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Evaluation of the Effects of e-Cigarette Aerosol Extracts and Tobacco Cigarette Smoke Extracts on RAW264.7 Cells

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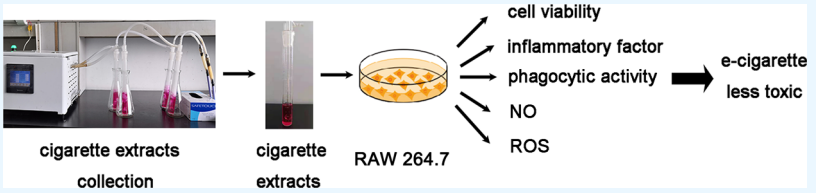

ABSTRACT: With the advancement of society, electronic cigarettes (e-cigarettes) have gained popularity among a growing number of individuals. While numerous toxicological studies have suggested that e-cigarettes are a safer alternative to traditional cigarettes, there is also a body of literature presenting contrasting findings. This *in vitro* study aimed to compare the effects of e-cigarettes and tobacco cigarettes (t-cigarettes) on RAW264.7 cells by using four e-cigarette aerosol extracts (ECA) and cigarette smoking extracts (CS) containing different nicotine concentrations. The results revealed that low concentration of nicotine in CS as well as in ECA with grape, watermelon, and cola flavors could promote cell viability. Conversely, high nicotine concentration in CS and ECA with four flavors decreased cell viability. Furthermore, our study demonstrated that CS significantly reduced the phagocytic capability of RAW264.7 cells and increased the levels of inflammatory cytokines (IL-6, TNF-α, and IL-1β) and reactive oxygen species (ROS) compared to ECA. Overall, our findings indicate all four e-cigarettes induced less cytotoxicity to RAW264.7 cells and might be safer than t-cigarettes.

1. INTRODUCTION

Electronic cigarettes (e-cigarettes), also known as electronic nicotine delivery systems (ENDS), have rapidly emerged as a new tobacco product in recent years. Originating in China in 2003, e-cigarettes gained popularity in the United States and Europe by 2006. Unlike tobacco cigarettes, which require combustion, e-cigarettes are electronic devices that consist of a rechargeable battery, a cartridge filled with e-liquid, a heating element (atomizer) to vaporize the e-liquid into aerosol, and a mouthpiece.[1,2] The e-liquid typically contains nicotine, flavorings, and other additives, such as propellants, solvents, and oils. Currently, e-cigarettes are considered as a smoking cessation or alternative product, but their safety and relative harm compared to traditional cigarettes (t-cigarettes) are still subjects of ongoing debate.

Tobacco smoke is known to contain over 5000 chemicals.[3] The combustion of t-cigarettes releases potentially toxic substances into mainstream smoke (MS), side-stream smoke (SS), second-hand smoke (SHS), third-hand smoke (THS), and discarded cigarette butts (CBs). Cigarette smoke comprises gaseous and particulate components, including tar.[4] Consequently, comprehensive studies are necessary to assess the safety and toxicity of e-cigarettes.

Macrophages are crucial cells involved in the body's inflammatory responses. Raw264.7 cells, derived from mouse peritoneal macrophages, are commonly used as a cellular model.[5] Macrophages contribute to the initiation and regulation of inflammatory responses by secreting various proinflammatory mediators and cytokines.[6] They are essential for controlling and eliminating infections, clearing debris and foreign antigens, and promoting tissue repair and wound healing. However, they also contribute to tissue damage and pathology during infections and inflammatory diseases.[7] Hence, macrophages play a significant role as immune cells in our bodies.

Cigarette smoke has been shown to impair macrophage phagocytic activity and activate macrophages to produce chemotactic molecules, which recruit additional inflammatory cells (neutrophils, monocytes, and lymphocytes) and perpetuates inflammation and oxidative stress.[8] Macrophages produce inflammatory mediators such as nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and

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interleukin-1 β (IL-1β).[9] Studies have indicated that rats exposed to e-cigarette aerosol exhibit similar vascular endothelial dysfunction as those exposed to t-cigarettes in vascular endothelial function. Macrophages also experience increased oxidative stress after exposure to e-cigarette aerosol.[10] Nicotine, a component of e-cigarette aerosol, has been suggested to potentially suppress inflammatory markers in e-liquids.[11] While reports indicate that both e-cigarettes and t-cigarettes can damage macrophages, few studies have directly compared the effects of e-cigarettes and t-cigarettes on macrophage functions.[9−12] In this study, we utilized different typical commercially available e-cigarettes and t-cigarettes to compare their differential effects on macrophages and examine their impacts on inflammatory responses and immune activity.

2. MATERIALS AND METHODS

2.1. Cell Culture. RAW264.7 cells (provided by Dr. Shuya Huang from the Second Hospital, Shandong University) were cultured in DMEM-H medium (Genview) supplemented with 10% fetal bovine serum (Applied Biosystems) in a humidified incubator at 37 °C with 5% CO_2. The culture medium was refreshed, and cells were subcultured as needed based on cell growth and morphology. For the experiments, cells in the logarithmic growth phase were seeded in cell culture plates at a density of 1×10^5 cells/mL.

2.2. Preparation of e-Cigarette Aerosol Extracts (ECA) and t-Cigarette Smoking Extracts (CS). The ECA and CS were prepared by passing the RELX e-cigarette aerosol of commercially available filter cigarettes or the commercially available t-cigarettes combustion through prewarmed (37 °C) cell culture medium without additives. The smoking machine used to generate the aerosol was calibrated to mimic the average puff duration for e-cigarette users, with a frequency of 10 puffs/h.[13] For each batch of aerosol, 160 puffs were collected into 20 mL of culture medium without serum. The nicotine concentration in the collected sample was examined using a 1260 Infinity HPLC-ELSD system equipped with a Sepax AAA column (4.6 150 mm, 5 μm) and calculated based on the standard curve obtained from nicotine liquid phase results. RAW264.7 cells were treated with different ECA or CS containing different nicotine concentrations (0.03125, 0.125, 0.5, 2, 8, and 32 μg/mL).

2.3. Determination of Cell Viability. Cell viability was assessed as previously described[14] using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, M5655 Sigma-Aldrich) assay. Briefly, RAW264.7 cells in the logarithmic growth phase were seeded in 96-well plates. The cells were treated with various ECA or CS with different nicotine concentrations (0.03125, 0.125, 0.5, 2, 8, and 32 μg/mL) for 20 and 44 h prior to incubation with 0.5% MTT for 4 h. After removing the medium, 100 μL of dimethyl sulfoxide (DMSO) solution was added to solubilize the formazan crystals, and the absorbance at 570 nm was measured using a SpectraMAX ABS microplate spectrophotometer (Molecular Devices). Cell viability was calculated using the following formula

$$\text{cell viability}(\%) = (\text{OD}_{\text{sample group}} - \text{OD}_{\text{blank group}}) / (\text{OD}_{\text{control group}} - \text{OD}_{\text{blank group}}) \times 100\%$$

2.4. Enzyme-Linked Immunosorbent Assay (ELISA). RAW264.7 cells in the logarithmic growth phase were seeded in 24-well plates. After treatment with CS or ECA with nicotine concentrations of 0.03125 and 0.125 μg/mL for 24 and 48 h, the cell supernatants were collected. The levels of IL-6, IL-1β, TNF-α, INF-β, and MCP-1 were determined using corresponding ELISA kits KE10007, KE10002, KE10003 (Proteintech Group Inc.), E12021767, and E11021768 (CUSABIO Ltd.), respectively, following the manufacturers' instructions.

2.5. Real-Time PCR (RT-PCR). RAW264.7 cells in the logarithmic growth phase were seeded in 6-well plates. Total RNA was extracted from the cells using TRIzol (Thermo Fisher Scientific Co. Ltd.) following a low temperature protocol. Subsequently, cDNA was generated using a commercial RT-PCR kit (ABclonal Co. Ltd., Wuhan, China). Real-time PCR was conducted using the SYBR Green QuantiTect RT-PCR kit (ABclonal Co. Ltd., Wuhan, China), and each sample was analyzed in triplicate with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal reference gene. The relative mRNA expression of the target genes was calculated using the $2^{-\Delta\Delta Ct}$ method. The specific primers were 5′-AGCTGGTGGTGCCATCAGAGG-3′ and 5′-TGGTAGGAGACGGCGATGCG-3′ for TNF-α, 5′-CACTGGTCTTTTGGAGTTTGAG-3′ and 5′-GGACTTTTGTACTCATCTGCAC-3′ for IL-6, 5′-GCAGAAGTACCTGAGCTCGCC-3′ and 5′-CCTTGCTGTAGTGGTGGTCGG-3′ for IL-1β, and 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGATTTC-3′ for GAPDH.

2.6. Neutral Red Uptake Assay. RAW264.7 cells in the logarithmic growth phase were seeded in 96-well plates and treated with CS and ECA with nicotine concentration of 0.03125 and 0.125 μg/mL for 24 and 48h. Then, the cells were rinsed twice with PBS before staining with 100 μL of 0.1% neutral red normal saline at 37 °C for 30 min. The supernatant was discarded, and the cells were washed twice with PBS. Subsequently, the cells were lysed with 100 μL of cell lysis solution (ethanol:glacial acetic acid, 1:1) at 37 °C for 4 h. Finally, the optical density at 540 nm was measured using a plate reader and used as ordinate for plotting.

2.7. NO Testing. RAW264.7 cells in the logarithmic growth phase were seeded in 24-well plates. After treatment with CS and ECA containing 0.03125 and 0.125 μg/mL nicotine for 24 and 48 h, the cell supernatant was used to determine NO concentrations using a kit (A013-2-1, Nanjing Jiancheng Bioengineering Institute) following the manufacturer's instruction.

2.8. Intracellular ROS Assay. RAW264.7 cells in the logarithmic growth phase were seeded in laser confocal petri dishes. The levels of intracellular ROS were detected using a fluorescent probe, 2′,7′-dichlorodihydrofluorescein (DCHF, Sigma). DCHF is capable of being oxidized into fluorescent 2,7-dichlorofluorescein (DCF) in the presence of intracellular ROS. The fluorescence was visualized and captured using a fluorescence microscope (Nikon Ci-L, Japan). The relative amount of ROS was quantified by determining the fluorescence intensity of DCHF per cell within the scanned area. Image-J software was utilized to calculate the average fluorescence intensity. The figure presented represents the relative fluorescence intensity, which indicates the level of ROS in the cells.

2.9. Statistical Analysis. All experiments were independently repeated at least 3 times. The normal distribution of data was analyzed using SPSS v11.5 (SPSS Inc., Chicago, IL).

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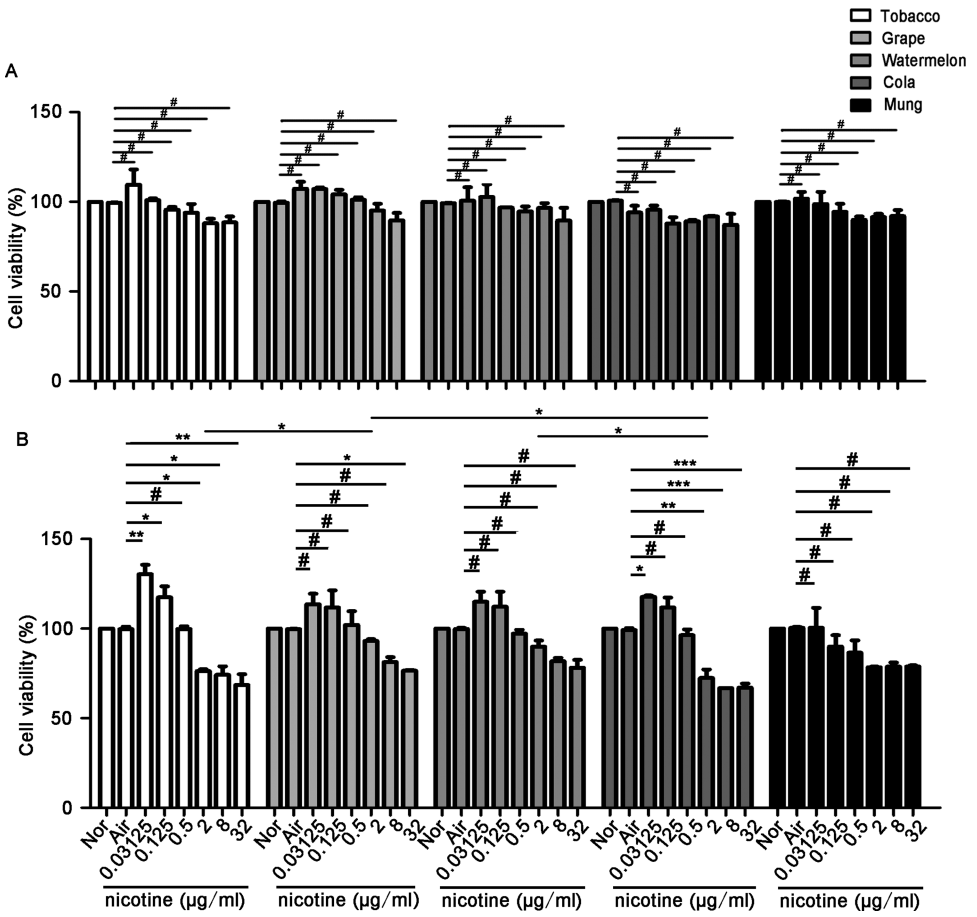

Figure 1. Viability of RAW264.7 cells treated with CS and ECA at different nicotine concentrations. (A) RAW264.7 cells were treated with CS or various ECA with different nicotine concentrations (0.03125, 0.125, 0.5, 2, 8, and 32 μg/mL) for 24 h. (B) RAW264.7 cells were treated with CS or various ECA with different nicotine concentrations (0.03125, 0.125, 0.5, 2, 8, and 32 μg/mL) for 48 h. Cell viability was measured using MTT assay. Data are expressed as means ± S.E. (#p > 0.05, *p < 0.05, **p < 0.01, and ***p < 0.001, n = 3).

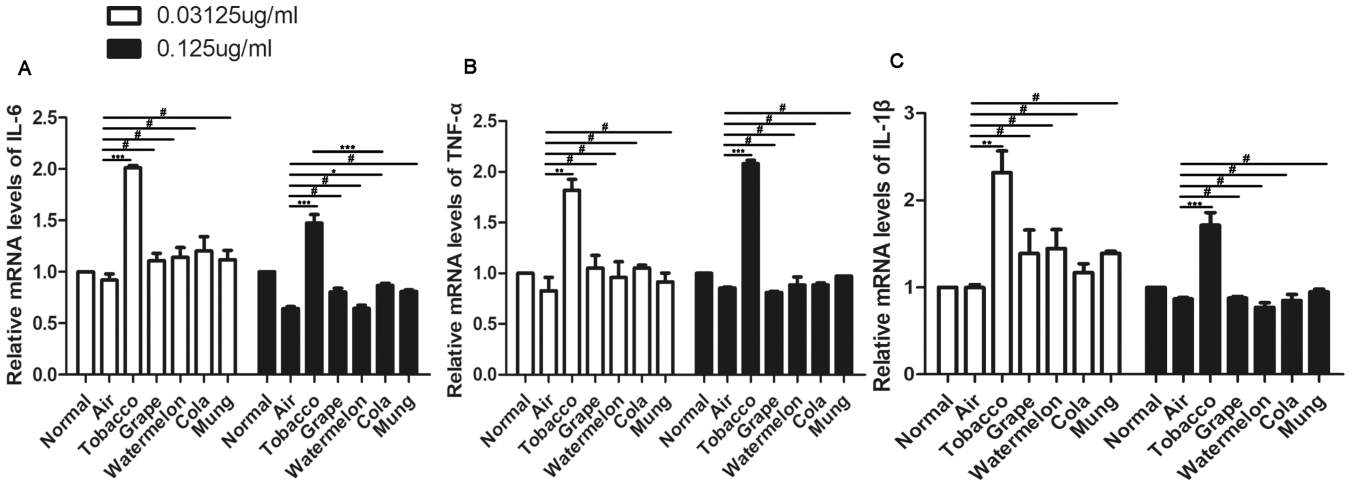

Figure 2. Impacts of ECA and CS on the mRNA levels of pro-inflammation cytokines in RAW264.7 cells. RAW264.7 cells were treated with various ECA or CS with nicotine concentrations of 0.03125 and 0.125 μg/mL for 24 h. The mRNA levels of (A) IL-6, (B) TNF-α, and (C) IL-1β in RAW264.7 cells were detected by RT-PCR assay. Data are expressed as means ± S.E. (#p > 0.05, **p < 0.01, and ***p < 0.001, n = 3).

Normally distributed data were expressed as mean ± SEM and compared using one-way ANOVA in SPSS v11.5. Post hoc comparisons were performed using the Tukey−Kramer multiple comparison procedure. Statistical significance was set at p < 0.05. The average fluorescence intensity was calculated using Image-J software, and images were processed using Graphpad Prism 5 (GraphPad Software, La Jolla, CA) and Adobe Photoshop CS6 (Adobe, San Jose).

3. RESULTS

3.1. Cell Viability Assessment. RAW264.7 cells were treated with ECA and CS containing different nicotine

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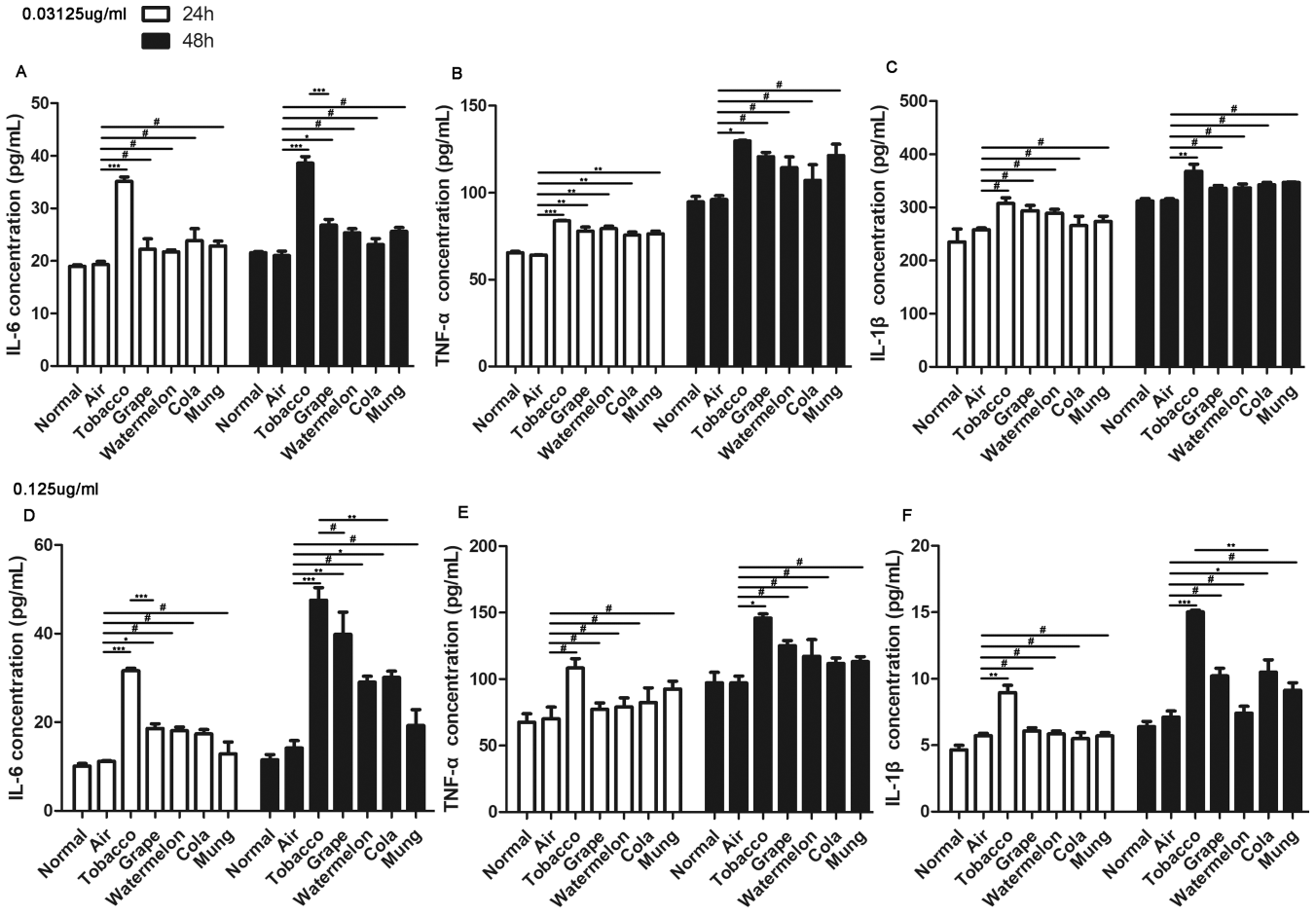

Figure 3. Impacts of ECA and CS on protein levels of pro-inflammation cytokines in RAW264.7 cells. RAW264.7 cells were treated with various of ECA or CS with nicotine concentrations of 0.03125 and 0.125 μg/mL for 24 and 48 h. The protein levels of (A) IL-6, (B) TNF-α, and (C) IL-1β in RAW264.7 cells at a nicotine concentration of 0.03125 μg/mL were detected using ELISA. The protein levels of (D) IL-6, (E) TNF-α, and (F) IL-1β at a nicotine concentration of 0.125 μg/mL in RAW264.7 cells were detected using ELISA. Data are expressed as mean ± S.E. (#$p > 0.05$, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, $n = 3$).

concentrations for 24 and 48 h, respectively. There was no significant difference between groups when treated with either CS or ECA for 24 h. But after 48h of treatment, we observed increased cell viability in the CS group but not in the ECA groups with different flavors at a nicotine concentration of 0.125 μg/mL. Moreover, cell viability treated with CS with nicotine concentrations at 0.03125 and 0.125 μg/mL increased to 130.3 and 117.5%, respectively, which were significantly different compared to the air groups ($p < 0.01$, $p < 0.05$). However, at nicotine concentrations of 2, 8, and 32 μg/mL, cell viabilities reduced in both CS and ECA with cola flavor groups (Figure 1B) to 77.82 and 72.4% at nicotine concentration of 2 μg/mL and to 74.25 and 66.85% at nicotine concentration of 8 μg/mL, respectively. At a nicotine concentration of 32 μg/mL, cell viability decreased to 68.54, 76.6, and 67.1% in CS, ECA with grape flavor, and ECA with cola flavor groups, respectively. These results suggested that at nicotine concentrations of 0.03125 and 0.125 μg/mL, cell viability significantly increased in the t-cigarette group but not in the e-cigarette groups. Therefore, nicotine concentrations of 0.03125 and 0.125 μg/mL were used in the following studies. Furthermore, at a nicotine concentration of 2 μg/mL, cell viability was higher in the ECA with grape flavor than in CS and ECA with cola groups ($p < 0.05$, $p < 0.05$) and higher in the ECA with watermelon flavor group than in the ECA with cola flavor group ($p < 0.05$). There was no significant difference among all groups at other nicotine concentrations.

3.2. IL-6, TNF-α, IL-1β, IFN-β, and MCP-1 Levels in RAW264.7 Cells. mRNA levels of IL-6, TNF- α and IL-1β were first examined using RT-PCR. After cells were treated with CS or ECA for 24 h, IL-6, TNF-α, and IL-1β mRNA levels increased significantly in the CS group but stayed steady in the ECA group (Figure 2). At a nicotine concentration of 0.125 μg/mL, the IL-6 level was higher in the ECA with cola flavor group than in the air group but still lower than in the CS group ($p < 0.05$, $p < 0.001$) (Figure 2A).

IL-6, TNF-α, IFN-β, MCP-1, and IL-1β protein levels were measured using ELISA. After 24 h of treatment, at a nicotine concentration of 0.03125 μg/mL, IL-6, MCP-1, and TNF-α protein levels were significantly higher in the CS group compared to the air group ($p < 0.001$) (Figures 3A,B, 4B), while the TNF-α level was higher in all ECA groups compared to the air group ($p < 0.01$). The MCP-1 level was higher in all ECA groups except the grape flavor group compared to the air group ($p < 0.01$) (Figure 4B). At a nicotine concentration of 0.125 μg/mL, the IL-6 protein level was higher in the ECA with grape flavor group than in the air group ($p < 0.01$) but lower than in the CS group ($p < 0.001$) (Figure 3D). The MCP-1 level was higher in all ECA groups except the grape

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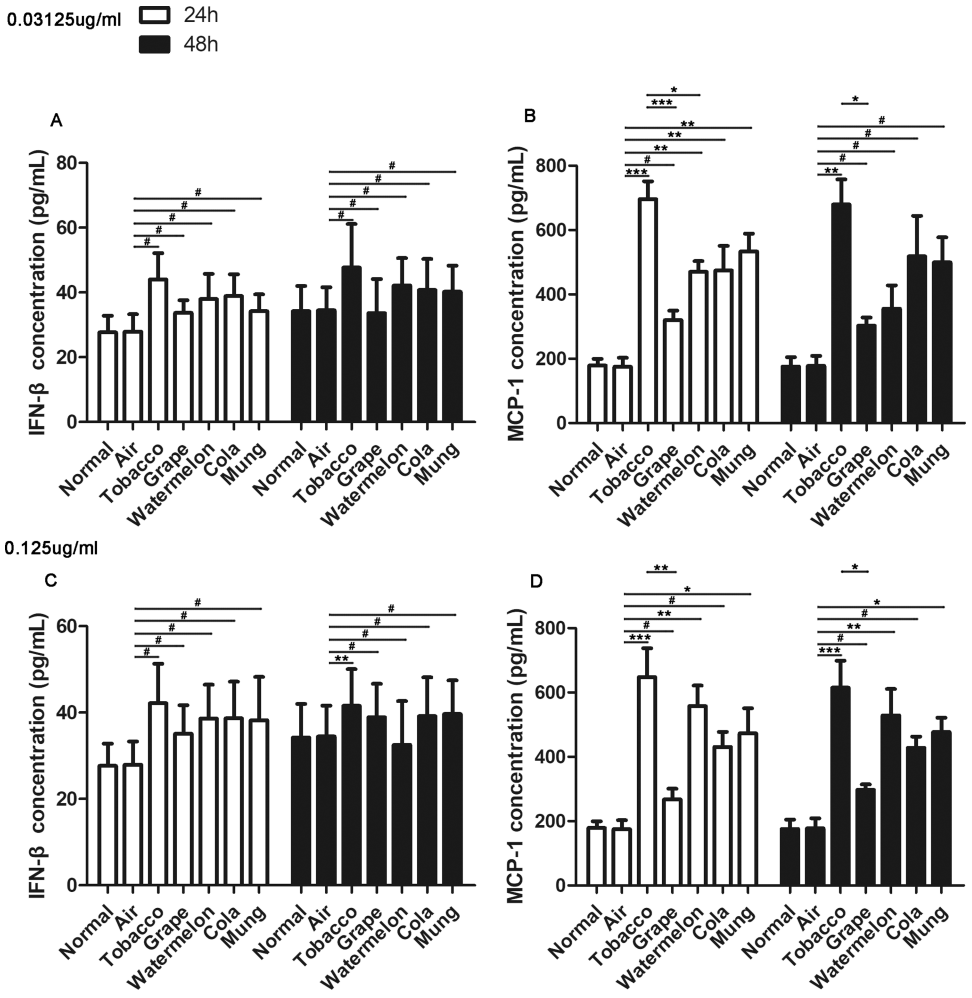

Figure 4. Impacts of ECA and CS on protein levels of pro-inflammation cytokines in RAW264.7 cells. RAW264.7 cells were treated with various ECA or CS with nicotine concentrations of 0.03125 and 0.125 μg/mL for 24 and 48 h. The protein levels of (A) IFN-β and (B) MCP-1 in RAW264.7 cells at a nicotine concentration of 0.03125 μg/mL were detected using ELISA. The protein levels of (C) IFN-β and (D) MCP-1 in RAW264.7 cells at a nicotine concentration of 0.03125 μg/mL were detected using ELISA. Data are expressed as mean ± S.E. (#$p > 0.05$, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, $n = 3$).

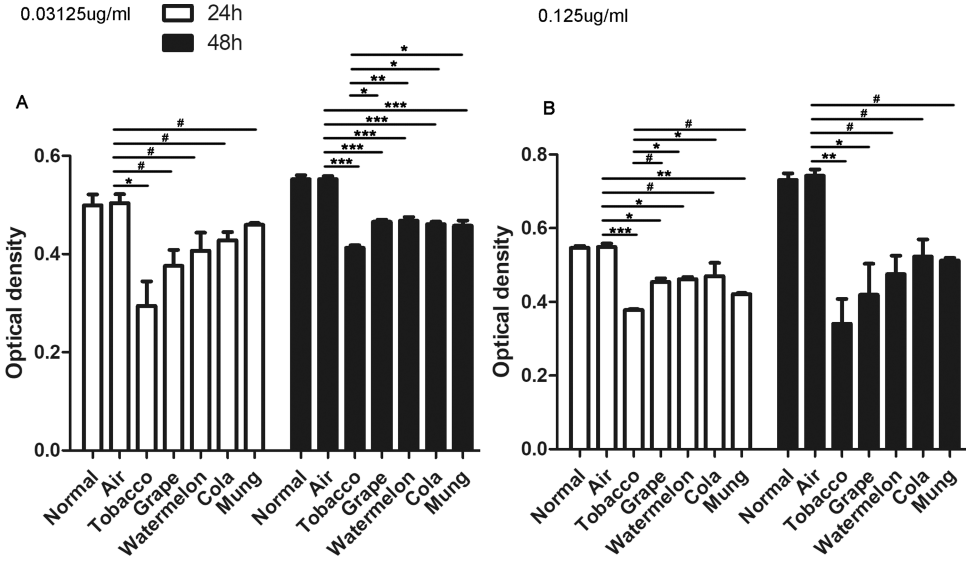

Figure 5. Impacts of ECA and CS on the phagocytosis of RAW264.7 cells. The RAW264.7 cells were treated with various ECA or CS with nicotine concentrations at 0.03125 μg/mL (A) and 0.125 μg/mL (B) for 24 and 48 h. Data are expressed as mean ± S.E. (#$p > 0.05$, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, $n = 3$).

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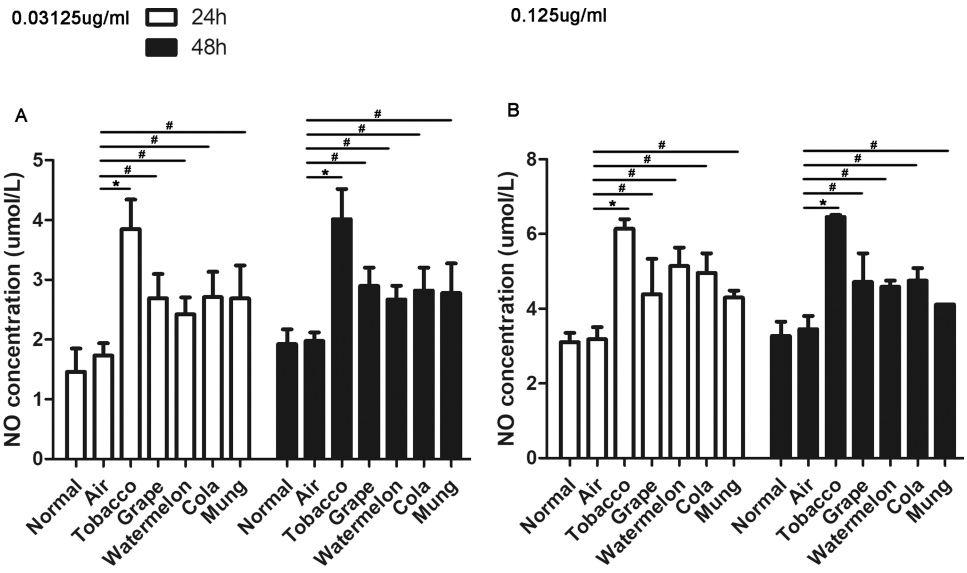

Figure 6. Impacts of ECA and CS on NO levels in RAW264.7 cells. RAW264.7 cells were treated with various ECA or CS with nicotine concentrations at 0.03125 μg/mL (A) and 0.125 μg/mL (B) for 24 and 48 h. Data are expressed as mean ± S.E. (#$p > 0.05$ and *$p < 0.05$, $n = 3$).

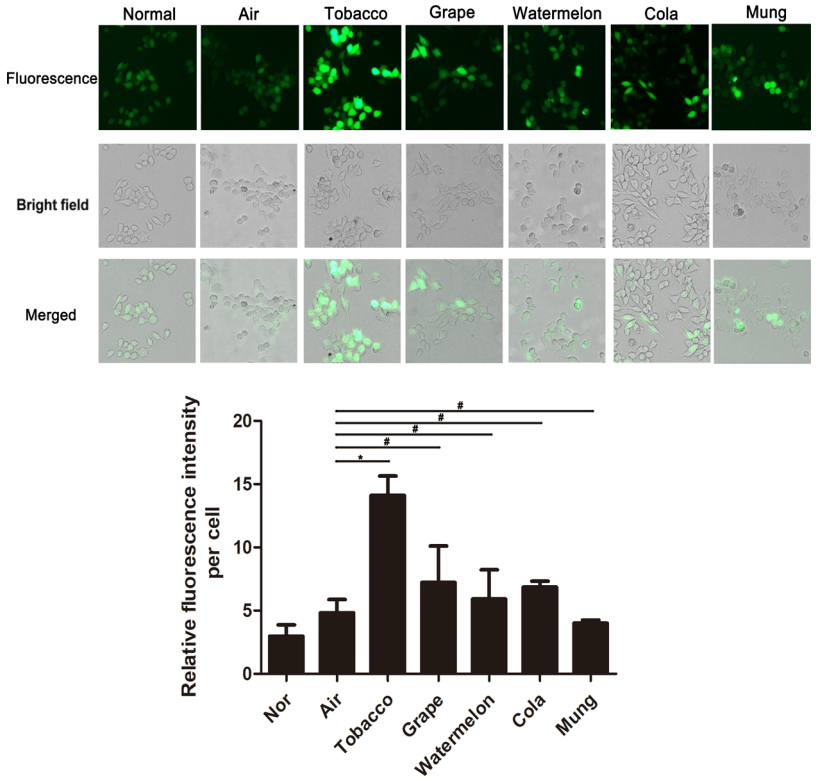

Figure 7. Impacts of ECA and CS on ROS levels in RAW264.7 cells. Cells were treated with various ECA or CS with a nicotine concentration at 0.125 μg/mL for 24 h. (A) Fluorescent images showing ROS levels in RAW264.7 cells. Scale bar: 10 μm. (B) The quantification of ROS levels in the aforementioned groups using a laser scanning confocal microcopy. Shown are the average fluorescence intensity calculated using Image-J software. Data are expressed as mean ± S.E. (#$p > 0.05$ and *$p < 0.05$, $n = 3$).

flavor group and the cola flavor group compared to the air group ($p < 0.01$) (Figure 4D)

After 48 h of treatment, at a nicotine concentration of 0.03125 μg/mL, IL-6, TNF-α, IL-1β, and MCP-1 protein levels were significantly higher in the CS group compared to the air group ($p < 0.001$, $p < 0.05$, $p < 0.01$, and $p < 0.01$, respectively). Moreover, the IL-6 protein level was higher in the ECA with grape flavor group compared to the air group but lower compared to the CS group ($p < 0.05$, $p < 0.001$) (Figure 3A−C). At a nicotine concentration of 0.125 μg/mL, IL-6, IL-1β, TNF-α, IFN-β, and MCP-1 protein levels were significantly higher in the CS groups than those in the air group ($p < 0.001$, $p < 0.01$, $p < 0.001$, $p < 0.01$) (Figures 3E and 4C,D). Moreover, IL-6, IL-1β, the protein level was higher in the ECA with cola flavor group than in the air group ($p < 0.05$ for all) but still lower than in the CS group ($p < 0.01$ for all) (Figure

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3D,F). The MCP-1 protein level was higher in the ECA with watermelon flavor group and the mung flavor group than in the air group ($p < 0.01$, $p < 0.05$) and lower in the grape flavor group than in the CS group ($p < 0.01$, $p < 0.05$) (Figure 4D). At nicotine concentrations of 0.03125 and 0.125 μg/mL, IL-6 and IL-1β show significant difference among ECA with watermelon, cola, and mung flavor groups (Figure 3A−E). These data suggested that compared with e-cigarettes, t-cigarettes could promote the release of inflammatory cytokines in macrophages.

3.3. Different Effects of ECA and CS on Phagocytic Activity of RAW264.7 Cells. Macrophages play a crucial role in the immune system by participating in the phagocytosis of foreign materials. Assessing the phagocytosis of RAW264.7 cells is an important means to investigate their immune activity. The neutral red uptake assay was used to determine the phagocytic activity of the RAW264.7 cells. Optical density was used as a measure to represent macrophages' phagocytosis capacity. At a nicotine concentration of 0.03125 μg/mL, after 24 h of treatment, the optical density of cells in the CS group reduced significantly. After 48 h of treatment, the optical density of cells in the CS and ECA groups with various nicotine concentrations significantly reduced compared to the air group ($p < 0.01$, $p < 0.001$). Moreover, the e-cigarette group exhibited higher cell phagocytosis compared to the t-cigarette group ($p < 0.05$, $p < 0.01$) (Figure 5A). At a nicotine concentration of 0.125 μg/mL, after 24 h of treatment, the optical density of cells in the CS group and the ECA with grape, watermelon, and mung flavor groups were significantly reduced ($p < 0.05$, $p < 0.01$, $p < 0.001$) compared to the cells in the air group. The cells in the ECA with watermelon and cola flavor groups exhibited higher phagocytosis than the cells in the CS group ($p < 0.01$). After 48 h of treatment, the optical density of cells in the CS group and the ECA with grape flavor group was reduced ($p < 0.01$, $p < 0.05$) (Figure 5B). Both CS and ECA demonstrated the ability to decrease macrophage phagocytosis, with macrophages in the t-cigarette group, showing lower phagocytic activity than those in the e-cigarette groups.

3.4. Effects of ECA and CS on NO Levels in RAW264.7 Cells. Nitric oxide (NO) plays crucial roles in various physiological processes depending on its sites of production. However, excessive NO production by macrophages can lead to the development of severe inflammatory diseases. At nicotine concentrations of 0.03125 and 0.125 μg/mL, after 24 and 48 h of treatment, NO levels released by RAW264.7 cells were significantly increased in the CS group compared to the air group ($p < 0.05$) (Figure 6). In contrast, NO levels in all ECA groups were not different from those in the air group ($p > 0.05$).

3.5. Impacts of ECA and CS on Reactive Oxygen Species (ROS) Levels in RAW264.7 Cells. After treating cells with different ECA or CS, the intracellular ROS level was assessed by observing fluorescence using a fluorescence microscope. In the CS group, the fluorescence intensity was much higher compared to the air group (Figure 7). The average fluorescence intensity was quantified using Image-J software. The relative ROS values for the normal group, air group, CS group, and CEA with grape, watermelon, cola, and mung groups were 2.978, 4.824, 14.115, 7.22, 5.91, 6.865, and 4.002, respectively. Compared with the air group, the ECA groups exhibited increased ROS levels without significant difference. These results demonstrated that incubation of RAW264.7 cells with CS promoted ROS production ($p < 0.05$) (Figure 7).

4. DISCUSSION

The emergence of e-cigarettes has been considered as an alternative to t-cigarettes. However, numerous studies have shown that both t-cigarettes and e-cigarettes are significant contributors to various diseases. For example, exposure to cigarette smoke has been linked to increased osteoclast numbers and bone remodeling *in vivo*, which can lead to osteoporosis.[15] Macrophages, a crucial play in chronic diseases, can be adversely affected by CS, causing dysfunction.[16] Repeated exposure to CS can also lead to macrophage infiltration and contribute to organ fibrosis development.[17] Macrophages are vital for immunity and protecting the body from pathogens. Studies have shown that both e-cigarettes and t-cigarettes can acutely affect heart rate, blood pressure, and arterial stiffness. Although ECA exposure induces oxidative stress in cultured macrophages, the level is comparatively lower than that induced by CS.[10] Furthermore, e-cigarette vapor has been shown to disrupt immune cell physiology impair the response to infectious challenges.[18] Both t-cigarettes and e-cigarettes have detrimental effects on the human body. Currently, e-cigarettes are being considered as smoking cessation or alternative products. Although studies have shown adverse effects of both e-cigarettes and t-cigarettes on macrophages, it remains unclear whether e-cigarettes are more or less harmful than t-cigarettes. Thus, we aimed to compare their toxic effects on macrophages.

In this study, RAW264.7 cells were treated with smoke water extract, simulating an e-smoking device used by humans. The system mimics the habits of an average smoker, allowing for nicotine concentration determination and maintaining a relatively constant chemical composition. Different types of ECA or CS were used, with varying nicotinic concentrations (0.03125, 0.125, 0.5, 2, 8, and 32 μg/mL). Nicotine, a harmful substance present in both e-cigarettes and t-cigarettes, was utilized as a quantitative substance in these experiments, investigating the effects of e-cigarettes and t-cigarettes on the human body.[19] The selected nicotine concentrations were within the average range nicotine concentration in normal human serum concentrations, considering that the average nicotine concentration in the plasma of smokers is reported to be 43.7 ± 38 ng/mL.[20] Given the relatively short duration of treatment in this study and the inability to fully simulate long-term smoking conditions, a slightly higher concentration (125 ng/mL nicotine) was chosen.

In the early stages of inflammation triggered by tissue injury, monocytes and macrophages initiate inflammation by expressing proinflammatory cytokines and interacting with other proinflammatory immune cells. However, persistent injury or unresolved inflammation can lead to chronic diseases, where macrophage activity becomes deleterious.[21] Research has shown that excessive proliferation of macrophages and the overproduction of inflammatory cytokines could keep the immune system in a constant state of battle, leading to immune cell exhaustion and compromised immune function.[21] Smoking is a chronic inflammatory factor in the body, and the overactivity of macrophages in response to chronic inflammation is harmful.[21] Our results showed that the cell viability and inflammatory factor levels were increased by CS at nicotine concentrations of 0.03125 μg/mL and 0.125 μg/mL. Similarly, exposure to ECA with cola flavor at nicotine concentrations of

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0.03125 μg/mL also promoted cell viability and inflammatory factor levels. However, the levels in CS groups were significantly higher than those in e-cigarette groups. These results indicate that t-cigarettes are more likely to activate the body's immune system, placing in a state of constant combat. Consequently, t-cigarettes are more likely to activate the induced immune cell exhaustion and weaken the immune system. IFN-β promotes the release of cellular inflammatory mediators.[22] MCP-1 plays a crucial role in regulating the migration and infiltration of monocytes/macrophages in inflammatory diseases.[23] Increased levels of MCP-1 and similar monocyte chemoattractant proteins may lead to extensive tissue damages.[24] Therefore, modulating these inflammatory mediators is important for the prevention and treatment of inflammatory diseases. Our results showed that the IFN-β and MCP-1 levels were increased by CS at nicotine concentrations of 0.03125 and 0.125 μg/mL. These results indicate that t-cigarettes are more likely to promote the development of inflammatory diseases.

In addition, reduced macrophage numbers also contributes to a weakened immune system and reduced immunity. In our study, the viability of RAW264.7 cells in the traditional cigarette group decreased when the nicotine concentrations were 0.03125 and 0.125 μg/mL. Cellular inflammation has adverse effects on chronic diseases. Notably, the inflammatory levels decreased in the CS and ECA with cola flavor groups when the nicotine concentration exceeded 2 μg/mL. This suggests that t-cigarettes with high nicotine levels can weaken the body's immune system. Overall, t-cigarettes pose greater harm to human health compared to e-cigarettes, regardless of the nicotine concentration being high or low.

Our findings revealed a decrease in cell phagocytosis in both t-cigarette and e-cigarette groups compared to the air group. Notably, the t-cigarette group exhibited even lower levels of phagocytosis compared to the e-cigarette group. Macrophages play a crucial role in eliminating invading bacteria, phagocytose foreign particles, clearing aging and damaged cells, and participating in immune responses.[25] Impairment of macrophage phagocytic function can lead to a decline in the ability to resist pathogens and phagocytose of foreign substances, ultimately resulting in weakened human immunity.[26] The defect in phagocytosis might be the upstream of cellular metabolism and could be associated with the reduced recognition of bacteria by macrophage surface receptors following CS exposure. This could also explain the higher frequency of infections in smokers.[27] Studies have indicated that monocyte-derived macrophages from healthy smokers exhibit impaired phagocytosis of *S. pneumonia* and *H. influenza*.[27,28] Additionally, blood mononuclear/macrophage cells from tuberculosis patients, especially those who smoke, have shown decreased phagocytosis.[29] Our results indicated that t-cigarettes significantly reduced macrophage phagocytic activity compared to e-cigarettes, suggesting that t-cigarettes may have a greater impact on reducing macrophage immune defense activity and overall human immunity.

NO is a signaling molecule involved in various physiological and pathophysiological processes. The prevailing hypothesis has suggested that NO contributes to toxicant-induced lung inflammation and injury.[30] Studies have described NO as a mediator of the inflammatory response, stimulating the production of inflammatory cytokines and peroxynitrite.[30] Excessive production of NO by iNOS in activated macrophages plays a key role in severe inflammatory diseases such as sepsis and arthritis.[31−33] Particularly, NO induces vascular reactivity collapse and pathological alterations.[34] Therefore, inhibiting NO production by activated macrophages has potential therapeutic effects on inflammatory diseases. Short-term exposure to CS has been shown to increase NO levels and potentially lead to acute lung inflammation.[30] Our results showed that t-cigarettes, but not e-cigarettes, significantly increased NO levels. Therefore, compared to e-cigarettes, t-cigarettes may contribute to the development of inflammatory diseases or exacerbate existing inflammatory diseases by elevating NO levels.

ROS, include oxygen radicals and peroxides, are oxygen-related species of high chemical reactivity. Their production is closely linked to the generation of NO, and ROS can reduce NO bioavailability,[35] contributing to oxidative stress and increased oxidative metabolism (ROS/RNS) response in pathological conditions.[35] Both NO and ROS are implicated in various diseases associated with host inflammation. Oxidative stress occurs when ROS production exceeds the antioxidant capacity, resulting in damage to cellular components such as DNA, lipids, and proteins. This damage can lead to cell death and extracellular matrix degradation.[36,37] Moreover, cellular oxidative stress triggers a cascade of inflammatory responses in humans.[38] CS is known to induce substantial ROS production, mainly released by activated cells, including macrophages, neutrophils, and structural cells.[39,40] Excessive ROS accumulation can also induce cell senescence, which is a significant risk factor for conditions like hypertension and chronic obstructive pulmonary disease (COPD).[41] Our findings revealed that t-cigarettes promoted ROS production in macrophages compared to e-cigarettes, suggesting that a potential link to inflammatory responses and increased susceptibility to COPD in humans.

5. CONCLUSIONS

This study compared the effects of t-cigarettes and e-cigarettes on RAW264.7 cells and concluded that e-cigarettes might serve as a viable alternative to t-cigarettes. However, it should be noted that e-cigarettes are not entirely without risks. Further research is needed to investigate the long-term effects of e-cigarettes on human health.

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