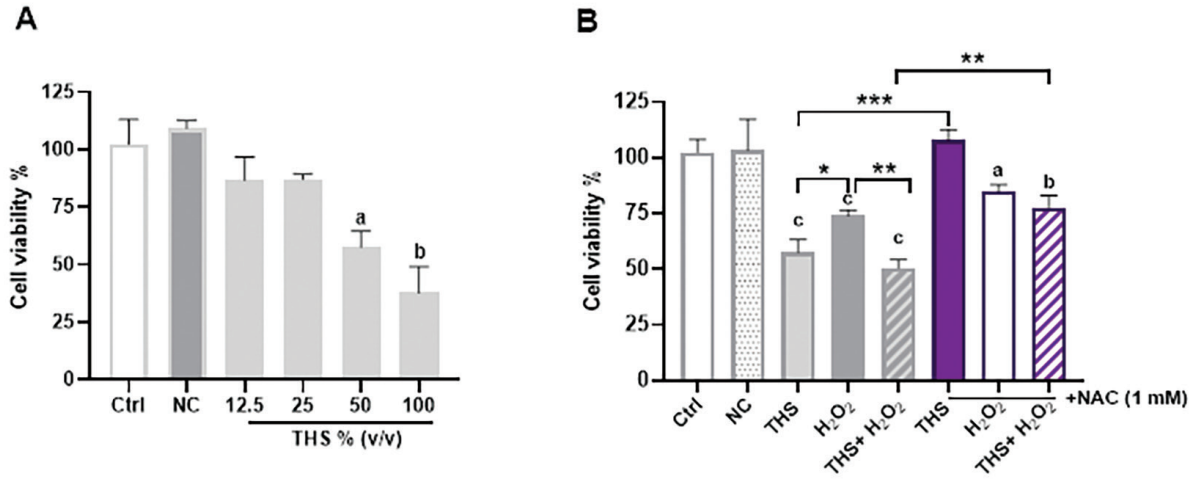

Figure 1. Cell viability of A549 cells exposed to THS with or without H_2O_2. A) Dose-dependent cytotoxicity profile of THS; B) Statistical significance between the Ctrl and groups $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$; the significance between the two groups $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$. NAC: (1 mM) applied a 2-hour pre-treatment; THS: extract (50%, *v/v*); H_2O_2: 100 µM. The data were shown as mean ± SD

NAC: N-acetylcysteine, THS: Third-hand smoke, SD: Standard deviation

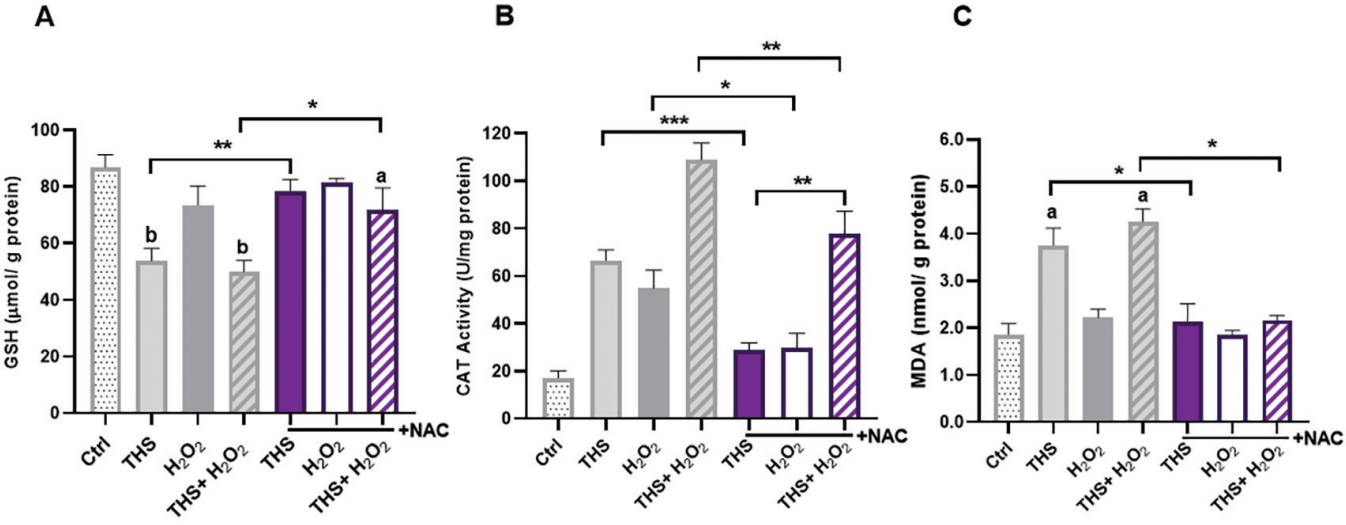

Figure 2. Modulation of oxidative stress and lipid peroxidation by THS and co-exposure with H_2O_2. A) Total GSH level; B) CAT activity; C) MDA level in A549 cells co-treated with THS and H_2O_2. Statistical significance between the Ctrl and groups $^{a}p < 0.05$, $^{b}p < 0.01$; the significance between the two groups $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$. NAC: (1 mM) applied as 2 hours pre-treatment; THS: extract (50%, *v/v*); H_2O_2: 100 µM. The data were shown as mean ± SD

NAC: N-acetylcysteine, THS: Third-hand smoke, SD: Standard deviation, GSH: Glutathione, CAT: Catalase, MDA: Malondialdehyde

Intracellular ROS levels

In the present study, total ROS levels were measured in A549 cells exposed to THS alone or in combination with H_2O_2. Based on our findings, THS exposure itself significantly elevated intracellular ROS production ($p < 0.05$) compared with the control group in A549 cells. Furthermore, this increase in ROS levels was intensified by co-exposure to H_2O_2 ($p < 0.01$) (Figure 3) Similar to the results of the oxidative stress assays, NAC pretreatment improved the elevated ROS production levels and decreased the relative ROS levels significantly in all THS-exposed groups ($p < 0.01$), possibly due to the replenishment of intracellular GSH deposits.

IL-6 level

The present findings revealed that residual THS exposure and its co-exposure to H_2O_2 significantly induced IL-6 release in A549 cells, and this response declined after NAC pre-treatment (Figure 4). The inflammatory response of the THS co-exposed COPD group was notably higher than that of the THS alone group despite NAC pre-treatment.

DISCUSSION

THS, a recent concept in environmental toxicology, can attach to surfaces for long periods, even after the smoke has cleared. Children are particularly susceptible to THS exposure because

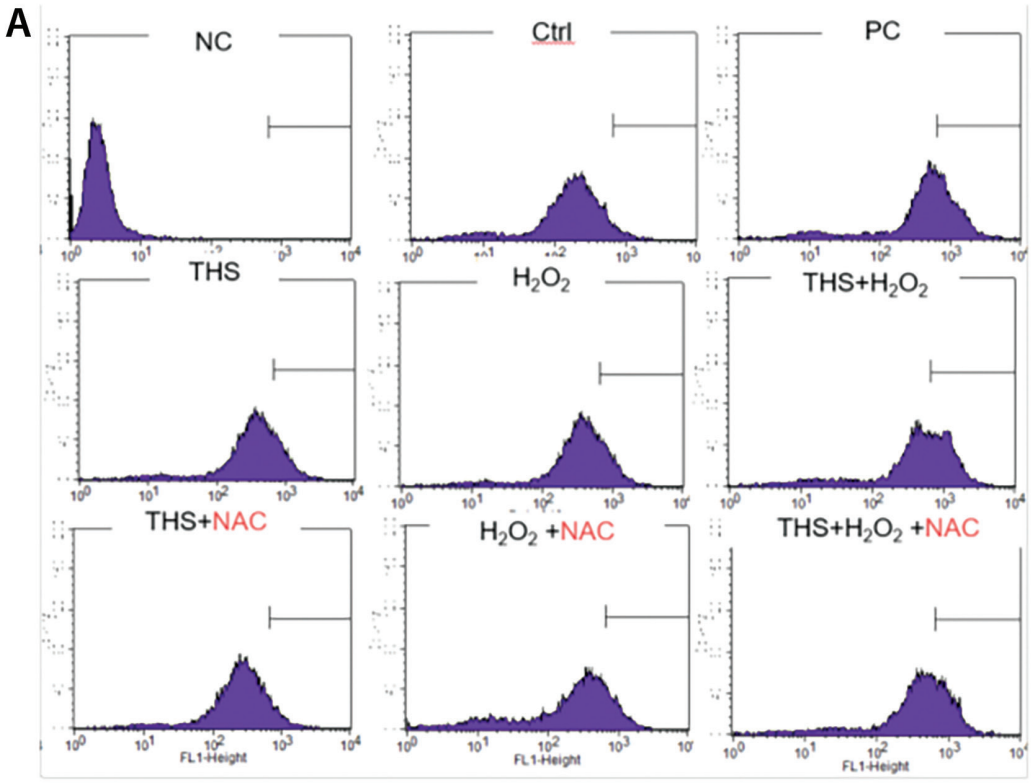

Figure 3. Intracellular ROS levels of A549 cells exposed to THS with or without H_2O_2. A) Representative histograms of the percentage increase in ROS accumulation in the groups. Enhancement of intracellular ROS levels was observed *via* the shift of the signal curve obtained for the THS and H_2O_2 treated cells to the right compared with that of the control. B) Relative ROS percentage of A549 cells exposed to THS with or without H_2O_2. Statistical significance between Ctrl vs. groups $^{a}p < 0.01$; $^{b}p < 0.05$; the significance between the two groups $^{*}p < 0.05$; $^{**}p < 0.01$. NAC: (1 mM) applied as a 2 hours pre-treatment; NC: Cells *w/o* DCFDA; THS: extract (50%, *v/v*); H_2O_2: 100 µM; THBP (100 µM)

THS: Third-hand smoke, PC: Positive control, NAC: N-acetylcysteine, ROS: Reactive oxygen species, DCFDA: 2,7-dichlorofluorescein diacetate, THBP: Tert-butyl hydroperoxide, NC: Negative control

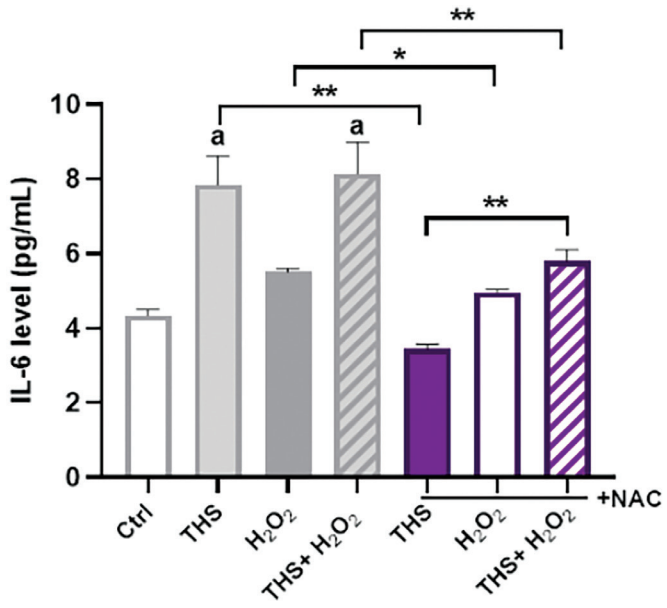

Figure 4. IL-6 release induced by THS and its co-exposure to H_2O_2 in A549 cells. Statistical significance between Ctrl vs. groups $^{a}p < 0.05$; the significance between the two groups $*p < 0.05$; $**p < 0.01$. NAC: (1 mM) applied a 2-hour pre-treatment; THS: extract (50%, *v/v*); H_2O_2: 100 µM. The data were shown as mean ± SD

NAC: N-acetylcysteine, THS: Third-hand smoke, IL-6: Interleukin-6, SD: Standard deviation

they are more likely to come into contact with smoke-embedded surfaces. Although parental precautions might protect this population against residual toxins, people with respiratory conditions, such as asthma and COPD, are still at increased risk of health problems due to involuntary THS exposure. In the present study, we have investigated the accelerative potential of THS in an H_2O_2-induced oxidative stress model of human airway epithelia *in vitro*.

Based on our findings, the present study showed that THS exposure can lead to cytotoxicity, oxidative stress, and an elevated proinflammatory response in human airway epithelia *in vitro*. Increased inflammation and oxidative stress due to tobacco smoke exposure either as second-hand or first-hand smoke are important indicators of chronic airway diseases in general and IL-6 plays a role in several inflammatory and immune responses, including the acute phase response, response to infections, and development of chronic diseases, such as rheumatoid arthritis and COPD.[25-27] In COPD, IL-6 levels are elevated in the blood and airways, contributing to the development and progression of COPD by promoting inflammation, impairing lung function, and increasing the risk of disease exacerbations.[26,27] Based on this context, exposure to THS may be a significant issue that must be addressed for people with respiratory diseases. Recent studies have shown that dose-dependent THS exposure decreases cell survival in human dermal fibroblasts, mouse neural stem cells, human palatal mesenchyme,[4] human hepatocellular carcinoma cells,[28] and male rodent reproductive cells.[29] In the present study, THS extracts reduced the viability of A549 cells, similar to previously reported data,[4,30,31] dose-dependently. Therefore, the complex chemical content of THS may lead to cytotoxicity in human airway epithelia, mostly *via* mitochondrial cell viability. In addition to its cytotoxic potential, co-exposure to H_2O_2 further increased the cytotoxicity response in the human airway epithelia. Moreover, it is well known that intracellular increases in the production of ROS are important parameters for evaluating the imbalance between the oxidative response and the body's ability to neutralize them. Since oxidative stress is believed to play a role in the development and progression of COPD and other respiratory disorders, detecting ROS levels might represent a preliminary marker for further lung tissue damage, which may lead to inflammation and impaired lung function.[12] Based on this finding, THS may interact with other environmental or exogenous oxidant factors to increase its toxicity. Moreover, THS induced the production of intracellular ROS, CAT activity, and depleted GSH deposits, which help detoxify ROS in A549 cells. In addition, THS increased lipid peroxidation, as well. However, these oxidative stress-induced toxicity responses were significantly alleviated by NAC pretreatment. Previously, Boskabady and Gholami Mahtaj[30] reported that the CS-induced COPD model in guinea pigs led to a significant decrease in the level of the thiol group in experimental animals, which was reversed by carvacrol pretreatment by boosting intracellular antioxidant capacity. As reported in our previous study,[21] exposure to CS and its components may lead to an excessive increase in CAT activity in the target organ, probably due to the presence of higher peroxide concentrations. In addition, the decreases in GSH and increased MDA levels in A549 cells clearly indicate that antioxidant defense mechanisms are not sufficient to prevent lipid peroxidation due to THS exposure either alone or in combination with H_2O_2. Similarly, *in vitro* studies using CS suggested a significant deposition of intracellular antioxidant enzymatic/non-enzymatic capacity[23] as well as an elevated inflammatory response.[23,31,32] It has been reported that overproduced free radicals react with cellular and humoral components, permanently impairing their function and triggering inflammatory responses.[33] However, there are limited studies on the residual portion of CS, such as THS, and its possible inflammatory and oxidant potency in respiratory disease conditions. In addition, the exposure and extraction conditions of THS might change the severity of these detrimental effects on the respiratory system under oxidative conditions. In the present study, the prepared THS extract had a nicotine level of approximately 0.28 mg/mL (0.28 mg/g fabric), whereas samples extracted from indoor or outdoor surfaces or under different extraction conditions might change this nicotine level dramatically from 2-12 g nicotine/g fabric.[4] Therefore, the observed toxicity outcomes in the literature might differ in proportion to the accumulation and extraction capacity of other expected chemicals present in THS such as VOCs, TSNAs, and PAHs.

THS in the environment might contribute to the inflammatory and oxidative complications of chronic airway conditions initially mediated by oxidative stress. It is known that the inhalation of

PAHs and various VOCs is responsible for oxidative damage and inflammation through the induction of mitochondrial free radical formation.[34] Furthermore, a major concern may arise due to environmental THS exposure because smoking with COPD has the highest correlation with all types of lung cancer and the development of small-cell lung cancer.[34,35] Therefore, as an indirect source of CS, THS might be a significant contributing risk factor for lung cancer development in this population. As a result, the present preliminary findings are important for elucidating the toxicological key points involved in cell survival/death in target organs with respiratory disease due to THS exposure.

CONCLUSION

Based on the present findings, co-exposure to THS may lead to more detrimental effects in human airways *in vitro*. Hence, it can be concluded that as an environmental residue, THS may play a role in the progression of chronic respiratory diseases, which are mediated through oxidative and inflammatory exacerbations in human airway epithelia. Further studies are needed to confirm these mechanisms by which THS is exerted and to help identify approaches to reduce environmental THS exposure.

Acknowledgment

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Ethics

Ethics Committee Approval: Not required.

Informed Consent: Not required.

Authorship Contributions

Concept: R.R., K.K., Design: R.R., K.K., Data Collection or Processing: R.R., K.K., G.P.C., Analysis or Interpretation: R.R., K.K., Y.Ö., G.P.C., H.S., Literature Search: R.R., K.K., Writing: R.R., H.S.

Conflict of Interest: No conflict of interest was declared by the authors.

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REFERENCES

1. Acuff L, Fristoe K, Hamblen J, Smith M, Chen J. Third-hand smoke: old smoke, new concerns. J Community Health. 2016;41:680-687.
2. Matt GE, Quintana PJ, Destaillats H, Gundel LA, Sleiman M, Singer BC, Jacob P, Benowitz N, Winickoff JP, Rehan V, Talbot P, Schick S, Samet J, Wang Y, Hang B, Martins-Green M, Pankow JF, Hovell MF. Thirdhand tobacco smoke: emerging evidence and arguments for a multidisciplinary research agenda. Environ Health Perspect. 2011;119:1218-1226.
3. Liu H, Chen H. The effects of thirdhand smoke on reproductive health. J Appl Toxicol. 2022;42:172-179.
4. Bahl V, Shim HJ, Jacob P 3rd, Dias K, Schick SF, Talbot P. Thirdhand smoke: Chemical dynamics, cytotoxicity, and genotoxicity in outdoor and indoor environments. Toxicol *In Vitro.* 2016;32:220-231.
5. Jacob P 3rd, Benowitz NL, Destaillats H, Gundel L, Hang B, Martins-Green M, Matt GE, Quintana PJ, Samet JM, Schick SF, Talbot P, Aquilina NJ, Hovell MF, Mao JH, Whitehead TP. Thirdhand Smoke: New Evidence, Challenges, and Future Directions. Chem Res Toxicol. 2017;30:270-294.
6. Matt GE, Quintana PJE, Hoh E, Dodder NG, Mahabee-Gittens EM, Padilla S, Markman L, Watanabe K. Tobacco smoke is a likely source of lead and cadmium in settled house dust. J Trace Elem Med Biol. 2021;63:126656.
7. Zhang J, Lin XF, Bai CX. Comparison of clinical features between non-smokers with COPD and smokers with COPD: a retrospective observational study. Int J Chron Obstruct Pulmon Dis. 2014;9:57-63.
8. Yoshida M, Minagawa S, Araya J, Sakamoto T, Hara H, Tsubouchi K, Hosaka Y, Ichikawa A, Saito N, Kadota T, Sato N, Kurita Y, Kobayashi K, Ito S, Utsumi H, Wakui H, Numata T, Kaneko Y, Mori S, Asano H, Yamashita M, Odaka M, Morikawa T, Nakayama K, Iwamoto T, Imai H, Kuwano K. Involvement of cigarette smoke-induced epithelial cell ferroptosis in COPD pathogenesis. Nat Commun. 2019;10:3145.
9. Safiri S, Carson-Chahhoud K, Noori M, Nejadghaderi SA, Sullman MJM, Ahmadian Heris J, Ansarin K, Mansournia MA, Collins GS, Kolahi AA, Kaufman JS. Burden of chronic obstructive pulmonary disease and its attributable risk factors in 204 countries and territories, 1990-2019: results from the Global Burden of Disease Study 2019. BMJ. 2022;378:e069679.
10. Crotty Alexander LE, Shin S, Hwang JH. Inflammatory diseases of the lung induced by conventional cigarette smoke: a review. Chest. 2015;1481307-1322.
11. CDC. What is COPD? Accessed at: https://www.cdc.gov/copd/about/index.html
12. Jiao Z, Zhang Q, Chang J, Nie D, Li M, Zhu Y, Wang C, Wang Y, Liu F. A protective role of sulforaphane on alveolar epithelial cells exposed to cigarette smoke extract. Exp Lung Res. 2013;39:379-386.
13. Schweitzer KS, Hatoum H, Brown MB, Gupta M, Justice MJ, Beteck B, Van Demark M, Gu Y, Presson RG Jr, Hubbard WC, Petrache I. Mechanisms of lung endothelial barrier disruption induced by cigarette smoke: role of oxidative stress and ceramides. Am J Physiol Lung Cell Mol Physiol. 2011;301:836-846.
14. Martins-Green M, Adhami N, Frankos M, Valdez M, Goodwin B, Lyubovitsky J, Dhall S, Garcia M, Egiebor I, Martinez B, Green HW, Havel C, Yu L, Liles S, Matt G, Destaillats H, Sleiman M, Gundel LA, Benowitz N, Jacob P 3rd, Hovell M, Winickoff JP, Curras-Collazo M. Cigarette smoke toxins deposited on surfaces: implications for human health. PLoS One. 2014;9:e86391.
15. Kolci K, Garipkuş SN, Reis R. Thirdhand smoke exposure and its toxicological impacts: A review on target organ based studies. Fabad J Pharm Sci. 2023;48:303-318.
16. Kirkham PA, Barnes PJ. Oxidative stress in COPD. Chest. 2013;144:266-273.
17. Polosa R, Thomson NC. Smoking and asthma: dangerous liaisons. Eur Respir. 2013;41:716-726.
18. Mims JW. Asthma: definitions and pathophysiology. Int Forum Allergy Rhinol. 2015;5(Suppl 1):2-6.
19. BSI. Biological Evaluation of Medical Devices Part 12: Sample Preparation and Reference Materials (ISO 10993-12:2007); 2009.
20. Reis R, Kolci K. Comparative toxicity responses of thirdhand smoke derived from conventional cigarette and heated tobacco products in human bronchial epithelial cells. Experimed. 2023;13:127-132.

21. Reis R, Kolci K, Bahcivan İ, Coskun GP, Sipahi H. Alpha-lipoic acid modulates the oxidative and inflammatory responses induced by traditional and novel tobacco products in human liver epithelial cells. Chem Biodivers. 2023;20:e202200928.

22. Hsu JY, Chu JJ, Chou MC, Chen YW. Dioscorin pre-treatment protects A549 human airway epithelial cells from hydrogen peroxide-induced oxidative stress. Inflammation. 2013;36:1013-1019.

23. Reis R, Orak D, Yilmaz D, Cimen H, Sipahi H. Modulation of cigarette smoke extract-induced human bronchial epithelial damage by eucalyptol and curcumin. Hum Exp Toxicol. 2021;40:1445-1462.

24. Aslani MR, Amani M, Moghadas F, Ghobadi H. Adipolin and IL-6 Serum Levels in Chronic Obstructive Pulmonary Disease. Adv Respir Med. 2022;90:391-398.

25. Mazarakis N, Higgins RA, Anderson J, Toh ZQ, Luwor RB, Snibson KJ, Karagiannis TC, Do LAH, Licciardi PV. The effects of the dietary compound L-sulforaphane against respiratory pathogens. Int J Antimicrob Agents. 2021;58:106460.

26. Fischer BM, Pavlisko E, Voynow JA. Pathogenic triad in COPD: oxidative stress, protease-antiprotease imbalance, and inflammation. Int J Chron Obstruct Pulmon Dis. 2011;6:413-421.

27. Zuo L, He F, Sergakis GG, Koozehchian MS, Stimpfl JN, Rong Y, Diaz PT, Best TM. Interrelated role of cigarette smoking, oxidative stress, and immune response in COPD and corresponding treatments. Am J Physiol Lung Cell Mol Physiol. 2014;307:205-218.

28. Hang B, Sarker AH, Havel C, Saha S, Hazra TK, Schick S, Jacob P 3rd, Rehan VK, Chenna A, Sharan D, Sleiman M, Destaillats H, Gundel LA. Thirdhand smoke causes DNA damage in human cells. Mutagenesis. 2013;28:381-391.

29. Hang B, Wang P, Zhao Y, Sarker A, Chenna A, Xia Y, Snijders AM, Mao JH. Adverse health effects of thirdhand smoke: from cell to animal models. Int J Mol Sci. 2017;18:9321.

30. Boskabady MH, Gholami Mahtaj L. Lung inflammation changes and oxidative stress induced by cigarette smoke exposure in guinea pigs affected by Zataria multiflora and its constituent, carvacrol. BMC Complement Altern Med. 2015;15:39.

31. Tan WSD, Liao W, Peh HY, Vila M, Dong J, Shen HM, Wong WSF. Andrographolide simultaneously augments Nrf2 antioxidant defense and facilitates autophagic flux blockade in cigarette smoke-exposed human bronchial epithelial cells. Toxicol Appl Pharmacol. 2018;360:120-130.

32. Lin XX, Yang XF, Jiang JX, Zhang SJ, Guan Y, Liu YN, Sun YH, Xie QM. Cigarette smoke extract-induced BEAS-2B cell apoptosis and anti-oxidative Nrf-2 up-regulation are mediated by ROS-stimulated p38 activation. Toxicol Mech Methods. 2014;24:575-583.

33. Lugrin J, Rosenblatt-Velin N, Parapanov R, Liaudet L. The role of oxidative stress during inflammatory processes. Biol Chem. 2014;395:203-230.

34. Wu JX, Lau ATY, Xu YM. Indoor secondary pollutants cannot be ignored: third-hand smoke. Toxics. 2022;10:363.

35. Taucher E, Mykoliuk I, Lindenmann J, Smolle-Juettner FM. Implications of the Immune Landscape in COPD and Lung Cancer: Smoking Versus Other Causes. Front Immunol. 2022;13:846605.

Supplementary Document of "Third-Hand Smoke Induces COPD Exacerbations in Human Airway Epithelia *In Vitro*"

Chromatographic conditions

Materials

(-)-Nicotine, acetonitrile, methanol, and glacial acetic acid were purchased from Sigma (USA). Deionized water was obtained from the Sartorius Arium Pro (Germany) water purification system and was used to prepare mobile phase solutions. All other chemicals used were of analytical grade.

Preparation of standard solutions

(-)-Nicotine is freely soluble in methanol. The stock solution was prepared as 9 mg/mL The stock solutions were then diluted with methanol within the concentration range of 0.70-1.62 mg/mL.

Instrumentation and chromatographic conditions

Agilent 1260 Infinity II series of LCMS systems (USA) equipped with a solvent pump, manual injection valve, and a diode-array detector were used. The quantitative analysis of nicotine was performed with the C18 column (particle size: 3 µm, pore size: 100A). The column temperature was adjusted to 30 ºC in the column compartment. The mobile phase consisted of an acetonitrile- 0.1% glacial acetic acid solution (95:5, v/v) mixture and was delivered at a 0.6 mL/min flow rate. The injection volume was 20 µL. The ultraviolet detector was operated at 254 nm.

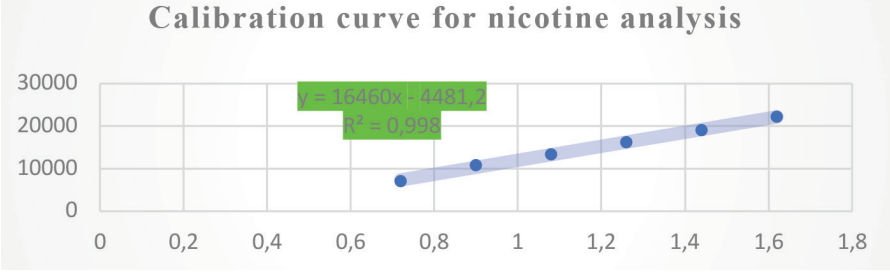
Calibration curve

The calibration curve was calculated according to area under the curve vs. concentration values. Six dilutions were used for the calculation of the equation and R2 value. The area of the standard peaks were calculated according to their mass values (Supplementary Figure S1, Supplementary Table 1).

Supplementary Table 1. Nicotine standard dilutions were used for the calibration curve.

Dilutions	Concentration (mg/mL)	Area (mAU)
1	0.70	7070.36
2	0.93	10774
3	1.08	13313.3
4	1.25	16146.6
5	1.43	19125,5
6	1.62	22231.2

mAU: Milli-absorbance units



Supplementary Figure S1. Calibration curve of nicotine analysis

Calibration curve equation:

y= 16460x-4481.2

R^2: 0.998

Detection of the nicotine content of third-hand smoke

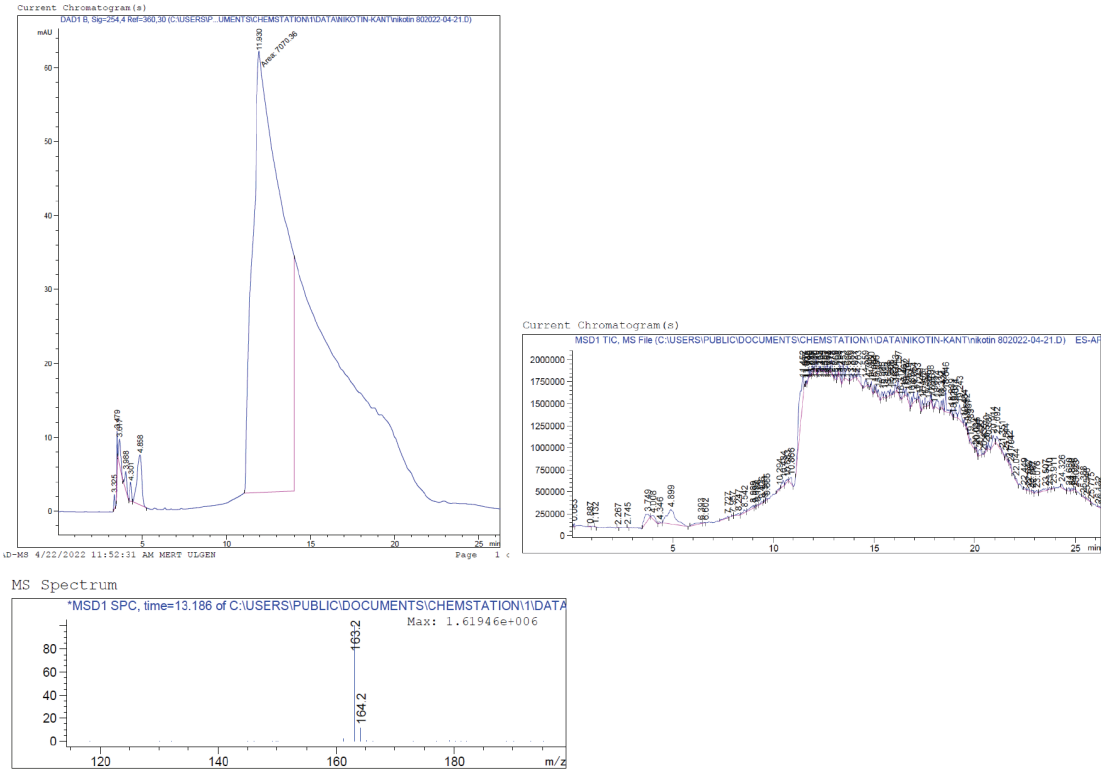
Nicotine analysis was performed for THS samples prepared from 2 of 3R4F research cigarettes and injected into the liquid chromatography-mass spectrometry system immediately. The area under the curve of the sample is given in Supplementary Table 2.

Supplementary Table 2. Peak area and nicotine concentration of THS

Test sample	Concentration (mg/mL)	Area (MAU)
Control	ND	-
THS sample	0.2872	246.965

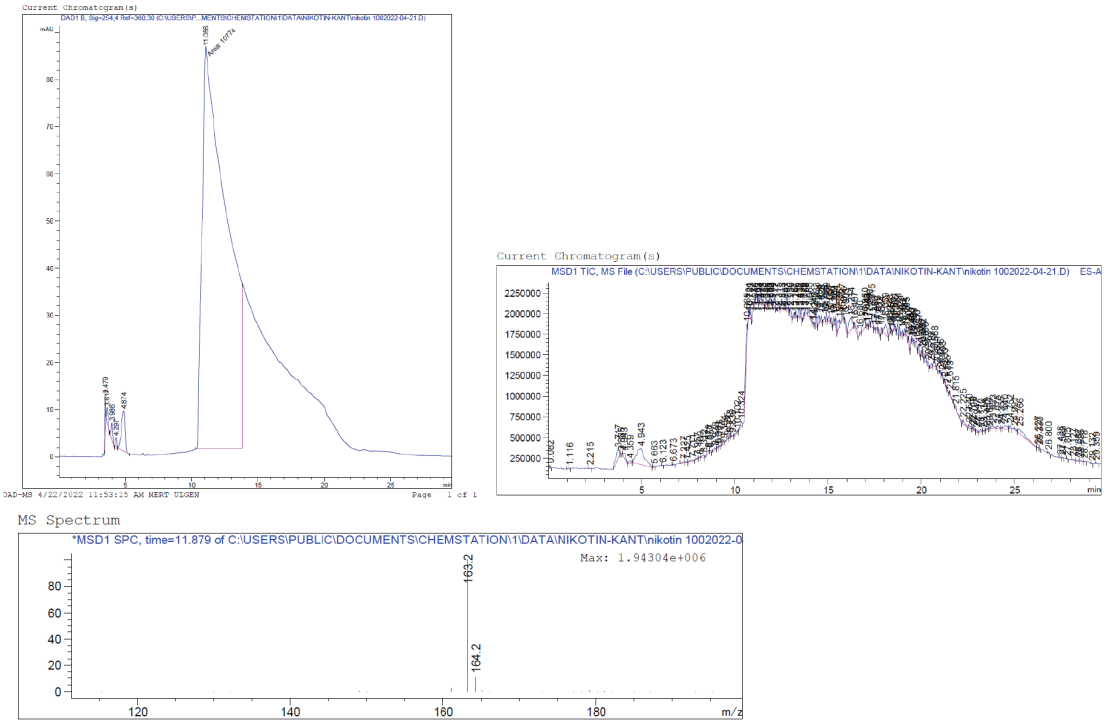
THS: Third-hand smoke

Supplementary Mass spectrum and chromatograms of nicotine standards and chromatograms of third-hand smoke samples are given below:

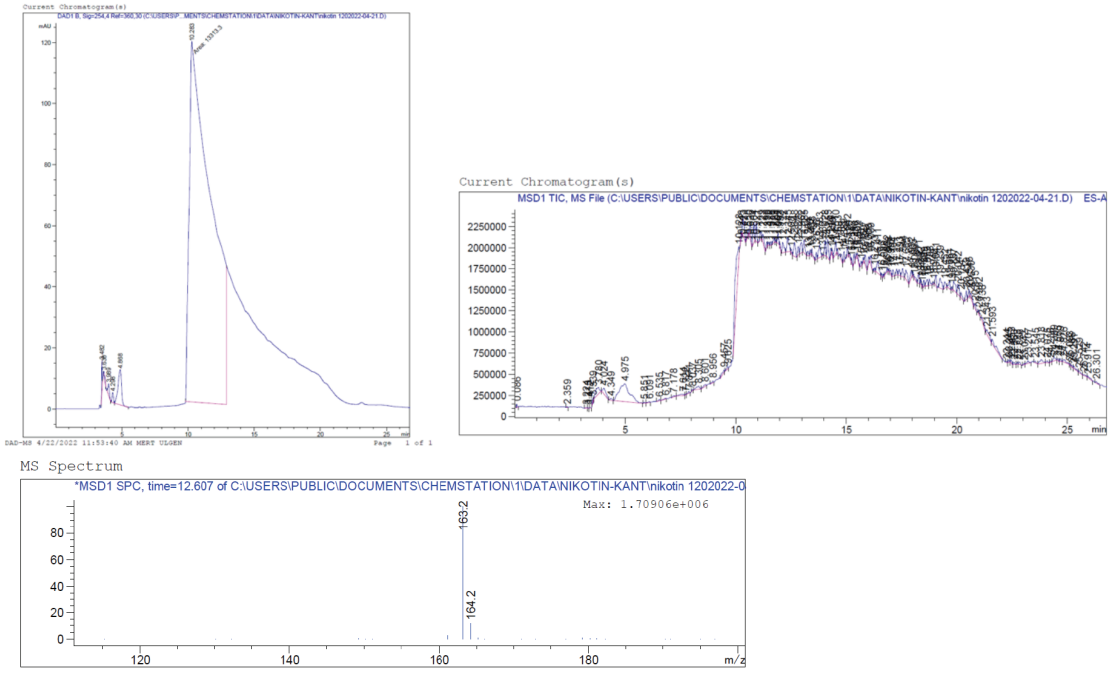


Supplementary Figure S2. AUC, Mass spectrum, and chromatogram of dilution 1

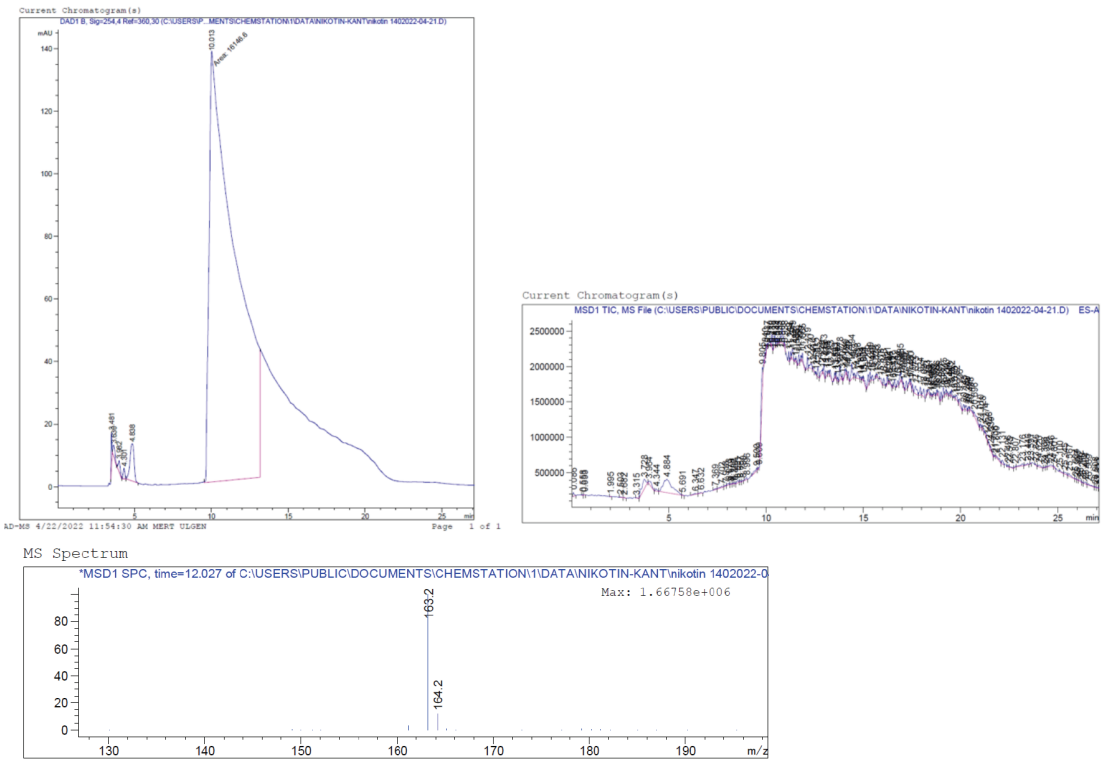
AUC: Area under the curve



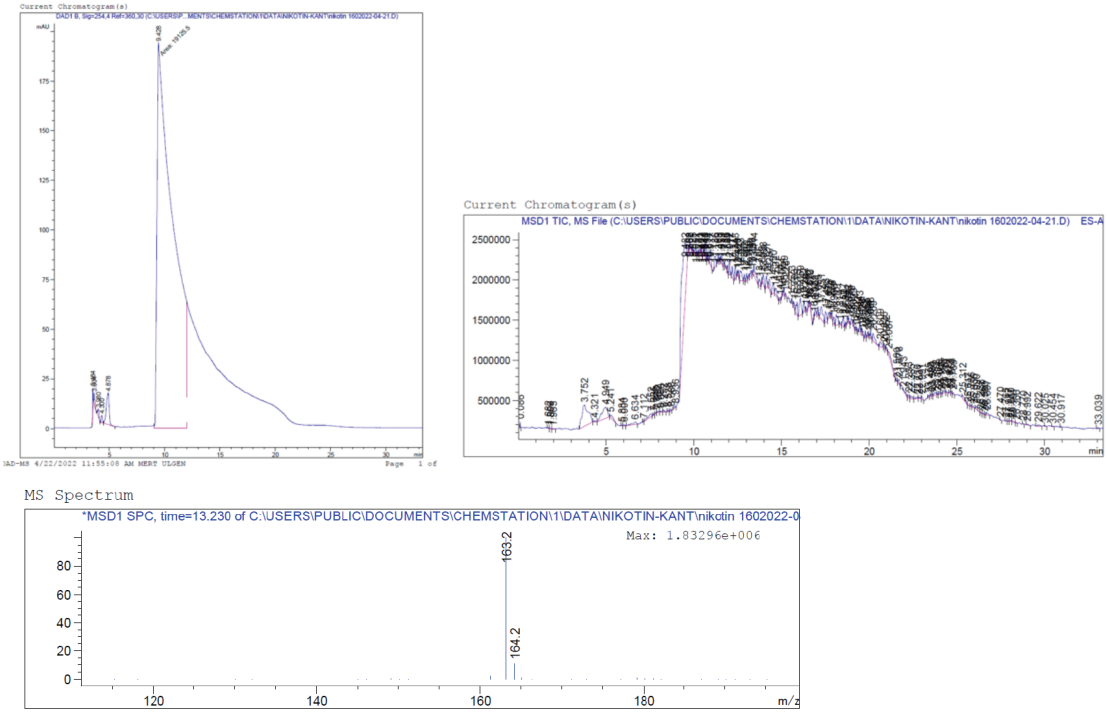
Supplementary Figure S3. AUC, Mass spectrum, and chromatogram of dilution 2
AUC: Area under the curve



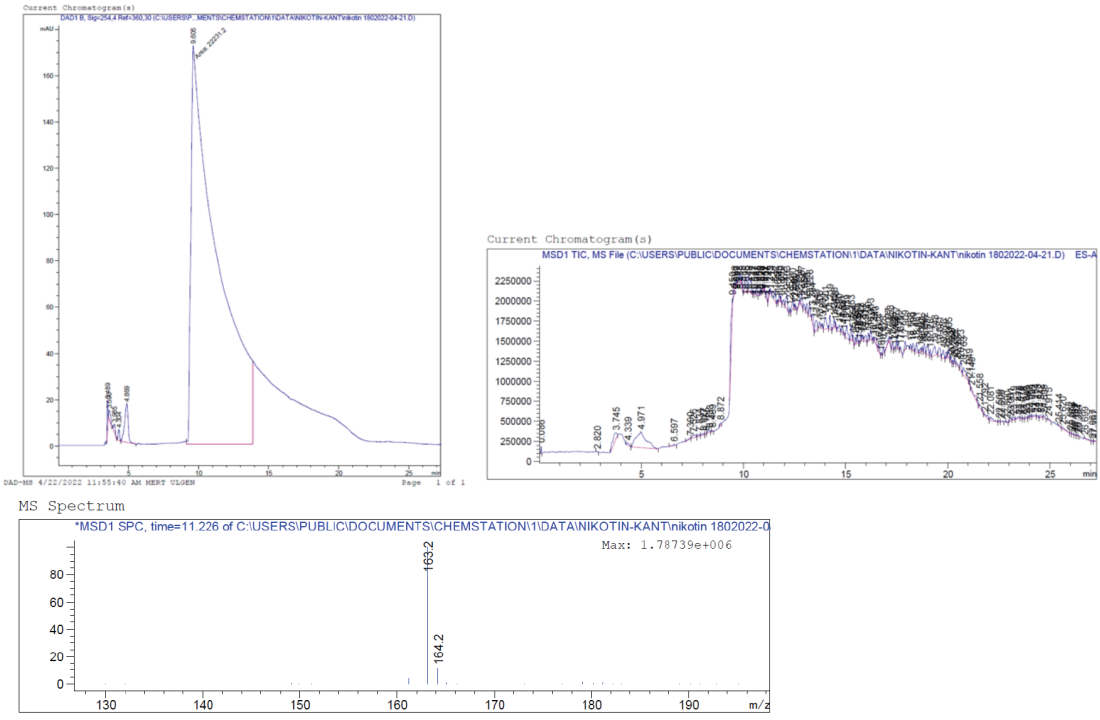
Supplementary Figure S4. AUC, Mass spectrum, and chromatogram of dilution 3
AUC: Area under the curve



Supplementary Figure S5. AUC, Mass spectrum, and chromatogram of dilution 4
AUC: Area under the curve

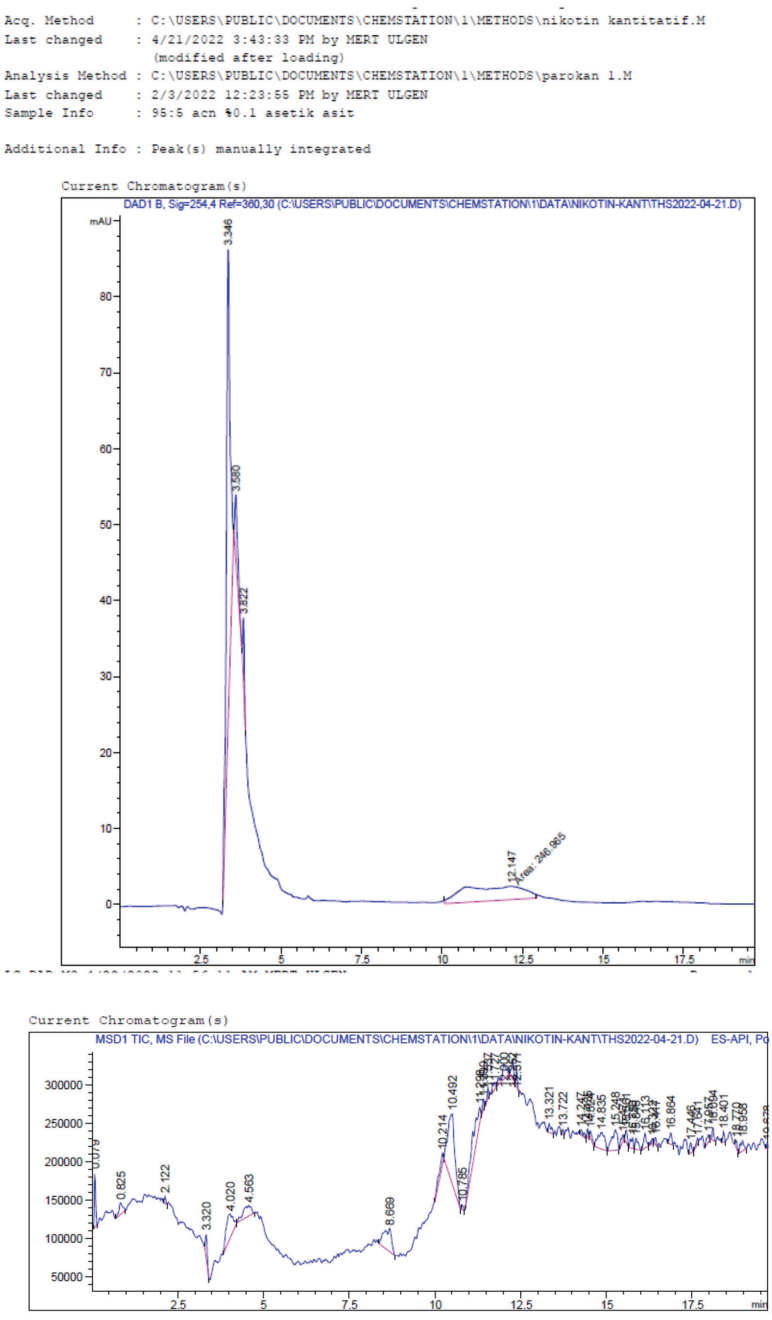


Supplementary Figure S6. AUC, Mass spectrum, and chromatogram of dilution 5
AUC: Area under the curve



Supplementary Figure S7. AUC, Mass spectrum, and chromatogram of dilution 6

AUC: Area under the curve



Supplementary Figure S8. Chromatogram and mass spectrum of THS sample
THS: Third-hand smoke