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Colchicine Protects against Ethanol-Induced Cellular Senescence and Inflammation in Endothelial Cells

Dissertation

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vorgelegt von
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Zusammenfassung

Inflammaging, ein bedeutender Risikofaktor für kardiovaskuläre Erkrankungen, ist mit der Entstehung von Thrombosen und Arteriosklerose verbunden. Die Akkumulation von seneszenten Zellen in Blutgefäßen trägt zum Inflammaging bei und fördert die Bildung und Ruptur von Plaques. Neben seiner bereits beschriebenen Rolle als Risikofaktor für kardiovaskuläre Erkrankungen löst Ethanol Entzündungen und Seneszenz aus, die beide mit kardiovaskulären Erkrankungen in Verbindung gebracht wurden.

In dieser Studie wird Colchicin als therapeutischer Eingriff angewendet, um die schädlichen Auswirkungen von Ethanol auf endotheliale Zellen zu mildern. Colchicin verhindert effektiv die Seneszenz und lindert den oxidativen Stress in von Ethanol exponierten Endothelzellen. Es verringert signifikant die Expression des Alterungs- und Seneszenzmarkers P21 und stellt gleichzeitig die Expression der DNA-Reparaturproteine KU70/KU80 wieder her. Colchicin inhibiert die Aktivierung des nukleären Faktors Kappa B (NFκ-B) und der mitogenaktivierten Protein-Kinasen (MAPKs) in Endothelzellen, die mit Ethanol behandelt wurden. Darüber hinaus reduziert es signifikant den durch Ethanol verursachten seneszenzassoziierten sekretorischen Phänotyp.

Zusammenfassend liefert die Studie überzeugende Beweise dafür, dass Colchicin die durch Ethanol ausgelösten molekularen Ereignisse verbessert, was zu einer Verminderung der Seneszenz und des damit verbundenen seneszenzassoziierten sekretorischen Phänotyps in Endothelzellen führt.

Summary

Inflammaging, a noteworthy risk factor for cardiovascular diseases, is associated with the development of thrombosis and atherosclerosis. The accumulation of senescent cells within blood vessels contributes to vascular inflammaging, promoting plaque formation and rupture. Alongside its recognized role as an acquired risk factor for cardiovascular diseases, ethanol triggers inflammation and senescence, both of which have been implicated in cardiovascular diseases.

This study employed colchicine as a therapeutic intervention to mitigate the detrimental effects of ethanol on endothelial cells. Colchicine effectively prevented senescence and alleviated oxidative stress in ethanol-exposed endothelial cells. It markedly decreased the expression of the senescence marker P21 while restoring the expression of DNA repair proteins KU70/KU80. Colchicine inhibited the activation of nuclear factor kappa B (NFκ-B) and mitogen-activated protein kinases (MAPKs) in endothelial cells treated with ethanol. Moreover, it significantly reduced ethanol-induced senescence-associated secretory phenotype (SASP).

In summary, this study provides compelling evidence that colchicine ameliorates the molecular events triggered by ethanol, resulting in the attenuation of senescence and SASP in endothelial cells.

List of abbreviation

8-OHDG	8-hydroxy-2-deoxyguanosine	**IL-6**	Interleukin-6
AKT	Protein kinase B (PKB)	**IL-8**	Interleukin-8
CAT	Chloramphenicol acetyltransferase	**IκB**	Inhibitors of κB
CCR	C-C chemokine receptor type 2	**JNK**	c-Jun N-terminal kinases
CDK	Cyclin-dependent kinase	**NFκ-B**	Nuclear factor kappa B
CDKN1A	Cyclin-dependent kinase inhibitor 1A	**MAP2K**	Mitogen-activated protein kinase kinase
DAMPs	Damage-associated molecular patterns	**MAP3K**	Mitogen-activated protein kinase kinase kinase
DNA	Deoxyribonucleic acid	**MAPK**	Mitogen-activated protein kinase
DSBs	Double-strand breaks	**MCP-1**	Monocyte chemoattractant protein-1
ELK1	ETS Like-1 protein	**MDPI**	Multidisciplinary Digital Publishing Institute
ERK	Extracellular signal-regulated kinases	**mM**	Millimolar
FMF	Mediterranean fever	**MMP**	Matrix metalloproteinase
GM-CSF	Granulocyte-macrophage colony-stimulating factor	**mRNA**	Messenger ribonucleic acid
GPx-1	Glutathione peroxidase 1	**NEMO**	NF-Kappa-B essential modulator

HuR	Human antigen R	**SASP**	Senescence-associated secretory phenotype
HUVEC	Human umbilical vein endothelial cells	**SA-β-gal**	Senescence-associated β-galactosidase
ICAM-1	Intercellular Adhesion Molecule 1	**SMAD**	Suppressor of Mothers against Decapentaplegic
IKK	IκB kinase	**SOD2**	Superoxide dismutase 2
IL-1	Interleukin-1	**SP1**	Specificity protein 1
IL-1β	Interleukin-1β	**TLR4**	Toll-like receptor 4
NETs	Neutrophil extracellular traps	**TNF-α**	Tumor necrosis factor alpha
NHEJ	Non-homologous end joining	**VCAM-1**	Vascular cell adhesion protein 1
NO	Nitric oxide	**VEGF**	Vascular endothelial growth factor
PAMPs	Pathogen-associated molecular patterns	**X-Gal**	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside
Rb	Retinoblastoma protein	**β-gal**	β-galactosidase
ROS	Reactive oxygen species		

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1. Introduction

1.1. Motivation and Overview

Alcohol abuse is a global public health concern, ethanol as the primary component of alcoholic beverages, under chronic and excessive consumption, has been associated with various diseases, such as cardiovascular diseases[1], liver disease[2], certain cancers[3], and mental health disorders [4,5]. Prolonged and excessive exposure to ethanol can lead to the development of endothelial dysfunction by activating multiple molecular and cellular mechanisms [6-10].

Endothelial cells line the inner surface of blood vessels and play a crucial role in the formation of new vessels, fibrinolysis, and coagulation. They maintain vascular homeostasis by regulating vascular tone, blood flow, and the interactions between blood components and the vessel wall. Dysfunction of these cells, including endothelial senescence and inflammation contributes to the development and progression of various cardiovascular diseases, such as atherosclerosis, thrombosis, and hypertension[11-14].

Understanding the underlying mechanisms by which ethanol affects the endothelial cells can aid in the identification of potential targets for preventive and therapeutic strategies. By identifying key pathways involved in the process, we are exploring potential interventions to mitigate or reverse the detrimental effects of ethanol on endothelial function. This could include focusing on oxidative stress, inflammation, DNA damage, or senescence-associated signaling pathways. Furthermore, research into the relationship between ethanol and endothelial cell senescence can contribute to public health efforts by providing evidence-based information for policymakers, healthcare professionals, and the general public. Such knowledge can support making guidelines on safe alcohol consumption limits and help raise awareness about the potential risks associated with excessive alcohol consumption.

Overall, investigating the relationship between ethanol and endothelial cell dysfunction is driven by the desire to understand the mechanisms underlying alcohol-related vascular damage, identify potential therapeutic targets, and improve public health outcomes related to alcohol abuse and cardiovascular diseases.

1.2. Endothelial Cell Inflammaging and Ethanol Exposure

The intact vascular endothelium is regarded as a critical protector of cardiovascular health, whereas vascular endothelial cell dysfunction contributes to a wide range of cardiovascular disorders, including atherosclerosis, aging, and hypertension[15]. Endothelial cell inflammaging is defined as chronic low-grade inflammation within the endothelial cells that line the blood vessels as a result of aging[16]. Inflammaging refers to the phenomenon of chronic inflammation that occurs with advancing age and is believed to contribute to age-related diseases and functional decline[17,18]. The endothelium plays a crucial role in maintaining vascular health by regulating various physiological processes, including blood flow, vascular tone, and immune responses. However, with age, the endothelial cells can undergo structural and functional changes, leading to a pro-inflammatory state characterized by increased production and release of inflammatory molecules, and consequently results in cardiovascular diseases[19,20]. Interestingly, the elimination of senescent cells from the vessels led to ameliorating the progression of atherosclerosis [18,19,21].

Endothelial cell inflammaging is thought to be influenced by several factors, including oxidative stress, mitochondrial dysfunction, cellular senescence, and dysregulation of immune responses[19,20]. It can impair the endothelial function, increase the permeability of blood vessels, and create a pro-thrombotic environment, all of which contribute to the development and progression of age-related cardiovascular diseases like atherosclerosis, thrombosis, and cerebral aneurysms [22,23]. Experiments on animals have shown that removing senescent cells can extend lifespan and confer safeguards against cardiovascular and age-related ailments[24,25].

Studies have demonstrated that chronic and excessive exposure to ethanol can induce endothelial dysfunction by activating a substantial pro-inflammatory cascade, promoting the upregulation of cell adhesion molecules, inducing stiffness in the rat aorta wall, and fostering the formation of foam cells[26,27], which refers to impaired functioning of the endothelial cells that line the inner walls of blood vessels. Ethanol triggers oxidative stress within endothelial cells, leading to DNA damage, activation of cellular aging pathways[10], and increased production of reactive oxygen species (ROS)[9]. ROS can accelerate cellular aging processes and promote senescence[8]. Ethanol-induced endothelial cell senescence is also associated with elevated endothelial nitric oxide (NO) production. NO is a vital signaling molecule involved in regulating blood vessel dilation, reducing inflammation, and maintaining vascular homeostasis. Increased NO level contributes to endothelial cell dysfunction and promotes the progression of vascular diseases[6,7].

It's important to note that the effects of ethanol on endothelial cell dysfunction can be influenced by various factors, including the duration and amount of alcohol consumed[1,26,28], and co-existing risk factors such as smoking[29]. Additionally, the reversibility of ethanol-induced endothelial cell dysfunction may differ depending on the extent of vascular damage and the cessation of alcohol consumption[30]. Further research is needed to better understand the dose-dependent effects of ethanol on endothelial dysfunction and the potential interactions with other risk factors. This will help in the prevention and management of alcohol-related vascular diseases.

1.3. Senescence and Oxidative Stress Markers

1.3.1. β-Galactosidase Expression

β-galactosidase (β-gal) is an enzyme commonly associated with senescence, specifically with the senescence-associated β-galactosidase (SA-β-gal) activity. SA-β-gal staining is used as a biomarker to identify and characterize senescent cells[31-34]. During cellular senescence, there is an increase in the expression and activity of the β-galactosidase enzyme in the lysosomes of senescent cells[31,32]. This increased activity of

β-galactosidase in senescent cells allows for the detection and visualization of senescence through SA-β-gal staining[34].

It is important to note that SA-β-gal staining is not a definitive marker of senescence, as some limitations and caveats exist. Not all senescent cells may exhibit SA-β-gal activity, and non-senescent cells may show positive staining in certain conditions[35,36]. Therefore, SA-β-gal staining is often used in conjunction with other markers and assays to more accurately characterize senescent cells[36]. Nevertheless, SA-β-gal staining has proven to be a valuable tool in the field of cellular senescence research[31-34]. It provides a visual and quantitative method to identify and analyze senescent cells, contributing to our understanding of the senescence process and its implications in various biological phenomena, aging, and age-related diseases, also presents significant prospects for the advancement of diagnostic and therapeutic strategies targeting inflammatory disorders, precancerous states, and cancer[37].

1.3.2. Ku70 and Ku80 Expression

Proteins Ku70 and Ku80, collectively known as the Ku heterodimer, are key components of the DNA repair pathway known as non-homologous end joining (NHEJ), a major mechanism responsible for repairing DNA double-strand breaks (DSBs) in eukaryotic cells[38]. Studies have shown that Ku70 and Ku80 play important roles in maintaining genomic stability and preventing senescence-associated DNA damage[39]. During cellular senescence, the activity and expression of Ku70 and Ku80 can be affected. Changes in Ku protein levels have been observed in senescent cells, suggesting their potential involvement in senescence-associated alterations in DNA repair capacity[40]. Dysregulation or depletion of Ku proteins may lead to the accumulation of unrepaired DNA damage and the induction of senescence[41].

Moreover, studies have indicated that the Ku heterodimer can influence the telomere maintenance pathway, interact with telomeres and modulate telomere length, stability, and function[39,42]. Telomeres, the protective caps at the ends of chromosomes, gradually

shorten with each cell division, dysfunctional telomeres are known to trigger cellular senescence[43].

The involvement of Ku70 and Ku80 in both DNA repair and telomere maintenance underscores their importance in cellular senescence[41]. Dysregulation or malfunction of these proteins can contribute to genomic instability, cellular aging, and the development of age-related diseases[38,39,43]. Overall, Ku70 and Ku80 are essential components of the Ku heterodimer involved in DNA repair and telomere maintenance. Their roles in maintaining genomic stability make them potentially significant factors in cellular senescence and age-related processes. Further studies are needed to unravel the precise mechanisms by which Ku proteins influence senescence and their implications for human health and disease.

1.3.3. P21 Expression

The p21 protein, also known as cyclin-dependent kinase inhibitor 1A (CDKN1A), is a cyclin-dependent kinase inhibitor that acts as a negative regulator of cell cycle progression, to be considered a key regulator of cellular senescence[44]. During cellular senescence, p21 plays a crucial role in mediating growth arrest and irreversible cell cycle exit. It is induced by various cellular stressors, including DNA damage, oncogene activation, and telomere dysfunction, which are known triggers of senescence[45,46].

The activation of p21 leads to the inhibition of cyclin-dependent kinases (CDKs), specifically CDK2 and CDK4, which are essential for cell cycle progression[47]. By inhibiting CDK activity, p21 prevents the phosphorylation of retinoblastoma protein (Rb), a critical regulator of the G1/S transition, and prevents the cells from progressing through the cell cycle[48]. In addition to its role in cell cycle regulation, p21 is involved in various senescence-associated processes, it contributes to the maintenance of genomic stability by facilitating DNA repair mechanisms[44]. P21 promotes DNA damage response pathways, including the activation of p53, a well-known tumor suppressor protein, and the subsequent activation of DNA repair enzymes[49]. Furthermore, p21 is involved in the senescence-associated secretory phenotype (SASP), which refers to the secretion of

various pro-inflammatory cytokines, chemokines, growth factors, and proteases by senescent cells[18,24]. The SASP can influence the behavior of neighboring cells, promote inflammation, and impact the tissue microenvironment, thereby contributing to age-related diseases[50,51].

Overall, p21 is a critical protein in cellular senescence, functioning as a potent regulator of cell cycle progression and DNA repair[49]. Its induction and activation play a significant role in the establishment and maintenance of senescence, contributing to the preservation of genomic integrity and the prevention of uncontrolled cell proliferation[52]. Revealing the mechanisms underlying p21-mediated senescence can provide insights into the fundamental processes of aging and age-related diseases and may have implications for therapeutic interventions targeting senescence-related disorders.

1.3.4. 8-hydroxy-2-deoxyguanosine

8-OHDG, namely 8-hydroxy-2-deoxyguanosine, is a modified base that is derived from the oxidation of guanine in DNA first reported by Kasai H et al. in 1984[53]. It is considered a biomarker of oxidative DNA damage and has been widely studied in the field of oxidative stress and its implications in various diseases[54]. The evaluation of 8-OHDG levels is commonly used as a quantitative and qualitative indicator of oxidative DNA damage and diagnostic biomarker in research and clinical settings[55].

8-OHDG serves as a valuable tool in oxidative stress research, enabling the assessment of DNA damage caused by oxidative processes. Its application extends to disease risk assessment[54], environmental exposure monitoring[56], antioxidant evaluation[54], and therapeutic monitoring[57]. The measurement of 8-OHDG contributes to our understanding of the role of oxidative stress in disease development and aids in the development of preventive and therapeutic strategies.

1.4. Senescence-Associated Secretory Phenotype

Cellular senescence is a state of irreversible growth arrest that cells enter in response to various stressors, such as DNA damage, telomere shortening, or oncogenic signaling[47,58,59]. This inherent type of senescence arises from the progressive depletion of telomeres during cellular replication, ultimately uncovering an unprotected free double-stranded chromosome end and initiating an irreversible DNA damage response[60,61]. Senescence serves as a protective mechanism against the proliferation of damaged or potentially cancerous cells[58]. However, senescent cells can also have significant effects on their surrounding microenvironment through the secretion of various SASP factors [51]. The SASP is characterized by the secretion of numerous pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α), chemokines like MCP-1 and IL-8, growth factors (VEGF and others), cell adhesion molecules, such as E-selectin, ICAM-1, and VCAM-1, matrix metalloproteinase, and other bioactive molecules[18,24]. These secreted factors can have both beneficial and detrimental effects, depending on the context and the specific components of the SASP, it plays a crucial role in maintaining tissue homeostasis and promoting tissue repair during normal development, wound healing, and a series of other conditions[62].

The SASP is now recognized as a key contributor to aging and age-related diseases[50]. By secreting growth factors and matrix metalloproteinase, senescent cells can stimulate neighboring cells to undergo tissue regeneration and remodeling[50,51]. However, in chronic diseases associated with aging, such as cardiovascular disease, cancer, and neurodegenerative disorders, the SASP can become dysregulated and contribute to disease progression[63-69] and the lack of inhibition of these molecules has been shown to reduce atherosclerosis formation[63-66] and decrease aneurysm formation and rupture[66-68] in different animal models. In the context of age-related diseases, the SASP has been implicated in promoting chronic inflammation, tissue fibrosis, and immune dysfunction[62,70]. The pro-inflammatory cytokines and chemokines released by senescent cells can attract immune cells and promote a chronic state of inflammation, this sustained

inflammatory environment can lead to tissue damage and impair the normal functions of surrounding cells[71]. Furthermore, the SASP exerts influence on neighboring cells by promoting cell proliferation, modifying cell signaling pathways, and shaping the microenvironment to facilitate tumor growth and progression. The SASP has been implicated in the development of age-related cancers, thus targeting senescent cells and their SASP components has emerged as a promising therapeutic approach[72].

In recent years SASP has been actively studied to better understand its composition, regulation, and functional consequences. Efforts are underway to identify specific SASP components that contribute to age-related diseases, as well as to develop interventions that can modulate the SASP for therapeutic purposes. Understanding the SASP and its contribution to disease pathology will open up new avenues for interventions aimed at mitigating the detrimental effects of cellular senescence and improving health outcomes in aging populations.

1.5. Signaling Pathways Involved in Inflammaging

1.5.1. NF-κB

The NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway is a critical signaling cascade involved in regulating a wide range of cellular processes, including immune responses, inflammation, cell survival, and cell proliferation[73]. It plays a central role in coordinating the transcriptional activation of genes involved in these processes. The NF-κB pathway comprises a family of transcription factors, including p65 (RelA), RelB, c-Rel, p50 (NF-κB1), and p52 (NF-κB2). In an inactive state, NF-κB is sequestered in the cytoplasm through binding to inhibitory proteins known as IκBs (inhibitors of κB), the most well-known IκB protein is IκBα. The activation of the NF-κB pathway occurs in response to various extracellular stimuli, such as pro-inflammatory cytokines (e.g.TNF-α, IL-1)[74], pathogen-associated molecular patterns (PAMPs)[75], damage-associated molecular patterns (DAMPs)[76], and oxidative stress[77]. These stimuli trigger the activation of a multi-protein complex known as the IκB kinase (IKK) complex,

which consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ (also known as NEMO)[78]. Upon activation, the IKK complex phosphorylates IκBα, leading to its ubiquitination and subsequent degradation by the proteasome. This allows the liberated NF-κB proteins to translocate into the nucleus, where they bind to specific DNA sequences called κB sites and activate the transcription of target genes[74].

The target genes regulated by NF-κB encompass a wide array of proteins involved in immune responses, inflammation, cell survival, and proliferation. These include pro-inflammatory cytokines, chemokines, adhesion molecules, growth factors, anti-apoptotic factors, and components of the immune system[73]. The NF-κB pathway is tightly regulated to ensure precise control of its activity. In addition to the IκB proteins, negative feedback mechanisms involving other regulatory proteins, such as A20[79] and IκBζ[80], also play roles in terminating NF-κB signaling and preventing excessive or prolonged activation. Aberrant activation or dysregulation of the NF-κB pathway has been implicated in a variety of diseases, including chronic inflammatory disorders, autoimmune diseases, cancer, neurodegenerative diseases, and metabolic disorders [73,75,76,80], targeting the NF-κB pathway has emerged as a potential therapeutic approach for these conditions.

NF-κB activation in senescent cells induces the expression of SASP components, including IL-6, IL-8, TNF-α, IL-1, and various chemokines[24,81]. These factors can stimulate the recruitment and activation of immune cells, promote tissue remodeling, and modulate the senescence-associated phenotype[71]. The interplay between the NF-κB pathway and the SASP creates a feedback loop. The SASP components, particularly IL-1 and IL-6, can further activate NF-κB signaling in an autocrine and paracrine manner, amplifying the inflammatory response and sustaining the senescent state[24]. NF-kB is considered the principal regulatory factor of the SASP by exerting a positive regulatory influence on multiple genes encoding inflammatory cytokines[82].

The NF-κB pathway is a complex signaling cascade that regulates diverse cellular processes, particularly immune responses, and inflammation[73,82]. Its activation and subsequent transcriptional activity play crucial roles in modulating gene expression and

shaping cellular responses in various physiological and pathological contexts. The NF-κB pathway and the SASP contribute to the complex interplay between senescence, inflammation, and age-related diseases[24]. Exploring the regulation and interaction of these pathways may provide insights into the development of therapeutic strategies aimed at mitigating the detrimental effects of senescence and chronic inflammation.

1.5.2. MAPKs

The MAPKs (Mitogen-Activated Protein Kinases) pathway is a highly conserved signaling cascade that regulates a wide range of cellular processes, including cell growth, proliferation, differentiation, survival, and response to stress and extracellular signals[83]. It is an essential signaling network found in eukaryotic organisms, including humans. The MAPK pathway consists of a series of protein kinases that sequentially activate each other through phosphorylation. The core components of the pathway include three major kinases: MAPK kinase kinase (MAP3K), MAPK kinase (MAP2K), and MAPK[84]. In mammals, the most well-known MAPKs are extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 MAPK[85].

The activation of the MAPK pathway typically begins with the stimulation of cell surface receptors, such as growth factor receptors[86], cytokine receptors[87], or G-protein coupled receptors[88], by their respective ligands. This leads to the activation of the MAP3K, which in turn phosphorylates and activates the downstream MAP2K. The activated MAP2K then phosphorylates and activates the final effector MAPK, which can translocate into the nucleus and phosphorylate specific target proteins, including transcription factors and other kinases. The MAPKs pathway plays a critical role in translating these signals into appropriate cellular responses by modulating gene expression, cytoskeletal rearrangements, and activation of other signaling pathways Each MAPK pathway branch (ERK, JNK, and p38) is associated with distinct cellular responses and is regulated by specific upstream signals. The ERK pathway is predominantly involved in cell growth, differentiation, and proliferation, whereas the

JNK and p38 pathways are primarily associated with stress responses, apoptosis, and inflammation[88].

Aberrant regulation of the MAPK pathway has been implicated in various diseases, including cancer, inflammatory disorders, neurodegenerative diseases, and cardiovascular diseases[89-91]. The MAPK pathway components, including ERK, JNK, and p38 MAPK, have been implicated in different aspects of senescence regulation. MAPKs serve as upstream regulators of NF-κB, a critical transcriptional coordinator of the senescence-associated secretory phenotype (SASP). When cells are exposed to stimuli that induce senescence, p38 MAPK enhances the transcriptional activity of NF-κB driven by DNA damage. Consequently, NF-κB activates the transcription of SASP genes, including interleukin-6 (IL-6), interleukin-8 (IL-8), and granulocyte-macrophage colony-stimulating factor (GM-CSF)[92-94]. The MAPK pathway also participates in enforcing the irreversible growth arrest associated with senescence. Activation of p38 MAPK, in particular, contributes to the establishment and maintenance of senescence by promoting cell cycle arrest through the upregulation of cyclin-dependent kinase inhibitors, such as p16 and p21[95].

Overall, understanding the intricate crosstalk between the MAPK pathway and cellular senescence provides insights into the regulatory mechanisms underlying senescence development and its associated phenotypes[95]. Targeting specific components of the MAPK pathway represents a potential avenue for therapeutic interventions aimed at modulating cellular senescence and its implications in age-related diseases and pathological conditions.

1.6. Colchicine and Its Perspective Use in Cardiovascular Disease

Colchicine has been used for centuries due to its medicinal properties[96]. Derived from the autumn crocus plant (*Colchicum autumnale*), this alkaloid compound has a rich history in traditional medicine[97]. Colchicine exerts its therapeutic effects by inhibiting microtubule formation, which plays a crucial role in cellular division, migration, and

inflammation[98]. By interfering with these processes, colchicine effectively reduces the inflammatory response in various diseases. One of the primary clinical applications of colchicine is the treatment and prevention of acute gout attacks[96,97,99]. Gout is a form of arthritis caused by the buildup of uric acid crystals in the joints, leading to intense pain and inflammation[100,101]. Colchicine acts by disrupting the migration of neutrophils, a type of white blood cell, to the affected joints. This reduces the release of inflammatory mediators, providing relief from pain and swelling associated with gout[99-101].

In addition to gout, colchicine has demonstrated efficacy in the treatment of familial Mediterranean fever (FMF), a hereditary autoinflammatory disorder. FMF is characterized by recurrent episodes of fever, abdominal pain, and joint inflammation. Colchicine has been shown to significantly reduce the frequency and severity of these episodes, improving the quality of life for individuals with FMF[102]. Furthermore, colchicine has shown promise in the management of pericarditis, an inflammation of the membrane surrounding the heart. Clinical trials have demonstrated its effectiveness in reducing the recurrence of pericarditis and relieving symptoms, leading to its inclusion in current treatment guidelines[103]. Colchicine's anti-inflammatory properties have also been explored in other conditions, such as Bechet's disease, dermatologic disorders, and even certain cardiovascular conditions[98,104]. Ongoing research continues to shed light on its potential benefits and expand its clinical applications.

Although traditionally known for its use in the treatment of gout and inflammatory conditions, colchicine has gained considerable attention in recent years for its potential benefits in cardiovascular diseases. Emerging evidence suggests that colchicine may have a positive impact on various aspects of cardiovascular health and could serve as an adjunct therapy in the management of certain cardiovascular conditions[104-108]. One area where colchicine has shown promise is in the prevention and treatment of acute coronary syndromes and myocardial infarction. Two clinical trials (*LoDoCo*, namely the second Low Dose Colchicine trial for secondary prevention of cardiovascular disease and *LoDoCo2*, namely Colchicine in Patients with Chronic Coronary Disease) led by Stefan M. Nidorf *et al.* have demonstrated that the addition of a 0.5mg/day low-dose colchicine

to standard therapy reduces the risk of recurrent cardiovascular events in patients who have experienced a recent heart attack and is effective for the prevention of cardiovascular events with stable coronary disease[107,108]. Another clinical trial (*COLCOT,* namely Low-Dose Colchicine after Myocardial Infarction) performed by Jean-Claude Tardif *et al.* identified that in patients who recently experienced a heart attack, the administration of a daily dose of 0.5 mg of colchicine resulted in a notable reduction in the occurrence of ischemic cardiovascular events compared to the placebo group[106]. Colchicine's anti-inflammatory properties are believed to contribute to these cardioprotective effects by mitigating inflammation within the arterial walls, stabilizing atherosclerotic plaques, and improving endothelial function[105]. Colchicine has also shown potential in the management of other cardiovascular diseases, such as atrial fibrillation and heart failure. Studies have suggested that colchicine may reduce the occurrence of atrial fibrillation after cardiac surgery[109], but did not improve outcomes in heart failure patients even though reducing inflammatory markers in a clinical trail[110].

While colchicine holds promise in the treatment of cardiovascular diseases, it is important to note that further research is needed to establish its precise role, optimal dosing, and long-term safety. Colchicine may have potential side effects, particularly at higher doses, and can interact with certain medications, necessitating careful consideration and monitoring when used in cardiovascular patients[98].

In conclusion, colchicine's anti-inflammatory properties and its ability to modulate various aspects of cardiovascular health make it an intriguing therapeutic option in the management of cardiovascular diseases. Ongoing research is expected to further define its role and guide its appropriate use in specific cardiovascular conditions, potentially leading to improved outcomes for patients with broader implications.

1.7. Aims of the Thesis

By studying the effects of ethanol on endothelial senescence and dysfunctions, we are aiming to uncover the specific molecular and cellular pathways that are disrupted or

activated in response to chronic alcohol abuse. Also, to find insights into the mechanisms underlying alcohol-related vascular damage and help identify potential therapeutic targets. Furthermore, the application of colchicine in the intervention and protection of ethanol-induced cellular dysfunction was evaluated.

1.8. Ethics approval

This project has been approved by the ethics committee of the Medical Faculty of Heinrich Heine University Düsseldorf (Study-No.: 2019-787-bio).

2. Publication - Colchicine Protects against Ethanol-Induced Senescence and Senescence-Associated Secretory Phenotype in Endothelial Cells.

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Colchicine Protects against Ethanol-Induced Senescence and Senescence-Associated Secretory Phenotype in Endothelial Cells

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Abstract: Inflammaging is a potential risk factor for cardiovascular diseases. It results in the development of thrombosis and atherosclerosis. The accumulation of senescent cells in vessels causes vascular inflammaging and contributes to plaque formation and rupture. In addition to being an acquired risk factor for cardiovascular diseases, ethanol can induce inflammation and senescence, both of which have been implicated in cardiovascular diseases. In the current study, we used colchicine to abate the cellular damaging effects of ethanol on endothelial cells. Colchicine prevented senescence and averted oxidative stress in endothelial cells exposed to ethanol. It lowered the relative protein expression of aging and senescence marker P21 and restored expression of the DNA repair proteins KU70/KU80. Colchicine inhibited the activation of nuclear factor kappa B (NFκ-B) and mitogen activated protein kinases (MAPKs) in ethanol-treated endothelial cells. It reduced ethanol-induced senescence-associated secretory phenotype. In summary, we show that colchicine ameliorated the ethanol-caused molecular events, resulting in attenuated senescence and senescence-associated secretory phenotype in endothelial cells.

Keywords: ethanol; HUVECs; cellular senescence; SASP; inflammation; colchicine; NFκ-B; MAPKs

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1. Introduction

Inflammaging develops in older individuals and is characterized by elevated levels of pro-inflammatory markers in the serum and different tissues of healthy individuals [1]. Inflammaging is considered a causal risk factor for cardiovascular diseases [1,2]. Throughout life, senescent cells accumulate in vascular tissue, resulting in the development of inflammaging, which consequently leads to cardiovascular diseases [1–4]. Senescent cells have been observed in atherosclerotic tissue, and by eliminating senescent cells, the progression of atherosclerosis could be prevented [1,2,5]. The abolition of senescent cells has been shown to increase lifespan and provide protection against cardiovascular and age-related diseases in experimental animal studies [3,6].

Primary cells grow to a certain limit, after which they stop proliferating and reach growth arrest, a phenomenon termed the Hayflick limit [4,6]. These cells are labeled replicative senescent [4,6,7]. The senescent cells acquire a pro-inflammatory phenotype, called senescence-associated secretory phenotype (SASP) [1,3,8]. These cells increase the expression of cytokines, such as interleukin-1β (IL-β), IL-6, and tumor necrosis factor-α (TNF-α); chemokines, such as monocyte chemoattractant protein-1 (MCP-1) and IL-8;

cell adhesion molecules, such as endothelial selectin (E-selectin), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1); and matrix metalloproteinase (MMP) such as MMP-2 [1,3]. The SASP-associated molecules have been implicated in several cardiovascular diseases, including atherosclerosis, stroke, and myocardial infarction, and aneurysm formation and rupture [9–15]. The lack or inhibition of these molecules has been shown to reduce atherosclerosis formation [11–14] and decrease aneurysm formation and rupture [9,10,15] in different animal models. The expression of SASP-associated molecules is regulated by P38 via nuclear factor kappa B (NF-κB) transcriptional activity [3,16,17]. Previous studies have shown that inhibiting the activation of P38 and NF-κB delayed cellular senescence and attenuated SASP [18–20].

In addition to replicative senescence, other factors such as oxidative stress, DNA damage, oncogene activation or inactivation, epigenetic alterations, mitochondrial dysfunction, and exposure to damage-associated molecular patterns (DAMPs) released by stressed cells can also induce senescence in cells [1,6,7], contributing to hypertension, arterial stiffness, and atherosclerosis, and thereupon leading to cardiovascular diseases [2,4,5,21]. In addition to age, smoking, alcohol abuse, and hypertension are potential acquired risk factors for cardiovascular diseases. Previously, ethanol has been shown to induce senescence in endothelial [22] and other cell types [23,24], increase the expression of SASP molecules [22], and activate NF-κB and mitogen-activated protein kinases (MAPKs) [23,25,26]. Colchicine is an alkaloid known to impede inflammation and extenuate the expression of pro-inflammatory molecules. Colchicine dampens inflammation and attenuates extracellular remodeling by inhibiting endothelial dysfunction, platelet activation, and platelet aggregation; inhibiting the interaction between endothelial cells and platelets, inflammatory cells and endothelial cells, and platelets and inflammatory cells; and blocking NF-κB activation, resulting in the reduced expression of inflammatory and extracellular remodeling molecules [27]. Colchicine has been used to treat gout flares, familial mediterranean fever, calcium pyrophosphate disease, Adamantiades–Behcet's syndrome, and pericarditis [28,29]. The results from published trials have shown that colchicine has provided benefits against cardiovascular diseases [27,28].

Here, we show that colchicine can reduce ethanol-induced cellular senescence and SASP by inhibiting NFκ-B and MAPKs activation.

2. Methods

2.1. Cell Culture

In the current study, we used human umbilical vein endothelial cells (HUVECs) purchased from Promocell (Heidelberg, Germany). The endothelial cell medium (C-22010, Promocell, Heidelberg, Germany) was added to the endothelial cell growth factors (C-39215, Promocell, Heidelberg, Germany) and used to maintain HUVECs. The cells were kept at 37 degrees Celsius (°C) in a 95% humidified atmosphere containing 5% CO_2. After thawing, the cells were seeded in a T75 cell culture flask. The cells were passaged when they reached 90% confluency. For passaging, the cells were trypsinized at 37 °C for 4 min. A total of 5000 cells/cm^2 were seeded in 10 cm culture plates for protein analysis and 6-well plates for mRNA analysis. For all experiments, endothelial cells at passage 7 were treated with endothelial cell medium containing either 400 millimolar (mM) ethanol (EtOH), 50 nanomolar (nM) colchicine, or 400 mM ethanol combined with 50 nM colchicine. The endothelial cells were treated with a higher concentration of ethanol (400 mM) to induce senescence and SASP over a short period. Controls were treated with endothelial cell medium only. For all experiments, the endothelial cells were treated with different conditions for 24 h, with the exception of immunofluorescence staining, for which the duration of treatment was 2 h. Colchicine was purchased from Sigma-Aldrich, St. Louis, MO, USA (C3915).

2.2. β-Gal Staining

β-Galactosidase (β-Gal) Reporter Gene Staining Kit (Sigma-Aldrich, St. Louis, MO, USA) was used for the detection of β-Gal expression. The manufacturer's instructions were followed to stain the endothelial cells treated for 24 h with different conditions, as described in the Section 2.1. The fixation buffer provided with β-Galactosidase Reporter Gene Staining Kit was used to fix the treated cells. The fixed cells were incubated with the freshly prepared staining solution at 37 °C for 7 h. After that, the staining solution was removed, and the 70% glycerol solution was used to overlay the cells for storage at 4 °C. Using optical microscope, the images were captured. To count the stained cells, ImageJ 1.53c (National Institute of Health, Bethesda, MD, USA) was used. For the experiment, biological triplicates were used.

2.3. Immunofluorescence Staining

Immunofluorescence staining was performed as previously described [22]. After three washing steps with phosphate-buffered saline (PBS) (Thermo Fisher, Waltham, MA, USA), the endothelial cells were fixed with 4% paraformaldehyde (Thermo Fisher, Waltham, MA, USA) for 15 min. For permeabilization, the cells were incubated with 0.2% Triton™ X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 10 min. Subsequently, the cells were treated with 5% bovine serum albumin (BSA) (VWR, Langenfeld, Germany) blocking solution for 1 h at room temperature (RT). After that, the cells were incubated with primary antibody 8-Hydroxydesoxyguanosin (8-OHDG) (1:500, Cat. No.: BSS-BS-1278R, BIOSS, Woburn, MA, USA) at 4 °C overnight. On the following day, the cells were washed three times with PBS and incubated with the secondary antibody (1:1000, Alexa Fluor 488, excitation 495 nm, emission 519 nm, Cat. No.: A48269, Thermo Fisher, Waltham, MA, USA) for 1 h at RT. SlowFade® Gold Antifade Mountant with DAPI (Cat. No.: S36938, Thermo Fisher, Waltham, MA, USA) was used for nuclear staining. The images were captured at 20× magnification. The images were analyzed with ImageJ.

2.4. Western Blot (WB)

For protein analysis, the endothelial cells were treated with different conditions, as described in the Section 2.1 for 24 h. Radioimmunoprecipitation assay buffer was used for total protein extraction. DC protein Assay Kit (500-0116, Bio-Rad, Hercules, CA, USA) was used according to the manufacturer's instructions to measure total protein concentration with the Paradigm micro-plate reader (Beckman Coulter, Krefeld, Germany). An amount of 25 microgram (μg) total protein in reducing conditions was loaded on 12% sodium dodecyl sulfate-polyacrylamide gel. The running conditions were 60 Volts for 20 min, followed by 110 Volts for 30–60 min. The polyvinylidene difluoride membranes were used for transfer at 250 milliampere for 120 min. The membranes were blocked with 5% BSA (0.05% tris buffered saline with tween (TBST)) for 1 h. Subsequently, membranes were incubated overnight at 4 °C with primary antibodies (Supplementary Table S1) and diluted in 5% BSA (0.05% TBST) on a shaking platform. On the next day, membranes were washed three times with TBST for 10 min. The membranes were incubated with secondary antibodies (Supplementary Table S1) at RT for 1 h. α-Tubulin was used as a loading control for all proteins except phosphorylated c-Jun N-terminal kinase (p-JNK), for which β-actin was used as the loading control. α-tubulin and β-actin were probed on different membranes. The probed WB membranes were scanned using Odyssey CLx Imaging system (LI-COR Biosciences, Lincoln, NE, USA). The untrimmed images of membranes have been provided in the Supplementary Material. ImageJ was used to calculate densitometry.

2.5. Quantitative Polymerase Chain Reaction (qPCR)

qPCR was performed as previously described [22]. Tri Reagent (T9424, Sigma-Aldrich, St. Louis, MO, USA) was used to extract total RNA. M-MLV Reverse Transcriptase kit (M1701, Promega, Walldorf, Germany) mixed with RiboLock RNase Inhibitor (EO0384, Thermo Fisher, Waltham, MA, USA) and Random Hexamer Primers (48190011, Thermo

Fisher, Waltham, MA, USA) was used to reverse transcribe 1.2 µg total RNA. AceQ SYBR qPCR Master Mix (Q111-03, Vayzme, Nanjing, China) was used to perform qPCR on BIO-Rad CFX Connect Real-Time PCR System. The primer sequences are provided in Supplementary Table S2. The qPCR protocol was an initial denaturation of 95 °C for 8 min, followed by 45 cycles of 95 °C for 15 s, 58.9 °C for 30 s, and 72 °C for 30 s, which was then followed by a melting curve. β-actin was used for normalizing to calculate relative mRNA expression. To quantify relative mRNA expression, comparative C_T method was used [30]. For the experiment, biological triplicates were used. For each biological replicate, we used three technical triplicates.

2.6. Statistical Analysis

For statistical analysis, we performed one-way analysis of variance (ANOVA) followed by Tukey's test. The level of significance was set to less than 0.05 (* $p < 0.05$).

3. Results

3.1. Colchicine Inhibited Ethanol-Induced Senescence

Ethanol is a known risk factor for cardiovascular diseases. We have already reported that ethanol increases cellular senescence in endothelial cells [22]. To investigate the effects of colchicine, we treated endothelial cells with either an endothelial cell medium alone (control) or an endothelial cell medium combined with either 400 mM ethanol, 50 nM colchicine, or 400 mM ethanol combined with 50 nM colchicine. After 24 h, we performed β-gal staining. Colchicine inhibited ethanol-induced cellular senescence in endothelial cells and lowered the percentage of β-gal positive cells (control = 11.01 ± 1.772%, EtOH = 37.21 ± 1.761%, colchicine = 10.76 ± 2.820%, EtOH + colchicine = 16.22 ± 2.630%, **** $p < 0.0001$, $n = 3$; Figure 1A,B). Next, we investigated the effect of colchicine on aging-associated biomarkers. Colchicine attenuated the relative protein expression of aging-associated biomarker P21 (control = 1.003 ± 0.018, EtOH = 2.398 ± 0.068, colchicine = 0.830 ± 0.060, EtOH + colchicine = 1.468 ± 0.075, **** $p < 0.0001$, $n = 3$; Figure 1C,D). P21 is a cyclin-dependent kinase (CDK) inhibitor, which establishes indefinite cell cycle arrest via the inhibition of CDK-2/4 [21]. The CDK-2/4 inhibition results in the active hypo-phosphorylated form of retinoblastoma protein, which, in turn, mediates cell cycle arrest and senescence phenotypes [21]. Colchicine also recovered the relative protein expression of DNA repair proteins KU70 (control = 1.000 ± 0.066, EtOH = 0.856 ± 0.060, colchicine = 0.995 ± 0.040, EtOH + colchicine = 0.995 ± 0.040, * $p < 0.05$, ** $p < 0.01$, $n = 3$; Figure 1C,E) and KU80 (control = 1.000 ± 0.072, EtOH = 0.838 ± 0.069, colchicine = 1.065 ± 0.004, EtOH + colchicine = 1.097 ± 0.058, * $p < 0.05$, $n = 3$, Figure 1C,F). The reduced expression of KU70 and KU80 has been observed in senescent cells compared to young cells [31]. KU70 and KU80 form a heterodimer and repair DNA double-strand breaks via a nonhomologous end-joining pathway [32]. KU70 and KU80 maintain telomere length, and the inactivation of KU70 and KU80 results in the shortening of telomere length in various primary cell types [33], leading to cellular senescence. Furthermore, KU80 has been shown to hinder oxidative stress-induced DNA damage [34].

Colchicine inhibited ethanol-induced endothelial senescence, ameliorated the relative protein expression of aging-associated biomarker P21, and restored the relative protein expression of the DNA repair proteins KU70 and KU80.

3.2. Colchicine Averted Ethanol-Induced Oxidative Stress in Endothelial Cells

The metabolism of ethanol produces reactive oxygen species (ROS) and reactive nitrogen species, resulting in increased oxidative stress [35]. Colchicine inhibited ethanol-induced oxidative stress in endothelial cells and lowered the percentage of oxidative stress-associated biomarker 8-OHDG positive cells (control = 8.569 ± 4.573%, EtOH = 48.62 ± 10.74%, colchicine = 8.553 ± 3.115%, EtOH + colchicine = 13.60 ± 6.001%, *** $p = 0.001$, $n = 3$; Figure 2). Colchicine obviated oxidative stress in ethanol-treated endothelial cells, which is in accordance with the previously reported findings [36,37].

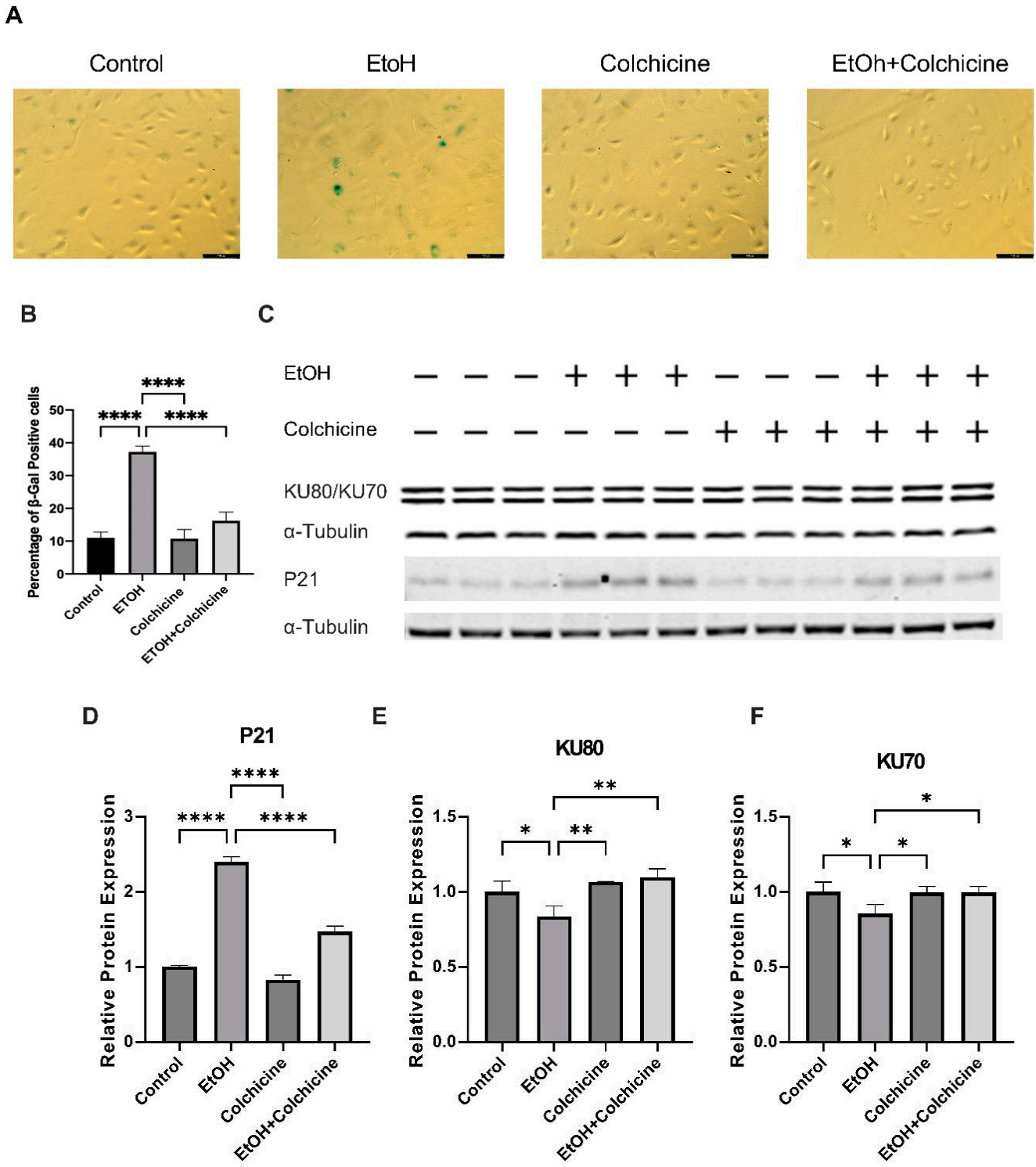

Figure 1. Colchicine inhibited endothelial senescence and restored the relative protein expression of aging-associated biomarkers. (**A**) Senescence in endothelial cells after 24 h of treatment with different conditions. (**B**) Colchicine subdued ethanol (EtOH)-induced senescence in endothelial cells. (**C**) Western blot showing protein expression of P21, KU80, and KU70. (**D**) Colchicine attenuated the relative protein expression of P21 in endothelial cells after ethanol exposure. (**E**,**F**) Colchicine recovered the relative protein expression of KU70 and KU80 in endothelial cells treated with ethanol. α-Tubulin was used as a loading control. Data are the mean of independent biological triplicates. The data was analyzed by performing one-way ANOVA followed by Tukey's test. Scale bar = 100 µm; error bars represent the SD (**** $p < 0.0001$, ** $p < 0.01$, and * $p < 0.05$).

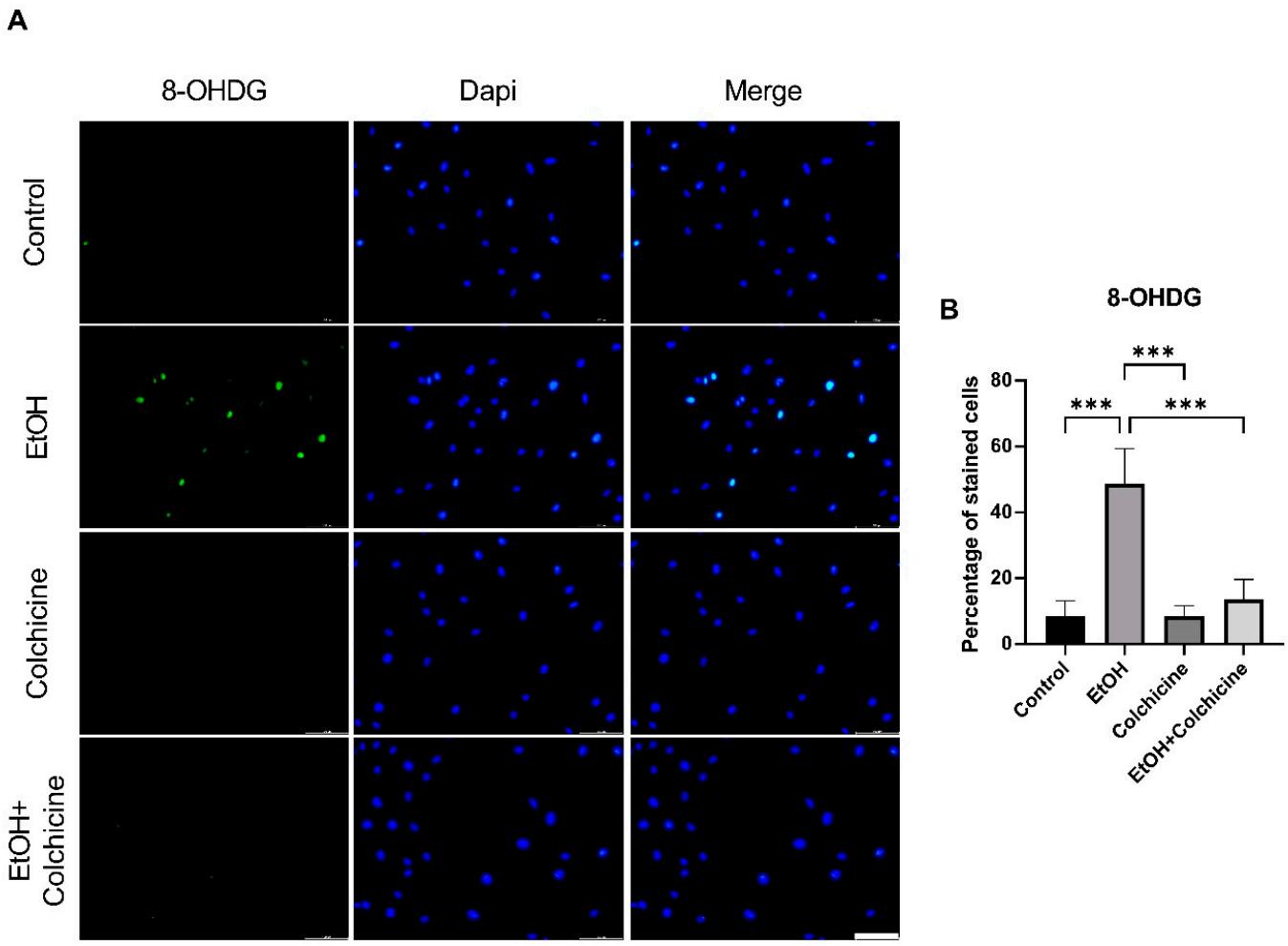

Figure 2. Colchicine restrained ethanol-induced oxidative stress in endothelial cells. (**A**) Immunofluorescence staining for oxidative stress marker 8-Hydroxydesoxyguanosin (8-OHDG) in endothelial cells 2 h after treatment with ethanol, colchicine, and ethanol combined with colchicine. Endothelial medium alone was used for untreated control. (**B**) Colchicine averted the expression of 8-OHDG in ethanol-treated cells. The experiment was performed with independent biological triplicates. The data was analyzed by performing one-way ANOVA followed by Tukey's test. Scale bar = 100 µm; error bars represent the SD (*** $p < 0.001$).

3.3. Colchicine Suppressed the Activation of NF-κB and MAPKs in Ethanol-Treated Endothelial Cells

To investigate the pathways of interest, we performed a protein analysis. NFκ-B has been suggested to play an important role in inflammation, aging, and cellular senescence [8,19]. Our protein analysis showed that colchicine inhibited the ethanol-induced relative protein expression of NF-κB subunit P65 (control = 1.000 ± 0.038, EtOH = 1.172 ± 0.049, colchicine = 1.019 ± 0.034, EtOH + colchicine = 1.053 ± 0.019, * $p < 0.05$, ** $p < 0.01$, $n = 3$; Figure 3A,B). Colchicine also abated the NF-κB activation, and reduced the relative protein expression of NF-κB subunit p-P65 (control = 0.9999 ± 0.083, EtOH = 2.580 ± 0.1417, colchicine = 1.310 ± 0.0488, EtOH + colchicine = 1.506 ± 0.0444, **** $p < 0.0001$, $n = 3$; Figure 3A,C) and *p*-P65/P65 (control = 1.002 ± 0.107, EtOH = 2.208 ± 0.208, colchicine = 1.285 ± 0.014, EtOH + colchicine = 1.431 ± 0.037, *** $p < 0.001$, **** $p < 0.0001$, $n = 3$; Figure 3A,D) was significantly reduced after colchicine treatment in ethanol-treated endothelial cells.

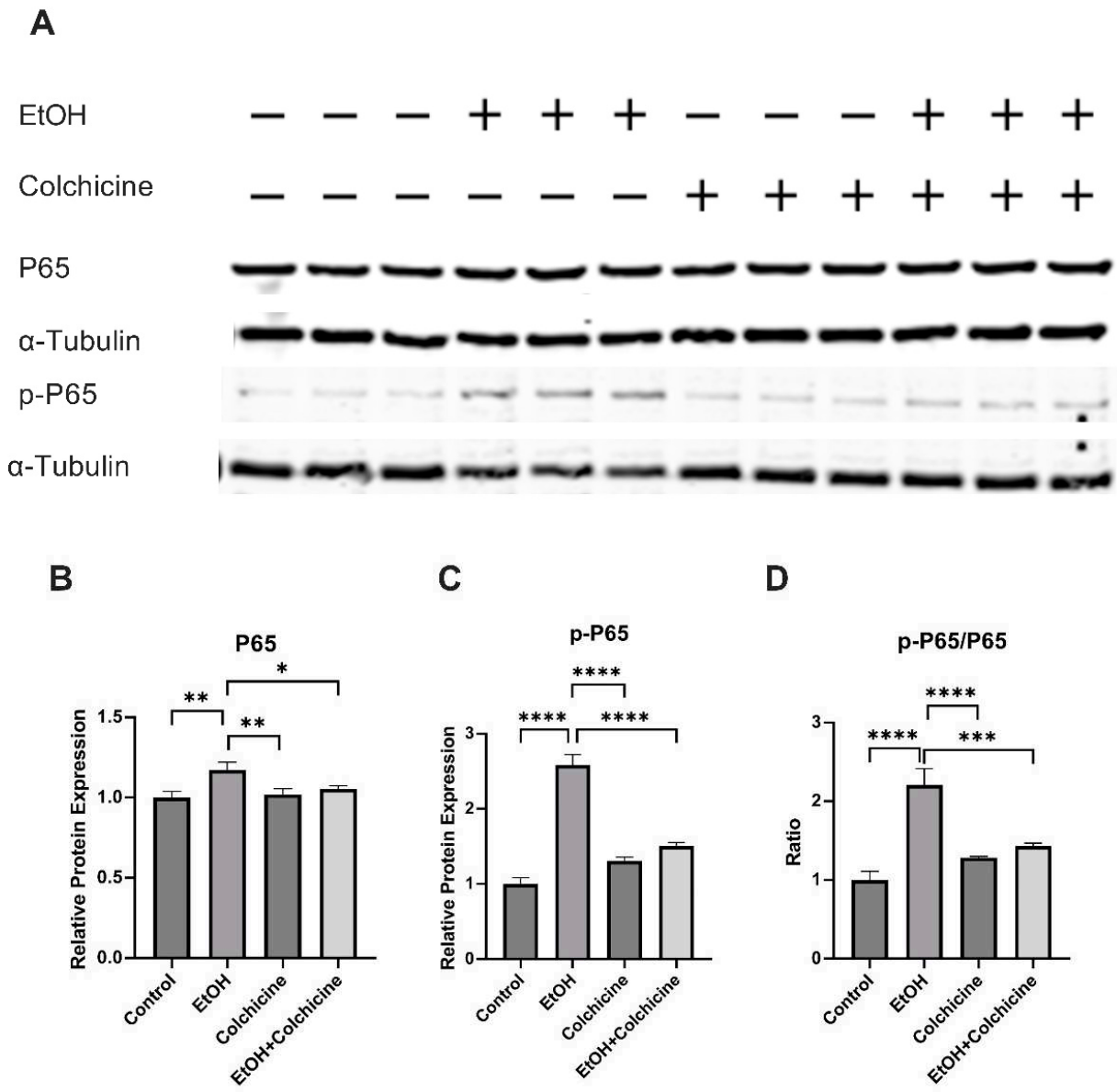

Figure 3. Colchicine inhibited nuclear factor kappa B (NF-κB) activation: (**A**) Western blots for proteins of P65 and p-P65. Colchicine obviated the relative protein expression of (**B**) P65 and (**C**) p-P65 in ethanol-treated endothelial cells. (**D**) The ratio of p-P65/P65 was significantly decreased in ethanol-treated endothelial cells exposed to colchicine. α-Tubulin was used as loading control. Biological triplicates were used for the experiment. Data was analyzed by performing one-way ANOVA followed by Tukey's test. Error bars represent the SD (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$).

It has previously been reported that ethanol activates MAPKs [25] and MAPKs have been implicated in cellular senescence. The protein analysis showed that colchicine impeded the ethanol-induced activation of MAPKs: the relative protein expression of p-P38 (control = 0.9988 ± 0.050, EtOH = 1.553 ± 0.098, colchicine = 0.814 ± 0.103, EtOH + colchicine = 1.070 ± 0.051, *** $p <= 0.001$, **** $p < 0.0001$, $n = 3$; Figure 4A,B), phosphorylated extracellular signal-regulated protein kinase (p-ERK) (control = 1.001 ± 0.1834, ETOH = 2.667 ± 0.4533, colchicine = 0.7198 ± 0.0671, EtOH + colchicine = 1.566 ± 0.1465, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $n = 3$; Figure 4A,C), and p-JNK (control = 1.000 ± 0.039, EtOH = 1.088 ± 0.035, colchicine = 0.899 ± 0.005, EtOH + colchicine = 0.976 ± 0.017, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 3$; Figure 4A,D).

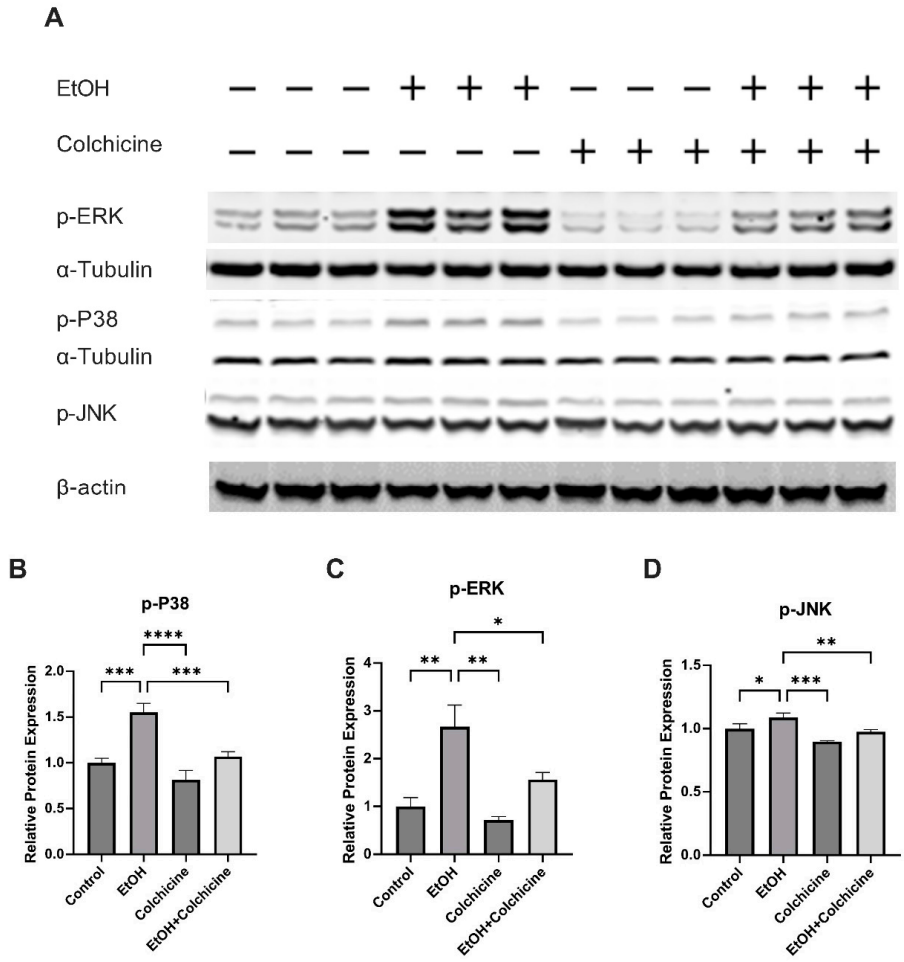

Figure 4. Colchicine inhibited mitogen activated protein kinases (MAPKs) activation: (**A**) Western blots for proteins of p-P38, phosphorylated extracellular signal-regulated protein kinase (p-ERK), and phosphorylated c-Jun N-terminal kinase (p-JNK). Colchicine averted relative protein expression of (**B**) p-P38, (**C**) p-ERK, and (**D**) p-JNK in ethanol-treated endothelial cells. α-Tubulin was used as a loading control for all proteins expression except p-JNK. β-actin was used as a loading control for only p-JNK on a separate membrane. The experiment was performed with independent biological triplicates. The data was analyzed by performing one-way ANOVA followed by Tukey's test. Error bars represent the SD (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$).

3.4. Colchicine Ameliorated Ethanol-Induced SASP in Endothelial Cells

Senescent cells acquire SASP, which is characterized by the increased expression and release of inflammatory cytokines, chemokines, proteases, and growth factors [1,3,8]. Because colchicine inhibited senescence and senescence-associated pathways in ethanol-treated endothelial cells (Figures 1, 3 and 4), we investigated the relative mRNA expression of SASP-associated cytokines, chemokines, and cell adhesion molecules. Colchicine curtailed the relative mRNA expression of the cytokines IL-1β (control = 1.067 ± 0.4931, EtOH = 4.146 ± 0.9146, colchicine = 0.7295 ± 0.1732, EtOH + colchicine = 0.3203 ± 0.09465, *** $p < 0.001$, **** $p < 0.0001$, $n = 3$; Figure 5A) and TNF-α (control = 1.023 ± 0.2783, EtOH = 2.089 ± 0.2934, colchicine = 0.5135 ± 0.0654, EtOH + colchicine = 0.6711 ± 0.2300, ** $p < 0.01$, *** $p < 0.001$, $n = 3$; Figure 5C). It also reduced the relative mRNA expression of the chemokines IL-8 (control = 1.004 ± 0.1091, EtOH = 2.398 ± 0.1235, colchicine = 0.9490 ± 0.0410, EtOH + colchicine = 0.9802 ± 0.0586, **** $p < 0.0001$, $n = 3$; Figure 5D) and MCP-1 (control = 1.013 ± 0.2085, EtOH = 3.341 ± 0.5194, colchicine = 0.7364 ± 0.1053, EtOH + colchicine = 0.4826 ± 0.1187, **** $p < 0.0001$, $n = 3$; Figure 5E). Finally, it decreased the relative mRNA expression of the cell adhesion molecules

ICAM-1 (control = 1.009 ± 0.1649, EtOH = 3.871 ± 0.2693, colchicine = 0.5126 ± 0.1318, EtOH + colchicine = 1.767 ± 1.133, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 3$; Figure 5F) and E-Selectin (control = 1.012 ± 0.1944, EtOH = 2.645 ± 0.3812, colchicine = 0.5963 ± 0.0850, EtOH + colchicine = 1.366 ± 0.3571, ** $p < 0.01$ *** $p < 0.001$, **** $p < 0.0001$, $n = 3$; Figure 5H). Ethanol did not significantly increase the relative mRNA expression of IL-6 (control = 1.024 ± 0.2673, EtOH = 3.092 ± 0.8467, colchicine = 0.4912 ± 0.1902, EtOH + colchicine = 1.756 ± 1.660, * $p < 0.05$, $n = 3$; Figure 5B) or VCAM-1 (control = 1.028 ± 0.3069, EtOH = 2.108 ± 0.7257, colchicine = 1.004 ± 0.2258, EtOH + colchicine = 0.6622 ± 0.1701, * $p < 0.05$, $n = 3$; Figure 5G). In conclusion, colchicine reduced the relative mRNA expression of SASP-associated cytokines, chemokines, and cell adhesion molecules in endothelial cells exposed to ethanol.

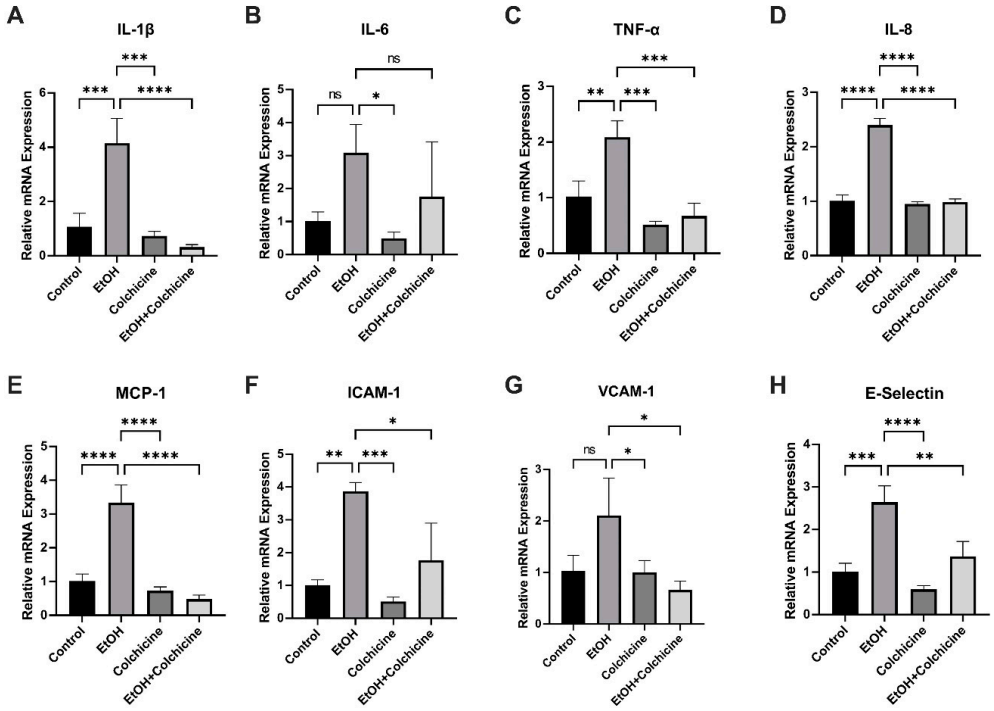

Figure 5. Colchicine attenuated the relative mRNA expression of SASP-associated inflammatory markers. Colchicine reduced the relative mRNA expression of (**A**) interleukin (IL)-1β, (**C**) tumor necrosis factor-α (TNF-α), (**D**) IL-8, (**E**) monocyte chemoattractant protein-1 (MCP-1), (**F**) intercellular adhesion molecule-1 (ICAM-1), and (**H**) endothelial selectin (E-Selectin) in ethanol-treated endothelial cells. Ethanol did not significantly increase the relative mRNA expression of (**B**) IL-6 or (**G**) vascular cell adhesion molecule-1 (VCAM-1). Colchicine significantly lowered the relative mRNA expression of VCAM-1 in ethanol-treated endothelial cells. β-actin was used as a loading control. qPCR data are the mean of three independent technical replicates. The data was analyzed by performing one-way ANOVA followed by Tukey's test. Error bars represent the SD (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$), ns: not significant.

3.5. Colchicine Mitigated Ethanol-Induced Relative mRNA and Relative Protein Expression of MMP-2

Colchicine did not decrease the ethanol-induced relative mRNA expression of MMP-1 (control = 1.000 ± 0.0285, ETOH = 2.617 ± 0.6381, colchicine = 1.847 ± 0.3830, EtOH + colchicine = 3.069 ± 0.9726, * $p < 0.05$, $n = 3$; Figure 6A) or MMP-11 (control = 1.003 ± 0.0929, ETOH = 3.795 ± 1.136, colchicine = 0.5490 ± 0.2415, EtOH + colchicine = 2.214 ± 1.214, * $p < 0.05$, ** $p < 0.01$, $n = 3$; Figure 6D). Both ethanol and colchicine alone or in combination did not significantly affect the relative mRNA expression of MMP-10 (control = 1.003 ± 0.0932, EtOH = 1.686 ± 0.0314, colchicine = 2.585 ± 0.8815, EtOH + colchicine = 1.693 ± 0.3770, $n = 3$; Figure 6C) or TIMP2 (control = 1.004 ± 0.1296, ETOH = 1.775 ± 0.3543, colchicine = 0.8472 ± 0.3676, EtOH + colchicine = 1.165 ± 0.7765, $n = 3$; Figure 6F).

Colchicine inhibited the ethanol-induced relative mRNA expression of TIMP1 (control = 1.001 ± 0.04815, EtOH = 1.773 ± 0.1194, colchicine = 0.5182 ± 0.0901, EtOH + colchicine = 0.7562 ± 0.02403, **** $p < 0.0001$, $n = 3$; Figure 6E) and MMP-2 (control = 1.000 ± 0.0212, EtOH = 2.761 ± 0.3196, colchicine = 0.6408 ± 0.0545, EtOH + colchicine = 1.591 ± 0.3091, *** $p < 0.001$, **** $p < 0.0001$, $n = 3$; Figure 6B). Colchicine also attenuated the relative protein expression of MMP-2 (control = 1.00 ± 0.127, EtOH = 5.161 ± 0.356, colchicine = 2.102 ± 0.381, EtOH + colchicine = 3.102 ± 0.177, **** $p < 0.0001$, $n = 3$; Figure 6G,H).

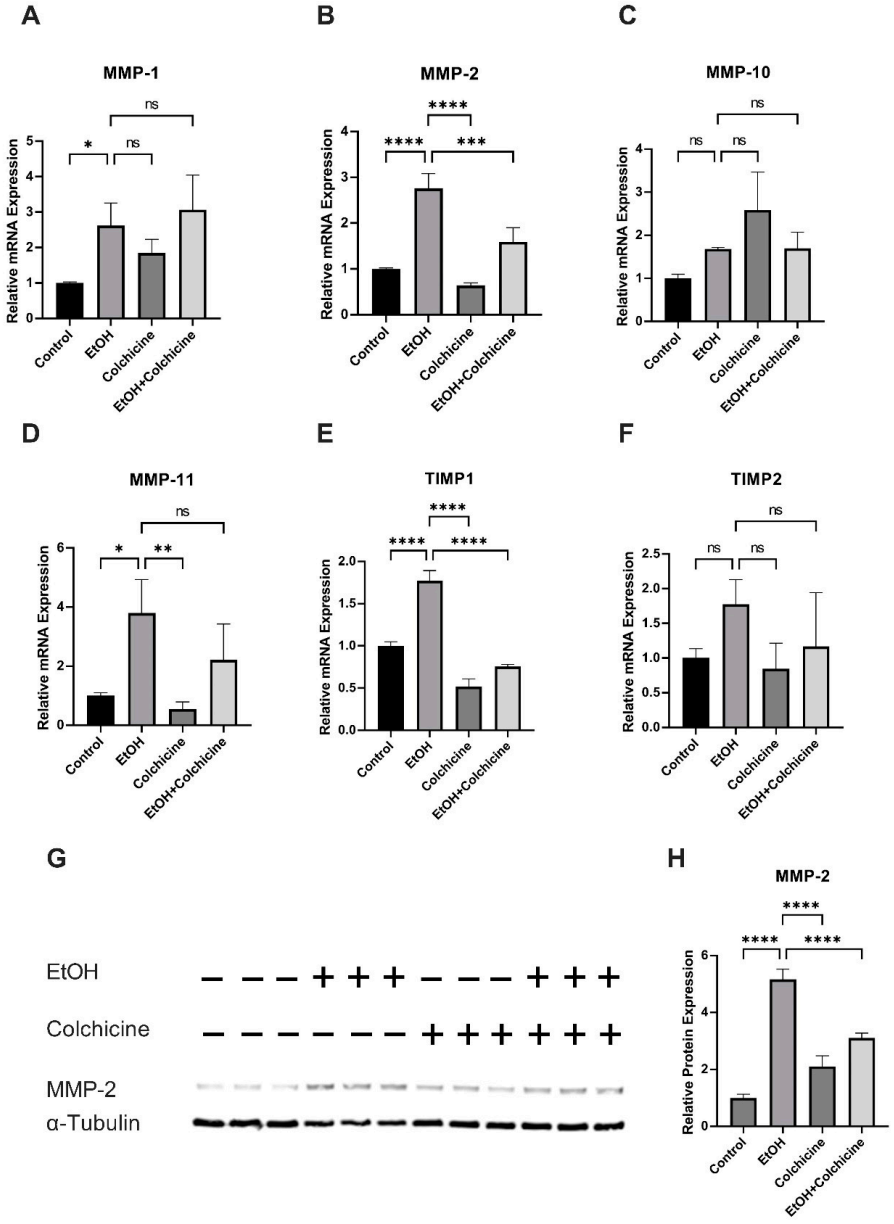

Figure 6. Colchicine abated the relative mRNA and relative protein expression of matrix metalloproteinase (MMP)2. Colchicine did not ameliorate the relative mRNA expression of (**A**) MMP1 or (**D**) MMP-11. Ethanol and colchicine did not alter the relative mRNA expression of (**C**) MMP-10 or (**F**) tissue inhibitor of metalloproteinase (TIMP)2. Colchicine attenuated relative mRNA expression of (**E**) TIMP1 and (**B**) MMP-2 and relative protein expression of (**G**,**H**) MMP-2 in ethanol-treated cells. β-actin for qPCR and α-tubulin for WB were used as a loading control. qPCR data are the mean of three independent technical replicates and WB data are the mean of the independent biological triplicates. The data was analyzed by performing one-way ANOVA followed by Tukey's test. Error bars represent the SD (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$), ns: not significant.

4. Discussion

We showed that colchicine averted cellular senescence and SASP in ethanol-treated endothelial cells. The pathway analysis showed that colchicine inhibited the activation of NF-κB, P38, ERK, and JNK pathways in endothelial cells exposed to ethanol. Ethanol is a potential risk factor for cardiovascular diseases. We, in addition to other researchers, have previously reported that alcohol causes cellular senescence [22,23]. Cellular senescence contributes to cardiovascular diseases via the increase in inflammaging in the vascular endothelium [1–4]. In the current study, we investigated the effects of colchicine on ethanol-treated endothelial cells.

The ethanol treatment induced cellular senescence in endothelial cells (Figure 1) [22–24]. Colchicine inhibited cellular senescence and attenuated oxidative stress in ethanol-treated endothelial cells (Figures 1 and 2). Previously, colchicine has been shown to exert anti-oxidative effects by upregulating the expression of anti-oxidant enzymes such as CAT, GPx-1, and SOD2 in platelets [38]. Ethanol metabolism results in the formation of ROS, which leads to an increase in oxidative stress [35]. The over-accumulation of ROS causes DNA damage and triggers cellular senescence. Colchicine, by inhibiting ROS generation in vivo and in vitro [36–38], can decrease oxidative stress and DNA damage. DNA damage and oxidative stress can activate NF-κB and MAPKs [8,39]. In addition to this, ethanol can activate NF-κB and MAPKs via TLR4/Type I IL-1 receptor signaling [25]. The pathway analysis showed that ethanol activated NF-κB, P38, JNK, and ERK pathways in HUVECs (Figures 3 and 4), which is in agreement with the previously reported findings [22,23,25,26]. These pathways modulate inflammation, contribute to cellular senescence [8,20,39–41], and increase the transcription and expression of cell cycle inhibitor protein P21 via different mechanisms [39,42–46]. The NF-κB activation enhanced the expression of cell cycle inhibitor P21 in response to DNA damage that was independent of the P53 pathway [42]. P38 increases P21 expression by enhancing the expression, stabilization, and promoter activity of P53 [43,44]. Moreover, P38 phosphorylates HuR, which, in turn, increases the cytoplasmic accumulation of HuR and the binding of HuR to P21, consequently improving the stability of P21 mRNA and, thus, enhancing P21 protein levels [45]. ERK1/2 promotes the transcription of P21 via the ELK1, SP1 and SMAD proteins [39,46]. The inhibition of NF-κB and MAPKs activation delayed cellular senescence [18–20], suggesting that inflammation can induce premature senescence in endothelial cells. Colchicine inhibited ethanol-induced senescence by attenuating oxidative stress, recovering the protein expression of KU70 and KU80, ameliorating P21 protein expression, and inhibiting the NF-κB, P38, ERK, and JNK pathways in endothelial cells.

Because Colchicine attenuated ethanol-induced senescence and inhibited the ethanol-induced activation of pro-inflammatory pathways, we investigated the effect of colchicine on SASP-associated cytokines (IL-1β, IL-6, and TNF-α), chemokines (IL-8 and MCP-1), and cell adhesion molecules (ICAM-1, VCAM-1, and E-Selectin) [1,3,8]. Colchicine inhibited the expression of these SASP-associated pro-inflammatory molecules (Figure 5) [38,47,48]. This pro-inflammatory response in senescent and dysfunctional endothelial cells is regulated by the NF-κB complex [3,8]. MAPKs have been suggested to be the upstream regulators of NF-κB [16]. P38 controls NF-κB activity in senescent cells and it induces SASP by increasing the mRNA expression of SASP molecules primarily via NF-κB transcriptional activity [3,16,17].

Senescent endothelial cells promote atherosclerosis and thrombosis by the increased expression and release of SASP-associated inflammatory factors and chemokines [3,8]. MCP-1, IL-8, and cell adhesion molecules E-selectin, VCAM-1, and ICAM-1 promote the extravasation of inflammatory cells from the blood stream across the endothelium [13,49–51]. These infiltrated inflammatory cells create a pro-inflammatory microenvironment, exacerbate inflammation, and lead to atherosclerotic plaque formation [50]. Colchicine reduced the adhesion of monocytes to HUVECs by inhibiting the expression of adhesion molecules VCAM-1 and ICAM-1 [38]. Colchicine decreased the recruitment of monocytes and neu-

trophils into the atherosclerotic plaque of mice aorta [47]. In addition to their role in the tissue infiltration of inflammatory cells, the SASP-associated molecules have the potential to activate the inflammatory cells [49,50]. MCP-1 activates immune cells, such as monocytes, and regulates the polarization of T-cells and the differentiation of monocytes into dendritic cells [50]. IL-1β and TNF-α released from senescent endothelial cells can activate inflammatory cells, neighboring endothelial and smooth muscle cells [52,53]. Both IL-1β and TNF-α have been shown to decrease collagen synthesis and increase the mRNA expression and activity of MMPs [54], which can consequently result in tissue remodeling [55,56]. The TNF-α-induced phenotype switch in smooth muscle cells can impair vasorelaxation [52]. TNF-α can cause endothelial cell apoptosis and smooth muscle cell proliferation and migration [53,57], leading to the initiation and progression of cardiovascular diseases. TNF-α increased the expression of E-selectin, VCAM-1, and ICAM-1 in HUVECs [58]. Colchicine reduced TNF-α, IL-1β, MCP-1, and ICAM-1 expression at mRNA and protein levels in vivo and in vitro [38,47,48]. Cerebral aneurysm formation and rupture were significantly reduced in MCP1-, TNF-α-, and TNF-α-R1 deficient mice [9,10,15]. The lack of MCP-1, MCP-1 receptor CCR2 inhibition, IL-1β deficiency, and TNF-α inhibition have been shown to decrease atherosclerosis formation [11–14]. The rupture of atherosclerotic plaque leads to thrombus formation. The aggregation, activation, and inflammatory response of platelets is known to play a key role in atherosclerosis and thrombus formation [59]. Colchicine blocked platelet–platelet aggregation in both whole blood and platelet-rich plasma, platelet activation (ROS generation), and procoagulant platelet formation [37,38,60]. The addition of colchicine in whole blood, in vitro, and in oral administration in healthy subjects, in vivo, decreased monocyte-platelet and neutrophil-platelet aggregation [60]. Colchicine has been shown to reduce NETs formation [61], which has been suggested to contribute to thrombosis by accumulating prothrombotic factors such as fibrinogen and von Willebrand factor, and by promoting platelet adhesion, activation, and aggregation [62]. In mice, colchicine inhibited carrageenan-induced thrombosis and ameliorated platelet activation [38]. The authors also showed that colchicine dampened human platelet activation by inhibiting the activation of AKT pathway, which subsequently blocked ERK1/2 activation [38]. These findings indicate that colchicine, by inhibiting senescence and curtailing SASP-induced sterile inflammation in the endothelium, can be potentially useful against cardiovascular diseases [27,28].

Previous studies have shown that senescent cells increase the expression of MMPs [1,3,8,21]. In addition, ethanol has been shown to increase the expression of MMPs in endothelial [22] and other cell types [63]. Alcohol consumption has been reported to increase serum levels of MMPs in alcohol abusers [64] and, in animal studies, alcohol elevated MMP expression in different tissues [65–67]. In the current study, ethanol increased the expression of MMPs (Figure 6). Colchicine inhibited the expression of MMP-2 at mRNA and protein levels (Figure 6). MMPs are known to contribute to cardiovascular diseases via tissue remodeling and scar formation, facilitating the migration and proliferation of smooth muscle cells, the infiltration of inflammatory cells such as monocytes and neutrophils into the endothelium, and promoting inflammation via their protease function on cytokines and chemokines [55,56]. These findings suggest that, by inhibiting MMP-2 mRNA and protein expression, colchicine can potentially suppress tissue remodeling and MMP-2-mediated inflammation in endothelial cells.

5. Conclusions

Alcohol is a potential risk factor for cardiovascular diseases. In the current study, we showed that ethanol induced premature senescence and SASP. Colchicine reduced ethanol-induced inflammaging in HUVECs possibly by inhibiting the activation of the NF-kB and MAPKs pathways. Thus, colchicine could be a potential pharmacological target for cardiovascular diseases.

6. Limitations

Our study has some limitations. The exposure of the endothelial cells to ethanol, for example, was acute and not chronic. The concentration of ethanol (400 mM) used was much higher and the ethanol evaporation from culture media was not prevented. This higher concentration of ethanol was used to induce senescence and SASP in endothelial cells over a short period. Moreover, the study was conducted using endothelial cells in vitro. In addition, we used HUVECs as endothelial cells. The data should be carefully interpreted.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox12040960/s1, Supplementary Table S1. The list of antibodies used in the study. Supplementary Table S2. The list of primers.

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Table S1. Primary and Secondary antibodies

Antibody	MW(kDa)	Brand	Catalog Number	Concentration
P21	21	Cell Signaling	# 2947S	1:1,000
KU70	70	Cell Signaling	#4588S	1:1,000
KU80	86	Cell Signaling	#2753S	1:1,000
P65	65	Abcam	ab16502	1:1,000
p-P65	65	Cell Signaling	#3033S	1:1,000
MMP-2	70	Invitrogen	436000	2 µg/mL
β-actin	45	Cell Signaling	#4970S	1:1,000
α-Tubulin	50	Sigma Aldrich	#T9026	1:500
p-P38	43	Cell Signaling	#4511S	1:1,000
p-JNK	46,54	Cell Signaling	#9255S	1:2,000
p-ERK	42,44	Cell Signaling	#4370S	1:2,000
8-OHDG		BIOSS	BSS-BS-1278R	1:500
Alexa Fluor Plus 488 Excitation: 495 nm Emission: 519 nm		Thermo Fisher	A48269	1:1000
IRDye 800CW Goat-anti-Rabbit Antibody Excitation: 778 nm Emission: 795 nm		LiCor	# LI-COR 92632211	1:10,000
IRDye 680RD Donkey anti-Mouse IgG Secondary Antibody Excitation: 676 nm Emission: 694 nm		LiCor	926-68072	1:10,000

Table S2. Primer list

Target gene	Gene Accession Number	Sense 5’ -3’	Antisense 5’ -3’
ICAM-1	NM_000201	CACAGTCACCTATGGCAACGA	TGGCTTCGTCAGAATCACGTT
VCAM-1	NM_080682, NM_001199834, NM_001078	AGTGGTGGCCTCCTGAATGG	CTGTGTCTCCTGTCTCCGCT
IL-8	NM_001354840, NM_000584	TGCCAAGGAGTGCTAAAG	CTCCACAACCCTCTGCAC
MCP-1	NM_002982	CACCAATAGGAAGATCTCAGTGC	TGAGTGTTCAAGTCTTCGGAGTT
MMP1	NM_001145938, NM_002421	CAGAGATGAAGTCCGGTTTTTC	GGGGTATCCGTGTAGCACAT
MMP2	NM_001302510, NM_001302509, NM_001127891, NM_004530, NM_001302508	ATAACCTGGATGCCGTCGT	AGGCACCCTTGAAGAAGTAGC
MMP10	NM_002425	CACAGTTTGGCTCATGCCTA	TGCCATTCACATCATCTTGC
MMP11	NM_005940	CCGCAACCGACAGAAGAGG	ATCGCTCCATACCTTTAGGGC
TIMP1	NM_003254	TGGCTTCTGGCATCCTGTTGTTG	CGCTGGTATAAGGTGGTCTGGTTG
TIMP2	NM_003255	GAATCGGTGAGGTCCTGTCCTGA	CCTGCACACAAGCCCGGATAAA
IL-1β	NM_000576	AGATGATAAGCCCACTCTACAG	ACATTCAGCACAGGACTCTC
E-selectin	NM_000450	CAAGAAGAAGCTTGCCCTATG	ACTTGAGTCCACTGAAGCCA
TNF-α	NM_000594	CCCGAGTGACAAGCCTGTAG	GATGGCAGAGAGGAGGTTGAC
IL-6	NM_001371096 NM_001318095 XM_011515391 NM_000600	CTGCAGGACATGACAACTCATC	ATCTGAGGTGCCCATGCTAC

3. Discussion

Ethanol represents a potential risk factor for the development of cardiovascular diseases. Consistent with previous research by our own research group and others [10,111], we established that ethanol exposure induces cellular senescence in endothelial cells[112]. The presence of cellular senescence contributes to the pathogenesis of cardiovascular diseases by promoting inflammaging within the vascular endothelium[18,19,24,113]. In the present study[112], our focus was to investigate the impact of colchicine on ethanol-treated endothelial cells, aiming to elucidate its effects and potential therapeutic implications. Our findings revealed that the administration of colchicine effectively prevented the occurrence of cellular senescence and SASP induction in endothelial cells subjected to ethanol treatment[112]. Pathway analysis demonstrated that colchicine exerted inhibitory effects on the activation of NF-κB, P38, ERK, and JNK pathways in ethanol-exposed endothelial cells[112].

After demonstrating that ethanol treatment induced cellular senescence in endothelial cells (Figure 1 in the publication[112]), we discovered that colchicine effectively inhibited cellular senescence and mitigated oxidative stress in ethanol-treated endothelial cells (Figures 1 and 2 in the publication). Earlier studies have shown that colchicine has anti-oxidative properties, as evidenced by its ability to increase the expression of antioxidant enzymes like CAT, GPx-1, and SOD2 in platelets[114]. Ethanol metabolism, on the other hand, gives rise to reactive oxygen species (ROS), thereby promoting oxidative stress[115]. Excessive ROS accumulation induces DNA damage and instigates cellular senescence. Colchicine has been shown to effectively inhibit ROS generation both *in vivo* and *in vitro*[114,116,117], leading to a reduction in oxidative stress and DNA damage.

Previous study showed that ethanol has the ability to activate NF-κB and MAPKs through TLR4/Type I IL-1 receptor signaling[118]. The activation of NF-κB and MAPKs can be triggered by DNA damage and oxidative stress[95,119]. Consistent with previously reported findings[10,111,118,120], our pathway analysis demonstrated that ethanol activated the NF-κB, P38, JNK, and ERK pathways in HUVECs (Figures 3 and 4 in the publication).

These pathways, through various mechanisms, play a crucial role in the modulation of inflammation, promotion of cellular senescence [95,119,121-123], and regulation of the transcription and expression of the cell cycle inhibitor protein P21 (Figure 1 in the publication) via diverse mechanisms[95,124-128]. NF-κB activation enhances P21 expression in response to DNA damage independently of the P53 pathway[124]. P38 increases P21 expression by upregulating the expression, stabilization, and promoter activity of P53[125,126]. Furthermore, P38 phosphorylates HuR, causing increased cytoplasmic accumulation of HuR, resulting in an increase in HuR binding to P21 mRNA, and consequently enhancing the stability of P21 mRNA and protein levels[127]. ERK1/2 promotes P21 transcription by interacting with ELK1, SP1, and SMAD proteins[95,128]. The delay in cellular senescence observed upon inhibition of NF-κB and MAPKs activation[121,129,130] indicates that inflammation can trigger senescence in endothelial cells. In our study, colchicine exhibited the ability to impede ethanol-induced senescence by mitigating oxidative stress, restoring the protein expression of KU70 and KU80, ameliorating P21 protein expression, and inhibiting the NF-κB, P38, ERK, and JNK pathways in endothelial cells (Figures 1, 3 and 4 in the publication).

Given that colchicine effectively mitigated ethanol-induced senescence and suppressed the activation of pro-inflammatory pathways, we proceeded to investigate its impact on the expression of SASP-associated cytokines (IL-1β, IL-6, and TNF-α), chemokines (IL-8 and MCP-1), and cell adhesion molecules (ICAM-1, VCAM-1, and E-Selectin)[18,24,119]. Remarkably, colchicine exhibited inhibitory effects on the expression of these SASP-associated pro-inflammatory molecules[114,131,132] (Figure 5 in the publication). It is worth noting that the pro-inflammatory response observed in senescent and dysfunctional endothelial cells is primarily regulated by the NF-κB complex[24,119]. Furthermore, MAPKs have been proposed as upstream regulators of NF-κB[133], with P38 playing a significant role in controlling NF-κB transcriptional activity in senescent cells and inducing SASP by promoting mRNA expression of SASP factors primarily through NF-κB transcriptional activity[24,93,133]. The enhanced expression and secretion of SASP-associated inflammatory factors and chemokines by senescent endothelial cells play a

pivotal role in promoting atherosclerosis and thrombosis[24,119]. MCP-1, IL-8, and cell adhesion molecules such as E-selectin, VCAM-1, and ICAM-1 facilitate the extravasation of inflammatory cells from the bloodstream across the endothelium[63,134-136]. Once infiltrated, these inflammatory cells create a pro-inflammatory microenvironment, exacerbate inflammation, and contribute to the formation of atherosclerotic plaques[135]. By inhibiting the expression of adhesion molecules VCAM-1 and ICAM-1, colchicine effectively reduced the adhesion of monocytes to HUVECs[114]. Moreover, colchicine demonstrated the ability to decrease the recruitment of monocytes and neutrophils into the atherosclerotic plaques of the mouse aorta[131]. In addition to their involvement in the tissue infiltration of inflammatory cells, SASP-associated molecules possess the potential to activate these inflammatory cells themselves[134,135]. MCP-1, for instance, plays a role in the activation of immune cells like monocytes, while also regulating T-cell polarization and monocyte differentiation into dendritic cells[135]. Senescent endothelial cells release IL-1β and TNF-α, which can activate neighboring endothelial and smooth muscle cells, as well as other inflammatory cells[137,138]. Notably, IL-1β and TNF-α have been shown to decrease collagen synthesis and increase MMPs mRNA expression and activity[139], ultimately leading to tissue remodeling[140,141]. The phenotype switch induced by TNF-α in smooth muscle cells can impair vasorelaxation[137] and TNF-α by promoting endothelial cell apoptosis, and smooth muscle cell proliferation and migration[138,142], which can contribute to the initiation and progression of cardiovascular diseases. Furthermore, TNF-α upregulates the expression of E-selectin, VCAM-1, and ICAM-1 in HUVECs[143]. Colchicine effectively reduced the expression of TNF-α, IL-1β, MCP-1, and ICAM-1 at both the mRNA and protein levels in *in vivo* and *in vitro* settings[114,131,132]. Notably, mice lacking MCP-1, TNF-α, and TNF-α-R1 exhibited significant reductions in cerebral aneurysm formation and rupture[66-68]. Similarly, inhibiting MCP-1, its receptor CCR2, IL-1β, and TNF-α has been demonstrated to decrease atherosclerosis formation[63-65,69] In our study, colchicine reduced the relative mRNA expression of SASP-associated cytokines (IL-1β and TNF-α), chemokines (IL-8 and MCP-1), and cell adhesion molecules (ICAM-

1, VCAM-1, and E-Selectin) (Figure 5 in the publication) which were induced by ethanol treatment. These findings are consistent with those of other researchers.

The rupture of atherosclerotic plaque triggers the formation of blood clots. Platelet aggregation, activation, and the subsequent inflammatory response are recognized as crucial factors in the development of atherosclerosis and thrombus formation[144]. Colchicine effectively inhibited platelet-to-platelet aggregation in both whole blood and platelet-rich plasma, as well as platelet activation characterized by reactive oxygen species (ROS) generation and the formation of procoagulant platelets[114,117,145]. Administration of colchicine, either in vitro using whole blood or *in vivo* in healthy subjects through oral administration, significantly reduced the aggregation of platelets with monocytes and neutrophils[145]. Moreover, colchicine demonstrated the ability to suppress the formation of neutrophil extracellular traps (NETs)[146], which are implicated in thrombosis due to their accumulation of prothrombotic factors such as fibrinogen and von Willebrand factor, as well as their promotion of platelet adhesion, activation, and aggregation[147]. In animal studies, colchicine exhibited inhibitory effects on carrageenan-induced thrombosis and mitigated platelet activation[114]. Additionally, the study revealed that colchicine attenuated human platelet activation by inhibiting the AKT pathway, subsequently blocking ERK1/2 activation[114]. Collectively, these findings highlight the potential of colchicine in combating cardiovascular diseases by suppressing cellular senescence, mitigating SASP-induced sterile inflammation in the endothelium, and modulating platelet functions[104,105]. Our study indicates that colchicine effectively mitigates the ethanol-induced upregulation of ERK1/2 (Figure 4 in the publication) and SASP factors (Figure 5 in the publication). These results carry important implications for the potential therapeutic efficacy of colchicine in the treatment of neurovascular diseases.

Previous research has demonstrated that senescent cells upregulate the expression of MMPs [18,24,47,119]. Moreover, it has been shown that ethanol exposure increases MMP expression in endothelial cells[10] as well as in other cell types[148]. Alcohol consumption has been associated with elevated serum levels of MMPs in individuals with alcohol abuse[149], and animal studies have shown that alcohol intake leads to increased MMPs expression

in various tissues[150-152]. In the current study, we observed that ethanol treatment resulted in increased MMPs expression (Figure 6 in the publication). However, the administration of colchicine effectively inhibited the expression of MMP-2 at both the mRNA and protein levels (Figure 6 in the publication). MMPs play a significant role in cardiovascular diseases by contributing to tissue remodeling, scar formation, the migration and proliferation of smooth muscle cells, the infiltration of inflammatory cells such as monocytes and neutrophils into the endothelium, and promoting inflammation through their proteolytic activity on cytokines and chemokines[140,141]. These findings suggest that colchicine, by inhibiting MMP-2 mRNA and protein expression (Figure 6 in the publication), has the potential to suppress tissue remodeling and MMP-2-mediated inflammation in endothelial cells.

Several clinical trials (*LoDoCo, LoDoCo2, and COLCOT*) have investigated the application of colchicine in the context of cardiovascular protection and illustrated that the administration of low-dose colchicine has significantly reduced the risk of cardiovascular events in patients with recent myocardial infarction and with coronary disease[106,108]. Meanwhile, low-dose of colchicine combined with other standard secondary prevention treatments effectively protects against cardiovascular events in patients with stable coronary disease[107]. The mechanisms underlying colchicine's cardiovascular benefits are not yet fully understood. The results of the CANTOS[153] trial, on the other hand, provide evidence for the efficacy of IL-1β monoclonal antibody therapy in lowering the incidence of myocardial infarction by targeting the IL-1β signaling pathway. These findings strongly support the notion that anti-inflammatory therapy represents a viable and successful approach for the treatment of myocardial infarction. Colchicine effectively suppressed the activation of the NFκB and MAPKs signaling pathways (Figures 3 and 4 in the publication) in the current study， which results in a decrease in the expression of SASP factors, including IL-1β (Figure 5 in the publication), thereby protecting against inflammation in HUVECs. Our findings, which are consistent with previous research, provide strong evidence for the anti-inflammatory properties of colchicine in endothelial cells.

3.1. Conclusion

Our study demonstrated that acute ethanol exposure induced senescence and the senescence-associated secretory phenotype (SASP) in human umbilical vein endothelial cells (HUVECs). However, the administration of colchicine effectively mitigated ethanol-induced inflammaging in HUVECs, potentially through the inhibition of the NF-kB and MAPK signaling pathways. These findings highlight the potential of colchicine as a promising pharmacological target for the treatment and prevention of cellular senescence in cardiovascular diseases. Further research *in vivo* experimental models and clinical studies are needed to fully explore the therapeutic benefits of colchicine in this context.

3.2. Limitations

It is important to acknowledge the limitations of this study. Firstly, the exposure of endothelial cells to ethanol was acute rather than chronic, which may not fully represent the long-term effects of ethanol on cellular senescence and SASP. Furthermore, the concentration of ethanol (400 mM) used in this study was higher than what is typically encountered in physiological conditions, and measures to prevent ethanol evaporation from the culture media were not implemented. This higher concentration was chosen to induce senescence and SASP in endothelial cells within a shorter timeframe. Additionally, it is crucial to note that this study was conducted using endothelial cells in an *in vitro* setting, specifically using human umbilical vein endothelial cells (HUVECs). Therefore, caution should be exercised when interpreting the data and extrapolating the findings to *in vivo* scenarios.

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