Aus der Klinik für Neurochirurgie
der Heinrich-Heine-Universität Düsseldorf
Direktor: Univ.-Prof. Dr. med Daniel Hänggi

Doxycycline Attenuated Ethanol-Induced Endothelial Cell Inflammaging: Implications for Alcohol-Mediated Vascular Diseases

Dissertation

zur Erlangung des Grades eines Doktors der Medizin
der Medizinischen Fakultät der Heinrich-Heine-Universität Düsseldorf

vorgelegt von
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Düsseldorf, 2022

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Faculty of Medicine at Heinrich Heine University Düsseldorf

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Parts of this work have been published:

Li, X., Khan, D., Rana, M., Hänggi, D., & Muhammad, S. (2022). Doxycycline Attenuated Ethanol-Induced Inflammaging in Endothelial Cells: Implications in Alcohol-Mediated Vascular Diseases. *Antioxidants (Basel, Switzerland)*, *11*(12), 2413.

Zusammenfassung

Erworbene Risikofaktoren, darunter übermäßiger Alkoholkonsum, Rauchen und oxidativer Stress, tragen zu Herz-Kreislauf-Erkrankungen bei. Übermäßiger Alkoholkonsum steht in engem Zusammenhang mit einer beschleunigten Alterung und letztlich mit einer Schädigung des Gefäßgewebes und der Organe, die zu zahlreichen Krankheiten führt, darunter Atherosklerose und die Bildung intrakranieller Aneurysmen. Die zellulären und molekularen Mechanismen hinter den Gewebeschäden, die zu Organerkrankungen führen, sind komplex, sodass therapeutische Möglichkeiten begrenzt sind. Zur Reduktion toxischer zellulärer Auswirkungen des Alkohols, könnten Arzneimittel eine pharmakologische Option bieten. Hier wurden die zellulären und molekularen Vorgänge nach Ethanol-Exposition an menschlichen Nabelvenen-Endothelzellen (HUVECs) untersucht indem ein Panel von 170 Medikamenten für das Medikamentenscreening verwendet wurde. Doxycyclin, ein klinisch verwendetes Antibiotikum, war außerordentlich wirksam bei der Abschwächung von ethanolvermittelten schädlichen molekularen Ereignissen in Endothelzellen. HUVECs wurden mit verschiedenen Konzentrationen (300 mM, 400 mM) Ethanol mit oder ohne Doxycyclin (10 µg/mL) behandelt, und die Zellalterung mit etablierten Methoden analysiert. Die Telomerlänge wurde als Verhältnis von Telomeren zu Einzelkopien von Genen (T/S) quantifiziert. Die Telomerlänge und die mRNA-Expression wurden mittels qRT-PCR quantifiziert und der Proteingehalt wurde mittels Western Blotting (WB) analysiert. Unsere Daten zeigten, dass die Behandlung mit Ethanol die Zellalterung beschleunigte und die Behandlung mit Doxycyclin die Telomerlänge wiederherstellte. Die Analyse der Zellsignalwege zeigte, dass Doxycyclin die Aktivierung von mTOR und NFκ-B hemmte. Doxycyclin stellte die Expression von altersassoziierten Proteinen wieder her, darunter Lamin b1 und die DNA-Reparaturproteine KU70 und KU80. Doxycyclin verringerte die Seneszenz und den Seneszenz-assoziierten sekretorischen Phänotyp (SASP) und verbesserte die Zellmigration in mit Ethanol behandelten HUVECs. Zusammenfassend lässt sich sagen, dass Doxycyclin die durch Ethanol ausgelöste Entzündung und Alterung in HUVECs verbessert.

Summary

Acquired risk factors, including excess alcohol consumption, smoking and oxidative stress, contribute to cardiovascular diseases. Excess alcohol consumption is strongly linked to accelerated aging and ultimately vascular tissue and organ damage leading to multiple diseases, including atherosclerosis and formation of intracranial aneurysms. Cellular and molecular mechanisms behind the tissue damage leading to diseases of organs are complex, and hence, therapeutic options are limited. Drug discovery may provide pharmacological therapeutic options to reduce the toxic cellular events of alcohol. Here, the cellular and molecular events after ethanol exposure on human umbilical vein endothelial cells (HUVECs) were investigated, and a panel of 170 drugs was used for drug screening. Doxycycline, a clinically used antibiotic, was exceptionally effective in attenuating ethanol-mediated detrimental molecular events in endothelial cells. HUVECs were treated with different concentrations (300 mM, 400 mM) of ethanol with or without doxycycline (10 μg/mL), and cellular aging was analyzed using established methods. Telomere length was quantified as the telomere to single-copy gene (T/S) ratio. Telomere length and mRNA expression were quantified by qRT–PCR, and protein levels were analyzed by Western blotting (WB). Our data showed that ethanol treatment accelerated cellular aging and that doxycycline treatment restored telomere length. Cell signaling pathway analysis showed that doxycycline inhibited mTOR and NFκ-B activation. Doxycycline restored the expression of aging-associated proteins, including lamin b1 and the DNA repair proteins KU70 and KU80. Doxycycline decreased senescence and senescence-associated secretory phenotype (SASP) and improved cell migration in ethanol-treated HUVECs. In conclusion, we report that doxycycline ameliorated ethanol-induced inflammation and aging in HUVECs.

List of abbreviations

pc3	procollagenase 3
GADD45	growth arrest and DNA damage
HUVECs	human umbilical vein endothelial cells
MMPs	matrix metalloproteinases
SASP	senescence-associated secretory phenotype
SMCs	smooth muscle cells
T/S	telomere to single-copy gene ratio
TIMP	tissue inhibitor matrix metalloproteinase
WB	western blot
ECs	endothelial cells
SMC	smooth muscle cells
mTOR	mammalian target of rapamycin
4EBP1	eukaryotic translation initiation factor 4E-binding protein 1
ILs	interleukin
AKT	protein kinase B
IKK	ikappaB kinase kinase
NFκ-B	nuclear factor kappa-light-chain-enhancer of activated B cells
CRP	C-reactive protein
IFNs	interferons
PI3K	phosphoinositide 3-kinases
NO	nitric oxide
PGI2	prostacyclin
EDHF	endothelium-derived hyperpolarizing factor
TXA2	thromboxane

ET-1	endothelin-1
P53	tumor protein 53
P21	cyclin-dependent kinase inhibitor 1
P16	cyclin-dependent kinase inhibitor 2A
MCP-1	monocyte chemoattractant protein 1
ICAM-1	intercellular adhesion molecule-1
VCAM-1	vascular cell adhesion molecule-1
E-selectin	endothelial-leukocyte adhesion molecule-1
FMD	flow-mediated dilation
MLC	myosin light chain
TJ	tight junction
TNF-α	tumor necrosis factor alpha
HUVECs	human umbilical vein endothelial cells
NHEJ	nonhomologous end joining

Table of contents

1. Introduction 9

1.1 Vascular Diseases and Inflammaging 9

1.2 Cellular signals in inflammation and aging 10

1.3 Endothelial Cells in Vascular Homeostasis and Pathology 10

1.4 Endothelial Senescence 11

1.5 Senescence-Associated Secretory Phenotype (SASP) Expression in Vascular Diseases 12

1.5.1 Cytokines 12

1.5.2 Chemokines 12

1.5.3 Receptor Ligand interaction in inflammation and aging 12

1.5.4 Extracellular Proteases and Regulators 13

1.6 The Effect of Alcohol on vascular Diseases 13

1.7 Doxycycline and Inflammaging 14

1.8 Aims of Thesis 14

2. Publication 16

3. Discussion 36

4. References 40

5. Appendix 49

5.1 Declaration of authors contribution 49

5.2 List of Figures 50

5.3 Acknowledgements 51

1. Introduction

The subject has been approved by the ethics committe (Study-No.: 2019-787-bio).

Cardiovascular diseases are the leading cause of death worldwide, and aging was recognized as a fixed risk factor that exceeded all other acquired risk factors, including blood lipids, blood pressure, and smoking, as predictors of clinical events [1]. Cardiovascular diseases, such as atherosclerosis and formation of arterial aneurysms leading to heart attack and stroke, are the leading cause of death in the United States, accounting for more than 40% of deaths in those 65 years and older. More than 80% of cardiovascular deaths occur in the same age group. Therefore, age itself is a major risk factor for cardiovascular diseases [2].

1.1 Vascular Diseases and Inflammaging

It is well known that the inflammatory response in the elderly is not as fast as in persons of younger age, facilitating long-term sustained inflammation [3]. In elderly individuals, most tissues are in a noninfectious chronic inflamed state, which is recognized as a crucial aspect of different cardiovascular diseases. Increased production of oxygen free radicals and consequent telomere shortening make older adults more susceptible to cardiovascular diseases. More importantly, in the process of aging, inflammatory molecules such as IL6 and IL8 [4] expressed by cells and the cellular microenvironment seem to be the relevant factors causing vascular diseases [5]. There is sufficient evidence in the scientific literature that supports the involvement of IL6 and IL-8 in the establishment and preservation of the inflammatory microenvironment of damaged vessel walls during various vascular disease processes [6, 7]. High levels of age-related proinflammatory markers, including IL-1, IL-1 receptor antagonist protein (IL-1RN), IL-6, IL-8, IL-13, IL-18, C-reactive protein (CRP), IFNα and IFNβ, have been reported in the elderly population, even in the absence of associated risk factors and clinically active diseases [3, 8]. Inflammaging is an emerging term and is characterized by elevated levels of blood inflammatory markers with high susceptibility to chronic diseases, disability, frailty and premature death [9]. Most recently, numerous studies have reported that cellular senescence was also detected in aneurysm tissue [10]. Moreover, immunosenescence and inflammaging have been highlighted as the origin of most diseases, such as infections, cancers, autoimmune diseases, and chronic inflammatory diseases, in the elderly [11]. Previous clinical trials have suggested that inflammaging is causally linked to the development and progression of vascular diseases, such as atherosclerosis, myocardial infarction, heart failure,

and aortic aneurysms [9]. This suggests that inflammaging could be the probable driver of age-related cardiovascular diseases and a potential therapeutic target in this setting [12]. Based on these findings, inflammation is considered a hallmark of accelerated aging and is considered one of the foundations of aging biology [9-13]. The hypothesis that inflammation affects cardiovascular and neurovascular diseases in multiple ways, including inhibition of growth factors, enhancement of metabolism, and intervention in homeostatic signaling, is supported by mechanistic studies but needs to be confirmed in humans [9].

1.2 Cellular signals in inflammation and aging

The mTOR pathway contributes to various signaling pathways to regulate cell proliferation, metabolism, autophagy, and apoptosis [14]. Activation of the mTOR pathway often occurs in tumors and vascular diseases, among many other diseases [15]. mTOR pathway activation initiates the aneurysm formation process and is accelerated by the chronic inflammatory cycle [16-19]. Interestingly, PI3K/AKT/mTOR pathway activation was reported in abdominal aortic aneurysm patients and rat models [20]. In further murine research, the mechanistic target of mTOR signaling was overactivated in abdominal aortic aneurysm smooth muscle cells [21]. The mTOR pathway is strongly associated with aging and life span. mTOR and its downstream signaling molecule S6 are activated, and 4EBP1 is inhibited in senescence [22-24]. Numerous reports have confirmed that mTOR is a lifespan regulator in vivo [25-28]. Deletion of the mTORC1 substrate S6K1 also extends lifespan conditionally, but only in female mice [29]. Likewise, the diseases associated with aging are often inseparable from the development of inflammation, and the mTOR causal relationship with NFκ-B via AKT and IKK regulation has also been confirmed in mechanistic studies [22]. The NFκ-B pathway plays a vital role in proinflammatory processes by regulating the transcription of a variety of genes encoding cytokines and chemokines to mediate the activation and differentiation of innate immune cells and inflammatory T cells [30, 31].

1.3 Endothelial Cells in Vascular Homeostasis and Pathology

Endothelial cells (ECs) play multiple roles in vascular biology, including blood vessel formation, endothelial cell barrier, coagulation and fibrinolysis, regulation of vascular tone, neoangiogenesis, and participation in inflammatory reactions [32]. The first step in vessel formation is the differentiation of hemangioblasts into ECs, forming the primary vascular

plexus. This process continues through angiogenesis, followed by the normalization of arterial or venous ECs, sprouting angiogenesis, and remodeling of the vasculature into a functional circulatory system [33-35]. Therefore, the dysfunction of new blood vessel formation leads to many malignant, ischemic, inflammatory, infectious, and immune diseases [36]. ECs serve as the transporter of molecules between blood and tissues [37]. Blood–brain barrier endothelial cells exhibit exceptional tight junctions and pinocytosis inertness. They are responsible for delivering blood glucose and insulin to the brain [38]. Endothelial cells form a monolayer covering the vascular endothelial surface. They maintain blood fluidity and hemostasis when vessels are damaged. Therefore, endothelial cells conditionally balance coagulation and fibrinolysis[39]. Under physiological conditions, endothelial cells constitute a nonadherent surface that effectively prevents platelet activation, and the coagulation cascade contributes to maintaining blood fluidity. At the site of vascular injury, endothelial cells act as supporting surfaces for procoagulant complex formation and coagulation [39]. The endothelium regulates vascular tone by releasing the factors nitric oxide (NO), prostacyclin (PGI2) and endothelium-derived hyperpolarizing factor (EDHF) or vasoconstrictive factors such as thromboxane (TXA2) and endothelin-1 (ET-1), which are involved in growth inhibition and stimulation [40]. Disruption of vascular homeostasis contributes to the development of endothelial dysfunction and subsequently activates the atherosclerosis process [41].

1.4 Endothelial Senescence

Endothelial senescence and related endothelial dysfunction are major causes of vascular diseases [42]. Cellular senescence can be triggered by many factors, including oxidative stress, radiation and telomere shortening [43, 44]. p53, p21, and p16 proteins have been reported as pro-senescence factors and have been suggested to play a vital role in vascular diseases and inflammation [45, 46]. Senescence is an irreversible state of growth arrest that can be either replicative senescence or stress-induced premature senescence. Telomere shortening leads to replicative senescence. Telomeres shorten with each cell division, and when these telomeres shorten to a certain extent, the cell becomes replicative senescent [47]. Stress-induced premature aging can be induced by a variety of stress signals, including telomere shortening and oxidative stress-induced DNA damage, metabolic stress, oncogenic activation, and constitutive activation by mitotic stimulation [48].

1.5 Senescence-Associated Secretory Phenotype (SASP) Expression in Vascular Diseases

Emerging advances in research suggest that senescent vascular cells that are characterized by irreversible cell cycle arrest and a pronounced senescence-associated secretory phenotype are strongly expressed in aged and diseased cardiovascular systems. These senescent cells contribute to endothelial and later cardiovascular dysfunction [49, 50]. Typically, senescent cells are recognized and cleared by the immune system[51]. However, the long-term presence of senescent cells and SASP can lead to cancer, atherosclerosis, and other vascular diseases [52, 53].

1.5.1 Cytokines

Multiple immune cells and senescent cells release different cytokines under different conditions and regulate tissue homeostasis. Interleukins (IL6, IL7, IL1a, IL1b, IL13, IL15) released by senescent cells establish the proinflammatory state [54]. In turn, the surrounding inflammatory microenvironment is formed to affect nearby cells and accelerate the development of multiple diseases [55]. Increased cytokine release facilitates endothelial dysfunction, which contributes to the activation of the primary stage of the atherosclerotic process [56] and then promotes the expression of atherosclerotic genes in macrophage foam cells and smooth muscle cells (SMCs) [57], suggesting a vital role of these cytokines in the development of atherosclerosis [58].

1.5.2 Chemokines

IL8 is the most common inflammatory cytokine in vascular diseases and is overexpressed in most senescent cells [54]. It was found in atherosclerotic tissue accompanied by overexpression of the pro-inflammatory chemokine MCP-1. IL8 and MCP-1 are strong initiators of monocyte and neutrophil fixative adhesion to the vascular endothelium and contribute significantly to the recruitment of inflammatory cells such as monocytes and neutrophils [59]. Then, the infiltrated neutrophils and macrophages release inflammatory cytokines and chemokines, leading to tissue damage and enhanced inflammation [60]. This process promotes the primary stages of several vascular diseases, including atherosclerotic plaques [56].

1.5.3 Receptor–ligand-ligand interactions in inflammation and aging

Vascular endothelial cells aligning all vessels are important regulators of vascular remodeling by releasing matrix metalloproteinases (MMPs), recruiting neutrophils, and inducing

macrophage differentiation. Destruction of the endothelium is the first event in aneurysm initiation and a signal of atherosclerosis in the early stage [61], which is characterized by increased expression of adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1), endothelial-leukocyte adhesion molecule-1 (E-selectin), and vascular cell adhesion molecule-1 (VCAM-1) [62]. They play a key role in mediating the firm adhesion of leukocytes to endothelial cells in various acute and chronic inflammatory conditions [63, 64].

1.5.4 Extracellular Proteases and Regulators

MMPs degrade extracellular matrix proteins and are regulated mainly by tissue inhibitors of metalloproteinases (TIMPs) [65]. MMP expression is associated with classic cardiovascular risk factors and inflammation. They strongly contribute to atherosclerosis, acute coronary syndrome, restenosis, aneurysm, and peripheral vascular diseases [66]. MMP1, MMP8, and MMP13 are related to collagenase. MMP2 and MMP9 are related to gelatinase. MMP3, MMP10, and MMP11 are related to stromelysin [67, 68]. MMP1, MMP3, and MMP10 are consistently upregulated in human and mouse fibroblasts undergoing replication or stress-induced senescence [69]. MMP9 was overexpressed in ruptured aneurysms compared with intact aneurysms established by E Petersen *et al.* [70] and associated with chronic inflammation [71]. Increased total MMP2 levels and MMP2 activity in aneurysm aortas compared to normal and atherosclerotic aortas have been confirmed by human and animal studies [72, 73]. Specifically, increased MMPs digest arterial structures and accelerate aneurysm formation [74], and they promote the development of multiple diseases through their disruptive effects on the intercellular connections of the vessel wall and significantly accelerate the inflammatory microenvironment [75]. MMPs have developed into important regulatory enzymes in pro- and anti-inflammatory pathways. There is increasing evidence that these proteases play a role in inflammation, primarily by modulating barrier function, cytokine/chemokine activity, or leukocyte influx [76].

1.6 The Effect of Alcohol on Vascular Diseases

Alcohol is a potential risk factor for cardiovascular diseases [77]. In animal experiments, small doses of alcohol can increase the release of nitric oxide and enhance endothelial-mediated vasodilation, while high doses of alcohol can impair the endothelium-dependent relaxation response [78]. Furthermore, chronic administration of alcohol to rats is usually related to the tolerance of the acute inhibitory effect of alcohol on endothelial-mediated vasodilation and may even lead to the enhancement of this response [78]. Haorah *et al.* suggested that blood–brain

barrier dysfunction occurring in brain endothelial cells could be associated with oxidative stress caused by ethanol metabolism in brain microvascular endothelial cells [79]. By high-frequency ultrasonography evaluation of brachial artery endothelial function among 92 long-term drinking males, Runlan Luo *et al*. [80] found that long-term moderate to severe drinking caused endothelial dysfunction, measured by high-frequency ultrasonography. Severe drinking even damaged vascular smooth muscle cells. Light drinking may have a beneficial effect on endothelial function, although the association is not significant [81-85]. Brachial artery blood flow-mediated dilation (FMD) measurement was used to assess endothelial function during reactive hyperemia among 404 men aged 30–79, and Aoi Tanaka *et al.* [82] reported that heavy alcohol consumption may be an independent risk factor for endothelial dysfunction. Vascular endothelial cells are the target of important mediation pathways of different ethanol concentrations, such as oxidative stress, lipoprotein, and insulin resistance. Alcohol-mediated oxidative stress leads to the activation of myosin light chain (MLC) kinases, phosphorylation of tight junction (TJ) proteins, reduced brain cell integrity, and enhanced monocyte migration across brain endothelial cells [79]. Alcohol-induced endothelial damage or protection may be related to the synthesis or effects of multiple markers, such as nitric oxide, cortisol, endothelin-1, adhesion molecule, tumor necrosis factor-alpha, interleukin-6, C-reactive protein, and hemostatic factors [83, 84, 86, 87]. Chronic ethanol exposure in vitro enhances agonist-induced TNF mRNA and protein expression in ECs. Functionally, the damage to various organs induced by chronic ethanol is related to the increase in TNF-α (TNF) release, and in the presence of ethanol, TNF-induced EC dysfunction, including proliferation, migration, and decreased expression of cyclin A, was significantly enhanced [85, 86].

1.7 Doxycycline and Inflammaging

Doxycycline is a semisynthetic tetracycline antibiotic [88, 89]. In vitro, doxycycline exhibits robust anti-inflammatory effects by increasing proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1α, IL-1β, IL-6, IL-8, and IL-17, and decreasing anti-inflammatory cytokines (IL-4 and IL-10) and MMPs [89-91]. Furthermore, doxycycline extends lifespan in an animal model, namely, *Caenorhabditis elegans* [92, 93].

1.8 Aims of Thesis

State-of-the-art research suggests that aging strongly contributes to vascular diseases. The inflammatory mechanism is associated with aging, which is greatly correlated with vascular

diseases such as atherosclerosis, aneurysm formation and stroke. Alcoholism is one of the potentially acquired risk factors for vascular diseases, and the most advanced research supports the idea that senescence acceleration is probably alcohol-mediated.

This thesis investigates the cellular and molecular signals of ethanol on human umbilical vein endothelial cells (HUVECs) and explores whether drug screening could discover potential candidates that attenuate ethanol-mediated molecular events in endothelial cells.

2. Publication

antioxidants

MDPI

Article

Doxycycline Attenuated Ethanol-Induced Inflammaging in Endothelial Cells: Implications in Alcohol-Mediated Vascular Diseases

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Abstract: Excess alcohol consumption is a potential risk factor for cardiovascular diseases and is linked to accelerated aging. Drug discovery to reduce toxic cellular events of alcohol is required. Here, we investigated the effects of ethanol on human umbilical vein endothelial cells (HUVECs) and explored if doxycycline attenuates ethanol-mediated molecular events in endothelial cells. Initially, a drug screening using a panel of 170 drugs was performed, and doxycycline was selected for further experiments. HUVECs were treated with different concentrations (300 mM and 400 mM) of ethanol with or without doxycycline (10 µg/mL). Telomere length was quantified as telomere to single-copy gene (T/S) ratio. Telomere length and the mRNA expression were quantified by qRT-PCR, and protein level was analyzed by Western blot (WB). Ethanol treatment accelerated cellular aging, and doxycycline treatment recovered telomere length. Pathway analysis showed that doxycycline inhibited mTOR and NFκ-B activation. Doxycycline restored the expression of aging-associated proteins, including lamin b1 and DNA repair proteins KU70 and KU80. Doxycycline reduced senescence and senescence-associated secretory phenotype (SASP) in ethanol-treated HUVECs. In conclusion, we report that ethanol-induced inflammation and aging in HUVECs were ameliorated by doxycycline.

Keywords: ethanol; HUVECs; inflammaging; telomere shortening; accelerated aging; cellular senescence; doxycycline; mTOR; NFκ-B

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Citation: Li, X.; Khan, D.; Rana, M.; Hänggi, D.; Muhammad, S. Doxycycline Attenuated Ethanol-Induced Inflammaging in Endothelial Cells: Implications in Alcohol-Mediated Vascular Diseases. *Antioxidants* **2022**, *11*, 2413. https://doi.org/10.3390/antiox11122413

Academic Editors: Jacopo Sabbatinelli, Angelica Giuliani and Stephen M. Black

Received: 17 October 2022
Accepted: 5 December 2022
Published: 7 December 2022

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1. Introduction

Aging is strongly associated with cardiovascular diseases. With increasing age, the risk of multiple cardiovascular diseases increases. The senescence of vascular cells is associated with age-related cardiovascular diseases [1–3]. Senescence is a state of irreversible growth arrest, which can be either replicative senescence or stress-induced premature senescence. Replicative senescence results from telomere shortening. With each cell division, telomeres shorten, and when these telomeres are shortened to a certain degree, cells become replicative senescent [4]. Stress-induced premature senescence can be induced by various stress signals, including telomere shortening and DNA damage by oxidative stress, metabolic stress, oncogenic activation, and constitutive activation of mitogenic stimuli [1,5].

The senescence and dysfunction of vascular endothelial cells play a vital role in vascular aging, which results in the initiation, progress, and advancement of cardiovascular diseases [1]. The senescence of endothelial cells is a potential link between aging and inflammation [2]. Senescent and dysfunctional endothelial cells actively express and release pro-inflammatory cytokines, chemokines, adhesion molecules, and matrix metalloproteinases (MMPs) [1,5–8], called senescent-associated secretory phenotype (SASP). Chronic

sterile inflammation can enhance this process and consequently promotes tissue remodelling [1,8]. Clinical and experimental studies suggest that chronic, sterile, and low-grade inflammation is a sign of the aging process, namely inflammaging [2]. With increasing age, the pro-inflammatory shift in the gene expression of vascular endothelial and smooth muscle cells (SMCs) forms a pro-inflammatory microenvironment in the vascular wall, which consequently contributes to vascular diseases [2]. Senescent cells have been found in atherosclerotic tissue, and the progression of atherosclerosis could be prevented by eliminating senescent cells [2,9]. Animal studies have shown that the elimination of senescent cells provided protection against age-related diseases and increased life span [5]. Risk factors such as oxidative stress, alcohol abuse, smoking, obesity, diabetes, and aging can induce endothelial senescence and dysfunction, which contributes to atherosclerosis, arterial stiffness, and hypertension leading to cardiovascular diseases [1,2,8,9]. According to World Health Organization, an estimated 2.3 billion people are current alcohol drinkers, and 3 million people died as a result of the harmful use of alcohol in 2016 alone. Alcohol abuse is one of the most potentially acquired risk factors for cardiovascular diseases, and state-of-the-art research supports the notion that alcohol abuse accelerates aging [10,11]. However, hitherto the direct effect of alcohol on cellular aging and the underlying mechanisms have not been investigated.

Doxycycline is a broad-spectrum antibiotic belonging to the tetracycline class, and it has an anti-inflammatory effect. It has been reported to inhibit the activation of NF-κB [12,13]. Both NF-κB and mTOR pathways play an important role in inflammation and aging [7,14–19]. Inhibition of NF-κB and mTOR pathways curtails inflammation, reduces cellular senescence and delays aging [7,14–19].

This is the first report showing that ethanol accelerated cellular aging and senescence in HUVECs. Doxycycline treatment reduced ethanol-enhanced aging and cellular senescence by inhibiting NFκ-B activation and the mTOR pathway.

2. Methods

2.1. Cell Culture

HUVECs were obtained from Promocell (Heidelberg, Germany) and maintained in the medium consisting of endothelial cell medium (C-22010, Promocell, Heidelberg, Germany) supplemented with endothelial growth factors (C-39215, Promocell, Heidelberg, Germany) at 37 °C in a 95% humidified atmosphere containing 5% CO_2. Cells were seeded in the T75 cell culture flask after thawing. When the cells were 90% confluence, they were incubated with trypsin for 4 min at 37 °C, and then cells were seeded in 10 cm cell culture plate, 5000 cells/cm^2. For experiments, the endothelial cells were used at passage 7 and were seeded in 6-well plates. After 24 h, the medium was changed, and the new medium containing either ethanol (300 mM or 400 mM), doxycycline (10 μg/mL), or 400 mM ethanol combined with doxycycline (10 μg/mL) was added to the culture; the medium was changed after two days. Doxycycline was purchased from Sigma-Aldrich (D1822).

2.2. Telomere Length Measurement

In the first experiment, the endothelial cells were treated with an endothelial cell medium containing either 300 mM or 400 mM ethanol. In the second experiment, the endothelial cell medium was supplemented with either 400 mM ethanol or 400 mM ethanol combined with doxycycline (10 μg/mL). The endothelial cell medium alone was used for the control group. The medium was changed after two days. After four days of treatment, DNA was extracted from cell pellets using innuPREP DNA Mini Kit (845-KS-1042050, Analytik Jena, Jena, Germany). For telomere length measurement, 1 ng DNA was utilized. For qPCR the primers (Table S2) telomere (TEL) and single-copy gene interferon beta 1 (IFNB1) were used as previously described [20]. Quantitative PCR was performed with AceQ SYBR qPCR Master Mix (Q111-03, Vayzme, Nanjing, China) on Bio-Rad with an initial denaturation of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by melting curve. The relative telomere length was calculated as

telomere to single copy-gene (IFNB1) ratio. The T/S was quantified using the comparative ΔCT method. All experiments were performed with three biological triplicates, and each biological replicate had three technical triplicates.

2.3. CellTiter-Glo (CTG) Assay

For the CTG assay, the cells were seeded in 96-well plate (5000 cells/cm^2). The medium was changed the next day with a new medium alone (for control) or medium containing ethanol (200 mM, 300 mM, 400 mM, 600 mM, and 800 mM), doxycycline (1 μM, 10 μM, 50 μM, and 100 μM) and combination of ethanol (400 mM) and doxycycline (10 μM). The medium was changed after two days. The readout of the cell survival was performed using luminescence-based CTG assay (Promega, Walldorf, Germany) according to the manufacturer's instructions, except that the reaction agent was diluted in 1:1 with PBS. The CTG assay was performed immediately after changing the medium on day 0 and on day 1, day 2, and day 4.

2.4. Western Blot

For protein analysis, endothelial cells were treated with endothelial cell medium supplemented with either 400 mM ethanol, doxycycline (10 μg/mL), or 400 mM ethanol combined with doxycycline (10 μg/mL). For the control group, the endothelial cells were treated with endothelial cell medium alone. After 24 h of treatment, the total protein was extracted using RIPA Buffer, then determined calorimetrically using the DC Protein Assay Kit (500-0116, Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions, and measured with the Paradigm micro-plate reader. Total protein (25 μg, in reducing conditions) was loaded on 12% sodium dodecyl sulfate-polyacrylamide gel and run at 60 volt for 20 min, then continued with 110 Volt for 30–60 min, which was further transferred onto a polyvinylidene difluoride membrane at 250 mA for 120 min. The non-specific binding was blocked with 5% skim milk (0.05% TBST) for 1 h. The membranes were incubated with primary antibodies (as reported in Supplementary Table S1) overnight at 4 °C on a shaking platform. The membranes were washed thrice for 10 min with TBST and then incubated with secondary antibodies (Table S1) for 1 h at RT. All antibodies were diluted in the blocking solution containing either 5% bovine serum albumin (BSA) for phosphorylated proteins or 5% skim milk for the rest of the non-phosphorylated proteins. The antibodies were diluted in Tris-buffered saline with Tween20 (TBST). The densitometry was calculated with NIH-Image J (National Institutes of Health, Bethesda, MD, USA) by correcting for β-actin. All WBs were performed with three biological triplicates.

2.5. Quantitative PCR

For quantitative PCR (qPCR), total RNA was extracted from endothelial cells treated with either endothelial cell medium alone (control group) or endothelial cell medium containing either 400 mM ethanol or 400 mM ethanol combined with doxycycline (10 μg/mL) for 24 h. The Nucleo Spin RNA kit (740955.50, MACHEREY-NAGEL, Düren, Germany) was utilized to extract total RNA following the manufacturer's instructions. RNA (1.2 μg) was utilized to reverse transcribe with M-MLV Reverse Transcriptase kit (M1701, Promega), Random Hexamer Primers (48190011, Thermo Fisher, Waltham, MA, USA), and RiboLock RNase Inhibitor (EO0384, Thermo Fisher, Waltham, MA, USA). qPCR was performed with AceQ SYBR qPCR Master Mix (Q111-03, Vayzme, Nanjing, China) on Bio-Rad with 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, followed by melting curve. The relative mRNA expressions were calculated after normalizing them to β-actin expression. The primer sequences are listed in Table S2. Relative mRNA expression was quantified using the comparative ΔCT method. All experiments were performed with three biological triplicates, and each biological replicate had three technical triplicates. The data are shown for one biological replicate.

2.6. Migration Assay

HUVECs were seeded in a 6-well plate. Once the cells were more than 95% confluence, a 10 µL sterile pipette tip was used to make a scratch on the monolayer of endothelial cells. After the scratch, the endothelial cells were washed 3 times with PBS, and a new endothelial cell medium containing either 400 mM ethanol or 400 mM ethanol combined with doxycycline (10 µg/mL) was added to the culture. For the control group, the endothelial cells were treated with endothelial cell medium alone. At 0 h, 6 h, 12 h, and 24 h following the scratch assay, images of cell migration in three randomly selected fields were taken for each well using an optical microscope. Finally, the area of the wound was measured by Image J. All experiments were performed with three biological triplicates, and each biological replicate had three technical triplicates.

2.7. β-Gal Staining

β-Gal staining was detected using a Senescence Cells Histochemical Staining Kit (GALS, Sigma, St. Louis, MO, USA), following manufacturer instructions. The endothelial cells were treated with endothelial cell medium alone (control group) or endothelial cell medium supplemented with either 400 mM ethanol or 400 mM ethanol combined with doxycycline (10 µg/mL). The medium was changed after two days. After 4 days, the cells were fixed in the fixation buffer provided with the Senescence Cells Histochemical Staining Kit. The fixed samples were stained with fresh solution for SA-beta-galactosidase activity at 37 °C for 7 h, followed by aspiration with the staining solution. After that, the cells were overlaid with a 70% glycerol solution and stored at 4 °C. The images were taken with an optical microscope. Finally, the number of stained cells was counted using Image J. The experiment was performed with three biological triplicates.

2.8. Immunofluorescence Staining

The cells were seeded in a 96-well plate (5000 cells/cm^2). The medium was changed the next day with a new medium alone (for control) or a medium containing ethanol (400 mM), doxycycline (10 µM), and a combination of ethanol (400 mM) and doxycycline (10 µM). After two hours of incubation, immunofluorescence staining was performed. The cells were washed 3 times with PBS and fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton™ X-100 for 10 min, and blocked with 5% BSA for 1 h at RT. The cells were incubated overnight at 4 °C with primary antibody 8-OHDG (1:500, Cat. No.: BSS-BS-1278R, BIOSS, Woburn, MA, USA). The next day, the cells were washed three times and then labeled with Secondary antibody (1:1000, Alexa Fluor 488, Cat. No.: ab150077) for 60 min at RT. Nuclei were stained with SlowFade® Gold Antifade Mountant with DAPI (Product # S36938) for 10 min. The images were captured at 20× magnification.

2.9. Statistical Analysis

Student's *t*-test was performed to compare two groups, and one-way ANOVA followed by Tukey's test was used to analyze more than two groups. The level of significance was set at * $p < 0.05$.

3. Results

3.1. Ethanol-Enhanced Molecular Aging in Endothelial Cells

Here, we investigated the effects of two different concentrations (300 mM and 400 mM) of ethanol on the telomere length of endothelial cells. The endothelial cells were treated with an endothelial cell medium containing either 300 mM or 400 mM ethanol. The endothelial cell medium without ethanol was used for the control group. Telomere length was quantified as T/S. The telomere length was shortened by more than 50% in ethanol-treated HUVECs as compared to that in the untreated control (Control = 1.03 ± 0.03 T/S, 300 mM ethanol day 2 = 0.18 ± 0.13 T/S, 400 mM ethanol day 2 = 0.14 ± 0.17 T/S, 300 mM ethanol day 4 = 0.32 ± 0.17 T/S, 400 mM ethanol day 4 = 0.18 ± 0.10 T/S, n = 3, *** $p < 0.001$, Figure 1A). Next, we investigated the protein expression of aging-related protein lamin-b1

and DNA repair proteins KU70 and KU80 after 24 h of 400 mM ethanol treatment. Protein analysis showed a remarkably reduced relative protein expression of lamin b1 24 h after ethanol exposure (Control = 100 ± 13.85, ethanol = 55.56 ± 12.95, n = 3, * $p < 0.05$, Figure 1B,C), indicating a disruption in nuclear structure. The ethanol treatment decreased the relative protein expression of DNA repair protein KU70 (Control = 100 ± 4.52, ethanol = 68.50 ± 18.69, n = 3, * $p < 0.05$, Figure 1B,D), and KU80 (Control = 100 ± 7.77, ethanol = 69.57 ± 1.3, n = 3, ** $p < 0.01$, Figure 1B,E), showing the diminished capacity of DNA repair. Ethanol treatment accelerated aging by shortening telomere length and reducing the relative protein expression of aging-associated protein lamin b1 and DNA repair proteins KU70 and KU80.

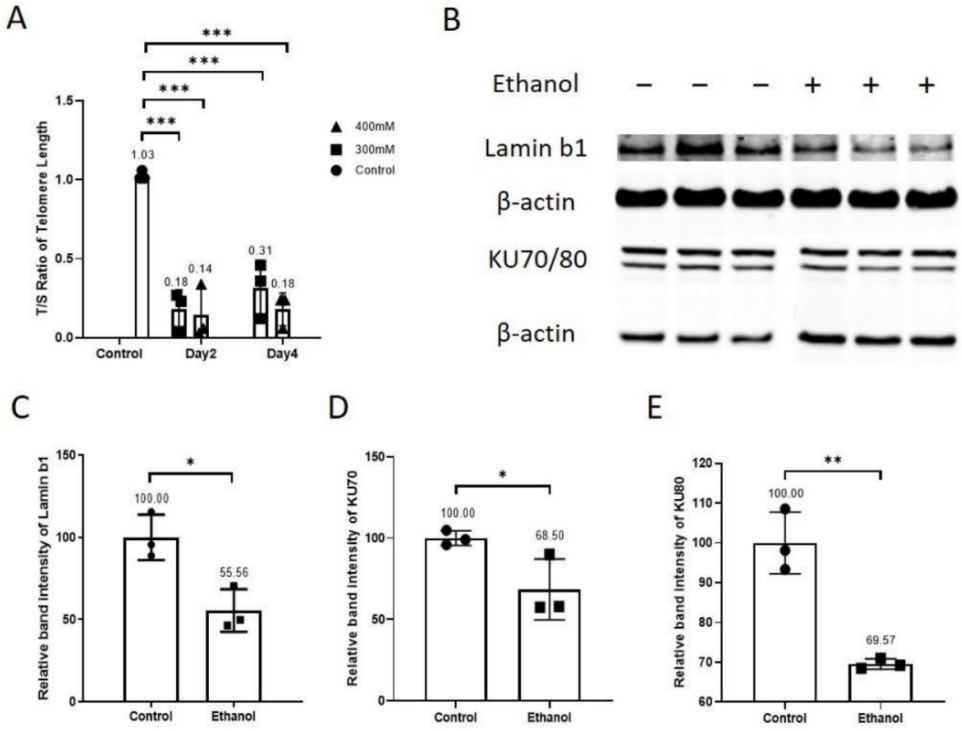


Figure 1. Ethanol accelerated molecular aging in endothelial cells. (**A**) The telomere length was shortened after ethanol (300 mM and 400 mM) treatment in HUVECs. (**B**) WB showing protein expression of lamin b1, KU70 and KU80 in HUVECs 24 h after ethanol treatment (400 mM). Ethanol exposure reduced the relative protein expression of (**C**) lamin b1, (**D**) KU70, and (**E**) KU80. β-actin was used as a loading control. Data are the mean of three independent biological triplicates. Student's *t*-test was performed to compare two groups, and one-way ANOVA followed by Tukey's test was used to analyze more than two groups. Error bars represent the SD (*** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$).

3.2. Doxycycline Reduced the Toxic Effects of Ethanol

To test the toxicity of ethanol, doxycycline and a combination of ethanol and doxycycline, we performed CellTiter-Glo (CTG) assay. The cells were treated with different concentrations of ethanol, doxycycline, and combined ethanol (400 mM) and doxycycline (10 μM). The medium was changed after two days. The CTG assay was performed on day 0, day 1, day 2, and day 4. On day 0, no difference was observed between the groups (Day 0: Control = 47.54 ± 8.84%, ethanol (200 mM) = 49.26 ± 9.34%, ethanol (300 mM) = 48.05 ± 8.93%, ethanol (400 mM) = 50.22 ± 15.67%, ethanol (600 mM) = 51.76 ± 10.25%, ethanol (800 mM) = 54.26 ± 9.97%, doxycycline (1 μM) = 48.32 ± 11.54%, doxycycline (10 μM) = 46.72 ± 10.73%, doxycycline (50 μM) = 46.71 ± 12.83%, doxycycline (100 μM) = 48.30 ± 11.91%, ethanol + doxycycline = 43.12 ± 9.23%, $p > 0.05$, n = 3, Supplementary Figure S1). On day 1 control did not show the difference in all concentrations of ethanol. Doxycycline (50 μM and 100 μM) showed improved viability than control and ethanol (300 mM). Ethanol 400 mM showed reduced growth than doxycycline (10 μM, 50 μM, and 100 μM) and the combination of ethanol (400 mM) and doxycycline (10 μM). Ethanol (600 mM and 800 mM) showed reduced growth than ethanol (400 mM) and doxycycline (10 μM) combined, and all concentrations of doxycycline used (Day 1: Control = 49.62 ± 3.11%, ethanol (200 mM) = 51.30 ± 2.37%, ethanol (300 mM) = 46.31 ± 1.24%, ethanol (400 mM) = 44.41 ± 1.22%, ethanol (600 mM) = 40.58 ± 1.08%, ethanol (800 mM) = 40.88 ± 3.10%, doxycycline (1 μM) = 51.97 ± 2.81%, doxycycline (10 μM) = 54.23 ± 3.58%, doxycycline (50 μM) = 59.25 ± 3.32%, doxycycline (100 μM) = 61.01 ± 4.36%, ethanol + doxycycline = 54.09 ± 1.69%, $p < 0.05$, n = 3, Supplementary Figure S1). On day 2, ethanol (300 mM, 400 mM, 600 mM, and 800 mM) showed decreased growth than the control, doxycycline (100 μM), and the combination of ethanol (400 mM) and doxycycline (10 μM) (Day 2: Control = 66.32 ± 1.74%, ethanol (200 mM) = 61.34 ± 3.44%, ethanol (300 mM) = 59.32 ± 2.10%, ethanol (400 mM) = 58.53 ± 1.84%, ethanol (600 mM) = 59.21 ± 1.58%, ethanol (800 mM) = 59.04 ± 2.32%, doxycycline (1 μM) = 64.95 ± 2.30%, doxycycline (10 μM) = 64.90 ± 1.35%, doxycycline (50 μM) = 63.44 ± 1.32%, doxycycline (100 μM) = 67.77 ± 3.47%, ethanol + doxycycline = 66.75 ± 3.36%, $p < 0.05$, n = 3, Supplementary Figure S1). On day 4, ethanol (300 mM, 400 mM, 600 mM, and 800 mM) and doxycycline (1 μM and 100 μM) showed impeded growth than the control. Ethanol (300 mM) showed reduced growth than doxycycline (10 μM, 50 μM) and a combination of ethanol (400 mM) and doxycycline (10 μM). Ethanol (400 mM) showed decreased growth than doxycycline (1 μM, 10 μM, and 50 μM) and a combination of ethanol (400 mM, 600 mM, and 800 mM) and doxycycline (10 μM) (Day 4: Control = 100.00 ± 0.00%, ethanol (200 mM) = 95.90 ± 2.14%, ethanol (300 mM) = 82.48 ± 2.51%, ethanol (400 mM) = 80.75 ± 2.50%, ethanol (600 mM) = 76.04 ± 2.10%, ethanol (800 mM) = 79.77 ± 3.19%, doxycycline (1 μM) = 88.81 ± 1.37%, doxycycline (10 μM) = 98.61 ± 1.42%, doxycycline (50 μM) = 95.11 ± 5.13%, doxycycline (100 μM) = 77.00 ± 2.11%, ethanol + doxycycline = 104.15 ± 3.32%, $p < 0.05$, n = 3, Supplementary Figure S1). For statistical analysis, ANOVA was used. The experiment was performed with three biological triplicates. Doxycycline 10 μM, the concentration that had no cytotoxic effects was used for further experiments.

3.3. Doxycycline Reduced Ethanol-Accelerated Aging

HUVECs were treated with either 400 mM ethanol or 400 mM ethanol combined with doxycycline (10 μg/mL) in endothelial cell medium. The control group was treated with endothelial cell medium alone. The medium was changed after two days. On day 4, we collected the cell pellets and extracted DNA. Telomere length quantification showed that doxycycline treatment ameliorated ethanol-accelerated telomere shortening in HUVECs

(Control = 1.01 ± 0.00 T/S, 400 mM ethanol = 0.22 ± 0.03 T/S, 400 mM ethanol + doxycycline = 0.66 ± 0.14 T/S, n = 3, *** $p < 0.001$, ** $p < 0.01$ Figure 2A). It is known that the activation of the mTOR pathway plays an important role in aging [7,18,19], therefore we investigated the effects of ethanol, doxycycline, and ethanol combined with doxycycline on the activation of mTOR and its downstream targets. For protein analysis, we treated the endothelial cells with endothelial cell medium supplemented with either 400 mM ethanol, 10 µg/mL doxycycline or 400 mM ethanol in combination with 10 µg/mL doxycycline for 24 h. The endothelial cell medium alone was used for the control group. Doxycycline inhibited ethanol-induced activation of mTOR, relative protein expression of p-mTOR (Control = 100 ± 6.49, ethanol = 174.87 ± 14.8, doxycycline = 122.57 ± 49.15, ethanol + doxycycline = 84.58 ± 14.05, n = 3,* $p < 0.05$, Figure 2B,C). Both ethanol and doxycycline increased the relative protein expression of mTOR downstream signaling molecule p-S6 but ethanol and doxycycline in combination reduced its relative protein expression (Control = 100 ± 1.83, ethanol = 108.95 ± 0.78, doxycycline = 116.67 ± 0.27, ethanol + doxycycline = 97.71 ± 1.35, n = 3, *** $p < 0.001$, Figure 2B,D). Ethanol decreased relative protein expression of S6 and doxycycline increased the relative expression of S6 protein in ethanol treated HUVECs (Control = 100 ± 3.59, ethanol = 89.39 ± 1.31, doxycycline = 96.80 ± 3.66, ethanol + doxycycline = 102.88 ± 1.15, n = 3, ** $p < 0.01$, * $p < 0.05$, Figure 2F). Both ethanol and doxycycline increased the ratio of p-S6/S6, but the combination of ethanol and doxycycline treatment decreased p-S6/S6 in HUVECs (Control = 100 ± 1.77, ethanol = 121.89 ± 1.05, doxycycline = 120.64 ± 4.68, ethanol + doxycycline = 94.98 ± 1.04, n = 3, ** $p < 0.01$, * $p < 0.05$, Figure 2G). Doxycycline alone increased the relative protein expression of *p*-4EBP1, but it did not affect its relative protein expression in ethanol-treated HUVECs (Control = 100. ± 0.40, ethanol = 85.05 ± 1.66, doxycycline = 113.16 ± 5.83, ethanol + doxycycline = 79.06 ± 7.94, n = 3, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$; Figure 2B,E). Doxycycline decreased telomere shortening and inhibited activation of mTOR and its downstream target S6 in ethanol-treated HUVECs.

To investigate ethanol-induced oxidative stress, we performed immunofluorescence staining for oxidative stress marker 8-OHDG. HUVECs were treated with ethanol (400 mM), doxycycline (10 µM), and a combination of ethanol (400 mM) and doxycycline (10 µM) for 2 h. The endothelial cell medium alone was used for control. Ethanol increased oxidative stress, which was ameliorated by doxycycline (Control = 0.58 ± 0.53, Ethanol = 90.52 ± 2.65, doxycycline = 3.84 ± 4.94, ethanol ± doxycycline = 12.37 ± 15.51, $p < 0.001$, Supplementary Figure S2). The experiment was performed with three biological triplicates, and each biological replicate had three technical triplicates. For statistical analysis, ANOVA was used (*** $p < 0.001$).

3.4. Doxycycline Inhibited Ethanol-Induced NFκ-B Activation

NFκ-B is known to play an important role in inflammation, aging and cellular senescence [14,15]. Ethanol can activate NFκ-B [21–23], and p-mTOR has been shown to activate NFκ-B [6]. Doxycycline reduced relative protein expression of NFκ-B p65 subunit in ethanol-treated HUVECs (Control = 100 ± 0.86, ethanol = 106.46 ± 1.14, doxycycline = 95.85 ± 2.15, ethanol + doxycycline = 97.67 ± 3.01, n = 3, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ Figure 3A,B). Ethanol increased activation of NFκ-B and doxycycline attenuated ethanol-induced NFκ-B activation in HUVECs, relative protein expression of p-p65 subunit of NFκ-B (Control = 100 ± 17.4, ethanol = 398.65 ± 33.54, doxycycline = 244.45 ± 72.76, ethanol + doxycycline = 256.93 ± 39.90, n = 3, ** $p < 0.01$, * $p < 0.05$, Figure 3A,B). Both ethanol and ethanol combined with doxycycline reduced mRNA expression of NF-κB (Control = 1.0 ± 0.09, ethanol = 0.74 ± 0.04, ethanol + doxycycline = 0.84 ± 0.04, ** $p < 0.01$, * $p < 0.05$, Figure 3D). Doxycycline impeded NFκ-B activation in ethanol-treated HUVECs.

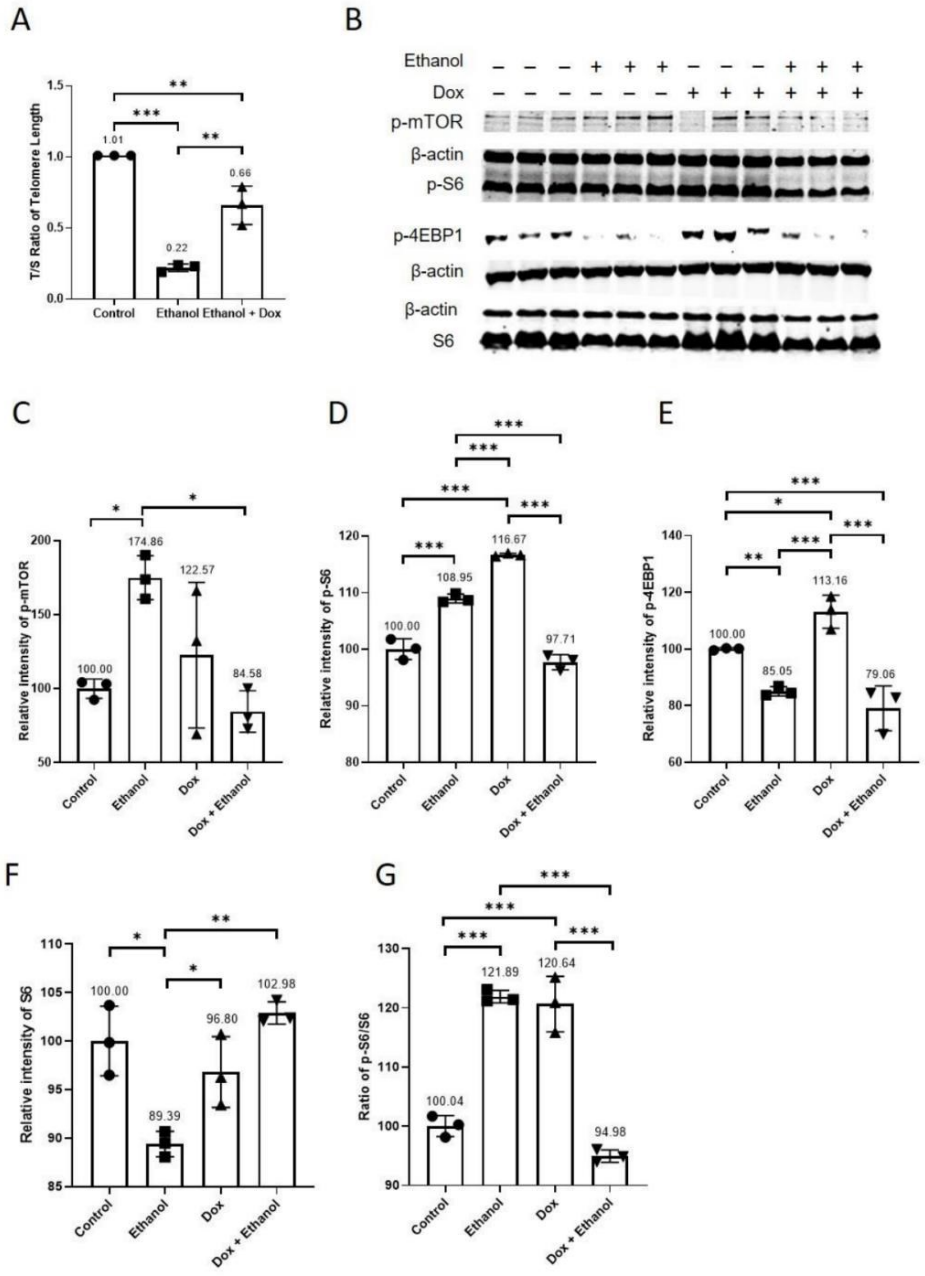


Figure 2. Doxycycline decreased ethanol-accelerated aging and inhibited ethanol-induced mTOR activation in HUVECs. HUVECs were treated with ethanol (400 mM), doxycycline (10 μM), and combination of ethanol (400 mM) and doxycycline (10 μM) for 24 h. The endothelial cell medium

alone was used for control. (**A**) Doxycycline reduced ethanol-induced shortening of telomere length. (**B**) WB showing protein expression of mTOR pathway proteins. Doxycycline inhibited ethanol-induced activation of (**C**) mTOR and mTOR downstream signaling molecule (**D**) S6. (**E**) Doxycycline did not affect the relative protein expression of p-4EBP1 in ethanol-treated HUVECs. (**F**) Ethanol alone decreased relative protein expression of S6, and doxycycline combined with ethanol increased the relative protein expression of S6 more than only ethanol-treated HUVECs. (**G**) Both doxycycline and ethanol increased p-S6/S6 more than control and combination of doxycycline and ethanol decreased p-S6/S6 more than ethanol alone and doxycycline alone. β-actin was used as a loading control. Data are the mean of three independent biological triplicates. One-way ANOVA followed by Tukey's test was used to analyze more than two groups. Error bars represent the SD (*** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$).

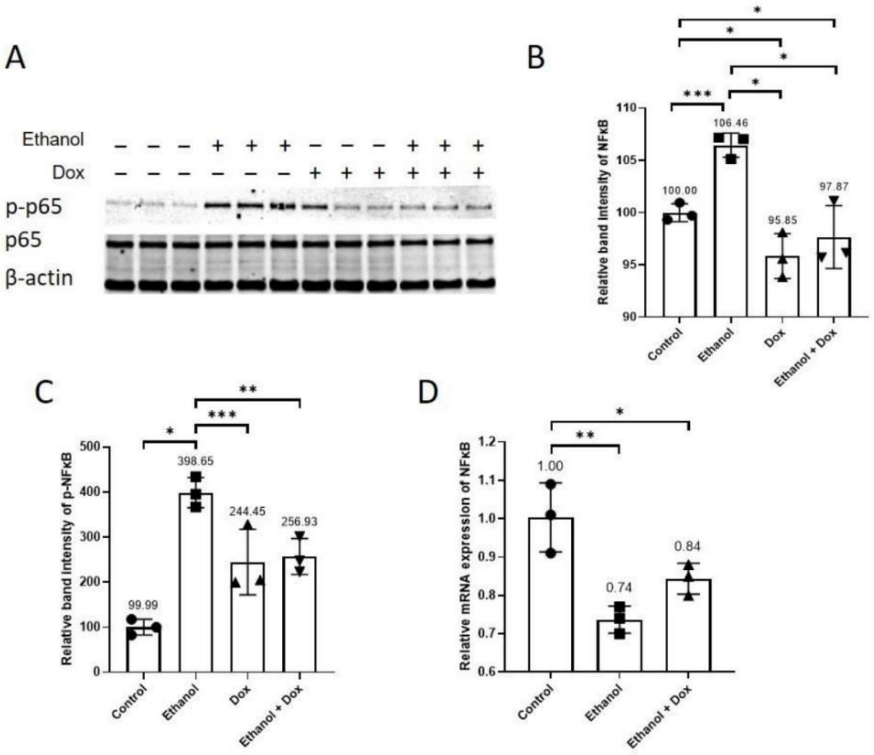


Figure 3. Doxycycline suppressed ethanol-induced NFκ-B activation in HUVECs. HUVECs were treated with ethanol (400 mM), doxycycline (10 μM), and combination of ethanol (400 mM) and doxycycline (10 μM) for 24 h. The endothelial cell medium alone was used for control. (**A**) WB showing the protein expression of p65 and p-p65. Doxycycline abated ethanol-induced relative protein expression of (**B**) p65 and (**C**) p-p65 in HUVECs. (**D**) Both ethanol (400 mM) and ethanol (400 mM) combined with doxycycline reduced mRNA expression of NF-κB. β-actin was used as a loading control. qPCR data are the mean of three technical triplicates, and WB data are the mean of three independent biological triplicates. One-way ANOVA followed by Tukey's test was used to analyze more than two groups. Error bars represent the SD (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

3.5. Doxycycline Recovered the Relative Protein Expression of Aging-Related Biomarkers in Ethanol-Treated HUVECs

Doxycycline restored the relative protein expression of DNA repair proteins KU70 (Control = 100 ± 10.12, ethanol = 80.38 ± 2.71, doxycycline = 91.53 ± 0.17, ethanol + doxycycline = 99.31 ± 9.67, n = 3, * $p < 0.05$, Figure 4A,B), and KU80 (Control = 100 ± 2.63, ethanol = 83.91 ± 4.28, doxycycline = 93.13 ± 1.22, ethanol + doxycycline = 94.92 ± 5.57, n = 3, ** $p < 0.01$, * $p < 0.05$, Figure 4A,C) in ethanol-treated HUVECs. The reduction in relative protein expression of aging marker lamin b1 after 24 h in ethanol-treated HUVECs was also reversed by doxycycline treatment (Control = 100 ± 7.95, ethanol = 58.93 ± 7.78, doxycycline = 84.27 ± 5.61, ethanol + doxycycline = 82.86 ± 3.56, n = 3, *** $p < 0.001$, ** $p < 0.01$ * $p < 0.05$, Figure 4A,D). Doxycycline did not reduce ethanol-induced relative mRNA expression of aging markers P16 (Control = 1.01 ± 0.14, ethanol = 2.77 ± 0.38, ethanol + doxycycline = 2.88 ± 0.10, n = 3, *** $p < 0.001$, Figure 4E) and P21 (Control = 1.01 ± 0.20, ethanol = 2.50 ± 0.38, ethanol + doxycycline = 2.51 ± 0.19, n = 3, ** $p < 0.01$, Figure 4F). Doxycycline increased the relative mRNA expression of growth arrest and DNA damage-inducible (GADD45) gene in HUVECs exposed to ethanol (Control = 1.00 ± 0.07, ethanol = 0.34 ± 0.09, ethanol + doxycycline = 0.59 ± 0.06, n = 3, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, Figure 4G). Doxycycline rescued the relative protein expression of aging-associated biomarker lamin b1, DNA repair proteins KU70 and KU80, and the relative mRNA expression of GADD45 in ethanol-treated HUVECs.

3.6. Doxycycline Attenuated Ethanol-Induced Cellular Senescence and Improved Functional Status of Endothelial Cells

It has already been known that increased inflammation and telomere shortening lead to cellular senescence [3,4,14]. To investigate if the already observed reduced inflammation and telomere recovery by doxycycline is translated into cellular senescence, β-gal staining was performed. Doxycycline inhibited ethanol-induced senescence in HUVECs, percentage of β-gal positive cells by total cells (Control = 16.66 ± 4.51%, ethanol = 65.06 ± 8.10%, ethanol + doxycycline = 18.20 ± 9.37%, n = 3, *** $p < 0.001$, Figure 5A,B).

As doxycycline inhibited ethanol-induced senescence in HUVECs, we further investigated if doxycycline also reduces SASP in HUVECs exposed to ethanol. The ethanol-treated endothelial cells showed increased relative mRNA expression of pro-inflammatory molecules IL-1β, IL-8, MCP-1, E-selectin, ICAM-1, and VCAM-1 (Figure 6A–F). Doxycycline did not affect the relative mRNA expression of IL-1β (Control = 1.15 ± 0.78, ethanol = 8.37 ± 2.07, ethanol + doxycycline = 10.41 ± 3.06, n = 3, ** $p < 0.01$, * $p < 0.05$ Figure 6A) and IL-8 (Control = 1.00 ± 0.07, ethanol = 1.79 ± 0.13, ethanol + doxycycline = 1.80 ± 0.11, n = 3, *** $p < 0.001$, Figure 6B) in ethanol-treated HUVECs. Doxycycline reduced the relative mRNA expression of MCP-1 (Control = 1.00 ± 0.08, ethanol = 2.90 ± 0.10, ethanol + doxycycline = 2.57 ± 0.11, n = 3, *** $p < 0.001$, * $p < 0.05$, Figure 6C), E-selectin (Control = 0.95 ± 0.05, ethanol = 7.54 ± 0.36, ethanol + doxycycline = 6.00 ± 0.71, n = 3, *** $p < 0.001$, * $p < 0.05$, Figure 6D), ICAM-1 (Control = 1.00 ± 0.13, ethanol = 8.52 ± 0.21, ethanol + doxycycline = 7.21 ± 0.38, n = 3, *** $p < 0.001$, ** $p < 0.01$, Figure 6E) and VCAM-1 (Control = 1.00 ± 0.08, ethanol = 1.95 ± 0.19, ethanol + doxycycline = 1.41 ± 0.15, n = 3, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ Figure 6F) in HUVECs treated with ethanol. The senescent cells are known to show impaired migration [24,25]; therefore, we investigated the effects of ethanol and ethanol combined with doxycycline on endothelial cell migration. Ethanol retarded the migration of HUVECs in wound assay, and doxycycline recovered the migration of HUVECs exposed to ethanol, percentage area covered by migrating endothelial cells at

different time points after making a scratch (at 0 h: Control = 0.00 ± 0.01%, ethanol = 0.00 ± 0.00%, ethanol + doxycycline = 0.00 ± 0.00%, after 6 h: Control = 36.27 ± 7.03%, ethanol = 24.20 ± 5.36%, ethanol + doxycycline = 33.00 ± 25.87%, after 12 h: Control = 78.18 ± 7.57%, ethanol = 50.83 ± 9.41%, ethanol + doxycycline = 79.65 ± 4.35%, after 24 h: Control = 99.77 ± 0.23%, ethanol = 82.30 ± 13.40%, ethanol + doxycycline = 96.56 ± 5.64%, n = 3, ** $p < 0.01$, Figure 6G,H). Doxycycline curtailed ethanol-induced endothelial SASP and dysfunction.

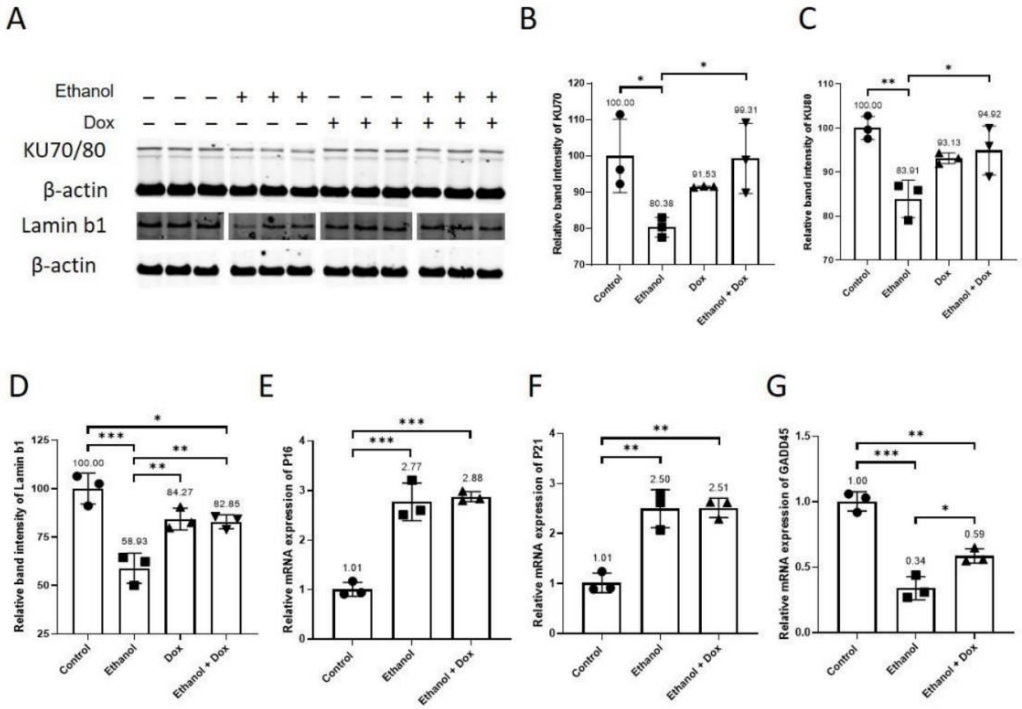


Figure 4. Doxycycline restored the relative protein expression of aging biomarkers. HUVECs were treated with ethanol (400 mM), doxycycline (10 μM), and combination of ethanol (400 mM) and doxycycline (10 μM) for 24 h. The endothelial cell medium alone was used for control. (**A**) WB showing protein expression of aging-associated biomarker lamin b1 and DNA repair proteins KU70 and KU80. Doxycycline recovered the relative protein expression of (**B**) KU70, (**C**) KU80 and (**D**) lamin b1 in ethanol-treated HUVECs. Doxycycline did not affect the ethanol-induced relative mRNA expression of aging markers (**E**) p16 and (**F**) p21. (**G**) Doxycycline ameliorated the relative mRNA expression of GADD45 in ethanol-treated HUVECs. β-actin was used as a loading control. qPCR data are the mean of three technical triplicates, and WB data are the mean of three independent biological triplicates. One-way ANOVA followed by Tukey's test was used to analyze more than two groups. Error bars represent the SD (*** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$).

The senescent cells release MMPs [8,15], which are known to play an important role in vascular remodeling. Therefore, we investigated the effects of ethanol alone and the combined effect of ethanol and doxycycline on the mRNA expression of MMPs and their inhibitors, namely tissue inhibitor matrix metalloproteinase (TIMP)-1 and TIMP-2. Ethanol increased the relative mRNA expression of MMP-1, MMP-2, MMP-8, MMP-10, MMP-11, and TIMP-2 (Figure 7A–E,G). Doxycycline did not affect the ethanol-induced relative mRNA expression of MMP-1 (Control = 1.01 ± 0.12, ethanol = 1.63 ± 0.15, ethanol + doxycycline = 1.73 ± 0.33, n = 3, * $p < 0.05$, Figure 7A), MMP8 (Control = 1.05 ± 0.35, ethanol = 2.93 ± 0.41, ethanol + doxycycline = 3.96 ± 0.97, n = 3, ** $p < 0.01$, * $p < 0.05$ Figure 7C), MMP10 (Control = 1.00 ± 0.08, ethanol = 1.37 ± 0.12, ethanol + doxycycline = 1.59 ± 0.06, n = 3, *** $p < 0.001$, ** $p < 0.01$ Figure 7D), MMP-11 (Control = 1.00 ± 0.11, ethanol = 2.41 ± 0.35, ethanol + doxycycline = 2.72 ± 0.24, n = 3, *** $p < 0.001$, ** $p < 0.01$ Figure 7E). Doxycycline increased the relative mRNA expression of TIMP-1 (Control = 1.00 ± 0.10, ethanol = 1.12 ± 0.08, ethanol + doxycycline = 1.63 ± 0.09, n = 3, *** $p < 0.001$, Figure 7F), TIMP2 (Control = 1.00 ± 0.07, ethanol = 1.59 ± 0.02, ethanol + doxycycline = 2.01 ± 0.23, n = 3, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, Figure 7G), and procollagenase 3 (pc3) (Control = 1.01 ± 0.12, ethanol = 2.17 ± 0.49, ethanol + doxycycline = 2.83 ± 0.77, n = 3, * $p < 0.01$, Figure 7H) in ethanol-treated HUVECs. Doxycycline reduced the ethanol-induced relative mRNA expression of MMP-2 (Control = 1.00 ± 0.04, ethanol = 1.99 ± 0.24, ethanol + doxycycline = 1.39 ± 0.02, n = 3, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ Figure 7B). The protein analysis showed that doxycycline also impeded the relative protein expression of MMP2 (Control = 100 ± 2.43, ethanol = 151.32 ± 4.32, doxycycline = 100.8 ± 14.76, ethanol + doxycycline = 95.94 ± 21.17, n = 3, ** $p < 0.01$, Figure 7I,J) in ethanol-treated HUVECs. Doxycycline inhibited ethanol-induced relative mRNA and relative protein expression of MMP2 in HUVECs.

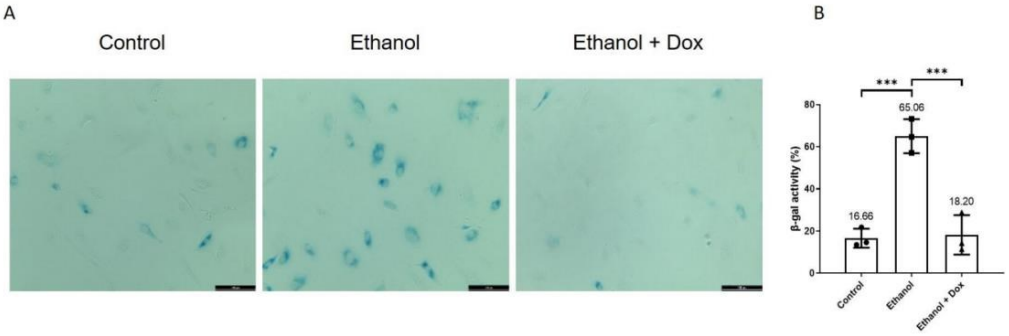


Figure 5. Doxycycline inhibited ethanol-induced cellular senescence in HUVECs. HUVECs were treated with ethanol (400 mM) and combination of ethanol (400 mM) and doxycycline (10 μM) for 4 days. The endothelial cell medium alone was used for control. The medium was changed after two days. (**A**) Images showing increased senescence only in ethanol-treated HUVECs. Scale bar = 100 μm. (**B**) Doxycycline inhibited senescence in HUVECs exposed to ethanol treatment. Data are the mean of three independent experiments. One-way ANOVA followed by Tukey's test was used to analyze more than two groups. Error bars represent the SD (*** $p < 0.001$).

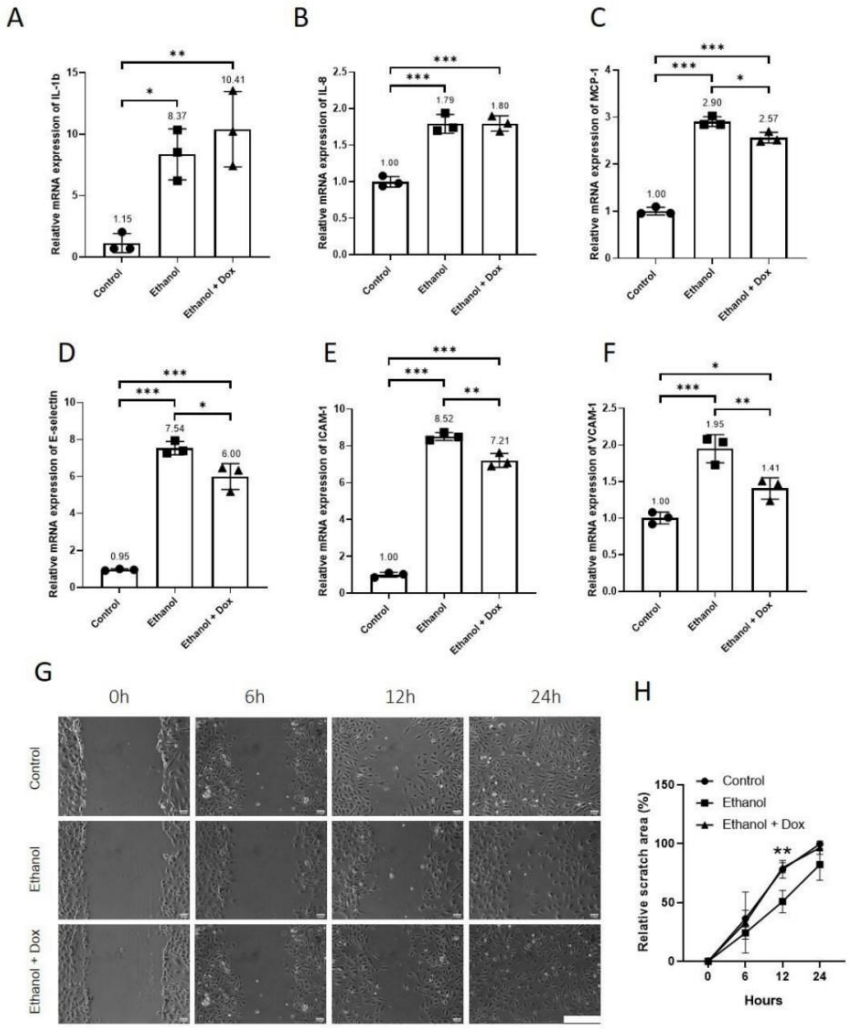


Figure 6. Doxycycline reduced endothelial SASP and dysfunction in ethanol-treated HUVECs. HUVECs were treated with ethanol (400 mM), doxycycline (10 μM), and combination of ethanol (400 mM) and doxycycline (10 μM) for 24 h. The endothelial cell medium alone was used for control. (**A–F**) Ethanol treatment increased the relative mRNA expression of L-1β, IL-8, MCP-1, E-selectin ICAM-1, and VCAM-1 after 24 h in HUVECs. Doxycycline did not alter the relative mRNA expression of (**A**) IL-1β and (**B**) IL-8, but it abated the relative mRNA expression of (**C**) MCP-1, (**D**) E-selectin, (**E**) ICAM-1, and (**F**) VCAM-1 after 24 h in ethanol-treated HUVECs. β-actin was used as a housekeeping gene. (**G**) Images showing HUVECs migration under different conditions at different time points after making a scratch using 10 μL pipette tip. Scale bar = 300 μm. (**H**) Quantification analysis showed that doxycycline ameliorated the migration of ethanol-treated HUVECs. qPCR data are the mean of three technical triplicates, and WB data are the mean of three independent biological triplicates. One-way ANOVA followed by Tukey's test was used to analyze more than two groups. Error bars represent the SD (*** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ compared to controls).

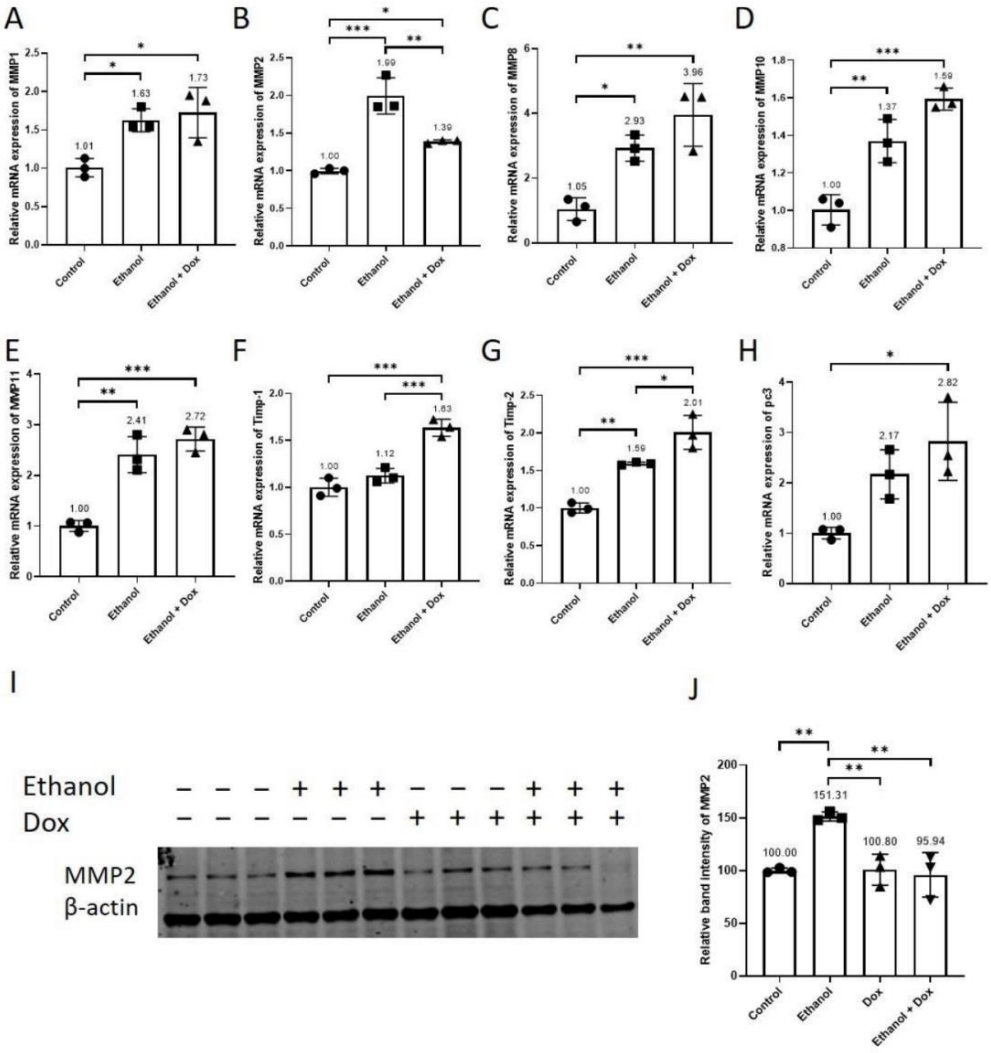


Figure 7. Doxycycline restrained MMP-2 relative mRNA and relative protein expression in ethanol-treated HUVECs. HUVECs were treated with ethanol (400 mM), doxycycline (10 μM), and combination of ethanol (400 mM) and doxycycline (10 μM) for 24 h. The endothelial cell medium alone was used for control. (**A–E**,**G**) Ethanol increased the relative mRNA expression of MMP-1, 2, 8, 10, 11, and Timp2 in HUVECs. (**F–H**) Doxycycline elevated TIMP-1, TIMP-2, and pc3 relative mRNA expression in HUVECs treated with ethanol. (**B**,**I**,**J**) Doxycycline suppressed ethanol-induced relative mRNA and relative protein expression of MMP2 in HUVECs. β-actin was used as a loading control. qPCR data are the mean of three technical triplicates, and WB data are the mean of three independent biological triplicates. One-way ANOVA followed by Tukey's test was used to analyze more than two groups. Error bars represent the SD (*** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$).

4. Discussion

In this study, we report that ethanol accelerated molecular aging caused cellular senescence and induced endothelial SASP and dysfunction. Doxycycline dampened ethanol-mediated inflammation and aging in endothelial cells and attenuated cellular dysfunction. Alcohol abuse is one of the potential risk factors associated with cardiovascular diseases. Studies suggest alcohol abuse causes aging [10,11], but the evidence is lacking showing the direct effect of ethanol on cellular aging. In this study, we investigated the effects of ethanol on cellular aging and found that doxycycline is a potential drug to reduce ethanol-induced accelerated aging and cellular senescence in endothelial cells.

We treated HUVECs with two different concentrations of ethanol. Ethanol accelerated aging in HUVECs (Figure 1). The telomere length in ethanol-treated HUVECs was shortened by more than 50% as compared to that in untreated controls (Figure 1A). Constitutive telomere shortening is known to trigger cellular senescence, induce apoptosis and reduce the proliferative capacity of cells [3]. Telomere loss is associated with the pathogenesis of cardiovascular diseases [3,9]. Telomere shortening is linked to vascular aging, and the factors that inhibited telomere shortening also retarded vascular aging [3]. The concentration of doxycycline (10 μM), which alone had no cytotoxic effects and inhibited the cytotoxic effects of ethanol (400 mM) (Section 3.2, Supplementary Figure S1), was used in the experiments. Doxycycline reduced ethanol-accelerated aging in HUVECs (Figure 2A) and inhibited ethanol-induced activation of mTOR (Figure 2B,C). Ethanol activated the mTOR pathway in vivo and in vitro [26,27], and mTOR has been implicated in aging [7,18,19]. Similar to rapamycin inhibition of the mTOR pathway in most cell types [28,29], in our study, doxycycline decreased p-S6 relative protein expression, and it had no effect on 4e-BP1 in ethanol-treated HUVECs (Figure 2B,D,E). Previous studies have shown that rapamycin inhibition of mTOR and depletion of p-S6 delayed the pathogenesis of age-related diseases and increased life span in different animal models [2,7,18,19,30]. Rapamycin inhibition of mTOR reversed age-associated arterial dysfunction and decreased vascular stiffness [2]. In addition to this, mTOR activation has been shown to promote inflammaging via activating NF-kB [6]. Additionally, ethanol has been reported to activate NF-kB in vivo and in vitro [21–23]. It is noteworthy that both ethanol and doxycycline reduced mRNA expression of NF-kB, which suggests that ethanol regulates NF-kB differently at transcriptional and posttranslational levels. NF-kB activation has also been implicated in aging [14–17]. NF-kB activation has been shown to regulate DNA repair protein KU80 [16]. Interestingly, the inhibition of NF-kB delayed and even reversed aging in animal models [14,17]. Our data demonstrate that doxycycline treatment inhibited NF-kB activation (Figure 3A,B), confirming the findings reported previously [12,13]. The constitutive NF-kB activation induced telomere shortening via KU80 dysregulation and increased DNA damage on telomeres [16]. KU80 forms a heterodimer with KU70. KU80/KU70 heterodimer binds to broken DNA and initiates its repair via non-homologous end joining [31,32]. In our study, ethanol decreased the relative protein expression of DNA repair proteins KU70 and KU80, which were recovered after doxycycline treatment. (Figure 4A,C). Animal experiments have shown that the deletion of KU80 and KU70 leads to accelerated aging [33,34]. In a mice study, complete loss of KU80 resulted in premature aging, and loss of a single allele caused accelerated aging in skeletal muscle in mice [35]. The lack of KU70 or KU80 activity resulted in telomere shortening in different mouse cell types [36]. KU80 is essential for human somatic cells [37], and loss of KU80 causes cell death due to massive telomere loss [38]. A decrease in KU70 and KU80 proteins has been reported in senescent cells [39]. The reduced KU70 and KU80 after ethanol treatment can lead to telomere loss and accumulation of DNA damage [36,38]. Moreover, lamin b1 was lost in human and murine cells when DNA damage induced cellular senescence [40]. Doxycycline reduced ethanol-induced cellular senescence and increased the protein expression of lamin b1 (Figures 4D and 5). Loss or reduction in lamin b1 has been reported to increase mTOR activation [41]. Moreover, inhibition of mTOR and its downstream signalling molecule p-S6 reduced cellular senescence [19]. Furthermore, p-mTOR negatively regulates autophagy, which results in the

accumulation of damaged proteins and organelles that consequently accelerates the progression of cellular senescence [18,19]. Inhibition of p-mTOR increases autophagy, which protects from proteotoxicity and thus delays cellular senescence [18,19]. In addition to this, p-mTOR inhibition improves mitochondrial function and decreases ROS levels [19], which can provide protection against oxidative stress-induced premature cellular senescence and telomere shortening. Moreover, doxycycline reduced ethanol-induced oxidative stress (Supplementary Figure S2), which suggests doxycycline can provide protection through multiple mechanisms. Multiple studies suggest that telomere shortening and endothelial senescence contribute to vascular aging, and this phenomenon has been implicated in heart failure [2,3]. Senescent cells induced senescence in young cells via a process termed as bystander effect [42]. Senescent cells contribute to age-related diseases by damaging the local environment and promoting tissue remodelling [1], suggesting that doxycycline, by eliminating senescent cells and reducing or inhibiting cellular senescence, can potentially provide protection against age-related vascular diseases [1,5].

Doxycycline did not affect the relative mRNA expression of aging markers P16 and P21 (Figure 4E,F), but it increased the relative mRNA expression of GADD45 (Figure 4G). GADD45 is implicated in stress signalling response, which can lead to cell cycle arrest, DNA repair, cell survival and senescence, or apoptosis, depending on the extent of cellular and DNA damage [43]. NF-kB activation has been shown to promote cellular senescence and aging [6,15,44,45]. Doxycycline inhibited NF-kB activation (Figure 3A,B) [12,13], and inhibition of NF-kB activation delayed cellular senescence and Aging [14,17], which suggests that inflammation can induce premature senescence and aging in endothelial cells [3]. Reciprocally, senescence induces inflammation as senescent cells become more pro-inflammatory and acquire SASP contributing to an amplifying effect [2,5,14,15]. These cells secrete cytokines such as IL-1β, chemokines such as IL-8 and MCP-1, and increase the expression of cell adhesion molecules, namely ICAM-1, VCAM-1 and E-selectin [3,6]. This pro-inflammatory response in senescent and dysfunctional endothelial cells is related to NFκ-B activation [2,8], and inhibition of NFκ-B activation reduces systemic inflammation and improved vasculature [2]. Doxycycline did not affect the mRNA expression of IL-1β and IL-8 (Figure 6A, B), but it inhibited NFκ-B activation (Figure 3A,B) and reduced the mRNA expression of MCP-1, E-selectin, ICAM-1 and VCAM-1 (Figure 6C–F). This direct effect of ethanol on the transcription of cell adhesion molecules in cell systems has not been reported before, although there are reports which suggest chronic consumption of alcohol increased the serum level of E-selectin and ICAM-1 [46]. These molecules promote migration, adhesion and infiltration of leukocytes to vascular endothelium contributing to chronic sterile inflammation [3]. These findings indicate that doxycycline can directly curtail inflammation via suppressing NFκ-B activation, and by reducing the transcription of MCP-1, E-selectin, ICAM-1, and VCAM-1, doxycycline can indirectly reduce sterile inflammation by decreasing the migration and infiltration of inflammatory cells to the arterial wall.

The migration of endothelial cells is an important process for angiogenesis and repair/healing of damaged tissue. Previous studies have reported that the senescent cells show retarded migration and are not efficient in the repair/healing of damaged tissue [24,25]. Ethanol treatment reduced the migration of HUVECs, and doxycycline significantly recovered the migration of ethanol-treated HUVECs (Figure 6G,H). In the literature, contradictory findings have been reported on the effects of ethanol on cell migration. Wei et al. reported that ethanol promoted the migration of cancerous cells but had no effect on normal cells [47]. Morrow et al. reported that ethanol in low concentration promoted endothelial cell migration [48]. In the same study, they showed that by increasing ethanol concentration, the angiogenic activity in HUVECs decreased [48]. In animal study, ethanol has been shown to impair angiogenesis and wound healing [49]. Doxycycline at higher concentrations (20 μg/mL, 100 μg/mL, and 500 μg/mL) has previously been shown to decrease the migration of epithelial cells and human dermal vascular endothelial cells [50]. The same study showed that doxycycline had a concentration-dependent effect

on the migration of these cells [50]. The current findings, together with previous reports, suggest that doxycycline in lower concentrations can promote tissue healing and repair via enhancing endothelial cell migration.

The senescent cells have been shown to release MMPs [8,15], and alcohol consumption has been reported to increase serum levels of MMPs in alcohol abusers [51]. Similar to humans, in animal studies, alcohol elevated MMPs expression in different tissues [52–55]. In accordance with the previous findings, ethanol increased the relative mRNA expression of different MMPs and the relative protein expression of MMP2 in HUVECs (Figure 7). Doxycycline reduced ethanol-induced relative mRNA and relative protein expression of MMP2 in HUVECs (Figure 7B,I,J). Previously, doxycycline has been shown to curtail MMP2 mRNA expression by reducing its stability and consequently decreasing the protein expression of MMP2 in aortic SMCs [56]. Doxycycline has been reported to impede MMP2 activity [57]. We show that doxycycline increased the relative mRNA expression of TIMP-1 and TIMP-2 in ethanol-treated HUVECs as compared to only ethanol-treated and untreated controls (Figure 7F,G). TIMPs are the most potent inhibitors of MMPs. MMPs are known to play an important role in cardiovascular diseases through different mechanisms, including ECM remodeling, promoting VSMCs migration by cleaving cadherin, and increasing vasoconstriction via cleaving vasoactive precursors such as endothelin-1 and adrenomedullin [8]. MMPs also contribute to inflammation [58]. MMP2 and MMP9 activated IL-1β by cleaving its precursor pro-IL-1β [59]. MMPs had both positive and negative effects on leukocyte recruitment and infiltration via cleaving chemokines [58]. Taken together, these findings suggest that doxycycline treatment can attenuate MMP-2-mediated inflammation and reduce tissue remodeling by directly inhibiting MMP-2 protein expression and indirectly by increasing the expression of its inhibitor TIMP-2.

5. Conclusions

Telomere shortening is associated with cardiovascular diseases, but this association does not state that telomere shortening has a causal link to cardiovascular diseases. It is possible that the risk factors that lead to cardiovascular diseases also caused telomere shortening, and the factors that reduced vascular aging also inhibited telomere shortening. Alcohol is a potential risk factor for cardiovascular diseases, and in the present study, we showed that ethanol induced telomere shortening and premature senescence. Doxycycline reduced ethanol-induced inflammaging in HUVECs, possibly by inhibiting activation of the mTOR and NF-kB pathways.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox11122413/s1, Figure S1: CTG assay showing the growth of HUVECs under different conditions. The cells were treated with different concentrations of ethanol (200 mM, 300 mM, 400 mM, 600 mM, and 800 mM,), doxycycline (1 μM, 10 μM, 50 μM, and 100 μM) and combined ethanol (400 mM) and doxycycline (10 μM). The medium was changed after two days. The CTG assay was performed at day 0, day 1, day 2, and day 4.; Figure S2: Immunofluorescence staining for oxidative stress marker 8-OHDG. The cells were treated with ethanol (400 mM), doxycycline (10 μM), and combination of ethanol (400 mM) and doxycycline (10 μM) for two hours. Endothelial medium alone was used for control. (**A**,**B**) Only ethanol-treated HUVECs showed increased oxidative stress. Scale bar = 100 μM, *** $p < 0.001$.; Table S1: Primary and Secondary antibodies.; Table S2: Primer list.

Author Contributions: Conceptualization, D.K. and S.M.; Investigation, X.L. and D.K.; Methodology, X.L. and D.K.; Project administration, D.K. and S.M.; Resources, S.M.; Writing—original draft, X.L. and D.K.; Writing—review and editing, M.R., D.H. and S.M. All authors have read and agreed to the published version of the manuscript.

Funding: Research commission grant of Heinrich Heine University Düsseldorf 2020 to SM. EANS research funds for vascular category 2019 and 2020 to S.M. Peek and Cloppenburg Stifftung 2021 to D.H. and S.M.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Data is contained within the article and Supplementary Material.

Conflicts of Interest: The authors declare no conflict of interest.

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3. Discussion

Our main findings describe the cellular and molecular mechanisms by which doxycycline ameliorates the deleterious signals initiated by alcohol, and mTOR/NFκ-B are the key molecular signaling targets to ameliorate inflammation in endothelial cells and EC-mediated diseases. In this study, we reported that ethanol-accelerated molecular aging contributed to cellular senescence and induced the endothelial senescence-associated secretory phenotype (SASP) and dysfunction. Doxycycline ameliorated ethanol-mediated endothelial cell inflammation and senescence and attenuated cellular dysfunction. The relationship between alcohol consumption and vascular diseases (e.g., hypertension, coronary heart diseases, stroke, peripheral arterial diseases, and cardiomyopathy) is well established [94]. This thesis aims to present the role of the mTOR/NFκ-B pathway in alcohol-induced inflammation and aging and doxycycline as a potential therapeutic pharmacological target for ethanol-induced vascular disease therapies.

In response to molecular damage, a toxic environment and reaching Hayflick's limit, cells enter a stage of senescence and stop replicating. As a natural defense mechanism, it is effective in the short term, but long-term cellular senescence disrupts tissue function and structure, in addition to creating chronic inflammation with all its attendant problems [95]. Lamin b1 loss is associated with multiple types of cellular senescence [96] and serves as a biomarker of senescence both in vitro and in vivo [97, 98]. Our protein analysis suggested alcohol-induced cellular senescence via decreased lamin b1 (Figure 1B). Moreover, upregulated lamin b1 maintains functional telomeres and thereby preserves genome integrity [99]. Our data indicate that doxycycline recovered alcohol-induced lamin b1 loss and delayed pathological senescence (Figure 4A, D and 5). Additionally, P21 and P16 are the first discovered aging markers during cellular senescence [100, 101] and were highly expressed when lamin b1 was knocked out at the protein level in the A549 cell line [102]. Our mRNA analysis demonstrated that ethanol treatment decreased GADD45 relative mRNA expression and increased the relative mRNA expression of P16 and P21. Doxycycline did not affect P16 and P21 relative mRNA expression, but it recovered GADD45 mRNA expression (Figure 4E, F, G). GADD45 is implicated in stress signaling responses, which may trigger multiple cellular programs, including cell cycle arrest, DNA repair, cell survival, and senescence or apoptosis [103]. Telomere loss is suggested to contribute to the pathogenesis of vascular diseases [104]. In our study, telomere length was reduced by more than 50% in ethanol-treated ECs compared to untreated controls (Figure 1 A). Moreover, our protein analysis illustrated that alcohol accelerated KU70/80 loss and

consequently damaged this DNA repair system, advanced telomere length shortening in HUVECs (Figure 1B, D, E), and doxycycline reduced KU70/80 loss and contributed to DNA repair and telomere maintenance (Figure 2A and 4A, B, C). The Ku70/80 heterodimer is a key molecule in the nonhomologous end joining (NHEJ) pathway and binds DNA ends with high affinity, protecting DNA ends from degradation and recruiting other NHEJ factors required for DNA repair [105]. Ku70/80 also contributes to DNA double-strand break repair and telomere length maintenance [106].

The mTOR pathway contributes to aging and longevity. Our results suggested that alcohol activated the mTOR pathway and p-S6 and inhibited p-4EBP1 (Figure 4B, C, D, E, F, G). Previous studies have reported that mTOR and its downstream signaling molecule S6 are activated and that its downstream signaling molecule 4EBP1 is inhibited in senescence [22-24]. Doxycycline inhibited the activation of mTOR and its downstream signaling molecule S6, but it did not affect 4EBP1 in ethanol-treated HUVECs (Figure 4B, C, D, E, F, G). It has been shown that abdominal aortic aneurysm formation is suppressed through mTOR inhibition [107], which suggests the critical role of mTOR in vascular diseases. Interestingly, mTOR modulates the DNA damage response and inflammation via the NF-κB-mediated pathway [108, 109]. Since Walker *et al.* first used the term inflammatory aneurysm in 1972 [110], inflammation has been a necessary factor in aneurysm research. The recruitment of macrophages, monocytes, and T lymphocytes has been found in aneurysmal walls [111, 112]. These inflammation-related cells can secrete collagenase, elastase, and gelatinase, similar to endothelial cells, to destabilize the intercellular matrix and vascular structure [113, 114]. NF-κB, as the most studied and classic inflammatory pathway, has been widely demonstrated to play a critical role in inflammatory activation [115]. Our study showed that alcohol activates the NFκ-B pathway and induces the release of inflammatory cytokines, which were attenuated by doxycycline treatment (Figure 5A, B, C, D). Moreover, activation or inhibition of NFκ-B induces or reverses, respectively, major features of aging organisms, and it has been considered a key transcription factor for aging drivers [116]. When the inflammatory process is activated, the NFκ-B dimer exposes the nuclear localization sequence (NLS), which quickly enters the nucleus from the cytoplasm and binds to specific sequences on nuclear DNA to promote the transcription of inflammatory cytokines such as IL-1β, chemokines such as MPC-1 and IL8, and cell adhesion molecules such as ICAM-1, VCAM-1, and E-selectin [117, 118]. Our study showed that doxycycline did not affect the mRNA expression of IL-1β and IL-8 but reduced MCP-1, ICAM-1, VCAM-1, and E-selectin mRNA expression in ethanol-treated HUVECs (Figure 6A, B, C, D, E, F). In response to inflammation in aneurysm pathology, MCP-1 upregulation by inducing the

migration and infiltration of monocytes and macrophages promotes their recruitment from the bloodstream to the vascular endothelium [119]. By decreasing macrophage recruitment, MCP-1 inhibition delayed the initiation of the primary stage of atherosclerosis [57], and blocking MCP-1 activity resulted in the inhibition of cranial aneurysm formation [120]. Increased MCP-1, IL8, and IL1β attract inflammatory cells such as neutrophils, basophils, and T cells during the inflammatory process [121], and cell adhesion molecules such as ICAM-1, VCAM-1, and E-selectin facilitate the recruitment of these cells to the endothelium [122]. The accumulation of these inflammatory cells can lead to endothelial cell dysfunction and the activation of atherosclerosis, thereby promoting the development of vascular diseases [123]. These findings suggest that doxycycline can directly inhibit inflammation by inhibiting NFκ-B activation, and by reducing the transcription of MCP-1, E-selectin, ICAM-1, and VCAM-1, doxycycline can indirectly reduce sterile inflammation by reducing the migration and infiltration of inflammatory cells into the arterial wall.

The migratory program of endothelial cells is a hallmark of angiogenesis and repair of damaged vessels [124]. Previous studies have reported that biological age-dependent cellular functional decline contributes to the dynamic features of migrating cell decrease [125]. In our study, ethanol treatment reduced the migration of HUVECs, and doxycycline significantly restored the migration of ethanol-treated HUVECs (Figure 6G, H). Conflicting findings on the effect of ethanol on cell migration have been reported. Morrow *et al.* reported that ethanol accelerated endothelial cell migration at low concentrations but decreased angiogenic activity at low concentrations [126]. It has also been reported that ethanol inhibits angiogenesis and wound healing in vivo [127]. At high concentrations, doxycycline inhibits cell migration, but at low concentrations, doxycycline accelerates cell migration in epithelial cells and human dermal vascular endothelial cells, so doxycycline has a concentration-dependent effect on cell migration [128]. The current findings, in conjunction with previous reports, suggest that lower concentrations of doxycycline can promote tissue healing and repair by enhancing endothelial cell migration.

MMPs are a family of proteins that degrade extracellular matrix proteins to destabilize the vascular structure [129] and participate in the entire aneurysm pathological process via angiogenesis, embryogenesis, morphogenesis, and wound repair [67]. Dysfunctional and senescent endothelial cells increase the expression of MMPs and release them [67]. In our study, ethanol increased the expression of MMPs in endothelial cells (Figure 7). All of the results confirmed that alcohol-induced MMP2 overexpression was reduced by doxycycline at both the mRNA and protein levels (Figure 7B, I, J). Moreover, MMP2 can increase leukocyte infiltration

by cleaving chemokines [130]. Taken together, doxycycline reduces vascular remodeling and inflammation by inhibiting MMP2 [130]. Our study also suggested that doxycycline increased the mRNA expression of Timp1 and Timp2 in ethanol-treated HUVECs compared to untreated controls (Figure 7F, G). Timp1 and Timp2 are both potent inhibitors of MMPs. MMPs are known to play important roles in vascular diseases through different mechanisms, including extracellular matrix remodeling and promotion of vascular smooth muscle cell migration by cleavage of cadherin and increased vasoconstriction by cleavage of vasoactive precursors such as endothelin-1 and adrenomedullin [131]. MMPs also play a critical role in the inflammatory process [132]. MMPs have opposite effects on leukocyte recruitment and infiltration mediated by cleaving chemokines [132]. More importantly, the activation of IL-1β can be mediated by MMP2 and MMP9 by cleaving its precursor pro-IL-1β [133]. These findings suggest that doxycycline treatment can attenuate MMP2-mediated inflammation and reduce tissue remodeling in HUVECs.

Thus, our study demonstrates that most of the indicators that influence vascular diseases caused by ethanol have been strongly recovered by doxycycline treatment. Here, we have shown that ethanol induced accelerated aging, senescence and SASP in HUVECs. Doxycycline ameliorated the effects caused by ethanol through mTOR/NFκ-B axis inhibition.

As a limitation, the present study focuses on the role of the mTOR/NFκ-B axis in inflammaging in HUVEC models. In vivo studies are needed to confirm the findings of the present study.

In summary, doxycycline recovered alcohol-mediated endothelial dysfunction, inflammation, cellular senescence, impact of DNA repair, and MMP overexpression [134]. Doxycycline could be a potential pharmaceutical target for alcohol-induced cardiovascular diseases regulated by the mTOR/NFκ-B axis.

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5. Appendix

5.1 Declaration of author contributions

Title: Doxycycline attenuated ethanol-induced inflammaging in endothelial cells: Implications in alcohol-mediated vascular diseases

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5.2 List of Figures

Figure 1. Ethanol accelerated molecular aging in endothelial cells.

.....20

Figure 2. Doxycycline decreased ethanol-accelerated aging and inhibited ethanol-induced mTOR activation in HUVECs

.....23

Figure 3. Doxycycline suppressed ethanol-induced NFκ-B activation in HUVECs.

.....24

Figure 4. Doxycycline restored the relative protein expression of aging biomarkers.

.....26

Figure 5. Doxycycline inhibited ethanol-induced cellular senescence in HUVECs.

.....27

Figure 6. Doxycycline reduced endothelial SASP and dysfunction in ethanol-treated HUVECs

.....28

Figure 7. Doxycycline restrained MMP-2 relative mRNA and relative protein expression in ethanol-treated HUVECs

.....29

5.3 Acknowledgments

I would like to express my sincere appreciation and gratitude to my supervisor PD Dr. med. Sajjad Muhammad for his continuous support, motivation and guidance throughout my time at the neurosurgery lab. I am grateful for the time and energy he put into supervising me through this entire process and especially for giving me the opportunity to perform part of the research in the Neurology Lab. I would like to thank Prof. Hänggi for the chance to perform my PhD thesis at the department of neurosurgery. My sincere thanks to Michael Gliem, Zippora Kohne and Goran Pavic from the Department of Neurology for immunohistochemistry support. My thanks to Carsten Hagenbeck from the Department of Gynecology and Obstetrics and Norbert Gerdes from the Department of Cardiology, Pulmonology, and Vascular Medicine for cooperation in providing the endothelial cells. In addition, my special gratitude goes to my second supervisor Majeed Rana for scientific guidance and support. A special thanks to Dilaware Khan for all the help and the great time in Düsseldorf. This work would not have been possible without them! Many thanks to all my former and current lab colleagues Ulf Khlert, Conny Uhlmann, Suad Yusuf, Philippe Aretz, Ann-Christin Nickel, Michael Hewera, Serdar Alpdogan, and Huakang Zhou for the great atmosphere working together every day and the nice time supporting each other and making lots of jokes. Finally, I would like to thank my family and friends for their never-ending love and support to achieve my goals. Thank you all.