

## Thirdhand Smoke Exacerbated H<sub>2</sub>O<sub>2</sub>-Driven Airway Responses in A549 Cells

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### ABSTRACT

Thirdhand smoke (THS) is residual smoke after the cigarette is extinguished and adheres to surfaces. Its re-emission into the air also makes THS a health concern for those who suffer from respiratory diseases. The present study evaluated THS's cytotoxic, oxidative, and inflammatory potential in the H<sub>2</sub>O<sub>2</sub>-driven respiratory responses in A549 human airway epithelial cells. Extracted THS from terrycloth exposed to 3R4F cigarettes was assessed via MTT to identify cytotoxicity. The reactive oxygen species (ROS) level was set via DCFDA fluorescence intensity in a flow cytometer, where GSH, MDA level, and CAT activity were assessed spectrophotometrically. IL-6 level is measured via ELISA. THS 50% (v/v) with significant cytotoxicity in A549 cells up-regulated the intracellular ROS level via right-shifted fluorescence intensity of DCFDA compared to the control (p<0.05), which was also amplified with H<sub>2</sub>O<sub>2</sub> co-treatment. MDA level remarkably increased with THS (p<0.05) whereas both THS and THS+H<sub>2</sub>O<sub>2</sub> led to a notable GSH depletion, increase in CAT activity as well as increase in IL-6 level, which were attenuated with negative control (NAC, 1mM) (p<0.05). The induction of oxidative stress might be defined as an important key event in THS-induced airway toxicity that may contribute to chronic respiratory disease progression.

**Keywords:** *chronic airway diseases, airway inflammation, oxidative stress, thirdhand smoke, cigarette.*

### INTRODUCTION

Cigarette smoke (CS) is the main preventable cause of death in the world, and it represents important health risks for both smokers and non-smokers<sup>[1]</sup>. Recently, a new toxicological concern has arisen due to the residual part of CS, thirdhand smoke (THS). THS is referred to as tobacco residue and stale or aged secondhand smoke. THS is not described as rigid smoke, but rather the byproducts of smoking and refers to the contamination of surfaces contacted with secondhand smoke (SHS)-emitted compounds<sup>[2]</sup>. The products of chemical transformations of these constituents, and the off-gassing of volatile substances into the atmosphere, thus might represent an important public and environmental issue. Apart from traditional tobacco smoke, people might be exposed to THS by three routes those are ingestion, inhalation, and dermal absorption. Particularly, the most important target population is the 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 be exposed to THS due to hand-to-mouth transfer via contaminated objects, indirectly<sup>[3]</sup>. THS has a complex chemical dynamic and consists of nicotine, and tobacco-specific nitrosamines (TSNAs), which are highly carcinogenic compounds form when tobacco burns and can react with other chemicals in the environment to create even more harmful substances, and volatile organic compounds (VOCs) such as benzene and formaldehyde<sup>[4,5]</sup>. In addition, polycyclic aromatic hydrocarbons (PAHs) and heavy metals were detected in the content of THS<sup>[6]</sup>.

Due to inhaling the re-released SHS-emitted compounds, the other vulnerable population might be considered as people with chronic airway disease such as chronic obstructive pulmonary disease (COPD)<sup>[7]</sup>. 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 tightly linked to the pathological process or prognosis of COPD<sup>[8]</sup>. COPD is considered a systemic disorder and is more common in individuals with a smoking history<sup>[9]</sup>. COPD is the most extensively studied inflammatory airway disorder induced by smoking and its incidence rate of all stages of COPD among active smokers was above 35% over 25 years [10]. According to the latest data from the Centers for Disease Control and Prevention (CDC), the disease is usually caused by smoking, and smoking accounts for as much as 80% of COPD-related mortality<sup>[11]</sup>. In addition, it was indicated that COPD

remains a socioeconomic burden, especially in countries with a low sociodemographic index between the years 1990-2019 [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 or necrosis, chronic inflammation, and other pathways that are not fully elucidated [12]. According to previous reports, TSNA and other reactive chemicals may damage lung cells directly, leading to inflammation and scarring. In addition, THS exposure might induce oxidative stress, which directly disrupts lung function and might promote further inflammation in the target organ. The amplified inflammatory response may contribute to the immune response in the respiratory system as well and trigger chronic inflammation in the airways, leading to thickening and narrowing of the bronchial tubes, which is a hallmark of COPD. Moreover, it is known that tobacco smoke can induce epithelial and mucus dysregulation due to damage to the lining of the airways leading to airway obstruction and difficulty breathing [5,13-16]. The precise contributions of each of these mechanisms are still being studied and thus, it is thought that THS may worsen the 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 revealed that with THS exposure in mice, the walls of alveoli in terminal respiratory bronchioles were thicker with increased pro-inflammatory cytokine levels in lung tissues than in non-exposed animals. In addition, THS toxins as a mixture exerted dose-dependent cytotoxicity in the A549 human lung epithelial cells mainly due to acrolein, phenol, and 2,5-dimethylfuran content [5]. In light of these limited findings, it might be suggested that THS exposure may cause a pro-inflammatory and oxidative environment in the lungs, which may increase the risk of disease progression in chronic airway diseases. It is known that when the free radicals overpower the 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 the mucus hyperproduction, tissue remodeling, inflammation, airway hyperresponsiveness, and tissue damage such as fibrosis and scarring in the lining of respiratory epithelia [17-19]. Hence, in the present study, it was aimed to elucidate the mechanistic pathways involved in THS-induced respiratory toxicity and the accelerative potential of THS in the H<sub>2</sub>O<sub>2</sub>-induced oxidative stress model of human airway epithelia *in vitro*.

## MATERIALS AND METHODS

### Materials

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

### Methods

#### The extraction of THS

THS was extracted from a terrycloth that was manually exposed to two 3R4F research cigarettes (11.0 mg total particulate matter (TPM) /cig; 9.4 mg tar/ cig; 0.73 mg nicotine/ cig, and 12 mg CO/ cig) in a polystyrene chamber according to (International Organization for Standardization British Standards Institution (ISO BSI 10993-12) [20] with a slight modification in our previous study [21]. According to the previously mentioned method, once the smoke exposure in the chamber ceased, the mainstream and sidestream smokes were mixed with minifan 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 provided through the weighing of the tar of different filter papers used for THS batches according to the method of Martins-Green and colleagues [22].

#### Nicotine level of THS

The nicotine content of prepared THS was assessed by liquid chromatography-mass spectrometry (LCMS, Agilent1260 Infinity II, USA) equipped with a solvent pump, manual injection valve, and a diode-array detector quantitatively as described previously in detail [23]. Six dilutions of nicotine standard were used for the calculation of the equation and R<sup>2</sup> value (*Supplementary Table S1*). The area of standard peaks was calculated according to mass values.

#### Cytotoxicity

A549 human lung epithelial-like cells (ATCC, CCL-185<sup>TM</sup>) 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 the previous data reported increased H<sub>2</sub>O<sub>2</sub> levels in expired breath condensates of patients with COPD [24] and oxidative stress contributes to the development and progression of chronic airway

diseases through numerous pathways such as mucus hyperproduction, tissue remodeling, and inflammation the *in vitro* oxidative stress in the respiratory system was demonstrated by the co-exposure of H<sub>2</sub>O<sub>2</sub> (100 μM) in A549 cells. As a negative control, a 2-hour pre-treatment of N-acetylcysteine (NAC, 1 mM) was used in all studies to observe its ameliorative effects against THS itself and with H<sub>2</sub>O<sub>2</sub>-driven oxidative stress conditions. After the incubation period, cytotoxicity was assessed by MTT assay as described previously [23].

#### Oxidative stress

##### Glutathione (GSH) level

GSH level was measured from cell lysates according to our previous study [25]. The exposed cell lysate prepared in PBS was mixed with DTNB solution and then mixed with EDTA buffer solution (pH 8.2). After incubation at 37°C at dark for 30 min, the absorbance of yellow-colored chromophore was measured at 412 nm spectrophotometrically (ThermoScientific, Finland). The results were expressed as μmol/g protein GSH and each measurement was performed in duplicates.

##### Catalase (CAT) activity

CAT activity was measured by using the correlation between carbonatocobaltate (III) complex and catalase enzyme. Briefly, the cell lysate is mixed with H<sub>2</sub>O<sub>2</sub> as described in our previous study [23] and incubated at 37 °C for 2 min. Followingly, the solution containing phosphate buffer (pH 7.4), sodium bicarbonate and cobalt (II) nitrate hexahydrate were added to the mixture and vortexed. The reaction tubes were kept in the dark for 10 min and absorbances for kinetic reaction were recorded at 440 nm spectrophotometrically for 2 minutes in duplicates. CAT activity was expressed as U/mg protein.

##### Intracellular reactive oxygen species (ROS) level

The oxidative effect of THS in H<sub>2</sub>O<sub>2</sub>-induced A549 cells was assessed with a cellular ROS assay kit by flow cytometry. As described previously [25], under oxidative conditions, 2,7-dichlorofluorescein diacetate (DCFDA), a fluorescence-sensitive dye, is deacetylated by cellular esterases and forms a non-fluorescent compound, is oxidized into 2,7-dichlorofluorescein (DCF) by the produced ROS. Briefly, cells pre-treated with NAC/ treated with THS/ treated with THS + H<sub>2</sub>O<sub>2</sub> were collected from 12-well plates and harvested in PBS solution, which was followed by 20 mM DCFDA addition to each flow cytometer tube under dark for 30 minutes at 37 °C with 5% CO<sub>2</sub>. The 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 h was used as the positive control. Data were analyzed in triplicates, and the intracellular ROS level was expressed as relative ROS content compared to the positive control (PC).

##### Interleukin-6 (IL-6) level

The inflammatory response induced by THS exposure in A549 cells co-treated with H<sub>2</sub>O<sub>2</sub> was determined with pro-inflammatory cytokine IL-6 release via human IL-6 ELISA kit according to the manufacturer's protocol as previously [25]. Cell supernatants were used to detect IL-6 release in A549 cells and each group was applied in duplicates. The results were expressed as pg/mL.

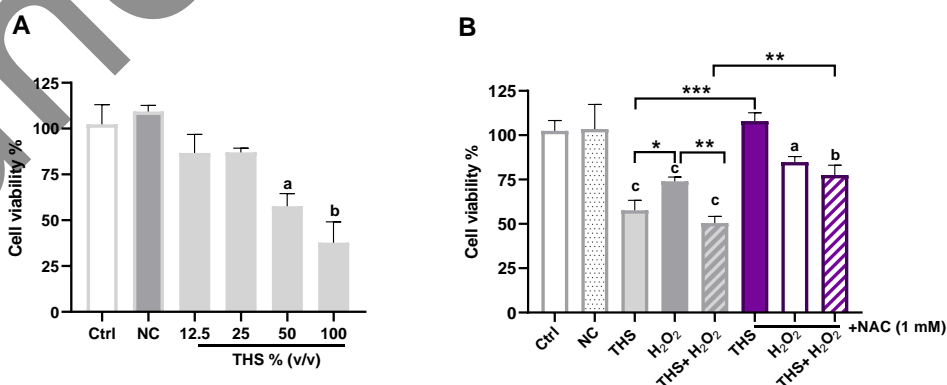
## RESULTS

#### Nicotine level of THS

According to LCMS analysis of THS extract (100 %, v/v), the nicotine concentration of samples was recorded as 0.33± 0.03 mg/mL. The results also showed that the nicotine content of the different batches of THS stored at - 80 °C was similar (Supplementary Table 1).

#### Cytotoxicity

Cytotoxicity of THS and its co-exposure with H<sub>2</sub>O<sub>2</sub> via MTT assay showed that THS induced a dose-dependent cytotoxicity in A549 cells, remarkably at 50-100% (v/v) (p<0.05) (Fig.1A). Based on this finding, THS 50% (v/v) (approximate IC<sub>50</sub> value) was selected for further studies on the assessment of cytotoxicity of THS in respiratory oxidative conditions *in vitro*.



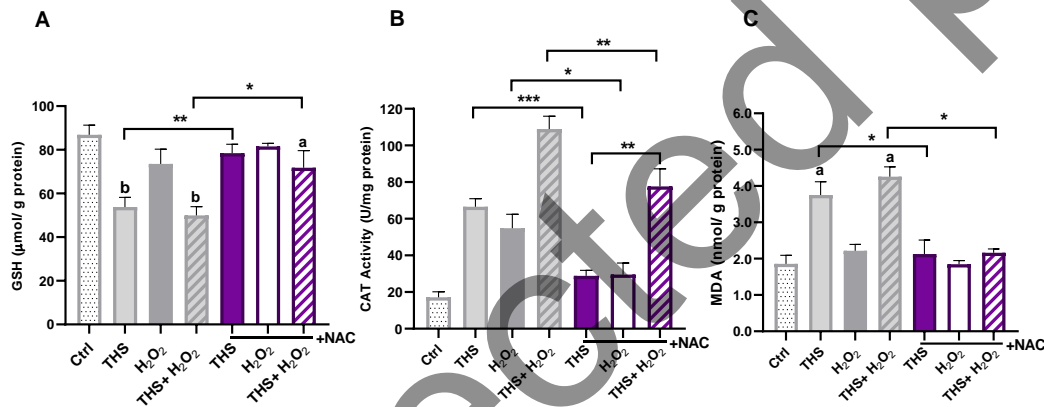
**Figure 1.** Cell viability of A549 cells exposed to THS with or without H<sub>2</sub>O<sub>2</sub>.

A. Dose-dependent cytotoxicity profile of THS; B. Statistical significance between Ctrl vs groups <sup>a</sup>p <0.05, <sup>b</sup>p <0.01, <sup>c</sup>p <0.001; the significance between two groups \*p <0.05; \*\*p <0.01 and \*\*\*p <0.001. NAC: N-acetylcysteine (1 mM) applied as 2 h pre-treatment; THS: Thirdhand smoke extract (50%, v/v), H<sub>2</sub>O<sub>2</sub>: 100 μM. The data were shown as mean ±SD.

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

#### Respiratory oxidative damage by THS in H<sub>2</sub>O<sub>2</sub>-stimulated cells

The oxidative stress conditions of A549 cells exposed to THS, H<sub>2</sub>O<sub>2</sub> and THS+H<sub>2</sub>O<sub>2</sub> after pre-treatments to NAC concluded 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 the CAT activity (p <0.01) (Fig. 2). In parallel, lipid peroxidation was significantly increased with THS exposure. However, the oxidative effect of H<sub>2</sub>O<sub>2</sub> exposure used in the *in vitro* modelling of COPD notably up-regulated CAT activity and MDA levels, which were also elevated with their co-exposure. Besides, pre-treatment with NAC significantly ameliorated the oxidative responses of THS alone and in COPD conditions induced by H<sub>2</sub>O<sub>2</sub> (Fig. 2).

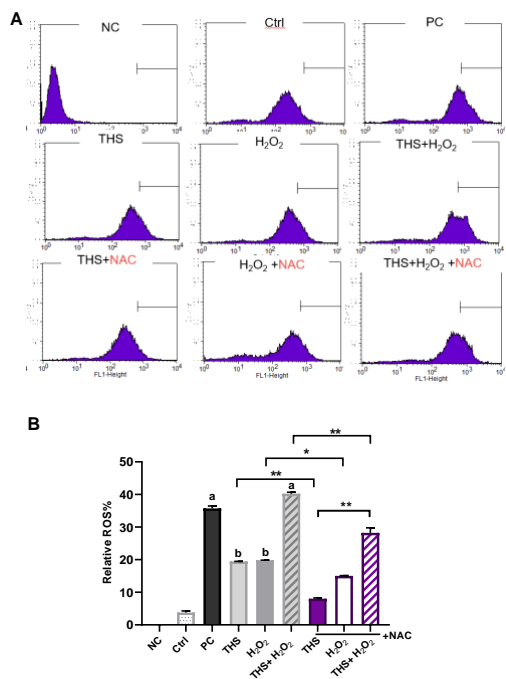


**Figure 2.** Modulation of oxidative stress and lipid peroxidation by THS and co-exposure with H<sub>2</sub>O<sub>2</sub>.

A. Total GSH level; B. CAT activity; C. MDA level in A549 cells co-treated with THS and H<sub>2</sub>O<sub>2</sub>. Statistical significance between Ctrl vs groups <sup>a</sup>p <0.05, <sup>b</sup>p <0.01; the significance between two groups \*p <0.05; \*\*p <0.01 and \*\*\*p <0.001. NAC: N-acetylcysteine (1 mM) applied as 2 h pre-treatment; THS: Thirdhand smoke extract (50%, v/v), H<sub>2</sub>O<sub>2</sub>: 100 μM. The data were shown as mean ±SD.

#### Intracellular ROS level

Intracellular increase in the production of ROS is an important parameter to evaluate the imbalance between the oxidative response and the body's ability to neutralize them. Since oxidative stress is thought to play a role in the development and progression of COPD and other respiratory disorders, detection of ROS level might represent a preliminary marker for further lung tissue damage, which may lead to inflammation and impaired lung function. In the present study, total ROS level was measured in A549 cells exposed to THS alone and in combination with H<sub>2</sub>O<sub>2</sub>. Based on our findings, it was shown that THS exposure itself significantly elevated the intracellular ROS production (p <0.05) compared to the control group in A549 cells. Furthermore, this increase in ROS level was exacerbated with co-exposure to H<sub>2</sub>O<sub>2</sub> (p <0.01) (Fig. 4). Similar to the results of oxidative stress assays, NAC pre-treatments improved the elevated ROS production level and declined the relative ROS levels significantly in all THS-exposed groups (p <0.01), possibly due to the replenishment of intracellular GSH deposits.



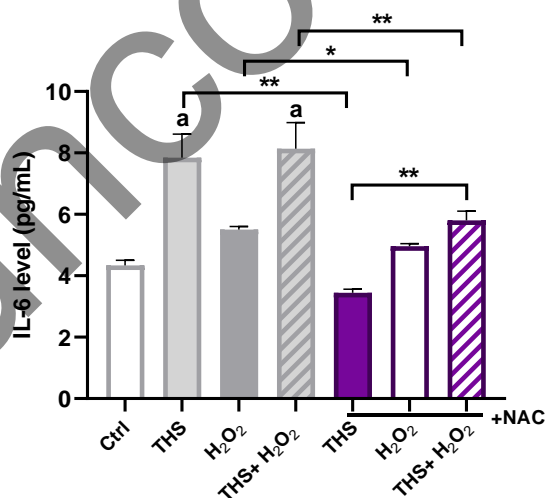
**Figure 4.** Intracellular ROS level of A549 cells exposed to THS with or without H<sub>2</sub>O<sub>2</sub>.

**A.** Representative histograms for percentage of increase of ROS accumulation in the groups. Enhancement of intracellular ROS level observed via the shift of the signal curve obtained for the THS and H<sub>2</sub>O<sub>2</sub> treated cells to the right compared with that of the control. **B.** Relative ROS% of A549 cells exposed to THS with or without H<sub>2</sub>O<sub>2</sub>.

Statistical significance between Ctrl vs groups <sup>a</sup>p < 0.01; <sup>b</sup>p < 0.05; the significance between two groups <sup>\*</sup>p < 0.05; <sup>\*\*</sup>p < 0.01. NAC: N-acetylcysteine (1 mM) applied as 2 h pre-treatment; NC: Cells w/o DCFDA; THS: Third-hand smoke extract (50%, v/v), H<sub>2</sub>O<sub>2</sub>: 100 μM; PC: positive control THBP (100 μM).

#### IL-6 level

IL-6 plays a role in several inflammatory and immune responses including the acute phase response, response to infections, and the development of chronic diseases such as rheumatoid arthritis and COPD [26,27]. In COPD, IL-6 levels are elevated in the blood and in the airways and contribute to the development and progression of COPD by promoting inflammation, impairing lung function, and increasing the risk of disease exacerbations. The present findings revealed that residual THS exposure and its co-exposure to H<sub>2</sub>O<sub>2</sub> significantly induced IL-6 release in A549 cells, where this response declined with NAC pre-treatment (Fig. 5). However, the inflammatory response of THS co-exposed COPD group was notably higher compared to the THS alone group despite the NAC pre-treatment.



**Figure 5.** IL-6 release induced by THS and its co-exposure with H<sub>2</sub>O<sub>2</sub> in A549 cells.

Statistical significance between Ctrl vs groups <sup>a</sup>p <0.05; the significance between two groups \*p <0.05; \*\*p <0.01. NAC: N-acetylcysteine (1 mM) applied as 2 h pre-treatment; THS: Thirdhand smoke extract (50%, v/v), H<sub>2</sub>O<sub>2</sub>: 100 μM. The data were shown as mean ±SD.

## DISCUSSION

THS, as a recent concept in environmental toxicology, can attach to the surfaces for long periods, even after the smoke has cleared. Children are particularly susceptible to THS exposure since they are more likely to come into contact with smoke-embedded surfaces. Even though parental precautions might be helpful to 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 investigated the accelerative potential of THS in the H<sub>2</sub>O<sub>2</sub>-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 elevated pro-inflammatory response in human airway epithelia *in vitro*. Since increased inflammation and oxidative stress are important indicators of chronic airway diseases in general [28,29], it might be suggested that THS exposure is a significant issue that must be addressed for people who have respiratory diseases. Recent studies showed that dose-dependent THS exposure decreases cell survival in human dermal fibroblasts, mouse neural stem cells, human palatal mesenchyme<sup>[4]</sup>, human hepatocellular carcinoma cells [30], and male rodent reproductive cells [31]. In the present study, THS extracts declined the cell viability of A549 cells, particularly at doses higher than 25% (v/v) (p<0.05). Therefore, it can be suggested that the complex chemical content of THS led to cytotoxicity in human airway epithelia mostly via mitochondrial cell viability. In addition to its cytotoxic potential itself, co-exposure with H<sub>2</sub>O<sub>2</sub> further increased the cytotoxicity response in the human airway epithelia. Based on this finding, it might be suggested that THS may interact with other environmental or exogenous oxidant factors to increase its toxicity. Moreover, THS induced the production of intracellular ROS formation, CAT activity, and depleted GSH deposits that help to detoxify ROS in A549 cells. In addition, THS increased lipid peroxidation, as well. However, these oxidative stress-induced toxicity responses were alleviated by NAC pre-treatment, significantly. Previously, Boskabady and colleagues (2015) 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 pre-treatment by boosting intracellular antioxidant capacity [32]. As reported in our previous study [23], CS exposure and its components may lead to an excessive increase in the CAT activity of the target organ, probably due to the presence of higher peroxide concentrations. In addition, the decrease in GSH and increased MDA levels in A549 cells clearly indicated that antioxidant defense mechanisms were not enough to prevent lipid peroxidation due to THS exposure either alone or with H<sub>2</sub>O<sub>2</sub>. Similarly, *in vitro* studies with CS suggested a significant deposition of intracellular antioxidant enzymatic/ non-enzymatic capacity [25] as well as elevated inflammatory response [25,33,34]. It is reported that overproduced free radicals react with cellular and humoral components, thus impairing their function permanently and can trigger inflammatory responses [35]. However, there are limited studies on the residual part of CS as in the form of THS and its possible inflammatory and oxidant potency in respiratory disease conditions. In addition, 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 extract of THS has nicotine level approximately 0.28 mg/mL (0.28 mg/ g fabric), whereas samples extracted from indoor or outdoor surfaces or different extraction conditions might change this nicotine level dramatically from 2-12 g nicotine/ g fabric [4]. Therefore, the observed results in the toxicity outcomes in the literature might be different in proportion to the accumulation and extraction capacity of the other expected chemicals present in the THS such as VOCs, TSNAs, and PAHs. As a preliminary finding, THS significantly up-regulated pro-inflammatory IL-6 release in human airway epithelial cells in the present study. Moreover, this exhibited inflammatory response was found to be higher in the THS and H<sub>2</sub>O<sub>2</sub> co-treated group even though the NAC pre-treatment (p<0.01). Therefore, it might be suggested that THS in the environment might contribute to the inflammatory and oxidative complications of chronic airway conditions initially mediated through oxidative stress. It is known that inhalation of polycyclic aromatic hydrocarbons (PAHs) and various volatile organic compounds is responsible for oxidative damage and inflammatory response through the induction of mitochondrial free radical formation [36]. Furthermore, a major concern may arise due to environmental THS exposure since smoking with COPD has the highest correlation with all types of lung cancer and to development of small-cell lung cancer [36,37]. Therefore, as an indirect source of CS, THS might represent a severe contributing risk factor for lung cancer development in this population. As a result, the present preliminary findings are important to elucidate the toxicological key points involved in the cell survival/death in the target organ with respiratory disease due to THS exposure.

## CONCLUSION

Based on the present findings, it might be suggested that co-exposure to THS may lead to more detrimental effects in human airways *in vitro*. Hence, it might be concluded that as an environmental residue, THS may have a role in the progression of chronic respiratory diseases, which were mediated through oxidative and

inflammatory exacerbations in human airway epithelia. Further studies are needed to confirm these mechanisms by which THS is exerted, and could help to identify approaches to reduce environmental THS exposure, as well. In addition, these preliminary findings might be used to develop new therapy methods that improve patient outcomes.

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**Author contribution statement:** Conceptualization: RR; experimental data analysis: RR, KK, YO, and GPC; writing and editing the original draft: RR, KK, YO, GPC, and HS.

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