Review

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Impact of CSE and THC on the Respiratory System Mucosa: Insights on Novel Experimental Models

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The significant role of tobacco smoking as a primary risk factor for respiratory diseases has been extensively substantiated. In addition, cannabis use has been linked to persistent respiratory symptoms and numerous lung disorders in several case studies. Tetrahydrocannabinol (THC), the major active principle in all cannabis products, exerts its effects by binding to cannabinoid type 1 (CB1) receptors and, to a lesser extent, cannabinoid type 2 (CB2) receptors, which are differentially expressed in both the central and peripheral nervous systems, as well as other anatomical systems, including the respiratory one. Research conducted to date has enabled a comprehensive understanding of the mechanisms involved in cigarette smoking and its role in the development of chronic respiratory conditions. Recent investigations have also postulated that smoking cannabis may increase the risk of airway obstruction. This review focuses on fully understanding the impact of cigarette smoke extract (CSE) and THC on the human respiratory mucosa by using a methodological approach to assess the administration techniques and experimental models used in this field. Traditional single-layer cell cultures are a cost-effective research solution. However, they fail to replicate the complex structure and function of human organs' microenvironments *in vivo*. Therefore, after analysing various experimental models proposed in the literature, we concluded that the three-dimensional *ex vivo* culture model could be considered the most suitable one for studying the effects of CSE and THC on the respiratory mucosa.

Keywords: COPD; THC; CSE; culture models

Introduction

The legalisation of cannabis in several countries and the increased popularity of vaping, especially among young people, has drawn attention to the impact of cigarette smoke extract (CSE) and tetrahydrocannabinol (THC) on the human respiratory system. This review examines the impact of CSE and THC on the human respiratory mucosa. By analysing the delivery methods and experimental models used in this field, we aim to provide a comprehensive understanding of the potential implications of CSE and THC on respiratory health.

Cigarette smoke, a complex aerosol of gaseous and particulate matter, is known to induce inflammation at both bronchiolar and interstitial levels, contributing to the development of Chronic Obstructive Pulmonary Disease (COPD) and other fibrotic interstitial lung diseases [1]. This review is focused on the pathogenic role of CSE, highlighting its association with increased oxidative stress, mucosal inflammation, and altered immune responses, which are central to the pathophysiology of COPD.

According to the World Health Organization, chronic obstructive pulmonary disease is the third leading cause of morbidity and mortality worldwide [2].

COPD is a widespread and manageable condition that entails a progressive limitation of airflow and damage to lung tissues, affecting approximately 384 million people and resulting in estimated costs of over one hundred billion dollars annually [3]. This condition is characterised by structural changes in the lungs, stemming from chronic inflammation caused by prolonged exposure to harmful substances, such as cigarette smoke. Chronic inflammation leads to the narrowing of the airways and reduced lung elasticity, highlighting COPD's extensive scope and significant impact on public health and healthcare costs [1].

Although COPD is primarily associated with the lungs, it is considered to be a complex and multicomponent disease, characterised by chronic systemic inflammation that often coexists with other conditions, known as comorbidities, emphasising the interconnected and multifactorial nature of COPD and its association with a range of additional conditions that influence the management and progression of the disease (Fig. 1) [1].

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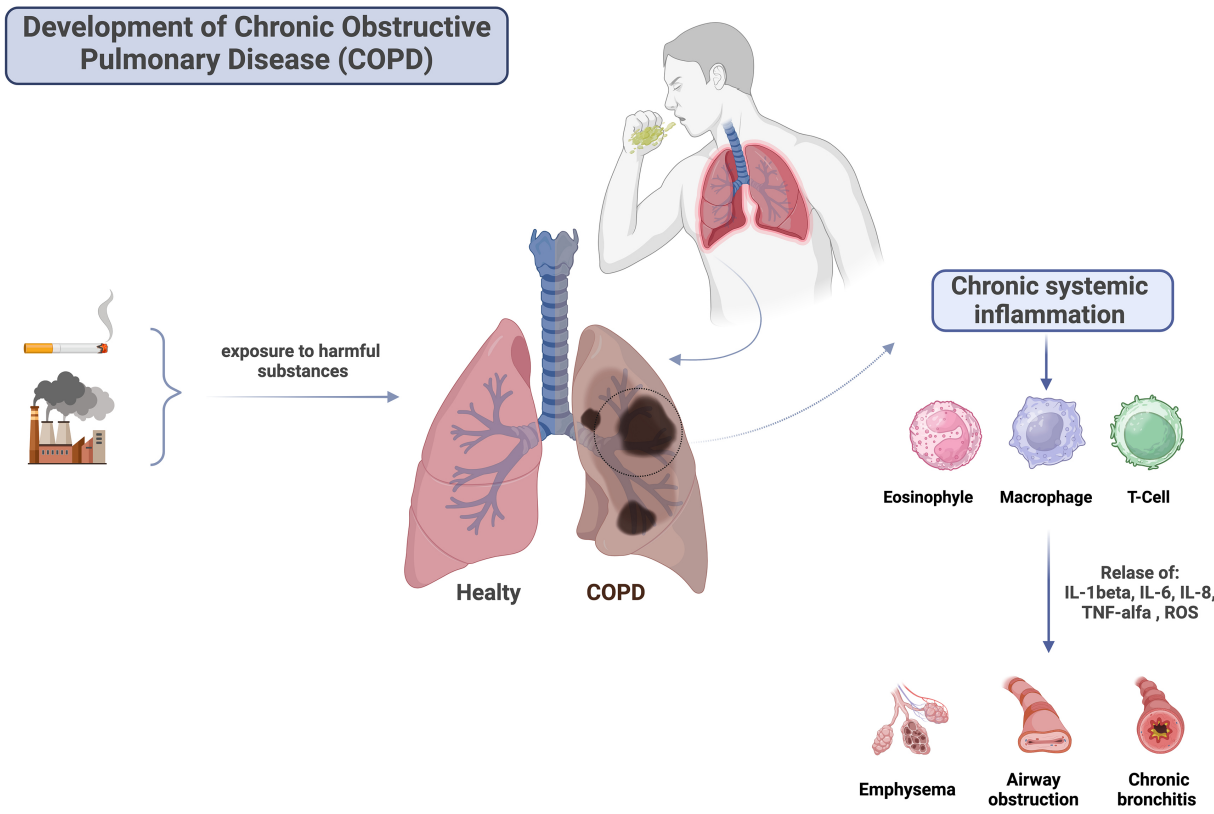

Fig. 1. Development of Chronic Obstructive Pulmonary Disease. Illustrative representation of the development of Chronic Obstructive Pulmonary Disease (COPD), highlighting the role of chronic systemic inflammation in influencing the condition's progression and its effects on respiratory health. Abbreviation: IL-1 beta, interleukin-1 beta; IL-6, interleukin-6; IL-8, interleukin-8; TNF-α, tumor necrosis factor α; ROS, reactive oxygen species. Created with https://www.biorender.com/.

The main causes of chronic bronchitis are attributed to cigarette smoking and environmental pollution. Smoking reduces ciliary motility and increases gland secretion, leading to mucus stagnation in the airways and triggering inflammatory processes [4]. The predominant symptoms include cough, bronchospasm resulting in airway obstruction and dyspnea. These symptoms are part of a negative clinical picture resulting from chronic bronchitis and emphasise the importance of identifying and mitigating the underlying causes to improve disease management and prevention. Symptoms of COPD may encompass chronic and progressive dyspnea, chronic cough, sputum production, and fatigue [5]. Patients often experience chronic cough, with or without sputum production, several years before the diagnosis of the disease, highlighting the relevance of this symptom as a precursor to airflow limitation. The onset of chronic cough in young smokers constitutes a risk factor for the development of COPD in adulthood [6]. On the other hand, dyspnea, and the perception of a decline in health status are not closely correlated with airflow limitation. This demonstrates the complexity and variety of symptoms associated with COPD and underscores the importance of a comprehensive symptom assessment for an accurate diagnosis and effective disease management.

Within the alveoli, the inflammatory response leads to the destruction of the intra-alveolar septa, resulting in a complication known as pulmonary emphysema. This pathological condition, often associated with smoking, is characterised by the destruction of elastin fibers due to the action of proteases, resulting in reduced antitrypsin. The rupture of the septa establishes communication between adjacent alveolar spaces, creating a less favorable ratio between air volume and alveolar membrane surface for gas exchange [7].

This paper provides an in-depth review of the impact of cigarette smoke extract (CSE) and THC on the human respiratory mucosa, which is crucial for understanding the progression and exacerbation of COPD and other respiratory diseases [8].

THC, the psychoactive component of cannabis, is also studied for its effects on the respiratory system. Despite its acute bronchodilatory effects, chronic exposure to THC has been linked to various respiratory symptoms. It may alter the function of alveolar macrophages, potentially compromising the defense mechanisms of the lungs. This review examines the similarities and differences between tobacco and marijuana smoke, emphasising the need for a nuanced understanding of their respective impacts on respiratory health.

Advanced *in vitro* and *ex vivo* models that mimic the human respiratory mucosa are essential to thoroughly assess the effects of CSE and THC. Traditional single-layer cell cultures are unable to accurately replicate the complex structure and function of the respiratory system. Therefore, this review advocates for the use of three-dimensional cell culture models, such as air-liquid interface cultures and lung organoids, which provide more physiologically relevant conditions to study the interactions between these substances and the respiratory mucosa.

This review also provides a comprehensive overview of the current understanding of how CSE and THC influence respiratory diseases. It emphasises the necessity of advanced *ex vivo* models to unravel the complex cellular and molecular mechanisms affected by these substances within the respiratory system, thus contributing to the development of targeted interventions for respiratory diseases.

The Impact of Tobacco Smoke on Respiratory Health and Disease Development

Tobacco smoke, an aerosol comprising gaseous and suspended particulate matter, contains liquid droplets with a wide range of condensed organic compounds [9]. Upon inhalation, compounds like nicotine can deposit in the respiratory tract through four mechanisms:

(1) direct gas deposition;
(2) evaporative gas deposition;
(3) particle deposition with evaporation;
(4) particle deposition with diffusion.

Tobacco smoke is acknowledged as a pathogenic or triggering agent for widespread lung conditions, marked by inflammation at both bronchiolar and interstitial levels [10]. Smokers are at an increased risk of developing fibrotic interstitial lung diseases, including idiopathic pulmonary fibrosis and interstitial lung disease associated with rheumatoid arthritis [11]. Additionally, some smokers may develop a combination of emphysema and pulmonary fibrosis [12]. Cigarette smoke, in conjunction with environmental pollution, is the primary causative factor for chronic bronchitis development [13]. Smoking diminishes ciliary movement and increases glandular secretion, resulting in mucus stagnation within the airways and inciting inflammatory processes. This combined impact significantly contributes to the chronic inflammatory state observed in chronic bronchitis [14]. Moreover, the activation of inflammatory cascades due to mucus stasis not only perpetuates chronic bronchitis, but also creates an environment conducive to recurrent respiratory infections and exacerbations, thereby aggravating the overall disease burden in affected individuals.

Several studies suggest that young smokers may develop respiratory symptoms in the early years of tobacco smoke exposure [14,15]. However, research on such respiratory symptoms focuses on individuals with significant exposure from many years of smoking. A more comprehensive understanding of the effects of smoking in young adults is still needed, and it is inappropriate to extrapolate the early effects of smoking on the respiratory tract in those under 30 from publications on older age groups. Therefore, a study conducted by the research team led by Vianna *et al*. [16] aimed to analyse the effects of tobacco smoke on lung function and respiratory symptoms in a young population (23–25 years old) in Ribeirão Preto, Brazil.

The most notable finding of this study was the marked association between tobacco consumption and respiratory symptoms among young adults, particularly in those who smoked 10 or more cigarettes per day. The study revealed that smoking was associated with wheezing, cough, breathlessness, and morning phlegm. Notably, the smoker group did not show an association with bronchial hyperresponsiveness or obstruction, as assessed by forced expiratory volume in 1 second (FEV1). Therefore, this study underscores the early health consequences of smoking among young adults and emphasises the potential reversibility of these effects upon smoking cessation. In 2016, another study involved 3108 high school students from the Mazovian Region in Poland to assess this population's smoking habits and respiratory diseases. The results unveiled that smoking is one of the primary causes of respiratory diseases in young individuals, with a notably higher prevalence of chronic bronchitis among smokers [17]. Smoking is linked to several potential respiratory complications, encompassing widespread lung disorders, such as desquamative interstitial pneumonia (DIP), respiratory bronchiolitis-associated interstitial lung disease (RB-ILD), and pulmonary langerhans cell histiocytosis (LCH) [7]. These conditions predominantly affect relatively young smokers and can lead to progressive lung damage. Furthermore, cigarette smoke is acknowledged as a precipitating factor for acute eosinophilic pneumonia and heightens the risk of developing fibrotic interstitial lung diseases, such as idiopathic pulmonary fibrosis (IPF) and diffuse lung disease associated with rheumatoid arthritis. Additionally, some smokers may develop a combination of upper lobe emphysema and lower lobe fibrosis [10].

Tobacco smoke has been extensively studied to comprehend its impact on immune responses, such as contributing to the development of lung diseases like Chronic Obstructive Pulmonary Disease (COPD) and other pulmonary conditions. Notably, a recent association has surfaced between environmental passive smoke (ETS) and the onset of asthma and allergic diseases in children. Exposure to cigarette smoke triggers an imbalance between oxidants and antioxidants, leading to oxidative stress, mucosal inflammation, and an upsurge in the expression of inflammatory cytokines, such as interleukin (IL)-8, IL-6, and tumor necrosis factor α (TNF-α) [18]. Moreover, cigarette smoke significantly alters the morphology, function, and polarisation of alveolar macrophages, leading to a compromised immune response and heightened susceptibility to infections. These alterations play a pivotal role in the impaired lung defense mechanisms observed in smokers.

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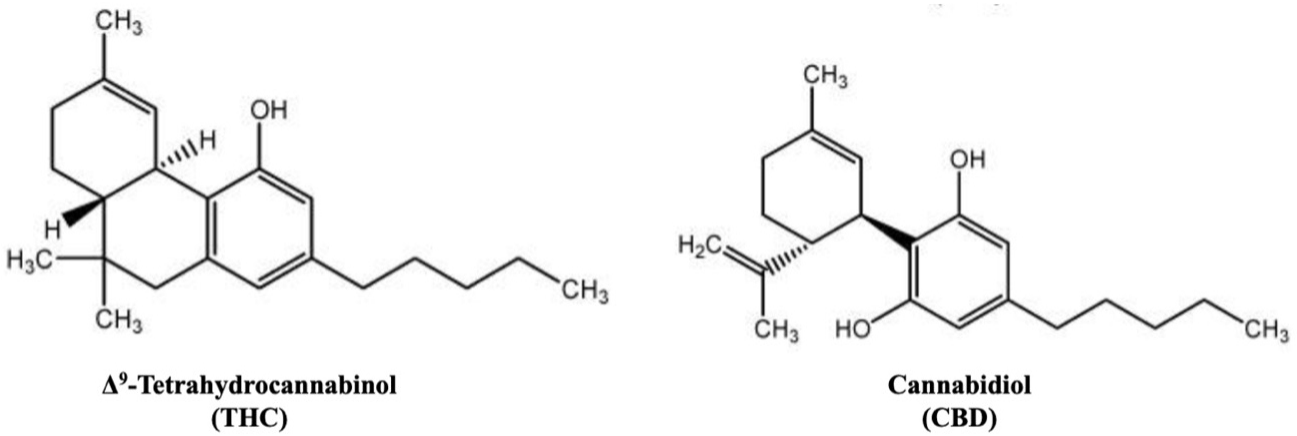

Fig. 2. Chemical structure of THC and CBD. Abbreviations: CBD, cannabidiol; THC, tetrahydrocannabinol.

A recent comprehensive study has delved into the intricate relationship between Chronic Obstructive Pulmonary Disease (COPD) and various parameters associated with smoking. The findings underscore an escalated risk of COPD in correlation with the duration and intensity of smoking, as well as the cumulative amount of tobacco consumed over a lifetime. Notably, heavy smokers exhibit a heightened susceptibility to developing COPD. However, the study revealed a substantial decrease in the risk of COPD with prolonged smoking cessation, with the risk potentially equating to that of a non-smoker after 15–25 years of abstinence. Furthermore, the study emphasises that the duration and intensity of smoking are the most significant predictive factors for the risk of COPD [7,18].

The Complex Relationship between Cannabis Use and Respiratory Health

Cannabis sativa, commonly known as marijuana, is a plant belonging to the Cannabaceae family, encompassing the dried leaves and flowers of the plant. Marijuana is composed of over 60 cannabinoids and more than 400 other compounds [19]. Among the cannabinoids isolated from cannabis, the two most significant compounds are cannabidiol (CBD) and Δ9-tetrahydrocannabinol (Δ9-THC) [20] (Fig. 2).

CBD was initially isolated from marijuana in 1940, and its structure was officially reported in 1963 [21]. Despite being overshadowed by THC due to its lack of psychoactive properties, CBD's significance has gradually gained recognition. On the other hand, the structure of the primary psychoactive phytocannabinoid, THC, was determined in Israel by Mechoulam and Gaoni in 1964, marking a pivotal milestone in cannabinoid research [22].

Cannabis use can significantly impact the response to stress and reward, ultimately contributing to a sense of well-being. However, prolonged cannabis use may compromise sensitivity to stress and reward, thereby heightening the risk of dependence and potential negative consequences, particularly in vulnerable individuals. Δ9-THC, a prominent component of cannabis, possesses analgesic properties and induces relaxing psychoactive effects, further influencing the overall impact of cannabis use [23]. Simultaneously, other components, such as cannabinol (CBN) and cannabidiol (CBD) have been shown to have antiepileptic, antiemetic and sedative effects, broadening the spectrum of potential therapeutic benefits associated with cannabis. However, the use of these substances, particularly THC, remains contentious due to the myriad of observed negative effects on human health, particularly at the neurobehavioral level, over prolonged periods.

Δ9-THC exerts its effects by interacting with cannabinoid type 1 (CB1) and cannabinoid type 2 (CB2) receptors in the endocannabinoid system, which are widely distributed throughout the human body, encompassing the brain, central nervous system, and immune system [24]. When THC activates CB1 receptors in the central nervous system, it elicits psychoactive and relaxing effects, influencing cognitive and emotional processes. On the other hand, its interaction with CB2 receptors impacts the immune system, potentially modulating inflammatory responses and immune function [25]. In the lungs, the activation of these receptors can significantly impact the inflammatory response, immune cell function, and airway contractility. THC's interaction with CB1 and CB2 receptors holds the potential to influence bronchodilation and may exert effects on inflammatory and immune responses within the respiratory system, thereby contributing to the complex interplay between cannabis use and respiratory health [26].

The long-term effects of cannabis have not been properly explored until relatively recently. Notably, the medicinal use of cannabis was thoroughly reviewed during a conference at the UCLA School of Medicine in Los Angeles, California, through discussions on its historical significance, therapeutic implications, and the comprehensive assessment of its short- and long-term effects on health [27]. Inhalation of cannabis has been linked to diminished physical performance during exercise and potential adverse im-

Table 1. Effects of tetrahydrocannabinol on the respiratory system.

Long-Term effects	Short-Term effects
Chronic bronchitis	Acute bronchitis
Increased risk of lung infections	Irritation of the airways
Reduced lung function	Cough
Respiratory symptoms, such as cough and phlegm production	Phlegm production

pacts on lung function and immune responses. Furthermore, cannabis can influence the cardiovascular system, potentially leading to tachycardia and hypotension. In the short term, cannabis demonstrates bronchodilatory effects, presenting the possibility of developing cannabinoid compounds for bronchodilation therapy. However, long-term studies indicate that smoking marijuana can result in lung damage, including severe bronchiolitis and squamous metaplasia of the tracheal mucosa, underscoring the need for a comprehensive understanding of the potential respiratory implications of cannabis use [24,26,28] (Table 1).

In 2002, Tashkin and colleagues [29] conducted a detailed analysis of the effects of habitual marijuana smoking on the respiratory and immune systems. Regular marijuana use has substantial clinical implications for the respiratory and immune systems, as it can lead to the development of chronic bronchitis symptoms, a heightened frequency of acute bronchitis episodes, and the presence of microscopic and immunohistochemical alterations in tracheobronchial biopsies, including airway lesions and unregulated growth, potentially impacting long-term respiratory health [30].

These changes are likely to compromise the mucociliary clearance function of the lungs, potentially increasing the susceptibility to lower respiratory tract infections and malignancies. Furthermore, regular marijuana use is associated with ultrastructural and functional alterations in alveolar macrophages, which serve as the primary resident immune cells in the distal lung [31]. Regular marijuana use may also compromise the lung's defense mechanisms against infections by inhibiting the antimicrobial activity of alveolar macrophages and impeding the production of essential proinflammatory cytokines required for immune activation, potentially contributing to an increased susceptibility to respiratory infections [32]. Furthermore, potential long-term effects on lung function have been reported, with negative implications for lung capacity and the presence of respiratory symptoms [33]. However, current evidence does not indicate a significant association between regular marijuana use and Chronic Obstructive Pulmonary Disease (COPD). While certain studies have hinted at a potential link between regular marijuana use and the onset of respiratory tract tumors, additional research is essential to definitively establish this relationship and comprehensively understand the potential long-term respiratory implications of marijuana use [29,30,34]. The short-term effects of THC on the respiratory system have been shown to encompass a substantial acute reduction in airway resistance and an elevation in specific airway conductance, implying an acute bronchodilatory response. A subsequent study conducted by Kaiser Permanente, involving 452 daily marijuana smokers and 450 non-smokers, revealed a significant increase in outpatient visits for respiratory diseases among marijuana smokers. The relationship between cannabis use and asthma, as well as allergies, is a complex issue that has proven very difficult to evaluate accurately [35]. Historically, cannabis has been utilised to alleviate asthma symptoms due to its mild bronchodilator effects. Nevertheless, recent research has brought to light a potential association between cannabis use and the aggravation of asthma symptoms, including an uptick in exacerbations and the emergence of new asthma-like symptoms, underscoring the need for a comprehensive understanding of the potential impact of cannabis use on respiratory health [35,36]. The acute bronchodilator effect is caused by THC. Given the diverse actions of cannabinoids, the precise effects of different types of cannabis, each containing varying concentrations of cannabinoids, on the airways remain uncertain. Recent analyses have delved into evidence indicating a heightened prevalence of respiratory symptoms among regular cannabis users, including chronic cough, sputum production, breathing difficulties, hoarseness, and chest tightness. It is necessary to continue studying the effects of cannabis to clarify the intricate relationship between cannabis use and respiratory health [30,37]. The similarities between marijuana smoke and cigarette smoke in terms of chemical characteristics suggests that smoking cannabis may also have the potential to cause respiratory symptoms. It is however essential to note the distinction in the composition of tobacco and cannabis smoke: while nicotine, the primary additive in tobacco, is only produced during the combustion of tobacco leaves, THC serves as the primary psychoactive component of cannabis. THC, along with other substances like CBN and CBD, is present exclusively when marijuana is burned. Although both smokes contain similar levels of volatile components, such as ammonia, hydrogen cyanide, and nitrosamines, their qualitative resemblance is primarily evident in tar components, including phenols, naphthalene, pre-carcinogenic benzo[a]pyrene, and benzo[a]anthracene. Therefore, further studies are needed to provide a comprehensive understanding of the potential respiratory implications of marijuana smoke in comparison to cigarette smoke [38].

The impact of cigarette smoke on pulmonary health is widely recognised, leading to symptoms such as cough,

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persistent sputum production (indicative of chronic bronchitis), wheezing, and breathing difficulties. Additionally, observable spirometric changes include a progressive and predominantly irreversible decline in the forced expiratory volume in 1 second (FEV1) and the ratio of FEV1 to forced vital capacity (FVC)—FEV1/FVC, highlighting the intricate and potentially detrimental effects of cigarette smoke on respiratory function [39]. This ratio is the most reliable parameter for assessing airflow obstruction [39]. Cytological variations in the airways, along with respiratory symptoms, such as wheezing, cough, persistent sputum production, and shortness of breath, as well as impact on overall lung function, have all been linked to cannabis smoking. Previous research has suggested that marijuana smoke may subject users to increased deposition of carbon monoxide and tar compared to tobacco smokers, and that the incidence of airway obstruction caused by marijuana smoke could be 2.5–5 times higher compared to tobacco smoke [40].

Acute and Chronic Impacts of CSE and THC on Respiratory Health

Studies conducted over the years have broadened our knowledge on the different responses of the body, and in particular the respiratory system, to CSE and THC. It is therefore possible to analyse and differentiate some of the acute and chronic effects of these substances on respiratory health. Firstly, acute exposure to CSE can cause immediate inflammation and irritation of the respiratory mucosa. This can lead to symptoms such as coughing, wheezing and shortness of breath. CSE induces oxidative stress that can damage the epithelial cells lining the airways. This oxidative damage can impair the mucociliary cleaning mechanism, thus making the lungs more susceptible to infection. In addition, it can cause bronchoconstriction, resulting in a temporary reduction in airflow and increased respiratory resistance.

THC, on the other hand, possesses acute bronchodilator effects, which may temporarily improve airflow and reduce airway resistance. Smoking a single cannabis joint with 2% THC causes acute bronchodilation that lasts about one hour in healthy subjects [41]. For this reason, cannabis has historically been used to alleviate asthma symptoms. Despite its bronchodilatory effects, acute exposure to THC can irritate the respiratory tract, causing coughing and production of phlegm. Acute THC exposure may modulate the immune response, potentially affecting the activity of alveolar macrophages, which are crucial in defending the lungs from pathogens.

Moreover, prolonged exposure to CSE is a significant risk factor for chronic bronchitis, characterised by persistent coughing and sputum production. Chronic exposure to CSE is associated with the development of COPD, which involves progressive and irreversible airflow limitation due to chronic inflammation and structural changes in the lungs. In addition, there is a reduction in lung function, evidenced by the reduction in forced expiratory volume in 1 second (FEV1) and the ratio of FEV1 to forced vital capacity (FVC)—FEV1/FVC.

Continuous exposure to CSE impairs mucociliary clearance and immune defense mechanisms, increasing susceptibility to respiratory infections.

According to a recent study, chronic cannabis use has been linked to the onset of chronic bronchitis symptoms, such as coughing, dyspnea, wheezing and increased sputum production. It is important to note that a significant proportion of the individuals experiencing these symptoms were joint tobacco smokers [42]. Cannabis smoke has also been associated with various forms of lung damage, such as bronchiolitis and squamous metaplasia of the tracheal mucosa. One study collected bronchial mucosa samples by biopsies from cannabis-smoking individuals and observed pathological changes, including basal cell hyperplasia, basement membrane thickening, calico cell hyperplasia and squamous cell metaplasia [43].

Chronic effects of cannabis smoke may include altering the function of alveolar macrophages, impairing the lungs' defense mechanisms and increasing the risk of infection. Some studies suggest that chronic marijuana smoking may increase the risk of airway obstruction, although the evidence is not as strong as for tobacco smoking [42,44].

Thus, the evaluation of the effects of cigarette smoke extract (CSE) and tetrahydrocannabinol (THC) on the human respiratory mucosa is functional to better understanding the progression of respiratory diseases. Consequently, advanced culture models that mimic the physiological conditions of the human respiratory system are essential to provide accurate results.

Molecular Mechanisms of THC and CSE in Lung Tissue: Inflammation, Damage, and Repair Pathways

THC and CSE exert their effects in the body by binding to specific receptors and triggering response mechanisms that contribute to the activation of inflammatory processes, cellular damage and repair mechanisms. THC acts mainly by binding to CB1 and CB2 cannabinoid receptors, which have different localisations. CB1 receptors are mainly found in the central nervous system, but are also present in the lungs, while CB2 receptors are primarily located in peripheral tissues, including immune cells. Once bound, THC activates the G-protein-coupled receptor (GPCr) pathway, leading to inhibition of adenylate cyclase, reduction of cyclic AMP (cAMP) levels and modulation of ion channels. Downstream, there will be alteration of neurotransmitter release and immune cell function [44].

At the inflammatory level, THC can modulate the production of both pro-inflammatory and anti-inflammatory cytokines. It has been shown to reduce the levels of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6,

while increasing those of anti-inflammatory cytokines, such as IL-10. This dual modulation can lead to a complex immune response in lung tissue.

A study by Huff *et al*. [45] shows how exposure to cannabis smoke extract increases the proinflammatory cytokine interleukin-8/CXCL8, an epithelial cell repair mediator. IL-8 transforms growth factor-α and oxidative stress gene expression in the cell culture medium of airway epithelial cells. This suggests a role for cannabis smoking in airway remodeling. Habitual smokers exhibit widespread macro- and microscopic metaplastic and inflammatory lesions in the central airway, including loss of ciliated epithelium and replacement with hypersecreting goblet cells [45]. Frequent exposure to THC also affects the function of alveolar macrophages, impairing their phagocytic activity, and can induce oxidative stress by generating reactive oxygen species (ROS), which can damage cellular components such as lipids, proteins and DNA.

This oxidative damage can impair the function of epithelial cells and other lung cells. THC has been shown to induce apoptosis and autophagy in lung cells. These processes are mediated through pathways involving caspases, B cell lymphoma-2 (Bcl-2) family proteins and autophagy-related genes (ATGs).

CSE, on the other hand, is a complex mixture of thousands of chemicals, including nicotine, tar, carbon monoxide and various carcinogens. In addition, smoking tobacco products exposes the body to acrolein, a highly reactive unsaturated aldehyde [46].

These components can interact directly with lung epithelial cells and immune cells. Nicotine, one of the main components of CSE, binds to the nicotinic acetylcholine receptors (nAChRs) of lung cells, leading to the activation of downstream signaling pathways.

Cigarette smoke may act by increasing the expression of mucin genes, particularly *MUC5AC* and *MUC7*, and to a lesser extent *MUC1* and *MUC2*. It may also synergistically increase the response to pro-inflammatory cytokines and bacterial infections [47].

Cigarette smoke activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, a key regulator of inflammation. NF-κB activation leads to the transcription of pro-inflammatory genes, including those encoding cytokines (e.g., TNF-α, IL-1β), chemokines (e.g., IL-8) and adhesion molecules. It also activates the mitogen-activated protein kinase (MAPK) pathway, which includes ERK, JNK and p38 MAPK. These kinases regulate the expression of inflammatory mediators and contribute to cellular responses to stress [48].

Like THC, CSE induces oxidative stress by generating ROS and reactive nitrogen species (RNS). This oxidative stress can lead to lipid peroxidation, protein oxidation and DNA damage, contributing to dysfunction and cell death. Components of CSE, such as polycyclic aromatic hydrocarbons (PAHs) and nitrosamines, can form DNA adducts and cause mutations. DNA repair mechanisms, including nucleotide excision repair (NER) and base excision repair (BER), are activated in response to this damage. However, chronic exposure can overload these repair systems, leading to genomic instability.

CSE can induce both apoptosis and necrosis in lung cells. Apoptosis is mediated by intrinsic (mitochondrial) and extrinsic (death receptors) pathways, involving caspases and Bcl-2 family proteins [18]. The necrosis process, on the other hand, is unable to repair lung cells.

The Evolution of *In Vitro* Models for Investigating Respiratory Diseases

Understanding the pivotal role of extracellular microenvironments in orchestrating cellular dynamics is of utmost importance in investigating the Epithelial-Mesenchymal Transition Unit (EMTU) in chronic inflammatory respiratory diseases [49]. Therefore, the establishment of an accurate and dependable model of human bronchial mucosa is imperative, marking a substantial step towards a comprehensive understanding of the pathological mechanisms involved in chronic respiratory diseases (Table 2, Ref. [49–56]).

Over time, the significance of respiratory tract cell culture models has been scrutinised in the field of respiratory pathology and drug delivery. The use of animal models in respiratory disease research has served as a valuable asset in understanding lung development and diseases, further underscoring the multifaceted approaches employed in respiratory research [54]. A variety of animal models have been developed for respiratory disease research, predominantly utilising rodents, such as genetically modified mice and rats, as well as guinea pigs. These small animal models effectively replicate specific aspects of COPD, making the selection of the most representative model a critical consideration in the advancement of novel COPD treatments [57]. These species are preferred due to their practicality, affordability, and the possibility of using genetically tailored strains to create models that are based on specific biological mechanisms. Research efforts are also extending to larger animals, including dogs and sheep, because their anatomical structures are more similar to humans [58]. Nevertheless, the physiological disparities between animals and humans pose a significant challenge in directly extrapolating findings from animal models to human conditions, thereby limiting the validity of data gathered in the context of human respiratory pathologies. This incongruity can affect the accuracy of the results. Furthermore, ethical concerns surrounding the use of animals in testing and experiments, genetic diversity across various animal species, the array of immunological responses, and the substantial costs and resources involved in the maintenance and execution of animal studies collectively present additional challenges in the extensive and effective application of animal models in respiratory disease research. Employing a suitable cell

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Table 2. Culture models.

Culture model	Advantages	Disadvantages	
Primary Cell	Can exhibit fully differentiated phenotypes and relevant markers, which are crucial for inhalation hazard identification and respiratory disease models. Can also be pooled to mitigate donor variability.	There is potential donor-to-donor variability, which can affect the consistency of results.	[49]
Air-Liquid Interface Cultures	Offer increased differentiation of morphology, barrier function, and a stable transepithelial electrical resistance (TEER). Provide a strong defense against toxins and are easier to expose to respirable substances.	More expensive due to the need for specialised culture inserts and have greater permeability to test substances, which may not always be desirable.	[50]
Co-Culture Systems	Can simulate the air-blood barrier more accurately by incorporating multiple cell types, such as macrophages, epithelial cells, and dendritic cells (DCs).	Can be complex to set up and may require validation to ensure their predictive value.	[51]
Precision-Cut Lung Slices	Can be generated under controlled conditions, contain all cell types in the lung, and maintain cell-cell communication.	Limited lifespan in culture, which restricts the duration of studies. There can also be variability in results between slices and donors.	[52]
EpiAirway Cells	A human primary differentiated cell culture that grows into an organotypic multilayer model with some barrier functions. Useful for assessing a range of responses to respiratory toxins.	May not represent the complexities of the intact respiratory system and are yet to be properly validated for regulatory acceptance.	[53]
Transgenic Knock-Out Mouse Models	Increasingly used to model asthma and can provide insights into specific biological mechanisms.	May not accurately represent human disease due to interspecies differences.	[54]
3D Co-Culture Models	Offer a more accurate representation of the air-blood barrier by including macrophages, epithelial cells, and DCs.	Complexity of the setup and the need for validation to confirm their relevance and accuracy.	[55]
Ex Vivo Human Systems	Show potential as useful models and are amenable to high-throughput experimental approaches.	Lack some functionality, have a limited lifespan, and may suffer from donor-to-donor variability.	[56]

By examining both the advantages and disadvantages of each model, this scheme provides a comprehensive overview of the benefits and limitations associated with various culture models in the context of studying the human respiratory system. 3D, three-dimensional.

culture model holds a paramount importance in assessing drug absorption and metabolism, as well as comprehending transport mechanisms across epithelial barriers. Leveraging *in vitro* models can expedite the advancement of new drugs, facilitating faster and more cost-effective testing processes, thereby streamlining the drug development pipeline [59]. Early efforts in culturing primary human lung cells involved a two-dimensional (2D) method, wherein primary epithelial cells from human nasal or bronchial brushings are cultured on a plastic plate submerged in media. These 2D monolayer cultures, known for their simplicity in generation, are used for diagnosing ciliary dysfunction [49].

It is essential to consider both the advantages and limitations of the three primary *in vitro* approaches for studying epithelial barriers:

(1) Primary culture models offer the advantage of providing a more faithful representation of native epithelium and a more accurate *in vitro* depiction. However, limitations include the limited availability of normal human tissue, a restricted number of cells, and donor variability.

(2) Transformed cell lines have the advantage of a known origin and the ability to be maintained in culture for extended periods. Nevertheless, limitations involve the lack of accurate representation of native epithelium and the potential to exhibit atypical phenotypes.

(3) Co-culture models offer the advantage of mimicking *in vivo* real-life conditions [51]. However, limitations consist of implementation complexity and the absence of a model that combines all representative features. For this reason, it is essential to select the most suitable cell culture model for the specific research objectives.

In vitro models encompass a diverse range of cell types, including human pluripotent stem cells (hPSCs) capable of differentiation into specific pulmonary cell lines, serving as an alternative to *in vivo* lung tissues for investigating lung development and diseases [60]. In 2D cultures, the organisation is typically lacking, with various cell types randomly distributed throughout the culture. To cultivate an enriched airway-like cell population of lung progenitors, hPSC-derived lung progenitors are cultured at the air-liquid interface (ALI) for an extended period. This extended culture period facilitates the differentiation of multiciliated, goblet and basal cells, including the development of functional beating ciliated cells [61]. Human tracheo-

bronchial epithelium (TBE) cell cultures are derived from primary cells obtained from medical waste, lung resections, donations, or autopsy, as well as from cell lines derived from cancers and primary cells transformed by viruses. Cell line systems, due to their continuous nature in culture, provide investigators with enhanced reproducibility and ease of use. Some of these culture models form lung epithelial cell monolayers, enabling the determination of transepithelial transport kinetics of test molecules to predict *in vivo* lung absorption [62]. Furthermore, immortalised human cell lines, while not entirely mirroring the *in vivo* environment, present an opportunity to explore intricate cellular interactions and pathological processes [39].

The conventional single-layer cell culture approach represents a straightforward and cost-effective research solution; however, it falls short in replicating the intricate structure and function of the specific microenvironment of human organs *in vivo*, stressing the need for more advanced and sophisticated *in vitro* models to capture the complexity of physiological processes accurately.

The advancements in creating three-dimensional models for studying respiratory diseases encompass a diverse array of *in vitro* and *ex vivo* approaches. Notable among these are the air-liquid interface (ALI), three-dimensional culture models derived from surgical resections, lung organoids [63], three-dimensional spheroids based on hydrogels or polymeric scaffolds, and integrated supports that combine the key advantages of each of the aforementioned models, such as lung-on-a-chip models (Fig. 3). These models facilitate the examination of mechanical forces and cellular interactions in a controlled environment, offering a more faithful representation of *in vivo* lung conditions and paving the way for a more comprehensive understanding of respiratory pathologies [39] (Fig. 3).

These diverse approaches offer unique research trajectories designed to yield a comprehensive understanding of the underlying pathogenetic mechanisms of chronic respiratory diseases. The creation of human "bronchospheres", which are three-dimensional (3D) spheres derived from primary human bronchiolar epithelial cells grown in a 3D matrix, is a recent advancement in this field. This method entails isolating basal stem cells from mouse or human epithelial tissue and embedding them in a 3D extracellular matrix (ECM) gel, resulting in the formation of spherical clonal colonies after a brief period in culture. These initial experiments have established a platform for conducting functional experiments on human tissue, demonstrating the self-renewal capability of human basal stem cells and their ability to generate proximal secretory and ciliated cells [64]. Organs-on-a-chip are microfluidic microphysiological systems designed to replicate the structural and functional properties of human tissues and organs *in vitro*. These systems consist of diverse organ-specific cell types that enable the simulation of corresponding organs [56]. The utilisation of the air-liquid interface (ALI) culture model for respiratory epithelial cell cultures presents notable advantages, including the facilitation of cellular differentiation, enhancement of the morphological and histological characteristics of airway epithelial cells, and the provision of pertinent insights for inhalation and pulmonary toxicology [65].

As we continue to develop new culture models, efforts have been focused on using a three-dimensional cell culture model that replicates the physiological air-liquid interface and incorporates differentiated epithelial cells and fibroblasts immersed in a cellular matrix. A model of this kind allows for a more in-depth study of respiratory epithelium alterations. The culture model developed by Professor Fabio Bucchieri (University of Palermo) in collaboration with Professor Donna Davies (University of Southampton) is one example of such ALI culture. This model features an air-liquid interface that allows for proper spatial orientation, differentiated mucociliary epithelium, and an extracellular matrix populated by metabolically active fibroblasts, providing a model capable of representing the complex microenvironment of the respiratory system [66].

This model, integrated into the landscape of three-dimensional culture models, opens new avenues in the study and understanding of respiratory diseases, as well as in the development of innovative therapeutic and preventive strategies [55]. The three-dimensional cell culture model provides an opportunity to study the interaction between the immune component and the respiratory epithelium, as well as allowing the creation of a co-culture where the outgrowth is exposed to a medium previously conditioned with the extracted immune component of interest. This approach enables an accurate evaluation of the interactions taking place at the level of the respiratory mucosa and provides valuable insights into the dynamics and immune responses involved in both physiological and pathological conditions.

While three-dimensional models like the air-liquid interface (ALI) and three-dimensional spheroids based on hydrogels or polymeric scaffolds excel in replicating the microenvironment, they are not without limitations. These include the lack of perfusion, stress, and limited vascularisation, highlighting the need for continued advancements in modeling techniques to more accurately capture the complexities of *in vivo* conditions for comprehensive respiratory research [67].

In light of these considerations, the choice of the most suitable cultivation model must be met by an in depth evaluation of the best extraction methods to preserve the purity and composition of ESC and THC extracts.

Administration Methods

The primary objective of this review was to explore the intricate impact of cigarette smoke extract (CSE) and tetrahydrocannabinol (THC) on human respiratory mucosa, with a specific focus on the administration methods and the specific experimental models employed. The aim of using

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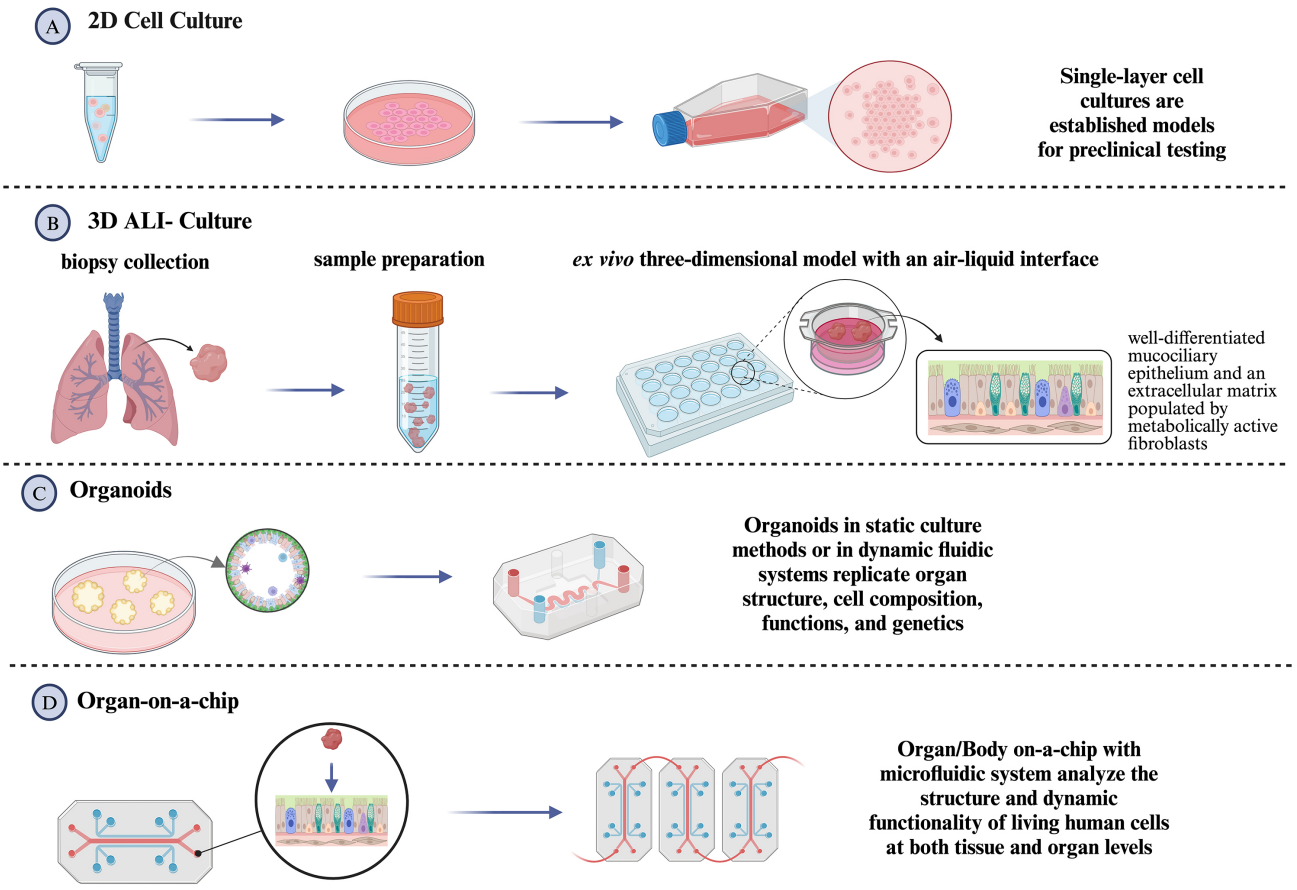

Fig. 3. Cultural models. Schematic representation of methodological approaches utilised in the study of airway diseases. Each panel presents a short workflow of each procedure. (A) 2D Cell Culture: establishes a single-layer cell culture model for preclinical testing. (B) Air-Liquid Interface Culture Models: developed from bioptic fragments grown on the bottom of transwell inserts, featuring well-differentiated mucociliary epithelium and an extracellular matrix populated by metabolically active fibroblasts. (C) Organoids: developed from adult cells, tissue, or induced pluripotent stem cells (iPSCs) grown in static culture methods or fluid dynamic systems, replicating organ structure, cell composition, functions, and genetics. (D) Organ/Body-on-a-Chip: uses a microfluidic system to analyse the structure and dynamic functionality of living human cells at both tissue and organ levels. Created with https://www.biorender.com/. 2D, two-dimensional.

CSE and THC is to elicit a response from the respiratory mucosa, enabling a comprehensive evaluation of the effects of these molecules on the respiratory system, thus shedding light on their potential implications for respiratory health.

Over the years, numerous methods of preparation and administration have been employed to assess the effects of cigarette smoke. The objective is to produce an extract that emulates the process of inhaling cigarette smoke.

One such method, pioneered by Carp and Janoff in 1978 [68] entails bubbling a mild MS cigarette in a beaker containing 25 mL of PneumaCult™-ALI medium. Subsequently, the resulting culture medium is filtered using a 0.22 µm Millex-GS filter (Millipore, Watford, UK) and adjusted to a pH of 7.4. This standardised approach is used in the preparation of CSE for research purposes.

An alternative method for preparing CSE, developed by Gellner *et al*. [69], builds upon the aforementioned approach. In this instance, eight mild MS cigarettes are bubbled in a Falcon containing 35 mL of Dulbecco's modified Eagle's medium (DMEM) with double antibiotics and without fetal bovine serum (FBS) (Fig. 4, Ref. [68]).

The process involves using a syringe to extract 35 mL of volume produced from a lit cigarette, simulating human inhalation through short puffs (2 seconds) and extended intervals between puffs (30 seconds). Notably, each cigarette is bubbled to a distance of 23 mm from the filter. The resulting extracts are employed to condition the cell culture medium and used to stimulate the cell cultures. This method of administration enables the comprehensive assessment of the impact of cigarette smoke in conjunction with all combustion products generated, providing a more holistic understanding of the potential effects on the respiratory system.

In 2003, a study was conducted to investigate the impact of cigarette smoke on a group of rats. In this study, 20 commercial cigarettes were administered daily for five days

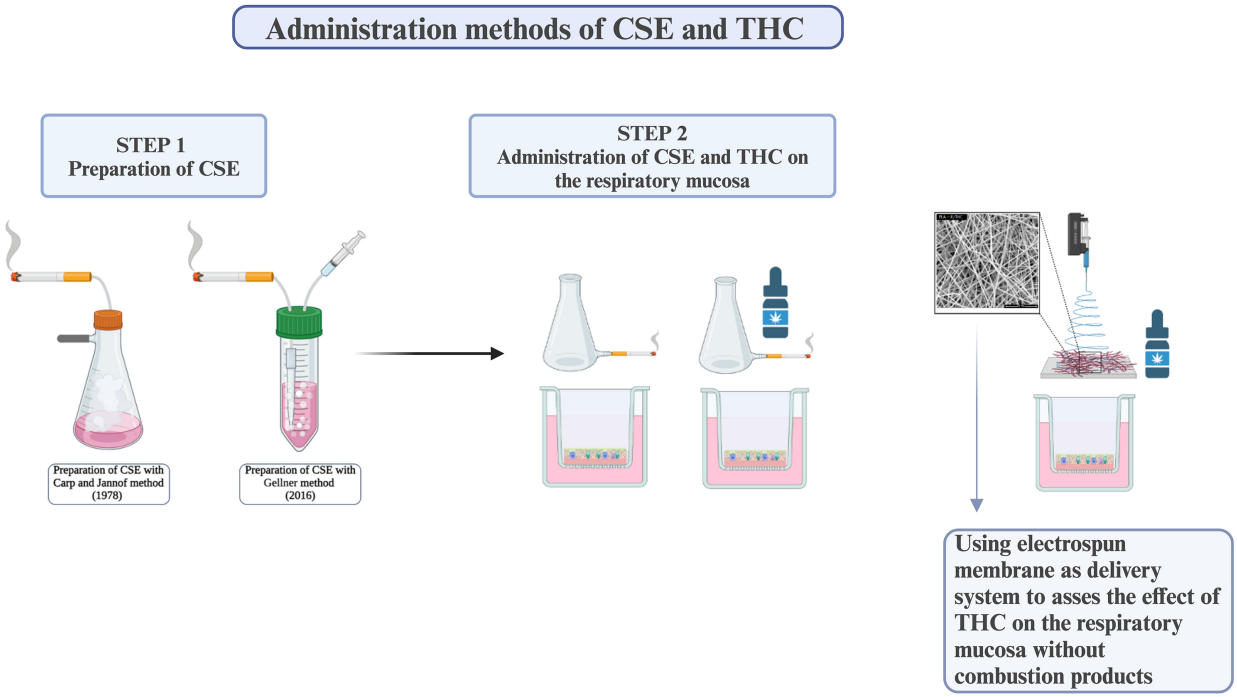

Fig. 4. Administration methods of CSE and THC. The visual illustration showcases the preparation of cigarette smoke extract (CSE) using Carp and Janoff's [68] as well as Geller's methods and the subsequent methods of administering CSE and THC. Notably, THC can be administered either in combination with CSE or independently, in the form of cannabis oil, utilising electrospun membranes. Created with https://www.biorender.com/.

a week, aiming to closely replicate the pattern of human smoking habits. This approach sought to simulate chronic exposure to cigarette smoke, providing valuable insights into the long-term effects of smoking.

Cigarette smoke exposure was meticulously regulated using a specialised plastic chamber, measuring 65 centimeters in length, 50 centimeters in width, and 45 centimeters in height. It featured three holes, with two designated for holding the cigarettes at one end, while the third hole, positioned on the opposite side, was connected to a tube linked to a Leeson vacuum pump. The exposure protocol was carefully structured to subject the rats to alternating periods of smoke and fresh air. Specifically, the rats were exposed to cigarette smoke for 5 minutes, followed by a 5-minute interval of clean air. This cycle continued until all 20 cigarettes were completely smoked. Each exposure session lasted approximately 1 to 1.5 hours [70].

This method ensured that the rats experienced a consistent and measurable amount of smoke, replicating a smoking environment while also allowing for intervals of fresh air. The use of the vacuum pump likely facilitated even distribution of the smoke within the chamber and cleared the smoke during the air intervals, creating a controlled setting for the study.

A wide range of administration methods have been explored to study the impact of THC impact on the respiratory mucosa. The choice of administration method is contingent upon the specific mechanism under scrutiny.

One such technique involves creating an extract in a similar manner to cigarette smoke extract. In this process, the cigarettes are hand-rolled, and the dried cannabis flower is ground using a plastic grinder. A slim, unrefined cellulose filter is then added to the end of the cigarette. Subsequently, cannabis smoke extract (CaSE) is generated using the method employed by Carp and Janoff [68] for CSE, where the lit cannabis smoke is passed through 10 mL of serum-free cell culture DMEM with or without 30% methanol (MeOH) or 30% ethanol (EtOH). The resulting CaSE is then filtered using a 0.45 µm filter [71].

Over time, diverse techniques have been devised for extracting cannabinoids, including THC, from Cannabis flos flowers, often involving high temperatures to achieve elevated cannabinoid concentrations. These extraction methods have undergone modifications to safeguard all cannabinoid properties, with recent advancements, such as the method developed by Bongiorno *et al*. [72], focusing on preserving terpene components and controlling the decarboxylation of cannabinoids to ensure the retention of the therapeutic properties of cannabis oil. This approach serves to maintain the integrity of the extracted compounds for research and therapeutic applications. The oil extracted from cannabis flowers can be administered through various methods.

When studying the effects of cannabis on the respiratory mucosa, it is crucial to assess the impact of cannabinoids, such as THC, without the presence of combustion

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products. One way to achieve this is by administering THC separately in the form of cannabis oil. However, due to the hydrophobic nature of the oil, a medium that facilitates the direct release of the molecule into the cell culture medium is needed. In recent years, electrospun membranes have gained attention as a suitable means of administering molecules in a biological context. Electrospinning is a technique used to generate nano/microscale fibers that can be tailored to specific requirements for compound administration. The resulting nanofibers possess significant plasticity, a flexible structure, and a high surface-area-to-volume ratio, which can enhance cell adhesion, proliferation, and differentiation activities [73].

Electrospun membranes are effective in delivering drugs, including THC oil, due to their ability to provide precise control over drug distribution kinetics and targeted localised treatments. Additionally, electrospinning allows for the modulation of the geometry and characteristics of the fibers to adapt them to the specific requirements for compound administration.

Overall, electrospun membranes offer a versatile and promising approach to personalised therapies and research in various fields. They have the potential to revolutionise drug delivery systems, offering a range of benefits, such as localised treatments and precise control over drug distribution kinetics [74].

The characteristics of electrospun membranes make them a potential tool for administering THC and studying the effects of cannabinoids outside of inhalation. To understand the release kinetics of the molecule in a culture medium, a segment of the cannabis oil-imbued membrane is placed in the medium and conditioned before being used to stimulate cell cultures. This approach allows for precise control over the molecule's release kinetics and facilitates the direct assessment of THC's impact on the respiratory mucosa. The biocompatibility of the membranes ensures favorable interaction with surrounding cells, which is crucial for accurate and reliable experimentation to study the effects of THC on the respiratory mucosa. However, electrospinning may not be suitable for electrically sensitive materials like biomolecules, which could limit the potential applications in some contexts. Overcoming this challenge may require the development of new techniques or approaches.

Conclusions

The review focused on the effects of cigarette smoke extract (CSE) and tetrahydrocannabinol (THC) on the human respiratory mucosa. It delved into the pivotal role of extracellular microenvironments in orchestrating cellular dynamics, particularly within the realm of chronic inflammatory diseases affecting the respiratory system.

It emphasized the need for precise human bronchial mucosa models to understand chronic respiratory diseases and discussed the limitations of using animal models for respiratory disease research. It also summed up the complexities and advancements in the development of three-dimensional cell culture models that faithfully replicate the air-liquid interface, incorporating differentiated epithelial cells and fibroblasts within a cellular matrix.

The review discussed the relationship between COPD and several parameters related to cigarette consumption, highlighting the increased susceptibility to COPD in correlation with smoking duration and intensity, while emphasising the potential risk reduction associated with prolonged smoking cessation. Comprehensive insights were provided into the specific delivery methods and experimental models used to study the impact of THC and CSE on the respiratory mucosa. It also covered the use of electrospun membranes for molecule delivery and the importance of selecting appropriate cell culture models for drug evaluation.

Research has increased our knowledge of how substances such as CSE and THC affect the respiratory tract. Research aims to understand their impact on human respiratory health, including potential cumulative and synergistic effects. A new research frontier could compare the respiratory effects of traditional smoking with those of modern e-cigarettes and vaporizers for both nicotine and cannabis. Furthermore, given the increasing tendency of young people to smoke these substances through vaporisers and e-cigarettes, long-term studies could be conducted on adolescents to assess how early exposure may affect lung function and development in adulthood. Future research could clarify the specific molecular pathways through which CSE and THC exert their effects on lung tissue. Investigations into the role of oxidative stress, inflammation and immune modulation could prove beneficial. Research on genetic and epigenetic factors that influence individual susceptibility to the harmful effects of CSE and THC could lead to the development of personalised prevention and treatment approaches.

Innovative therapies that specifically target the respiratory damage caused by THC and CSE should be developed. These could include pharmacological agents that attenuate inflammation, oxidative stress or airway remodelling, as well as research into natural or synthetic compounds that can protect lung tissue or repair damage.

Availability of Data and Materials

Not applicable.

Author Contributions

AC, FC and FB chose the methodological approach for writing the review. AC, SB, DP and OMM applied to the methodological topic of the work. FC and FB provided help and advice on drafting the manuscript. AC wrote the manuscript. OMM and DP contributed to the drafting of the manuscript. All authors contributed to important editorial changes in the manuscript. All authors read and ap-

proved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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