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Abbreviation

5-LO.	Arachidonat-5-Lipoxygenase
AMPK.	adenosine 5′ monophosphate-activated protein kinase
ATM	kinases ataxia telangiectasia mutated
BHT	butylated hydroxytoluene
CDKN2A.	cyclin-dependent kinase inhibitor 2A
CDKN2a/b	cyclin-dependent kinase inhibitor 2A/B
CVD	cardiovascular diseases
DDR	DNA damage response
DNA	Deoxyribose nucleic acid
DPI	diphenyleneiodonium
E2F	transcription factors
EC	endothelial cells
ECM	extracellular matrix
EDHF	endothel-derived hyperpolarizing factor
eNOS.	Endothelial nitric oxide synthetase
FIS	cell–cell fusion induced senescence
H_2O_2	hydrogen peroxide
HUVECs.	Human umbilical veinn endothelial cells
ICAM-1.	Intracellular adhesion molecules-1
IL	interleukin
iNOS	inducible mitric oxide synthetase
kB.	Kappa B
miRNAs	mitochondria Ribonukleinsäure
Mn-SOD	superoxide dismutase
MPO	Myeloperoxidase
mPGES	Microsomal prostaglandin E synthase
mTOR.	mammalian Target of Rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase
NF-κB	nuclear factor kB
NO	nitric oxide
NOS	nitric oxide synthase ()
Nrf2	nuclear factor erythroid 2-related factor 2
O_2^-	superoxide
OIS	oncogene-induced senescence
$ONOO^-$	peroxynitrite
P5.	Passage 5
PA.	perlatolic acid
PFA	paraformaldehyde
PKC	Proteinkinase C
RB1	retinoblastoma
RONS	reactive nitrogen pecies
ROS.	reactive oxygen species
SASP	senescence associated seretory
SIRT-1.	Sirtuin-1
TIS	Therapy-induced senescence
TNF	Tumor necrosis factor
UV	Ultraviolet
µM.	Micromolar

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Abstract

Background

Many studies have reported the relation between the oxidative stress and aging and how is this theory very important at the moment to understand aging and aging related diseases.
All the aerobic cells produce reactive oxygen (ROS) and play a major role both in normal cells, in aging and aging related diseases. Heart disease, stroke and hypertension are all known to be partly caused by arterial disease. ROS production is involved not only in determining harmful effects, but also in the extraction of energy from organic molecules, immune response, and signaling. In addition to elevated oxidative stress, inflammation is a decisive mechanism for vascular ageing. In fact, the function of these factors cannot be understood separately because several interactions occur between inflammation and oxidative stress and vice versa, as different redox-sensitive transcriptional factors such as NF-κB is activated by ROS, enhancing the expression of cytokines, adhesion molecules, and proinflammatory enzymes.

Aims

The free radical theory of aging asserts that organisms over time generate high level of free radicals, which accumulate and cause DNA damage and many age-related diseases. Our purpose of this study was to establish a model of chronic oxidative stress to induce cellular senescence in endothelial cells and to use it to test the preventive/reverting capacity of three anti-oxidative/anti-inflammatory compounds BHT, PA, DPI. As readouts we used cell number, ROS and NO production, expression of ICAM-1, proliferation marker Ki67 and DNA damage marker γ-H2AX.

Materials and method

H_2O_2 (50μM) was added to HUVEC cultures for an hour every 48 hours until day 9, followed by replacing the medium EC growth medium with or without our reagent (BHT, PA, DPI). One day after exposure the Cells were fixed and counted upon DAPI staining and the ROS-and NO-production as well as the expression of ICAM-1, Ki67 and γ-H2AX were measured. ROS was measured in HUVECs by using the fluorescent H_2-DCF (2',7' dichlorofluorescein). Detection of ICAM-1 expression was measured by cell ELISA. To assess the production of NO, the

supernatant was deproteinized by ethanol followed by centrifugation later supernatant infused with Griess reagents (sulfonamide and NED) and its absorbance measured was at 540nm after. Ki67 and γ-H2AX foci were detected by immunocytochemistry.

Result

Our data shows that exposure of HUVEC to H_2O_2 led to increased expression of the DNA damage marker γ-H2AX, reduced culture growth as well as to a lower portion of $Ki67^+$ cells. The elevate of ROS and NO production and ICAM-1 expression as one of SASP protein are strong hints of occurring oxidative stress and inflammation also, taken together all the analyzed parameters would be compatible with the interpretation that our cells indeed after this treatment regimen become senescent. The assessment of the preventive effects of BHT and PA, were shown by increasing in $Ki67^+$cells and decreasing in DNA damage marker γ-H2AX.
The ROS production and ICAM-1 expression were decreased after our treatment with BHT and PA while DPI caused increase in ROS production and ICAM-1 expression, which refer to the toxic effect of DPI in this concentration. Also, BHT and PA could reduce certain parameters assessed here but failed to prevent the loss of proliferation in the cultures exposed to chronic oxidative stress.

Conclusion

In our stressed cells the reduction in growth curve and $Ki67^+$ cells, and the elevated in ROS and NO production as well as the enhanced of ICAM-1 expression and DNA damage refer to that we have successfully employed an experimental regimen for the induction of endothelial senescence by exposure our cells to intermittent H_2O_2 (50μM). Then we utilized this model system to investigate the preventive effect of 3 substances (BHT, PA, DPI) for their capacity to counteract the stress induced premature senescence. The three compounds alleviated certain stress responses but seemed to fail to preserve the proliferative capacity of the stressed cells

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Zusammenfassung

Einleitung

Eine große Anzahl von Daten, die oxidativen Stress in vaskulären Alterungsprozessen enthalten, wurden veröffentlicht, seit Denham Harman zum ersten Mal die Theorie des freien Radikals des Alterns vorschlug. Alle aeroben Zellen produzieren reaktiven Sauerstoff (ROS) und spielen eine wichtige Rolle sowohl in normalen Zellen als auch Seneszenz Zellen. Herzerkrankungen, Schlaganfall und Bluthochdruck sind alle bekannt Erkranhungen, die teilweise durch arterielle Erkrankungen verursacht werden. Die ROS-Produktion ist nicht nur an der Bestimmung schädlicher Wirkungen beteiligt, sondern auch an der Gewinnung von Energie aus organischen Molekülen, der Immunantwort und der Signalisierung. Neben erhöhtem oxidativem Stress ist Entzündung ein entscheidender Mechanismus für die Gefäßalterung. Tatsächlich kann die Funktion dieser Faktoren nicht getrennt verstanden werden, da mehrere Wechselwirkungen zwischen Entzündung und oxidativem Stress und umgekehrt, da verschiedene Redox-sensitive Transkriptionsfaktoren wie NF-B durch ROS aktiviert werden, wodurch die Expression von Zytokinen, Adhäsionsmolekülen und pro-inflammatorischen Enzymen reguliert wird.

Ziele

Die Theorie des freien Radikals des Alterns behauptet, dass Organismen im Laufe der Zeit ein hohes Maß an freien Radikalen erzeugen, die sich ansammeln und DNA-Schäden und viele altersbedingte Krankheiten verursachen. Unser Ziel dieser Studie war es, ein Modell von chronischem oxidativem Stress zu etablieren, um zelluläre Seneszenz in Endothelzellen zu induzieren und es zu verwenden, um die präventive von drei antioxidativen/entzündungshemmenden Verbindungen BHT, PA, DPI zu testen. Als Auslese verwendeten wir Zellnummer, ROS- und NO-Produktion, Expression von ICAM-1, Proliferationsmarker Ki67 und DNA-Schadensmarker γ-H2AX.

Materialien und Methoden

H_2O_2 (50 µM) wurde den HUVEC für eine Stunde alle 48 Stunden bis zum 9. Tag ausgesetzt, gefolgt von Ersetzung des EC-Wachstumsmedium mit oder ohne Reagenz (BHT, PA, DPI). Einen Tag nach der Exposition wurden die Zellen fixiert und auf DAPI-Färbung gezählt und die ROS-, NO-Produktion sowie die Expression von ICAM-1, Ki67 und γ-H2AX gemessen. ROS wurde in HUVECs mit dem fluoreszierenden H2-DCF (2',7' Dichlorfluorescein) gemessen. Der ICAM-1-Expression wurde mit Zell-ELISA gemessen. Um die Produktion von NO zu messen, wurde der Überstand später nach Derproteinisierung durch Ethanol und Zentrifugation mit Griess-Reagenzien (Sulfonamid und NED) infundiert und bei 540nm gemessen. Ki67 und γ-H2AX wurden durch Immunzytochemie nachgewiesen.

Ergebnis

Unsere Daten zeigen, dass die Exposition von HUVECS gegenüber H_2O_2 zur Verringerung der Zellzahl sowie der Anzahl der Ki67+-Zellen und zur Erhöhung des DNA-Schadensmarkers γ-H2AX führte. Die erhöhte ROS- und NO-Produktion und die ICAM-1-Expression als eine von SASP-Proteinen sind auch stark Hinweise auf auftretenden oxidativen Stress und Entzündungen, zusammengenommen wären alle analysierten Parameter mit der Interpretation vereinbar, dass unsere Zellen tatsächlich nach diesem Behandlungsschema seneszent wurden.. Darüber hinaus zeigten wir die präventive Wirkung von BHT und PA, wie die Erhöhung des Anteils an $Ki67^+$ Zellen und die Verringerung des DNA-geschädigten Markers γ-H2AX zeigen. Die ROS-Produktion und die ICAM-1-Expression wurden nach unserer Behandlung mit BHT und PA verringert, während DPI zu einer Erhöhung der ROS-Produktion und der ICAM-1-Expression führte, die sich auf die Toxizitätswirkung von DPI in dieser Konzentration beziehen.

Schlussfolgerung

In unseren gestressten Zellen beziehen sich die Reduktion der Wachstumskurve und $Ki67^+$ Zellen, und die erhöhte ROS- und NO-Produktion sowie die verbesserte ICAM-1-Expression und erhöhen des DNA-Schäden Marker γ-H2AX

darauf, dass wir erfolgreich ein experimentelles Regime zur Induktion der endothelialer Seneszenz eingesetzt haben, indem wir unsere Zellen intermittierend H_2O_2 (50 µM) ausgesetzt haben. Dann nutzten wir dieses Modellsystem, um die präventive Wirkung von 3 Substanzen (BHT, PA, DPI) auf ihre Fähigkeit zu untersuchen, der stressinduzierten vorzeitigen Seneszenz entgegenzuwirken und stellten fest, dass zwar die meisten untersuchten Parameter durch diese Substanzen günstig beeinflusst wurden, nicht aber die Fähigkeit zur Proliferation erhalten werden konnte.

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1 Introduction

1.1 Cellular senescence

Cell senescence of diploid cells is an irreversible cell cycle arrest which limits the proliferative life span of cells [1]. It is an anti-proliferative process that regulate cellular propagation exposed to various stresses [1]. During the life cellular senescence represents a physiological or pathological process [2, 3]. Senescence has been observed in a variety of cell types, like keratinocytes, melanocytes, endothelial cells, epithelial cells, T lymphocytes, and tissue stem cells.[4, 5]. The average life span for a healthy person is 80 years.

The death rate increases, and pathological problems arise when one gets older [6].

In 1532, Muhammad ibn Yusuf al-Harawi published the first known

research on ageing, "Ainul Hayat."[7]. Almost five decades have elapsed, but the trigger and mechanism of ageing continue to stay unclear [8] .

In 2010 a research has shown that about 576 million individuals aged 65 and older [9].

There have always been age-prolongers that have captured the attention of people due to the creativity and stimulated our wondering about the phenomenon. The biological cause of aging is thus the most unresolved research issue to date. Also, to this day, the largest known challenge to science research is the quest for the reasons to the biological mechanisms of aging [10, 11].While several explanations describe ageing, none have been shown to be generalizable [12, 13].

In the 1960, Hayflick and Moorhead noted that cultured human diploid fibroblasts could proliferate for a limited number of times before stopping [14]. This biological clock, known as the “Hayflick limit,” is caused by the progressive shortening of telomeres with each cell division and represents a physiological response to prevent genomic instability and thus the accumulation of DNA damage [15].Different cellular stressors, such as telomere shortening, oxidative damage, DNA damaging agents, or oncogene activation, can cause cellular senescence and impaired in proliferation by initiates the senescent cell cycle arrest [16–18].Various pathways are activated, and fundamental distinct stages of senescence are attained, depending on the cell type and the trigger [16–18]. Hayflick and Moorhead 1 define the initial phenomenon of cell culture as a replicative senescence.

Also, cellular senescence is a stress response that led to cells degeneration and aging pathologies. The senescence response is stimulated by several different cellular damage-related stimuli [19, 20] and is accompanied by broad morphological and phenotypic changes [17, 21]

like increased cell size, a flat morphology in vitro, a more smoothed form, increase in a lysosomal enzyme known as senescence associated beta galactosidase [22], exhibit senescence-associated heterochromatin foci formation [23], DNA damage foci [24], upregulation of certain microRNAs (miRNAs) as well as secretion of a wide range of factors, including growth factors, cytokines, chemokines and proteases, identified as senescence associated secretory phenotype (SASP) otherwise senescence messaging secretome [25]. Senescent cells exhibit major transcriptional changes for instance upregulation of tumor suppressor genes and downregulation of cell cycle promoter genes [16, 17, 21]. As previously stated, the senescence-associated secretory phenotype (SASP) is a phenotype correlated with senescent cells. SASP has the potential to attract immune cells and can cause chronic inflammation [26–28]. Senescent cells communicate actively with surrounding cells and tissue through the SASP [1]. Through paracrine and autocrine mechanisms, the SASP factor transforming growth factor beta (TGF-b) can promote aging. Senescence is directly impairing cellular DNA and cellular antioxidants, promoting cellular oxidative stress by producing reactive oxygen species (ROS) which induces DDR signaling [29] . TGF-b signaling, in an autocrine manner, ensures a stable cell cycle arrest by establishing a positive feedback loop that leads to p15INK4b induction, even when p16INK4A is lost [30, 31].

SASP expression is induced by various transcriptional and posttranscriptional factors, the most important transcription factors are NF-κB (nuclear factor of kappa light) and C/EBPβ (CCAAT/enhancer binding protein. Both transcription factors are activated during senescence [26–28]. The IL1 has an effect on the induction of NF-kappa B expression, which in turn stimulates the expression of IL1A. positive feedback plays a significant role in the orchestration of SASP factors, which are itself important to stimulate

NF-κB activation and expression. Recently, it has been demonstrated that SASP can also be transmitted by paracrine means [29, 32]. Senescence mediates a secretory program that produces ROS and DNA damage, coordinate by an inflammasome and interleukin 1A activation, which develops permanent cell cycle arrest [26, 28, 33]

Cellular senescence and SASP were more likely to suppress cancer and to support tissue repair or regeneration against injuries by enforcing arrest and recruiting immune cells that remove damaged, alternatively SASP has strong paracrine activities that suggest that the reaction to senescence is not just a cancer prevention mechanism but are protumorigenic and immunosuppressive. Also, SASP has effects not only to suppress tumors [39] or healing injurie [34, 35] but it also can involve tumor promotion [36]. Cellular senescence may be started after long-term stress, accumulation of reactive oxygen species (ROS) known as stress-inducing

senescence.[37–39]. It is believed that excessive mitochondrial ROS are play an important role in development the cellular senescence. Excessive ROS production, disrupted mitochondrial dynamics, defects in electron transport chain, bioenergetic imbalances, reduction in mitochondrial nicotinamide adenine dinucleotide, enhanced adenosine monophosphate activated protein kinase activity, and mitochondrial calcium accumulation are all examples of mitochondrial homeostasis perturbations [40].

Pathologies such as atherosclerosis, heart failure, osteoporosis, renal failure, macular degeneration, pulmonary insufficiency, neural degeneration, Parkinson's Alzheimer's and many other age-related diseases, are all caused by accumulation of senescent cells and degeneration in cellular mechanisms. Although susceptibilities to specific age-related pathologies vary by species, they generally rise with exponential kinetics, beginning around the midpoint of the species' lifespan [40].

1.1.1 Senescence in physiology and disease

It has been suggested that cellular senescence may be responsible for some conditions that are characteristic of tissue disfunction and aging [40].

Cellular senescence is functionally coupled to many biological processes, such as wound repair, tissue remodeling, aging, and metastasis, and even though a paradoxically, it has been discovered to help stimulate tumor development. [41–43]. Cellular senescence needs both autonomous and non-cell-autonomous activities to be involved in these processes.

The SASP mediates most non-cell-autonomous effects, which can affect the tissue microenvironment, affect the surrounding cells and recruit immune cells to eliminate senescent cells [41, 42]. Cellular senescence decreased incidence of cancer and other diseases characterized by overgrowth. The accumulation of senescent cells with aging, however, leads to host toxicity and degeneration of cellular mechanisms and increased susceptibility towards certain age-related diseases. The Senescence Program involves a number of physiological and pathological processes requiring remodeling of tissues. Although senescence is largely manifesting in the way that can lead to general consequences, it is critical to know and understand the concrete issues and issues it impacts on the organism. Senescence has pleiotropic effects. Even though the effects of Senescent cells are imperfect classification, they may be considered beneficial or adverse for the physiology and disease of cells [44].

1.1.1.1 Tumor suppression

cellular senescence is more important than forms of cell death for tumor suppression [44]. cell senescence research history has lasted for more than half a century; the functional relevance of cell senescence has only been determined in vivo in the last 10 years. There is an accepted correlation between senescence and the treatment of cancer [16, 45–47]. An irreversible cell cycle arrest in OIS cells is an optimal method for preventing tumor development after oncogene activation [48]. Cell senescence was established as a mechanism of tumor suppressor in the first functional, in vivo studies [49–52]. Also, it was reported that OIS has proved important to prevent development of lymphoma and contribute to the therapy response [49–53]. Cellular senescence and SASP suppress cancer cells and support tissue repair or regeneration against injuries by enforcing arrest and recruiting immune cells that remove damage.

Finally, as we don't want to stray from our original point, it should be emphasized that cellular senescence plays important role in tumor suppression in mammals

1.1.1.2 Embryogenesis

Additionally, the programmed senescence has been found to be helpful for human embryogenesis [54–56]. It has been proposed that cell–cell fusion induced senescence (FIS) may have an important role in the placenta physiological function, such as supporting the embryonic development. ERVWE1, a fusion protein which participates in the formation of placenta syncytiotrophoblast, causes cell fusion and causes cell senescence both in cancer cells and normal fibroblast [57, 58]. SA-gal^{+}and ki-67^{-} senescent cells are characterized by WNT and Hedgehog activity and occur during development within various areas of the embryo, such as the apical square, the neural ceiling plate, the mesonephros and endolymph sac [25].

1.1.1.3 Wound Healing

The dramatic and rapid induction of p16INK4a activity has been observed by tissue wounding in various animal models [34, 35]. NF-kB activation and expression of SASP cytokines, in addition to p16INK4a expression, are found at wound sites and appear to be assist in optimal healing [34, 59].

Senescent cells have been shown to play a beneficial role in wound healing in a variety of situations. The deposition of ECM after wounding helps the healing process, but if it is excessive, it may cause fibrosis, which impairs proper repair. In a variety of tissues, including

the liver [60], skin [35], lung [61]and heart [62] , senescence has been shown to play a role in wound healing and the fibrotic response [63].
Senescent cells have been also linked to a variety of pathological processes in addition to normal development and physiology [25].

1.1.1.4 Senescence in age-related disease

Almost all multicellular organisms show signs of aging, which is currently characterized as a gradual decline in tissue and organ function over time. Last but not least, the loss of tissue functions can produce many age-related chronic diseases. As the incidence of such conditions increases later in life exponentially, the emergence of common basic molecular and cellular mechanisms may be underlined [44]. Although Increased expression of the CDKN2a/b (encoding p16INK4a, ARF and p15INK4b) has been linked to a lower risk of atherosclerotic vascular disease [3, 64, 65] . During repair processes a fibrotic response is activated and leads to excessive connective tissue formation. The accumulation of extracellular matrix (ECM) proteins leads to permanent scarring and affects the structure and functionality of the tissue that can in extreme cases lead to organ failure and death [66]. Senescence can suppress other forms of harmful proliferation in addition to neoplastic proliferation. In various tissues, it has been demonstrated that cellular senescence either promotes or inhibits the formation of scar tissue [25].

1.1.2 Cellular senescence

1.1.2.1 Therapy-induced senescence

It is well established that exposure to DNA damage agents such as UV, irradiation [67], tert-butyl hydroperoxide [67] or anticancer chemotherapy agents [68–70] induces senescence in both normal and cancer cells, a phenomenon called "therapy-induced senescence" (TIS) [31, 71–73].Senescence in the various cancer cell lines are also caused by ionizing radiation [74–76]. The mechanisms transforming tumor cells into senescence are generally associated with the increase in DNA damage [38]. Evidence for this mechanism has been observed in cancer cell lines, a cancer subtype following the discovery of OIS [77]. During the analysis of human biopsy senescence markers from patients who had been treated with chemotherapy, scientists discovered the presence of senescence and concluded that it is associated with response to treatment [78–81] . inhibiting the cellular proliferation by TIS, encourage occurrence the aging

process in cells of patient. It is extremely difficult to tell the difference between replicative aging, TIS, and stress-induced senescence, which is consistent with stress because the nomenclature simply reflects the range of various stimuli that can cause cells to become senescent [25].

1.1.2.2 Stress-induced senescence

Researchers have found a relationship between oxidative stress and aging. Smoking, poor nutrition, all of these are contributions of senescence and inflammation in the endothelium [82]. Senescence is associated with a variety of stresses, such as oxidative stress [83, 84].

Oxidative stress occurs when the accumulation of reactive oxygen species (ROS) overwhelms endogenous antioxidant systems and/or when endogenous antioxidant systems are damaged. ROS (reactive oxygen species) are a class of oxygen-based molecules that have a high chemical reactivity [85, 86]. ROS are composed of both free radicals such as superoxide ($O2-$) and hydroxyl radicals ($OH\cdot$), as well as nonradical species such as hydrogen peroxide (H_2O_2) [85, 86] and all these compounds are source of oxidative stress. Increased output of ROS is crucial in developing several signs of DR from vascular instability and vascular leakage to disease angiogenesis [87–92]. Also, oxidative stress is one of the factors that cause senescence to develop [84, 93] but the complete mechanisms of stress induced senescence, which occur by oxidative stress are not completely clear.

When ROS levels rise, the p53/p21CIP1 axis is activated through the p38 MAPK (mitogen activated protein kinase 14) [94]. This temporary growth arrest in cell senescence progresses through the upregulation of p16INK4A in the stressed cells [1] . When p16INK4A and p21CIP1 are up-expressed, phosphorylated RB1 prevents the cell cycle from proceeding to the next round of cell replication, which stops the cell in G1 phase [16].

1.1.2.3 Oncogene-induced senescence

In addition, activated oncogenes are popular inducers of senescence. The oncogene induced senescence first was observed in the form of human fibroblasts by expressing the oncogenic form of RAS (H-ras-V12) leading to cell cycle arrest [48]. Oncogenic RAS leads to aberrant DNA duplication, DNA double strand break, and DDR pathway activation, which also may drive senescence [19, 20]. Furthermore, 'oncogene-induced senescence' also causes the

CDKN2A locus depression [95, 96]. Functioning oncogenes will interestingly, cause senescence via various mechanisms.

1.1.2.4 Replicative senescence

The loss of telomere length causes replicative senescence[97]. Telomeres sequences exist at the ends of chromosomes where they are shown to be capable of preventing off genome instability. It includes repeated (TTAGGG) sequences covering many kilo bases of DNA [98]. When a telomere reaches a certain short length, the cells become senescence as a form of DNA damage which causes a DNA damage response (DDR) activation [99]. The most relevant mediators of the DDR are the DNA damage kinases ataxia telangiectasia mutated (ATM) and the Rad3-related and checkpoints 1 and 2 [38, 100], which inhibit cell-cycle progression by phosphorylation of p53. The inhibition of the cyclin dependent kinase by p21 CIP1 is achieved by the process of p53 activation [101, 102]. In addition, ‘replicative senescence' causes the CDKN2A locus [103], which contains two essential tumor suppressor genes: p14ARF and p16INK4A, to be expressed [104, 105]. The tumor suppressor RB1 (retinoblastoma) [106], which binds and inhibits E2F transcription factors, is hyperphosphorylated and activated by the combined inhibition of CDKs by p21CIP1 and p16INK4 [106], permanently arresting cells in the G1 phase [107].

1.1.3 Endothelial senescence

The main, luminal layer of blood vessels is made up of endothelial cells, and the outer vessel wall is made up of smooth muscle cells. Endothelial cells form the foundation of blood vessels, which rapidly expand and coalesce into capillary plexus, which are remodeled into arterial–venous networks capable of supporting systemic circulation[108].

Maintenance of blood fluidity, control of vascular tone, modulation of proinflammatory molecule activity, proinflammatory immune responses, and neovascularization are all essential physiological functions of endothelial cells in vascular homeostasis [109] .

The release of vasoconstrictor and vasodilator agents controls vascular tone and hence blood flow. ET-1 and other vasodilator compounds such as NO, prostacyclin, and EDHF control the fragile equilibrium between vasoconstriction and vasodilation [2] . Aging people have less flow-mediated vascular dilation, as well as less systolic volume and NO production [110]. The healthy endothelium acts as a barrier to control small molecules, inflammatory cells, and nutrients from moving in and out of the blood vessel wall [111] . Gaps between endothelial junctions (places) control during oxidative and inflammatory stimuli the vascular permeability.

Damaged endothelium enhances the permeability of the vessel to facilitate the passage of inflammatory cells and small molecular cell components into and out of the tissues [111]. Insulin resistance, smoking, and obesity are cardiovascular risk factors that induce changes in endothelial structure and function, allowing for the arteries to become stiff as well as cause hypertension, atherosclerosis and a vascular disease [109].

Studies have linked vascular impairment with nitric oxide declines, endothelial progenitor cell dysfunction, and vascular inflammation to vascular aging, and together they promote vascular disease [112–114].Cell senescence is thought to promote survival in early life by avoiding tumor growth and maintaining tissue integrity (somatic cells). Senescent cells, on the other hand, specifically impair tissue function in later life and can hasten the onset of age-related diseases such atherosclerosis [115].

1.1.3.1 Oxidative stress caused endothelium senescence

A large number of data, which include oxidative stress in vascular aging processes, have been published since Denham Harman first proposed the free radical theory of aging [116].

Denham Harman's suggestion that free radicals linked with the basic aging mechanism [117], as an interpretation of the ageing chemistry reactions, was increasingly accepted [118]. The free radical theory of aging in which free radicals are responsible for damage in the cellular and tissue levels due to their high reactivity become very popular. Evidence from several clinical trials show a strong links between oxidative stress and aging.

Endogenous oxygen-generating radicals have been identified as the cause for the aging and death of all living organisms [116, 119]. In reality, mitochondria, in which free radicals are continuously generated during cell life, and especially mitochondrial DNA, are the main targets of free radical attack. The cell that uses oxygen and therefore produces reactive oxygen species had to develop complex defense mechanisms to neutralize reactive oxygen species and protect them from free radical damage. In 1972, the free radical theory was revised after mitochondria had been established as the cause of the most free radical reactions that had taken place in cells. The life span of the cells was also asserted to be determined by the rate of free radical damage[117]. Therefore, the rising of oxidative stress in aging cells appears to be a result of the imbalance between free radical and antioxidant defenses [120]. An ideal oxidative balance has been identified with oxidants, antioxidants and biomolecules [121]. There is a balance between these three components in a regular situation. Excess production of free radical will lead to oxidation and further impairments in the cellular process [122, 123] . Free radicals

are atoms or molecules, where in their external shell exist unpaired electron therefore, they are extremely reactive and can developed if oxygen interacts with those molecules [124]. Free radicals in mammals may also be carbon or nitrogen centered such as nitric oxide that regulate various process such as vascular tone, but the main radicals in aerobic species are O2-centered radicals. This can therefore be made in cells through the loss or acceptance of one electron, which acts as oxidants or reduction agents [125]. As stated before, reactive oxygen species are primarily produced in mitochondria, which use the majority of the O2 absorbed for substrate metabolism and ATP production, converting the remaining O2 to water.

Cell signaling and bacterial defense require reactive oxygen species, which are released during normal aerobic metabolism. There tends to be an electron leakage from the mitochondrial electron transport chain in respiring cells, ultimately resulting a variety of free radicals and activated oxygen compounds known as reactive oxygen species [126]

All the aerobic cells produce reactive oxygen (ROS) and nitrogen (RONS) and play a major role both in aging and in age-related diseases [127]. Reactive oxygen species (ROS) and reactive nitrogen (RNS) refer to oxygen and nitrogen reactive radical and non-radical derivatives, respectively [128]. RNOS can generally irreversibly oxidize biological macromolecules that cause accumulated cell function disability. This is naturally seen in old people and in conditions like atherosclerosis or Alzheimer's disease with a high incidence in older people.

RONS and ROS production is involved not only in determining harmful effects, but also in the extraction of energy from organic molecules, immune response, and signaling [129]. ROS is produced within the cells by endogenous and exogenous sources. ROS is produced within the cell, mainly by endogenous sources like NADPH oxidase, myeloperoxidase, and lipoxygenase II [130]. Air and water contamination, tobacco, alcohol, heavy or transition metals, medications (e.g., cyclosporine, tacrolimus, gentamycin, and bleomycin), industrial solvents and radiation are all exogenous sources of ROS, which are metabolized into free radicals within the body [131]. Increased development of reactive oxygen species (ROS) by NAD(P)H oxidases [132–136] and mitochondria [137, 138] in laboratory animals [135, 136, 139–143] and humans [133, 144] leads to endothelial dysfunction and broad elastic artery stiffening with age.

NADPH oxidase, which is the dominant radical superoxide anion source (O2•), which is generated with NADPH supplied electrons during cellular breathing by the electron of one molecular oxygen reduction. The majority of the O2• is transformed by superoxide dismutase (SOD)into the hydrogen peroxide (H_2O_2) [129].

Although H_2O_2 is not a free radical because it has no unpaired electrons, it can transform through the Fenton or Haber–Weiss reactions to the highly reactive ROS hydroxyl ion (OH•). The hydroxyl radicals are very reactive and can make problems in cellular membranes and proteins in general. H_2O_2 can also be converted into hypochlorous acid in neutrophils, in the presence of chloride and MPO and particularly damaging the cellular proteins [129] .

The 2008 publication by Moosmann and Behl states that these findings support the free radical theory of aging [145] . And Nemoto and Finkel concluded in a paper published in 2004, "The most likely sign in our opinion will be to appear in any hypothetical center of aging right now, it is the free radicals. Indeed, free radical´s cellular effects are the most likely contributors to explaining aging in a wide variety of organisms [146].

All these papers have talked about the relation between oxidative stress and aging and how is this theory very important at the moment to understand aging and aging related diseases.

.

There is good proof that endothelial dysfunction resulting from elevated oxidative stress substantially leads to both compromised coronary arteries dilation [136] and promotes myocardial ischemia and neurovascular uncoupling [147, 148]

Also, endothelial dysfunction is a pathophysiological signature of coronary disease development. In industrialized nations, cardiovascular disease is the leading cause of death, and its presence is strongly linked to advanced age [149]. The phenotype of the aged vasculature is characterized by functional changes and age-related atherosclerosis [93]. Endothelial dysfunction is an important element of the changes occurring in the vasculature with age [133] . The failure of endothelial to trigger an adequate vasodilatory response because of inadequate natrium Oxide (NO) bioavailability is one of the hallmarks of endothelial dysfunction [150].

Three types of nitric oxide synthase (NOS) generate NO from L-arginine: epithelial NOS, which is involved in vasodilation and vascular control, neuronal NOS, which is involved in intracellular signaling, and inducible NOS, which is triggered in response to various endotoxin or cytokine signals [151]. NO has powerful anti-inflammatory, antithrombotic and Anti-leucocyte adhesion properties [152–154]. A relatively reactive molecule, peroxynitrite ($ONOO^-$), formed by reactions of O2 with NO [129, 130]. Thus, reduction of NO probably promotes a pro-atherogenic phenotype of vascular aging [152–154]. Also, oxidative stress can affect several aspects of age vascular function by oxidation essential proteins, or by redox sensitive transcription factors, but the inactivation of endothelium-based nitric oxide is one of its more powerful results (NO). Age associated decreasing in endothelium dilation, enhanced vasocontraction and dysregulation of tissue perfusion are caused by impaired bioavailability of

NO [135, 136, 139, 155–159]. Heart disease, stroke and hypertension are all conditions which are actually known to be partly caused by arterial disease [149, 160].

1.1.3.2 Relation between oxidative stress and chronic inflammation in aging

Increased synthesis of reactive oxygen species (ROS), decreased antioxidant defense, and increased proinflammatory factors are all linked to aging. All this led to chronic inflammation and oxidative stress, two processes which reinforce each other through activation of redox-sensitive, proinflammatory nuclear factor kappaB (NF-κB)

It was reported that oxygen free radical transformed to H_2O_2 through Mn-SOD in the mitochondria of vascular endothelial cells in aged rat arteries. This enhances in H_2O_2 level activate NF-κB in the cytoplasm of aged cells [143].

Also, there is an association between inflammation and oxidative stress, as maintained NF-κB is one of the redox-sensitive transcriptional factors that activated by excessive ROS production and led to increasing the gene expression of cytokines (TNF-α, IL-1, and IL-6), adhesion molecules (ICAM, VCAM), and proinflammatory enzymes (iNOS, COX-2) [161].

Also, ROS act as trigger that push cells into senescent and may contribute to widespread change in gene expression such as SASP that involved in inflammatory process.

Senescent endothelial cells impair not just the function of the vessel's endothelial line but also the surrounding cells by their secrets called Senescence-related materials (SASPs).

Oxidative stress, cellular senescence, and consequently, SASP factors are involved in several acute and chronic pathological processes, such as CVDs, acute and chronic kidney disease (CKD), neurodegenerative diseases (NDs), macular degeneration (MD), biliary diseases, and cancer. The pathways that contribute to vascular inflammation are likely to be complex. The interaction between increased oxidative stress and inflammatory process activation in the old vascular wall is significant [162]. ROS are intracellular signaling molecules that activate pro-inflammatory signaling pathways, including NF-κB and TNF, the stress-response system [143], which control the activity of endothelial paracrine mediators and promote the development of atherosclerosis. Aged endothelial and smooth muscle cells have strong NF-κB activation [143, 163] and selective NF-κB inhibition in the vasculature has been shown to enhance blood flow control, decrease systemic inflammation, exert beneficial metabolic impact, and extend health period. [164]. Inflammatory mediators (e.g., TNF activates NAD(P)H oxidase are powerful inducers of cellular oxidative stress [165].

As previously stated, this endothelial failure is caused by a decrease of nitric oxide bioavailability as a result of endothelial oxidative stress and inflammation, which can be modulated in older adults by CVD risk factors this age-related endothelial redox change activat NFkB, an inflammatory transcription factor. The activation of NFkB induces transcription of pro-inflammatory cytokines, which can further inhibit endothelial function. The two mechanisms, oxidative stress and inflammation, which contribute to advancing-age endothelial disease and to cell and molecular incidents. Another possible mediator of this endothelial pro-inflammatory phenotype is a rise of immune or senescent cells. It has been shown that the p53/p21 pathway causes cell senescence, genomic instability, telomere disruption, or DNA damage [166].

In conclusion, considering the strong relationship between oxidative stress, inflammation, and aging, the oxidation-inflammatory hypothesis of aging, also known as oxi-inflammaging, has been proposed: aging is a breakdown of homeostasis caused by chronic oxidative stress that affects the regulatory systems, including the nervous, endocrine, and immune systems. The immune system's activation causes an inflammatory response, creating a cycle in which chronic oxidative stress and inflammation feed off each other, increasing age-related morbidity and mortality [167].

1.2 Antioxidant and anti-inflammatory compounds

It was stated that in both vascular endothelial and smooth muscle cells from the ancient rat arteries, the over-produced oxygen free radical is transformed to H2O2 by Mn-SOD. Increased releases of H2O2 resulted in the cytoplasm of aging cells activated the NF-κB. Although NF-κB appears to be the key transcriptional factor mediating H2O2-induced vascular-cell response [168].Generally, it is postulated that antioxidants block or impede the process of oxidative damage to cellular membranes, slowing or blocking oxidative chain reactions. Most researchers believe that consuming antioxidants and free radical scavengers helps to prevent and cure different cardiovascular diseases [169]. Many studies supported the critical role of oxidative stress in the development of heart disease. The main source of death in the elderly in the Western countries is the atherosclerotic disease. Several antioxidants have been suggested to prevent the progression of this disease, including polyphenols and lycopene [126]. At low concentrations, antioxidants can slow down or even prevent the process of oxidation of a substrate. Non enzymatic and enzymatic antioxidant are known as endogenous defenses non-enzymatic such as glutathione, bilirubin, uric acid, thiols, albumin, and nutritional factors like

vitamins and phenols and enzymatic such as the superoxide dismutases, the glutathione peroxidases [GSHPx], and catalase). The endogenous antioxidant defense balances the generation of reactive oxygen species. The nutrition, many in the phenol family, is the most effective source of antioxidants [126]

1.2.1 Butylhydroxytoluene (BHT)

The antioxidants such as phenolic compounds have one or more aromatics rings with one or more hydroxyl groups and become more effective by increasing the number of hydroxyl groups [170]. In the late 1940s, synthetic phenolic antioxidants were developed [171].They've been used in the dairy, medicinal, cosmetic, and petrochemical industries to extend shelf life and enhance consumer product quality, freshness, flavor, and texture [172].They're commonly used in a variety of materials to bind free radicals and slow lipid oxidation [171]. BHT, which has two tert-butyl groups at the ortho and the para-position methylic group is the most widely used synthetic phenolic antioxidant in food, pharmaceuticals and cosmetics, as well as rubber additive, plastics, mineral oil and printing additives [173, 174]. Certain BHT intermediates derived from complex phenols are stabilized electrophilic Michael acceptors through conjugation. In biological systems, these acceptors can react with proteins and thiols that contain an SH group (nucleophiles), resulting in detoxification [175].

1.2.2 Perlatolic acid (PA)

Natural products derived from lichens have been extensively studied for their biological properties and have shown a strong inhibitory effect on NF-κB activation in the low micromolar range [176].The anti-inflammatory activity of PA described through the inhibition of the three major pro-inflammatory targets (mPGES, 5-LO, NF-κB) [176].

1.2.3 Diphenylene iodonium (DPI)

Diphenyleneiodonium (DPI) is commonly used as a flavoenzyme inhibitor, specifically NADPH oxidase. Previous researches has shown that endothelial cells like HUVEC can generate ROS via PKC and NADPH oxidase [177]. The effects of PKC and NADPH oxidase inhibitors on oxidative stress were investigated to explain the ROS synthesis cascade caused by these two stimulants. These findings showed a substantial reduction in the development of intracellular ROS in those oxidization-stimulated cells with incubation of diphenylene diodonium (DPI). O2 − and H2 O2 are produced by NADPH oxidases (NOXes) and double oxidases (DUOXes). The action of these enzymes is inhibited by diphenyeneiodonium (DPI) and also used as a specific inhibitor[178] .

2 Materials and methods

2.1 Materials (Chemicals & reagents)

Medium M199, fetal bovine serum (FBS), goat serum, gelatin, paraformaldehyde (PFA), Dimethyl Sulphoxide (DMSO), Sulfonamide, Naphthyl ethylenediamine dihydrochloride (NED), vanadium chloride (III) (VCl_3) were bought from Sigma-Aldrich (Saint Louis, USA). Hepes Buffer, Trypsin-EDTA and penicillin, streptomycin, fungizone were purchased from LONZA (Visp, Switzerland). Endothelial cell growth supplement (ECGS) with heparin was from PromoCell (Heidelberg, Germany). 2′,7′-dichlorodihydroflurescеindiacetate (H2-DCF) was obtained from ThermoFischer Scientific (Vienna, Austria). Hydrogen peroxide (H_2O_2), butylated hydroxytoluene (BHT), diphenyleneiodonium (DPI), perlatolic acid (PA), triton-x, primary antibody for Ki-67 and γ-H2AX, alexa flour 647 goat anti-rabbit and alexa flour 488 goat anti mouse from (life technologies, Columbus USA)

Chemicals and reagents	**Abbreviation**	**company**
Hydrogen peroxide	H_2O_2	Sigma-Aldrich (Saint Louis, USA)
Medium M199	M199	Sigma-Aldrich (Saint Louis, USA)
Fetal bovine serum	FBS	Sigma-Aldrich (Saint Louis, USA)
Goat serum		Sigma-Aldrich (Saint Louis, USA)
Gelatin		Sigma-Aldrich (Saint Louis, USA)
Paraformaldehyde	PFA	Sigma-Aldrich (Saint Louis, USA)
Dimethyl sulphoxide	DMSO	Sigma-Aldrich (Saint Louis, USA)
Sulfonamide		Sigma-Aldrich (Saint Louis, USA)
Naphthyl ethylenediamine dihydrochloride	NED	Sigma-Aldrich (Saint Louis, USA)
Vanadium chloride (III)	VCl3	Sigma-Aldrich (Saint Louis, USA)

HEPES buffer		LONZA (Visp, Switzerland)
Trypsin-EDTA		LONZA (Visp, Switzerland)
Penicillin		LONZA (Visp, Switzerland)
Streptomycin		LONZA (Visp, Switzerland)
Fungizone		LONZA (Visp, Switzerland)
Endothelial cell growth supplement	ECGS	PromoCell (Heidelberg, Germany)
heparin		PromoCell (Heidelberg, Germany)
2′,7′-dichlorodihydroflurescindiacetate	DCF	ThermoFischer Scientific (Vienna, Austria)
Perlatolic acid	PA	Sigma-Aldrich (Saint Louis, USA)
Diphenyleneiodonium	DPI	Sigma-Aldrich (Saint Louis, USA)
Butylated hydroxytoluene	BHT	Sigma-Aldrich (Saint Louis, USA)
I-CAM-1-antibody		
Primary antibody for KI67		Thermo scientific
Primary antibody for γ-H2AX		biolegend
Alexa Flour 647 goat anti-rabbit		(lifetechnologies,Columbus USA)
Alexa Flour 488 goat anti-maus		(lifetechnologies,Columbus USA)
Triton-x		
4’, 6-diamidino-2-phenylindole	DAPI	

2.2 Cell culture

2.2.1 HUVEC isolation

Cell culture flasks (T75) were coated with gelatin and placed in the incubator. In the bath for pre-heating purposes the EC growth medium (medium M199 contained; 20% FBS supplemented with 3 mg / ml ECGS and 22.5 mg / ml heparin, 2 mM L-glutamine, 100 µg / ml streptomycin, 100 U / ml penicillin and 0.25 µg / ml fungizone) is positioned to achieve the optimum cell temperature (37 °C). The first step was to wash the umbilical cord with HBSS to wash off all the blood. Then the vein was washed with HBSS and CMFH afterthat the cells were isolated from umbilical cord by using collagenase. After 20 minutes the cells were rinsed with CMFH several times to stop the activity of the collagenase. The separated cells were collected in 50 ml tubes and the tubes were centrifuged at 1200 rpm
for 10 min.
The supernatant was carefully removed, and the pellet of cells was resuspended in full EC growth medium and the cell suspension transferred into gelatin coated flasks. and incubated at 37°C in 95% air 5% CO2 atmosphere saturated with H2O.

2.2.2 Passaging/Splitting of the cells

Then cells were split up as they achieve a 100 percent confluency. EC growth medium was sucked out and the cells were washed with CMFH. And to separate the cells from the flask we used trypsin (conc?). We used 5 % media (5% serum in M199) to interrupt the activity of trypsin after separating the cells. Then the separated cells were centrifuged for 5 minutes. The supernatant was removed, and the pellet was suspended in EC growth medium. The cells have been counted and an aliquot was seeded onto a previously gelatin covered flask and the rest were centrifuged at 1200 rpm for 10 min and the pellet was resuspended in 2ml 5% DMSO in full media and freezed in liquid. Endothelial cells were then incubated at 37°C in a 95% air/5% CO2 atmosphere saturated with H2O.

2.3 Induction of oxidative stress in HUVECs by exposure to repeated low doses of H_2O_2

The cells were subsequently seeded in 96-well plates with EC growth medium and HUVECs were exposed to H_2O_2 (50µM) for one hour after 24-hour, except the cells for our negative control. After that, H_2O_2 was exchanged for either only EC growth medium, or EC growth medium with PA(7µM), DPI (10µM) or BHT inhibitor (40µM). The exposure was repeated on day two, four, six and eight. From day nine until day fifteen, the cells were cultivated in EC growth medium only.

2.4 Cell proliferation assessment in HUVEC-cultures by determining the cell number

The cell proliferation was evaluated by the cell count at various time points. The cells were seeded in EC growth medium and after incubation for 24 hours HUVECs were exposed to H_2O_2 (50µM) for one hour omitting our negative control. After that the H_2O_2 was replaced by either EC growth medium or by PA (7 µM), DPI (10µM) or BHT (40 µM) in EC growth medium. Cells were then fixed with 4% PFA after 10 minutes, 30 min, 60 min and on day one, three, five, seven, nine, eleven, fifteen days for 10 minutes and then washed with PBS (2x), stained with DAPI (1µM) in PBS for 30 minutes and washed again with PBS. The cells were counted using the image analysis software CellP, Soft (Imaging Systems Olympus, Münster, Germany).

2.5 Intracellular ROS measurement in HUVECs

We used H2-DCF as an indicator for reactive oxygen species (ROS). Supernatant was removed (collected for later NO assessment) and replaced by H2-DCF (10µM) diluted in PBS supplemented with 5% FBS. Then the ROS production was measured after 30 min incubation in the dark (at 37°C in the CO2-incubator). We used for ROS measurement the plate reader Synergy 3 (Bio Tek).

2.6 Detection of ICAM-1 by cell ELISA

After ROS measurement the cells were fixed with PFA 4% in PBS for 10 min and washed twice with PBS. Cells were exposed to H_2O_2 for one hour followed by adding EC growth medium only or adding our reagent PA (7 µM), DPI (10µM) or BHT (40 µM). Then we fixed cells with 4% PFA after that cells were washed with PBST (PBS+0,05%Tween20) and to block

nonspecific binding of the antibody we used 5% BSA (bovine serum albumin) and incubated at 4°C. Next the cells were washed again with PBST and incubated with the first antibody (anti-mouse CD54 antibody) at room temperature for one hour. The cells were washed again with PBST and incubated with the second antibody (F(ab)2 antibodies to mouse IgG+IgM peroxidase labeled) for 40 minutes. Then the cells were washed again with PBST and exposed to the peroxidase substrate the streptavidin-biotinylated horseradish peroxidase complex. By adding 0.5 N sulfuric acid, the color formation reaction was halted. We measured the absorbance at 450 nm by using plate reader Synergy 3. (From Bio Tek)

2.7 NO measurement in HUVECs

We deproteinized our previously collected supernatants by adding ethanol (EtOH) and incubating at room temperature for one hour. Then we centrifugated this mixture for 10 min at 10 000 rpm/11200 rcf. Into a 96-well plate, 50 µL of supernatant was pipetted and incubated for 30 minutes, upon adding 50µl VCl3 (2%) and 50µl Griess mixture (sulfonamide (1%) and NED (0.1%). The absorption at 540nm was then measured.

2.8 Immunostaining for Ki67 and γ-H2AX

HUVECs were exposed to H_2O_2 (50µM) with or without our reagent (BHT, PA, DPI) for the indicate time point as described above then the fixed cells were washed with PBS, followed by permeabilization with Triton-X (0,1%) for 10 min in room temperature , then the cells were washed 1x with PBS and blocked with goat serum (3%) for 2h, cells were then stained with primary antibody for γ-H2AX (mouse MAb) and Ki67 (rabbit MAb) overnight. After washing with PBS and staining with secondary antibody Alexa Flour 647 goat anti-rabbit (life technologies, Columbus USA) and Alexa Flour 488 goat anti mouse (life technologies, Columbus USA). fluorescent images were taken randomly after staining with Olympus live imaging microscope IX83 cellVivo (Olympus, Austria) using 10X magnification with farm size 4x4.

Counting of Ki67 positive cells was achieved by using the cellSens software version 1.18, while γ-H2AX positive cells were counted manually.

2.9 Statistical analysis

Data was statistically analyzed using the unpaired two-tailed t-test by using Microsoft Excel and presented as mean ± SD of six replicates for the untreated and H_2O_2-treated groups (C and H) and of triplicates for the inhibitor-treated groups

p-values < 0.05 were considered as significant, <0.01 as very significant and <0.001 very highly significant.

3 Aims

Many studies have reported the relation between the oxidative stress and aging and how is this theory is important to the understanding of aging and aging related diseases. All aerobic cells produce reactive oxygen (ROS) which plays a major role both in normal cells, in aging and aging related diseases. Pathologies such as atherosclerosis, heart failure, osteoporosis, renal failure, macular degeneration, pulmonary insufficiency, neural degeneration, Parkinson's Alzheimer's and many other age-related diseases, are all caused by accumulation of senescent cells and degeneration in cellular mechanisms. Thus, endothelial dysfunction is a pathophysiological signature of coronary disease development.

The main aims of this study are:

- Investigate, over a period of 15 days, the induction of endothelial senescence through repeated exposure to 50μM H_2O_2.
- Investigation the capacity of Butylhydroxytoluene, Perlatolic acid and Diphenylene iodonium to prevent the induction of senescence in stressed cells

4 Results

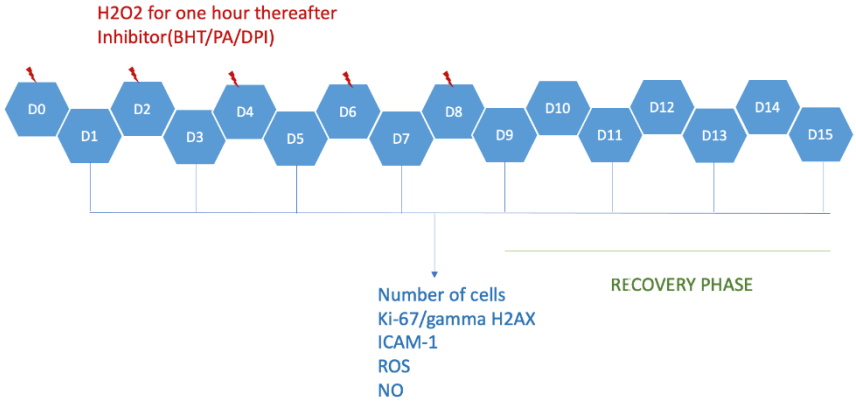
The most important risk factor for most chronic diseases is aging. Endothelial senescence can be induced by a variety of factors; among them, oxidative stress. A large number of data, which investigate oxidative stress in vascular aging processes, have been published since Denham Harman first proposed the free radical theory of aging [120]. Oxidative stress has a major role in causing senescence and inflammation in the endothelium. Oxidative stress occurs when the accumulation of reactive oxygen species (ROS) overwhelms endogenous antioxidant systems and/or when endogenous antioxidant systems are damaged. ROS are composed of both free radicals such as superoxide ($O2-$) and hydroxyl radicals ($OH\cdot$), as well as nonradical species such as hydrogen peroxide (H_2O_2) [86, 87] . All these compounds are source of oxidative stress. Senescent cells can communicate with surrounding cells via SASP (senescence-associated secretory) and can cause chronic inflammation through secretion of proinflammatory cytokines, chemokines and proteases. Oxidative stress can also affect several aspects of age vascular function by oxidation of essential proteins, or by redox sensitive transcription factors, but the inactivation of endothelium-based nitric oxide is one of its more powerful impacts (NO). Age associated decrease in endothelium dilation, enhanced vasocontraction and dysregulation of tissue perfusion are caused by impaired bioavailability of NO [142, 143, 146, 152–156].

In our experiment we wanted to investigate the senescence inducing effect of oxidative stress by exposure to H_2O_2 and the preventive antioxidant and anti-inflammatory effects of PA, BHT, DPI in HUVECs and examine if any of these reagents can inhibit the stress-induced development of endothelial senescence.

4.1 Response of HUVEC to intermittent oxidative stress over one week by exposure to H_2O_2 every other day for one hour

We have used in our experiment 50µM H_2O_2 for one hour (every 48 h for 8 days) to induce stress in endothelial cells, then the cells were cultured in EC growth medium without H_2O_2 for one week to detect the ability of the cells to proliferate after these 8 days and determine the portion of cells converting to senescence, which is characterized by a permanent growth inhibition.

For evaluation of senescence associated cell cycle arrest, we recorded growth curves, measured the expression of the proliferation marker Ki67, DNA damage marker γ-H2AX, ICAM-1 as one of SASP proteins as well as the ROS and NO production. Those parameters have been measured one day after H_2O_2 exposure, because we wanted to exhibit the long-term effect not the acute effect (Figure 1).

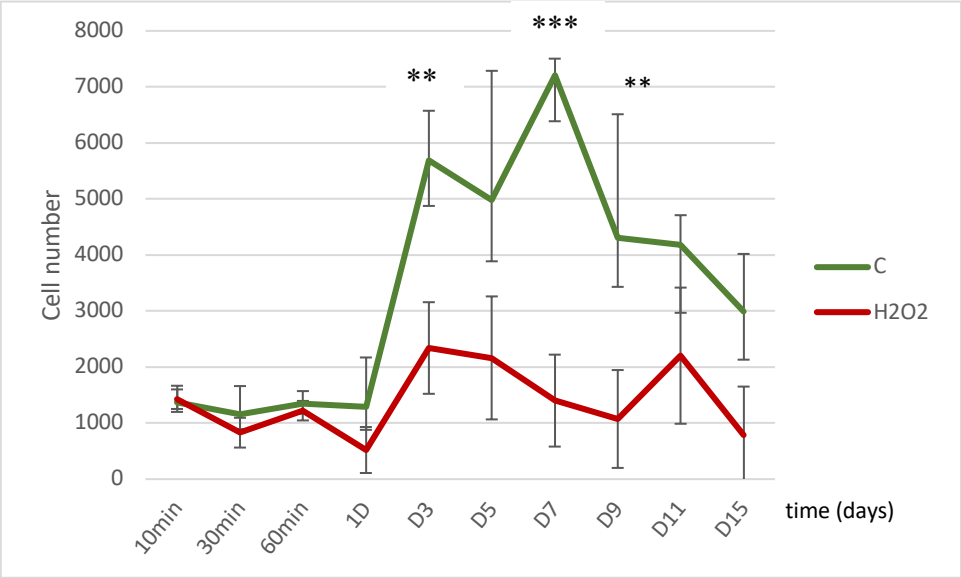


4.1.1 Intermittent exposure of H_2O_2-induces cell cycle arrest and inhibition of cell proliferation in HUVECs

Several groups have described the induction of cellular senescence in HUVECs by applying oxidative stress using H_2O_2. We have used 50µM H_2O_2 for one hour every 48 hours over a period of 8 days. We registered growth curves, the expression of proliferation marker Ki67 and the DNA damage marker gammaH2AX to evaluate the induction of senescence.

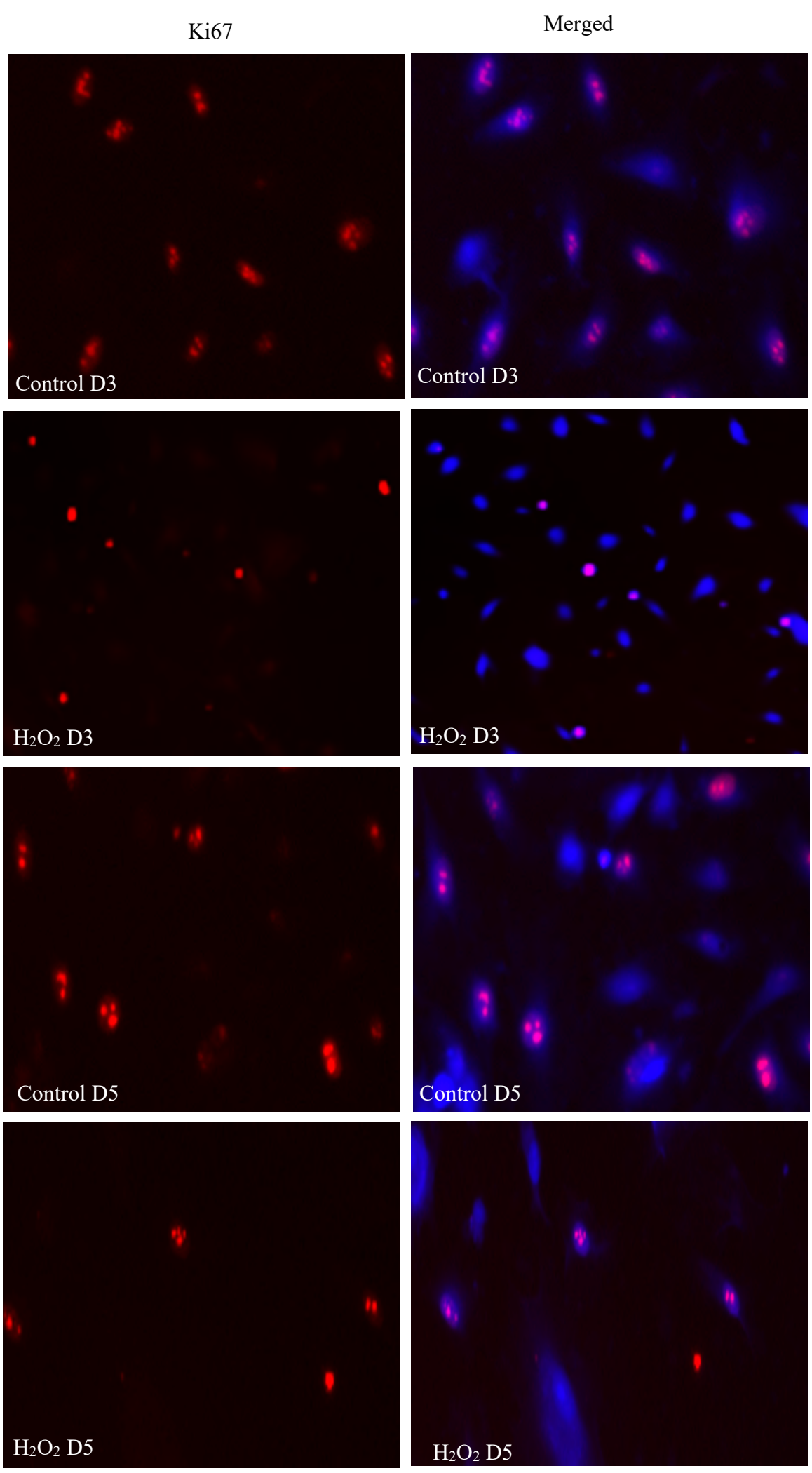
The Ki-67 protein is a nonhistone nuclear protein that is a convenient and reproducible biomarker for proliferation. Ki-67 protein is present during the active phases of the cell cycle but is absent during the inactive phase of the cell cycle. Ki-67 protein has been widely used as a proliferation marker. The phosphorylation of the Ser-139 residue of the histone variant H2AX, forming γH2AX, is an early cellular response to the induction of DNA double-strand breaks which accumulate in aging cells.

Repeated exposure of HUVECs (P5) to 50 µM H_2O_2 every two days for 1 hour (day 0, day two, day four, day six, and day eight), followed by culturing the endothelial cells for a period of 1 week. This "recovery period" was implemented to assess if the stressed cells would be able to proliferate again (and thus not being senescent) after ending the oxidative stress. As a control, cells were exposed to EC growth medium only.



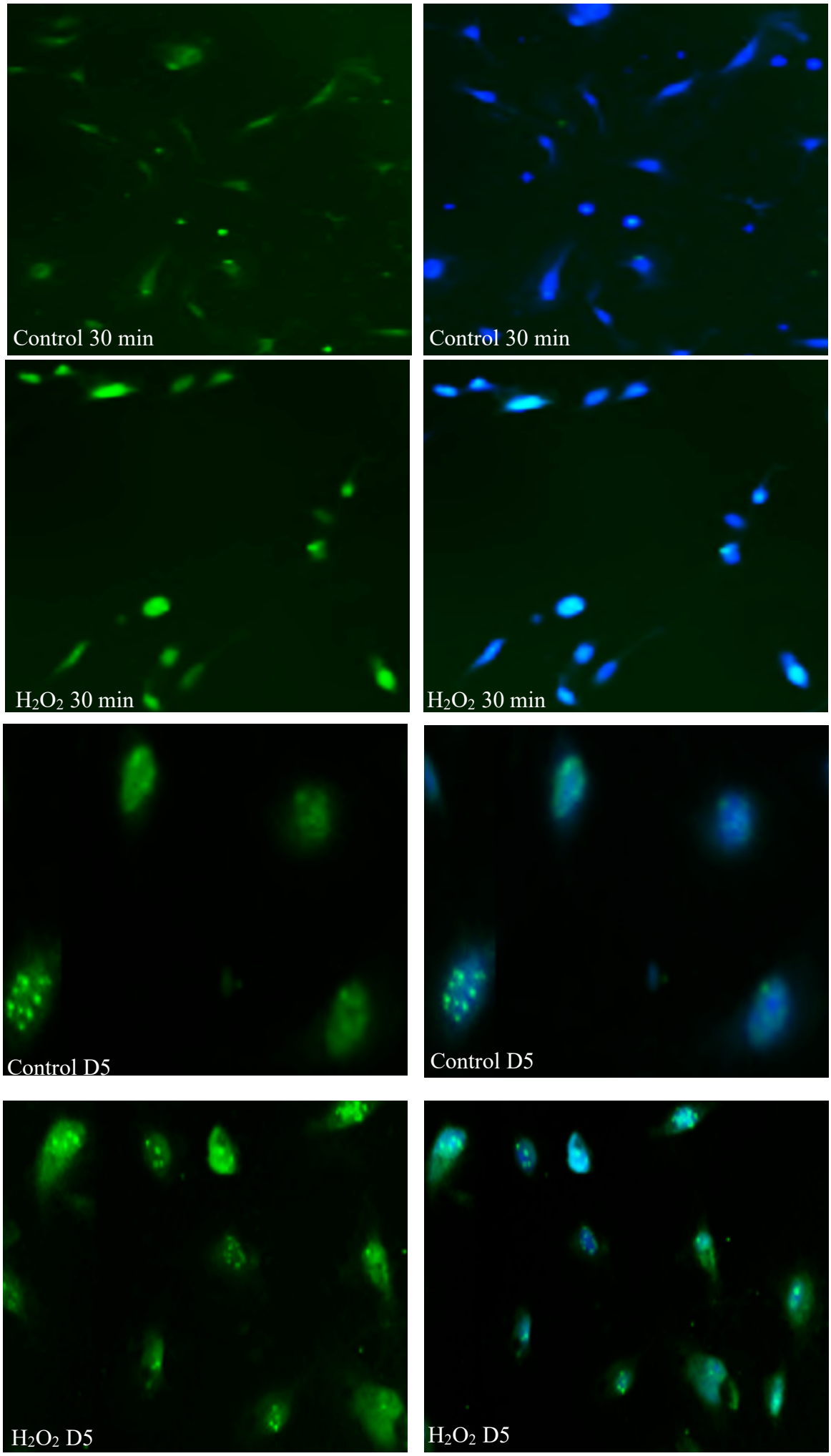
Our results show that from day 3 on there was a significant reduction in cell number by H_2O_2 exposed cells compared to control cells ($p<0.005$). On day 7, control cells reached their maximum number, then cell number decreased until day 15. The significant disparity in cell

number between control and stressed cells illuminate that H_2O_2 impaired the proliferation of HUVECs cells. We suggest that control cells could no longer proliferate after day seven because of the phenomenon of contact inhibition due to HUVEC confluency and that subsequently the culture became unstable displaying significant cell loss. This reduction of cell proliferation in control as well as H2o2 treated cells was supported by the results of immunofluorescent detection of the proliferation marker Ki67 showing that the portion of Ki67 positive cells in the stressed cultures decresed over the course of the experiment


Ki67
Merged
Control D3
Control D3
H2O2 D3
H2O2 D3
Control D5
Control D5
H2O2 D5
H2O2 D5

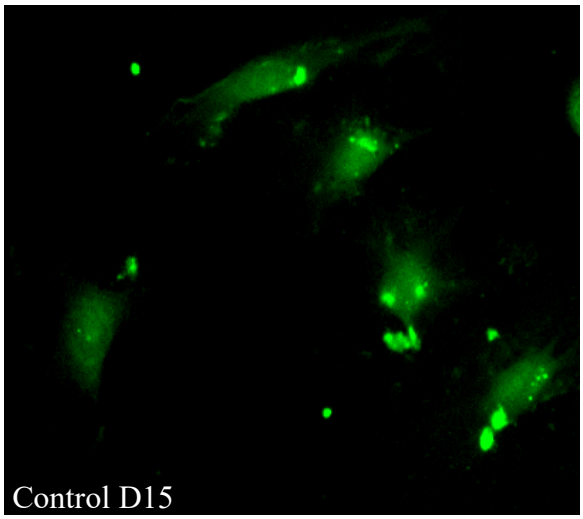
γ-H2AX

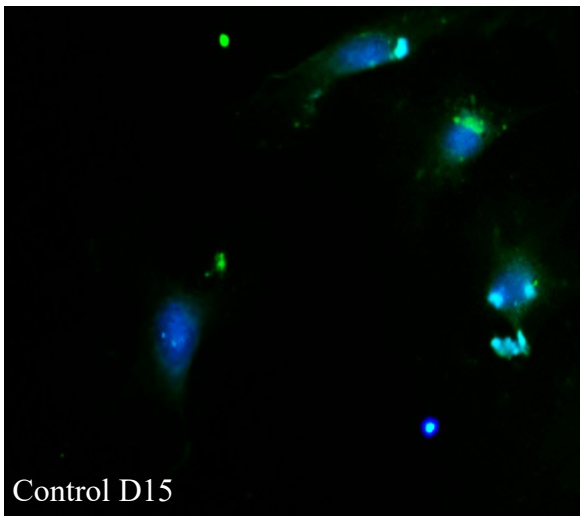
Merged

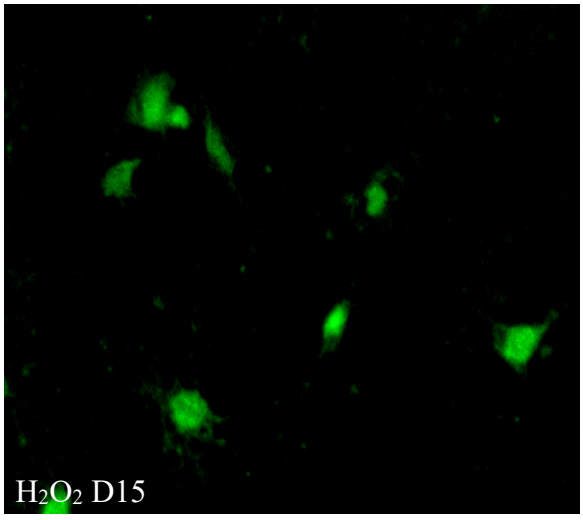


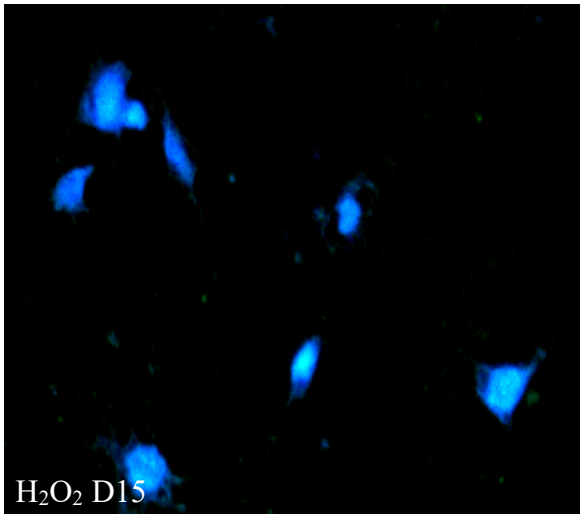
γ-H2AX

Merged

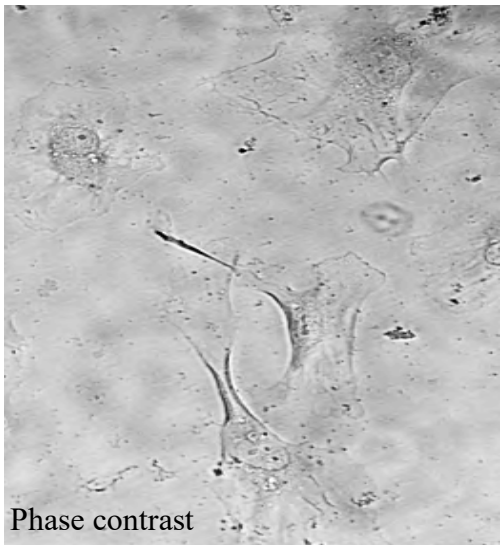


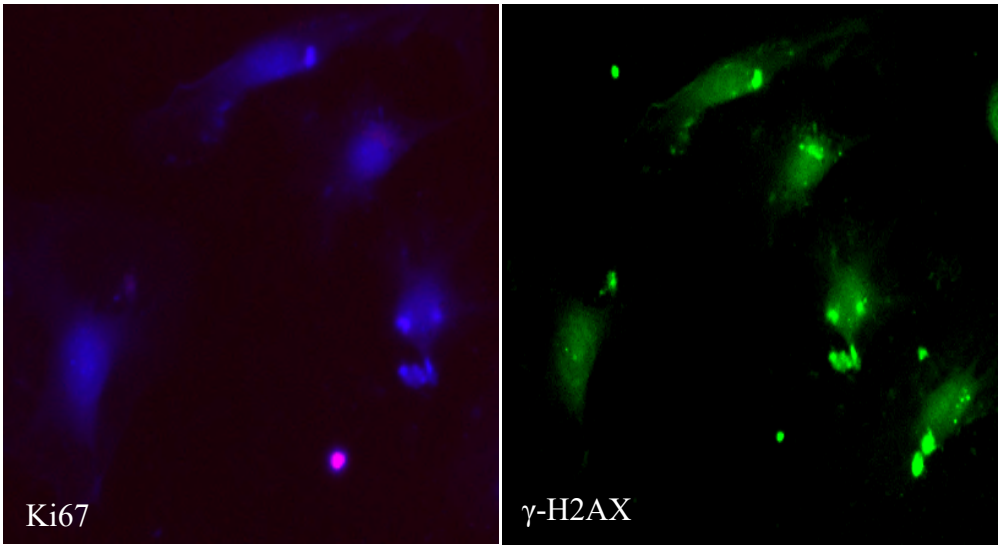


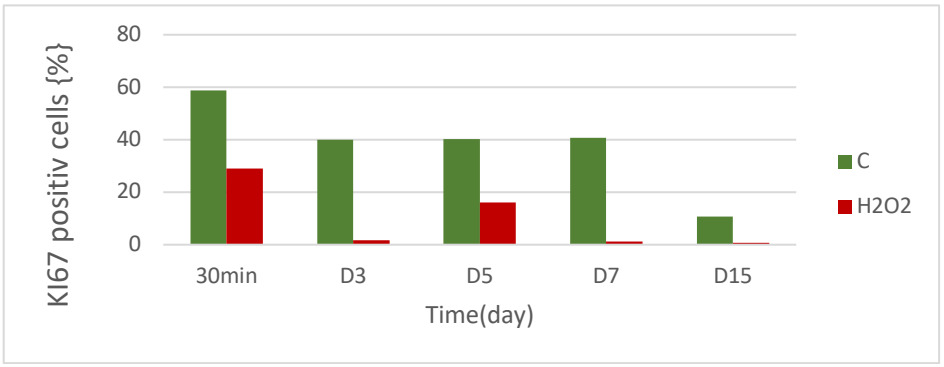




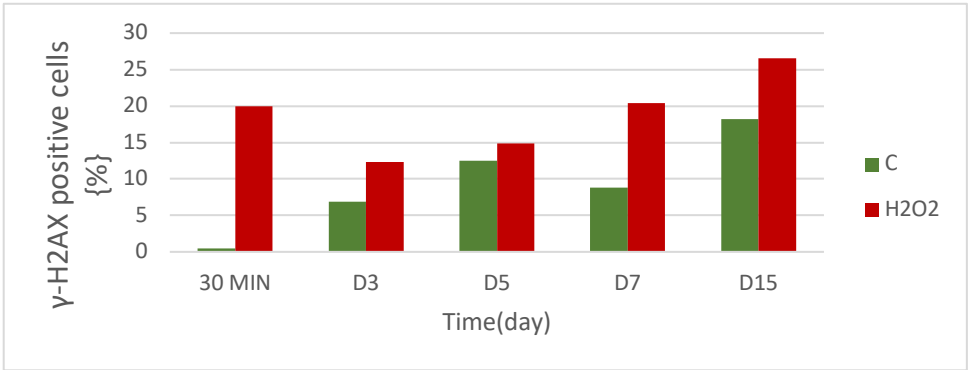
Control D15







In control cells the %age of $Ki67^{+}$ reached 40% and remained at this level until day 7 then decreased to about 10% by day 15, which is compatible with a cell growth curve, in which the cell number reached the maximum on day 7 and declined thereafter until day 15. The intermittent exposure of HUVECs to H_2O_2 (50µM), markedly reduced endothelial growth. This assessment reduction of proliferation in HUVECs exposed to H_2O_2 revealed that, on day 3, day 5, day7, day15, a lower percentage of cells expressed this marker when compared to control. The phase contrast photo of our control cells on day 15 shows characteristic features of the senescent morphology such as increased cell size, a flat morphology in vitro and more smoothed form.



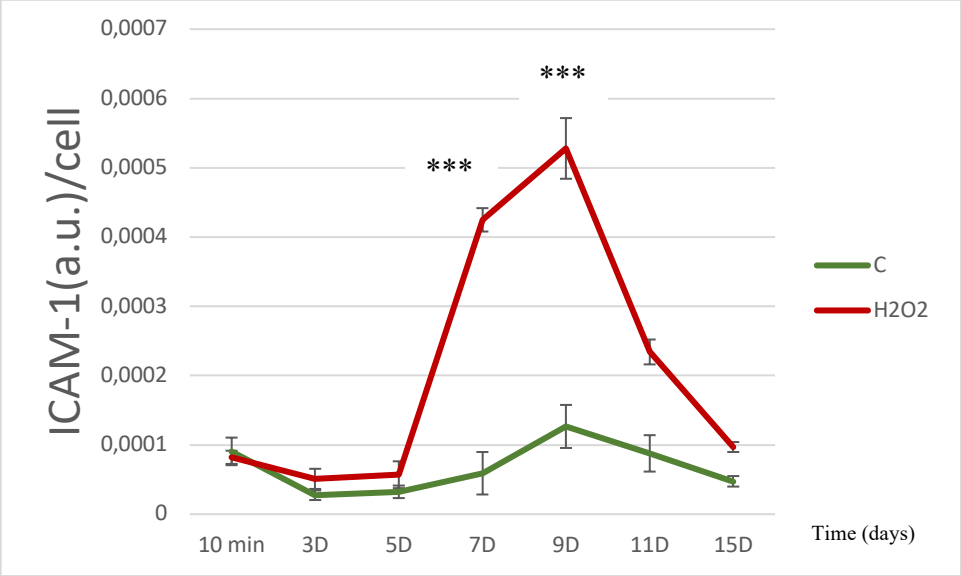
Then we have measured the γ-H2AX expression to detect the effects of H_2O_2 on DNA - if H_2O_2 induced DNA damage in HUVECs.

Immunofluorescence staining showed that in stressed cells compared to control cells the expression of γ-H2AX, a prominent marker of DNA damage, has been higher throughout the course of the experiment. In both control cells and stressed cells, the γ-H2AX expression gradually increased until day 15.

This data revealed that H_2O_2 stress led to DNA damage growth arrest and support the notion of senescence induction. Cell proliferation become impaired as indicated by first increase in γ-H2AX expression then a decrease in Ki67 expression and a decrease in cell number.We then progressed to evaluate another senescence associated feature, the activation of the senescence associated secretory phenotype (SASP) as assessed by the induction of the prolonged upregulation of ICAM-1.

4.1.2 Intermittent exposure of HUVECs to H_2O_2 stimulated lasting ICAM-1 expression

Cellular senescence is associated with the induction of a proinflammatory phenotype that causes chronic inflammation through secretion of proinflammatory cytokines, chemokines and proteases. One of these proteins named ICAM-1 is a cell surface protein and is critical for the firm arrest and transmigration of leukocytes out of blood vessels and into tissues. ICAM-1 is constitutively present on endothelial cells at very low levels, but its expression is increased by proinflammatory cytokines in senescent cells.



Cells were exposed to 50µM H_2O_2 for one hour every 48 hours over a period of 8 days thereafter the cells were cultured in EC growth medium only for 7 days.

In accordance with previously data, we suggest that this intermittent exposure to H_2O_2 (50 µM) has induced cell cycle arrest, as described above. To strengthen our concept that this intermittent exposure of our HUVEC to H_2O_2 (50 µM) led to induction of premature senescence, we measured ICAM-1 expression one day after every H_2O_2 exposure over a period of 15 days by cell ELISA.

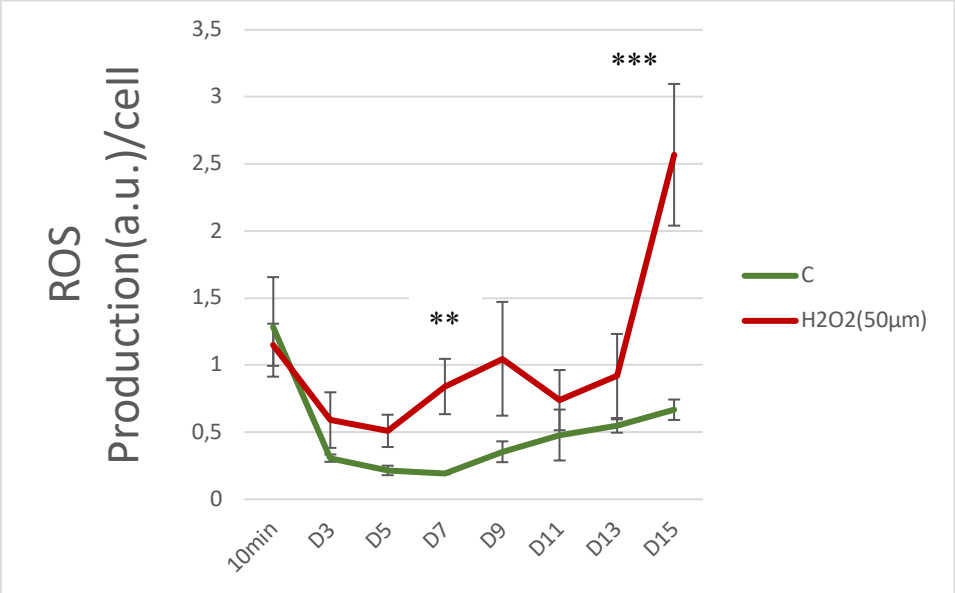
We observed a statistically significant ($p<0.001$) increased expression of this adhesion molecule in cells exposed to H_2O_2 (50 µM) for one hour every other day, compared to our control cells on days 7, 9. We observed an increased level of ICAM-1 from day five until day nine (reached the maximum), followed by a reduction on day eleven and day 15

The ICAM-1 expression by control cells increased slightly until day 9 but was still significantly lower than in H_2O_2 stressed cells over the whole observation period.

Our data show that intermittent exposure of HUVECs to H_2O_2 has not only induced cell cycle arrest but also upregulated the expression of SASP protein ICAM-1. Both results are in accordance with the notion that exposure of HUVECs to H_2O_2 induced senescence in our cells.

4.1.3 Intermittent exposure of HUVECs to H_2O_2 induced generation of ROS production

Reactive oxygen species (ROS) are reactive intermediates of molecular oxygen that act as important second messengers within the cells. However, an imbalance between the generation of reactive ROS and antioxidant defense systems represents the primary cause of endothelial dysfunction, leading to vascular damage. Increased production of reactive oxygen species (ROS), mostly produced by dysfunctional mitochondria in senescence cells.



We measured the cellular ROS production over a period of 15 days to assess if our cells exposed to H_2O_2 increase their level of ROS production. HUVECs (P5) cells stressed with H_2O_2 (50 µM) for 1hour every 48 hour until day 8 then H_2O_2 was replaced with EC growth medium only till the end. ROS measurements were performed one day after H_2O_2 exposure to investigate both the acute and long-term effects.

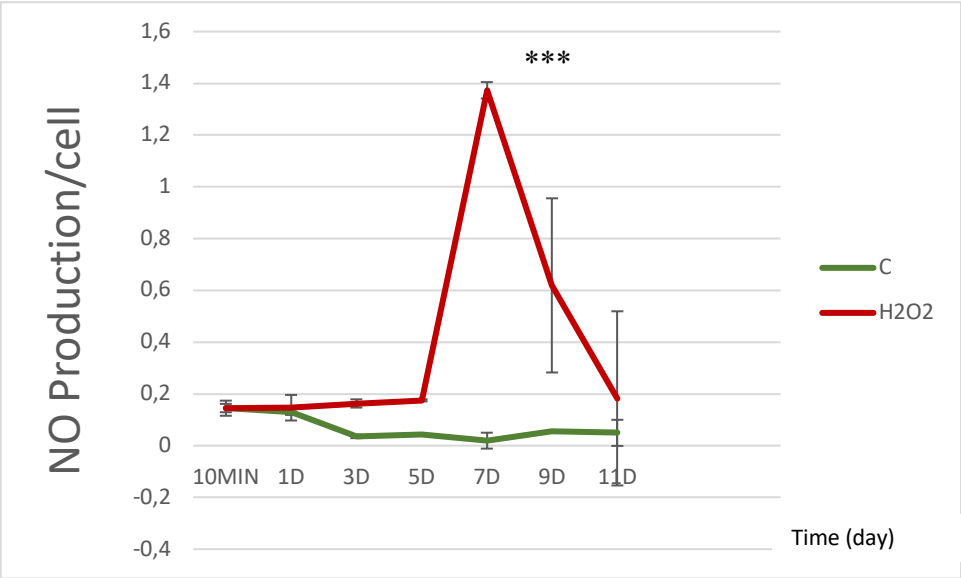
Treatment with H_2O_2 led to increased generation of ROS in HUVECs. In stressed cells the ROS production was somewhat higher than control until day 13 then the ROS generation increased even more significantly on day 15. ROS is short lived and even in recovery phase high ROS production have been detect specially on day 15, which mean that in stressed cells the expression level of Enzyme, which important for ROS balance (antioxidant enzyme) might be decreased.

These data revealed an increased also in ROS generation in HUVECs after exposure to H_2O_2 (50µM) for one hour, so this could also contribute to increased cell cycle arrest by damaging

DNA and decreased proliferation in HUVECs. Such upregulated ROS level would be compatible with our previous results (cell number, ki-67, γ-H2AX, ICAM-1) that endothelial cells became senescent

4.1.4 Intermittent exposure of HUVECs to H_2O_2 led to higher NO production

Nitric oxide (NO) is a soluble gas continuously synthesized from the amino acid L-arginine in endothelial cell. NO is a parameter that controls vascular tone and homeostasis, and it was originally identified as endothelium derived relaxing factor (EDRF). We evaluated NO because it plays a vital role in endothelial health and endothelial dysfunction is often associated with its loss.



The NO production in H_2O_2 treated cells reached the maximum at day 7 and decreased again until day eleven. For our control cells the NO level hasn´t changed much after day three. NO level was significantly higher in the H_2O_2-treated group compared to the control group. On day 11 however, when the cells were no longer stimulated with H_2O_2, the NO level dropped significantly. Unexpectedly, NO production increased by stressed cells between days 5 and 9 only temporarily before decreasing again. My interpretation would be that the cells may have attempted to maintain the NO production.

These results together with cell number, Ki67, γ-H2AX, ICAM-1, ROS demonstrate that changes

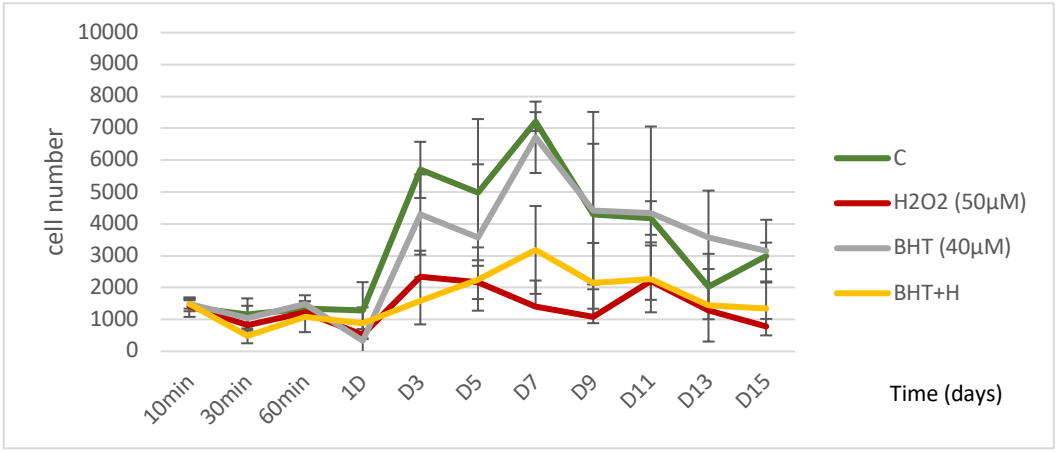
are induced because of oxidative stress and that these changes would be compatible with the interpretation that cells might have entered a state of senescence

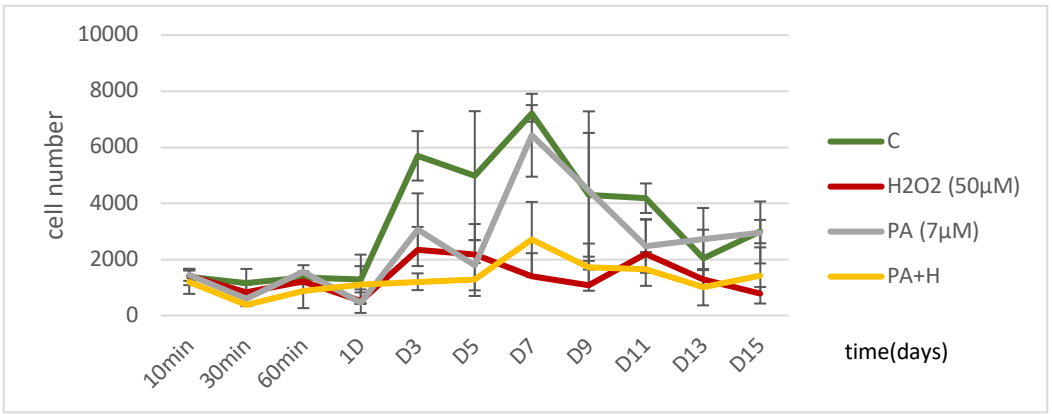
4.2 Assessment of BHT and PA for their potential to prevent oxidative stress-induced changes to HUVECs

Increased releases of H_2O_2 in the cytoplasm of aging cells resulted in activation of NF-κB [143]. NF-κB is one of the redox-sensitive transcriptional factors that is activated by excessive ROS production and leads to increasing the gene expression of cytokines (TNF-α, IL-1, and IL-6), adhesion molecules (ICAM, VCAM), and proinflammatory enzymes (iNOS, COX-2) [162]. the strong relationship between oxidative stress, inflammation, and aging, the oxidation-inflammatory hypothesis of aging, also known as oxi-inflammaging, has been proposed: aging is a breakdown of homeostasis caused by chronic oxidative stress that affects the regulatory systems, including the nervous, endocrine, and immune systems. The immune system's activation causes an inflammatory response, creating a cycle in which chronic oxidative stress and inflammation feed off each other, increasing age-related morbidity and mortality [1]. Then we here used antioxidant compounds like BHT and anti-inflammatory compound PA to assess their preventive effect the induction of senescent through oxidative stress. BHT is the most widely used synthetic phenolic antioxidant in food, pharmaceuticals and cosmetics and PA is a natural product derived from lichens have been extensively studied for their biological properties and have shown a strong inhibitory effect on NF-κB activation.

4.2.1 BHT and PA use may prevent H_2O_2-induced growth inhibition in HUVECs

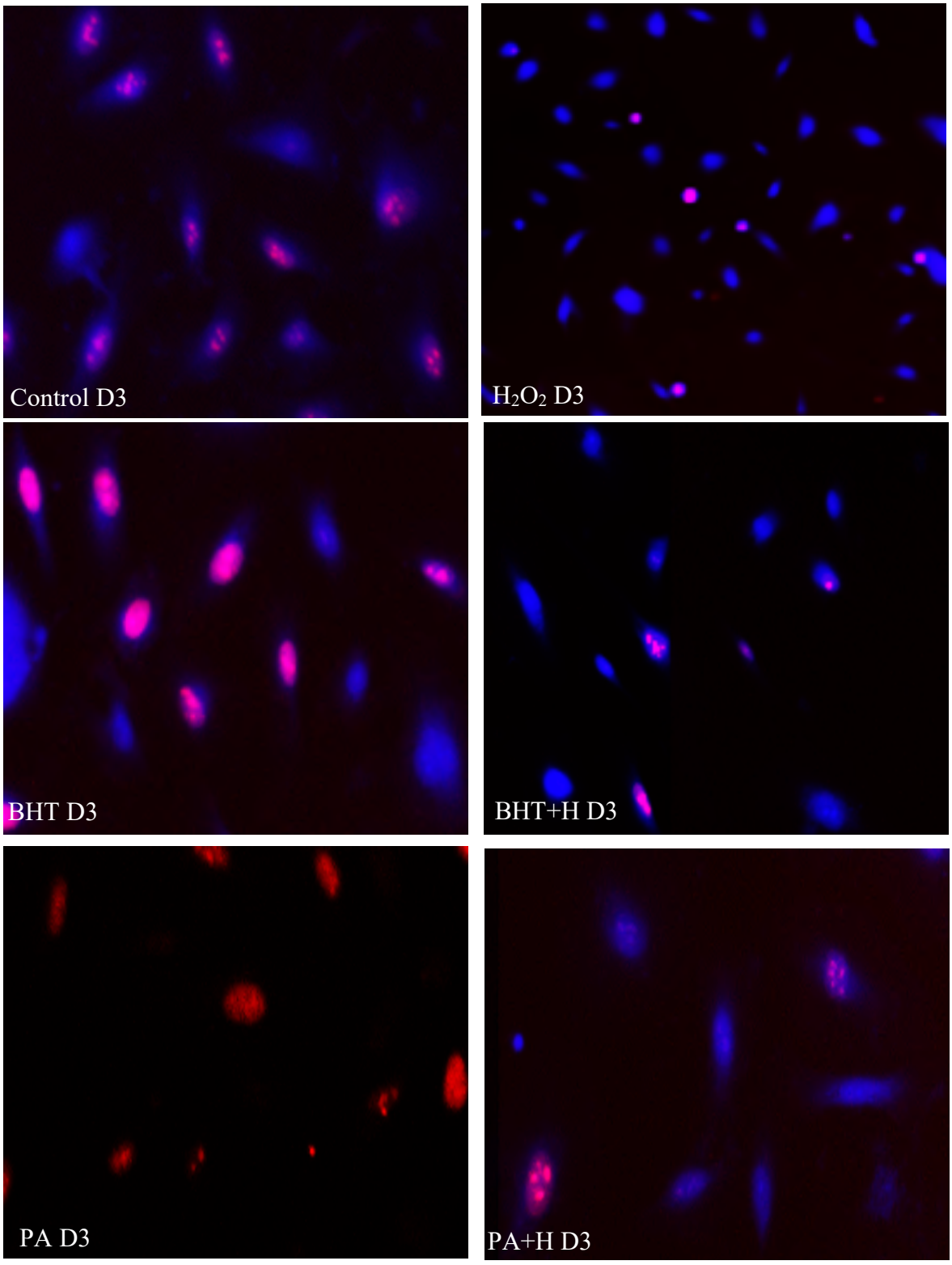
In this experiment we have used two antioxidant substances, BHT (40µM) and DPI (10µM) and anti-inflammatory PA(7µM) to assess, if these 3 substances might be able to prevent the progression from acute oxidative stress to cellular senescence, when applied after setting the stress. BHT is a synthetic antioxidant. BHT might be considered as positive control for the prevention of oxidation-induced effects although in this experimental setup, it was applied only right after the one-hour-long oxidative stress procedure and not during the exposure to H_2O_2 itself. We applied BHT (40µM), PA(7µM), DPI (10µM) to the EC growth medium in our experiment within the first eight days, each time after exposing the cells to H_2O_2.



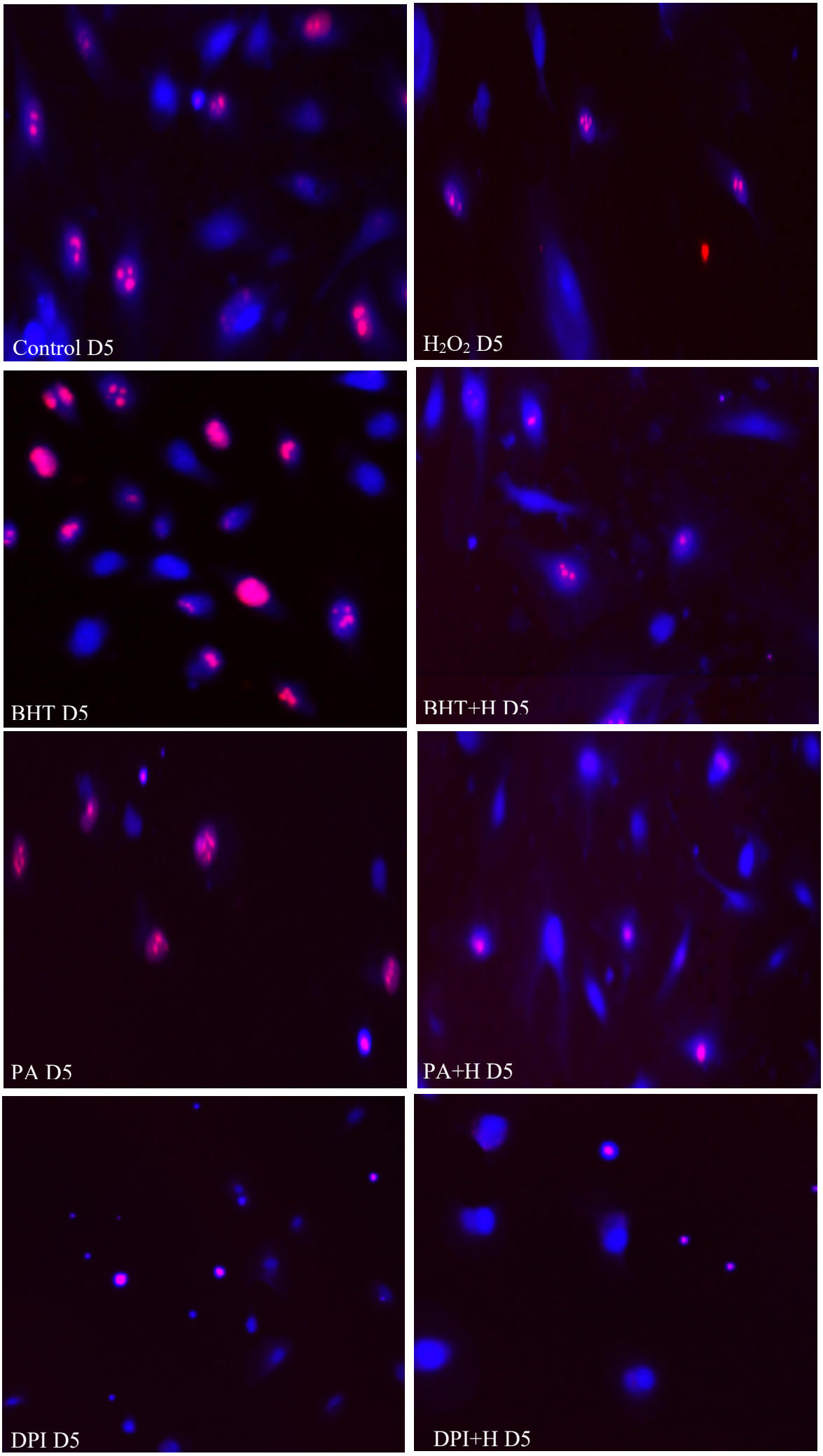


We observed that the treatment of H_2O_2 -stressed cells with BHT or PA led to somewhat increased cell proliferation on day 7 and 9. Compared to the H_2O_2 (50 µM) stressed cells, cells post treated with PA, still seemed to be able to proliferate between day 13 and 15. The growth curves of our post treated cells demonstrates this only mild preventive (if any) effect of our reagent

Ki67 DAPI
Day 3

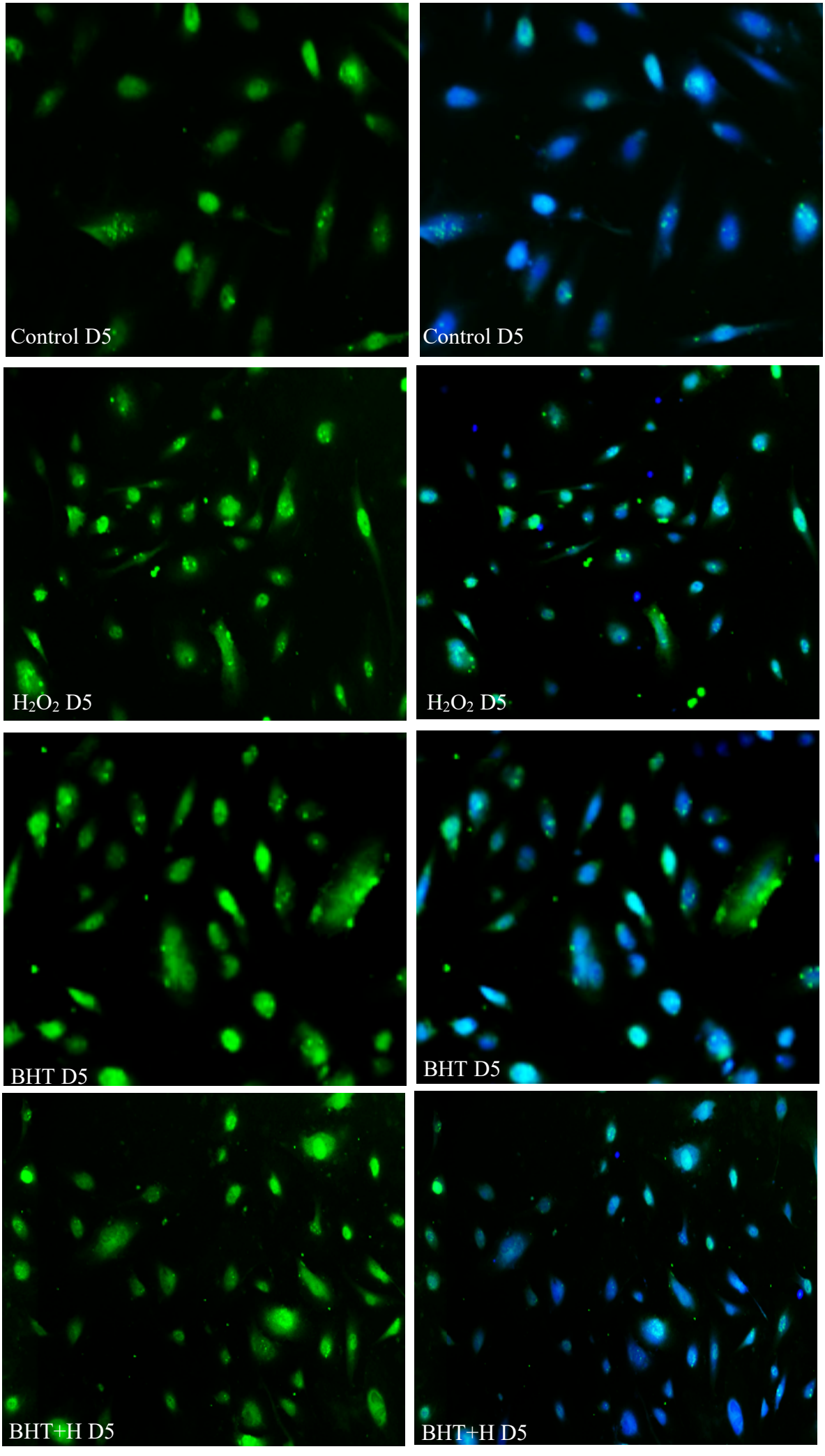


Ki67 DAPI



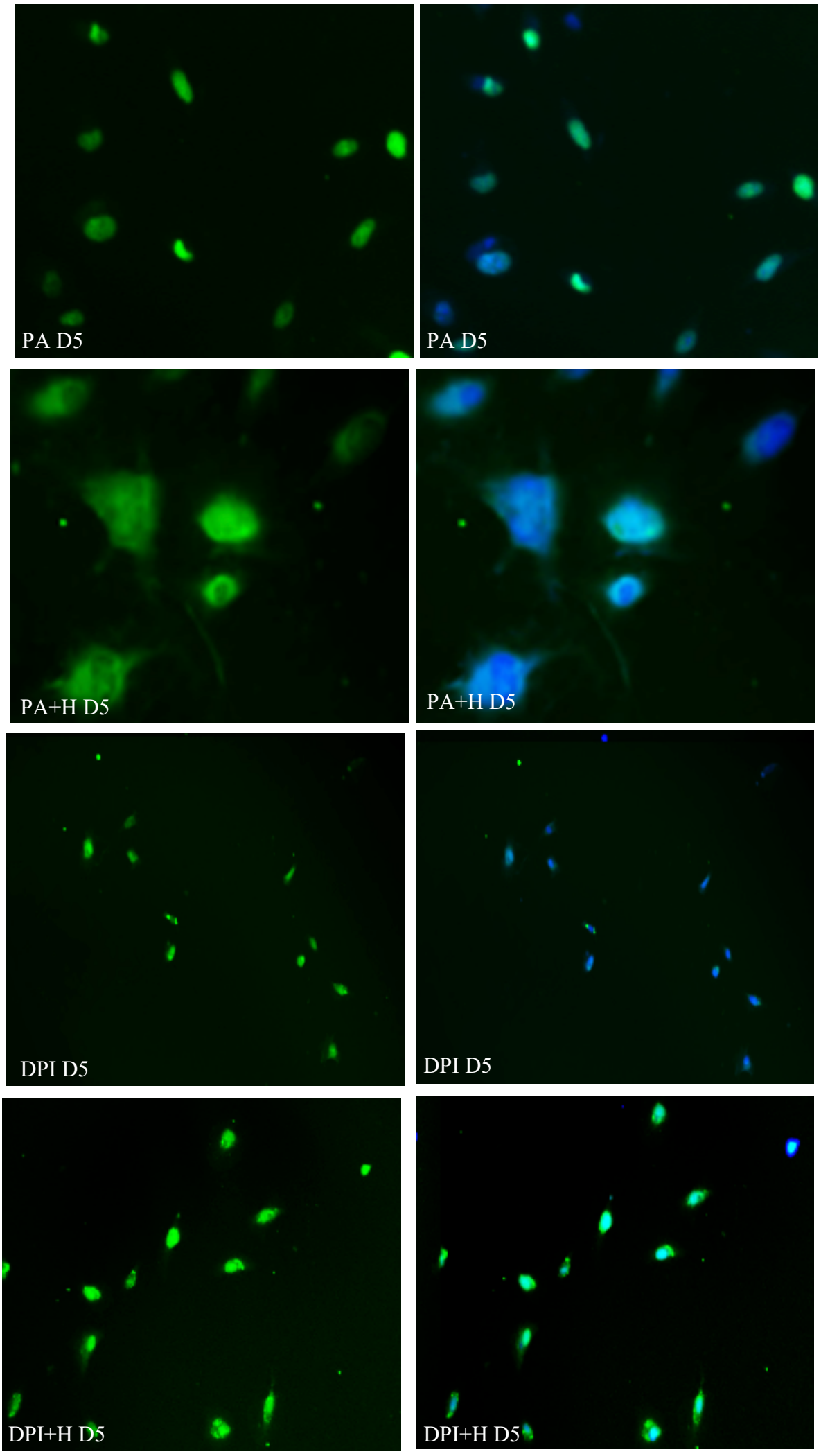
γ-H2AX

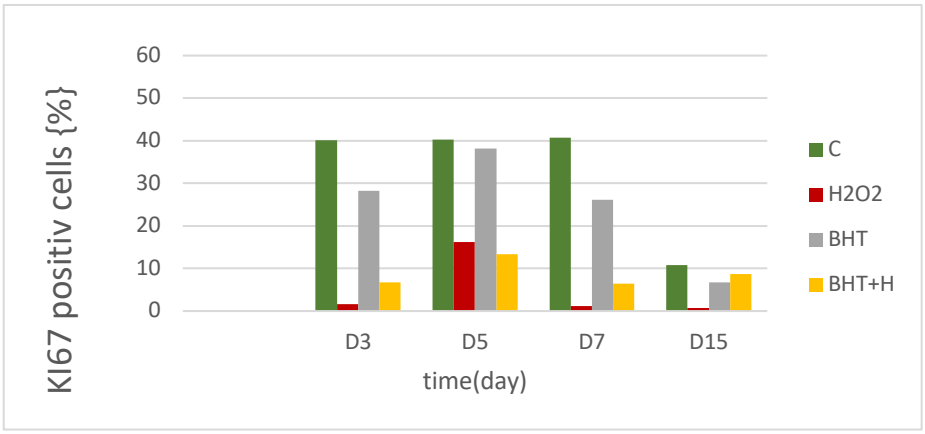
Merged

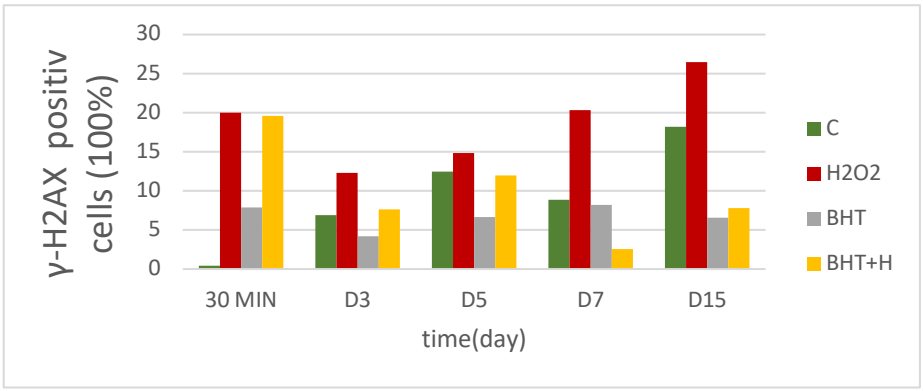


γ-H2AX

Merged




KI67 positiv cells {%}
60
50
40
30
20
10
0
D3
D5
D7
D15
time(day)
C
H2O2
BHT
BHT+H


γ-H2AX positiv cells (100%)
30
25
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D3
D5
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time(day)
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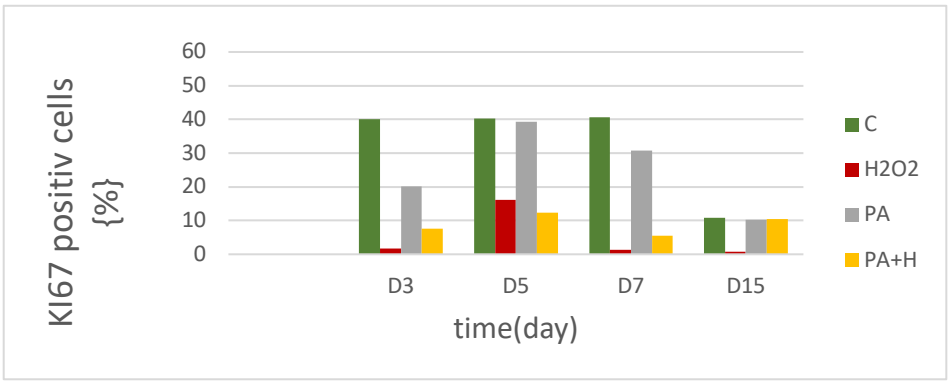
H2O2
BHT
BHT+H


KI67 positiv cells {%}
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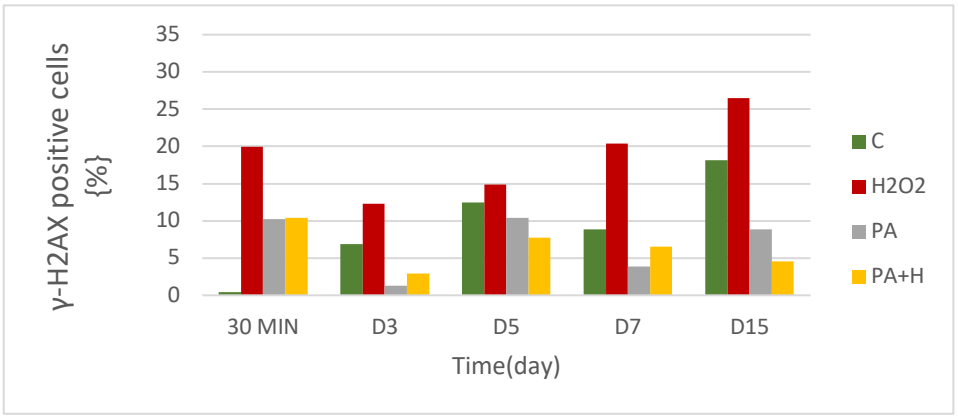
PA+H


γ-H2AX positive cells {%}
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D15
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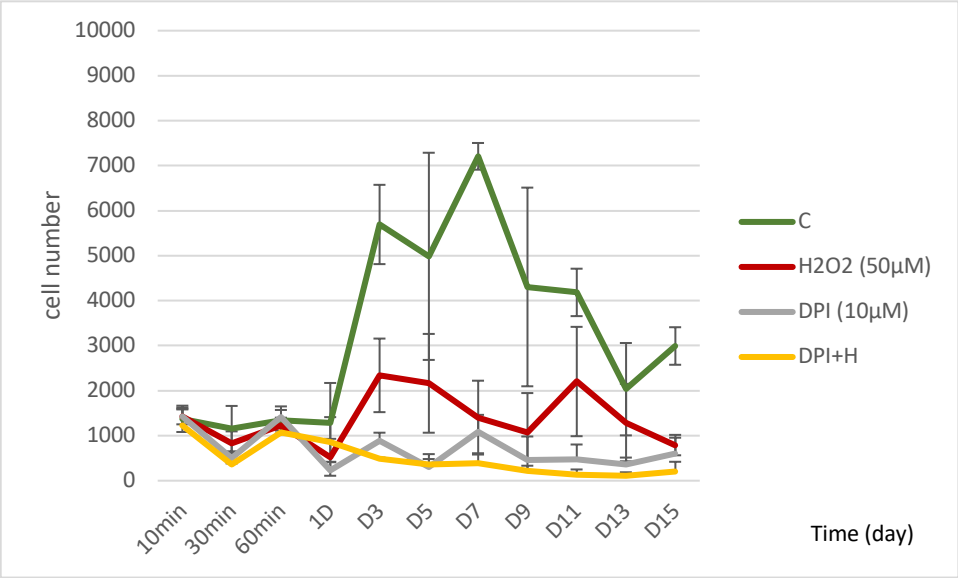
H2O2
PA
PA+H

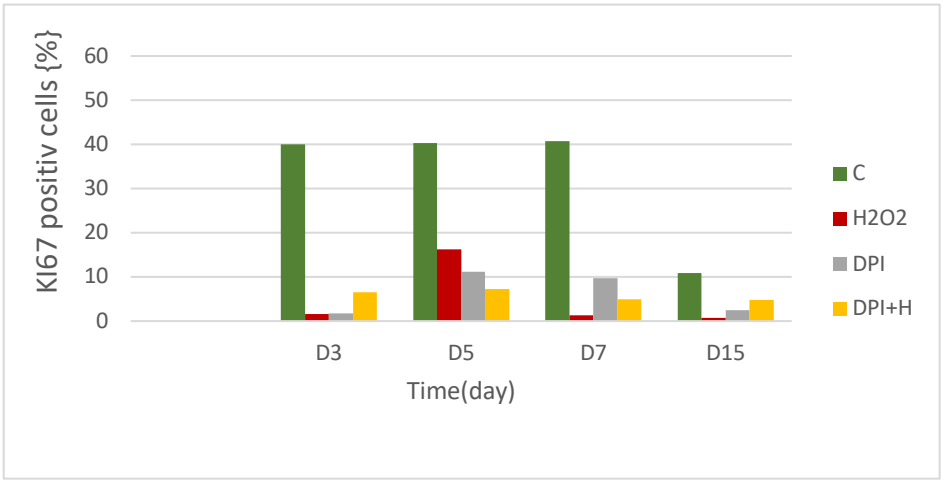
H_2O_2 impaired the proliferation of HUVECs from day 3 on and decreased the portion of cells expressing the proliferation marker Ki67 throughout the experimental period, while cells post treated with BHT and PA after H_2O_2 exposure, reduced the loss in the %age of $Ki67^+$ cells on days 3, 7 and 15.

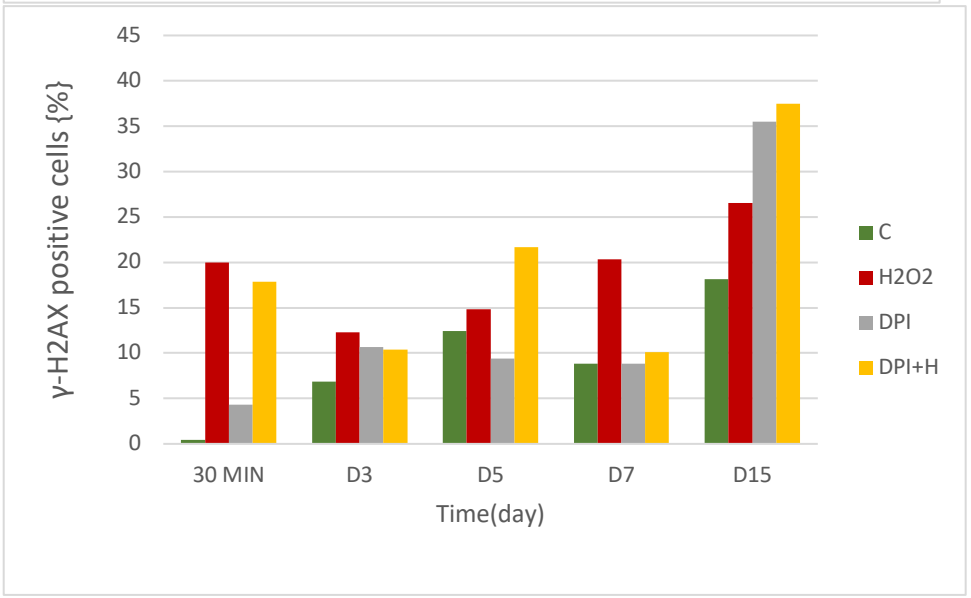
Also, BHT and PA, reduced the number of cells showing DNA damage (through expression of γ-H2AX. γ-H2AX is significant decreased on day 7 and 15 compared to stressed cells. Together with our previous data (cell growth curve) cells post-treated with the respective substances improved the cell proliferation and prevented at later time points DNA damage and thus the accumulation of γ-H2AX.



Diphenyleneiodonium (DPI) is an NADPH oxidase (NOX) inhibitor that is widely used as antioxidant. Here we wanted to assess whether DPI can prevent the H_2O_2 induced reduction in proliferation in HUVECs.

We applied our antioxidant, DPI, to the EC growth medium within the first 8 days, each time after exposure to H_2O_2. The results show that the use of DPI led to cell loss right from the beginning indicating that the concentration for DPI used here was toxic to our cells.





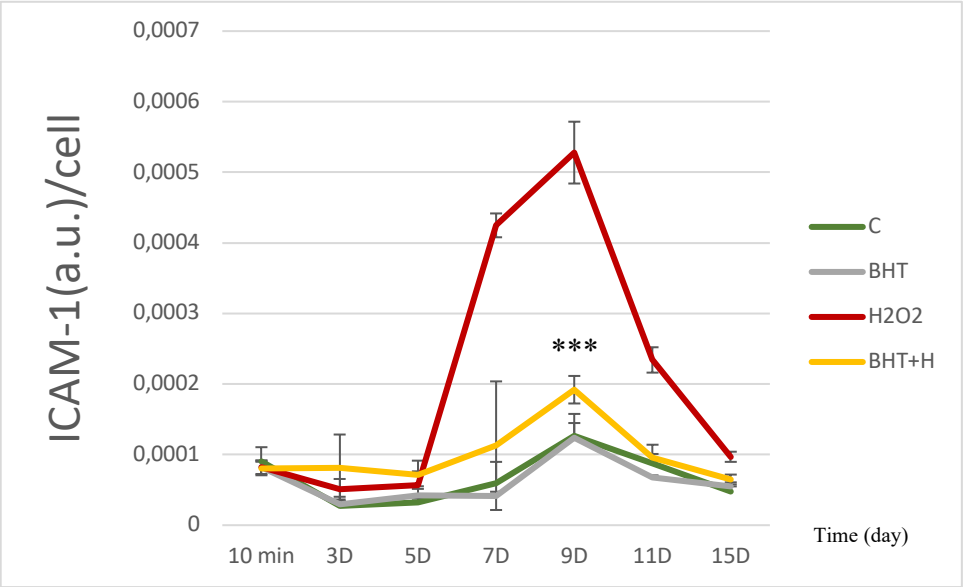
Our data showed that Ki67 expression in surviving DPI treated cells was drastically reduced at day 3 both, intensity per cell and %age in Ki67 expressing cells while by DPI as well as DPI+H the expression of γ-H2AX is high in comparison with positive control on day 5 and 15, which perhaps might be explained by the toxicity of DPI at this concentration. Exposure to DPI of HUVECs cells with or without H_2O_2 resulted in a significant decrease of cell proliferation as well as increase DNA damaged marker γ-H2AX.

Our cell number, Ki67and γ-H2AX outcome proves the toxic effect of DPI by this concentration. Next, we measured ICAM-1 expression as one of SASP protein to determine further evidence for the protective properties of our reagents.

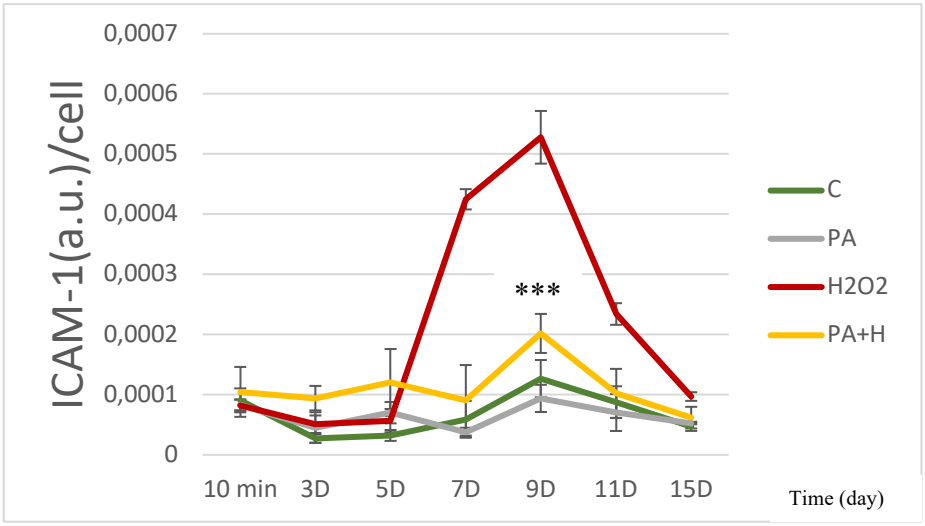
4.2.2 BHT and PA counteract the H_2O_2 induced expression of ICAM-1

We selected ICAM-1 as representative SASP-protein to show if our inhibitors would prevent the induction of elevated ICAM-1 levels associated with developing the senescence phenotype. As inhibitors we used, as before, BHT (40µM), PA (7µM), and DPI (10µM).

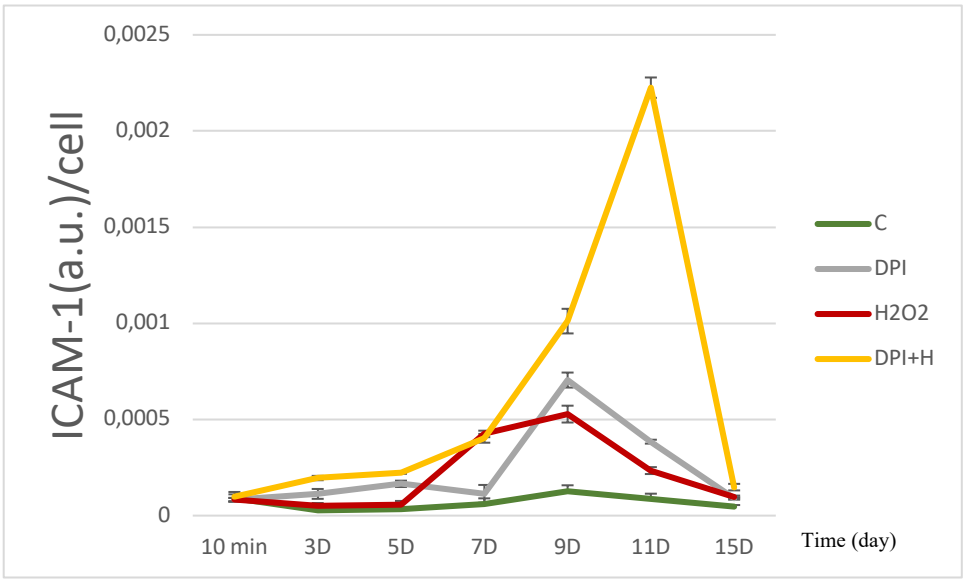
After treating the stressed cells with BHT, the ICAM-1 expression was lower than in "H_2O_2 only" treated cells, from day 7 on. During this period there is no great difference in the ICAM-1 expression between negative control and BHT alone.



By PA also, the expression of ICAM-1 is significantly decreased from day 7 on compared to stressed cells. These results showed a significant prevention of upregulation in these SASP protein in H_2O_2 stressed cells.

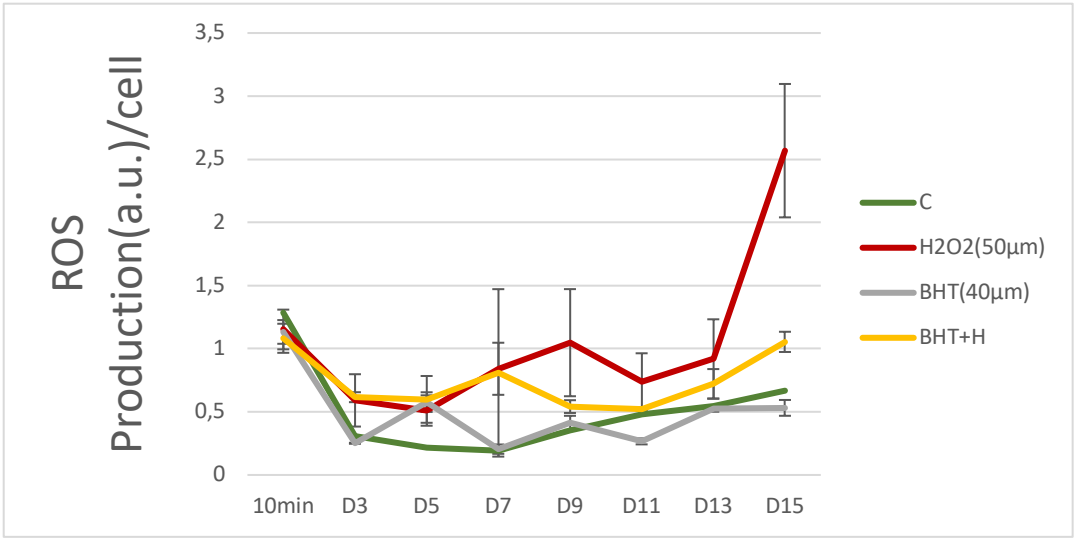


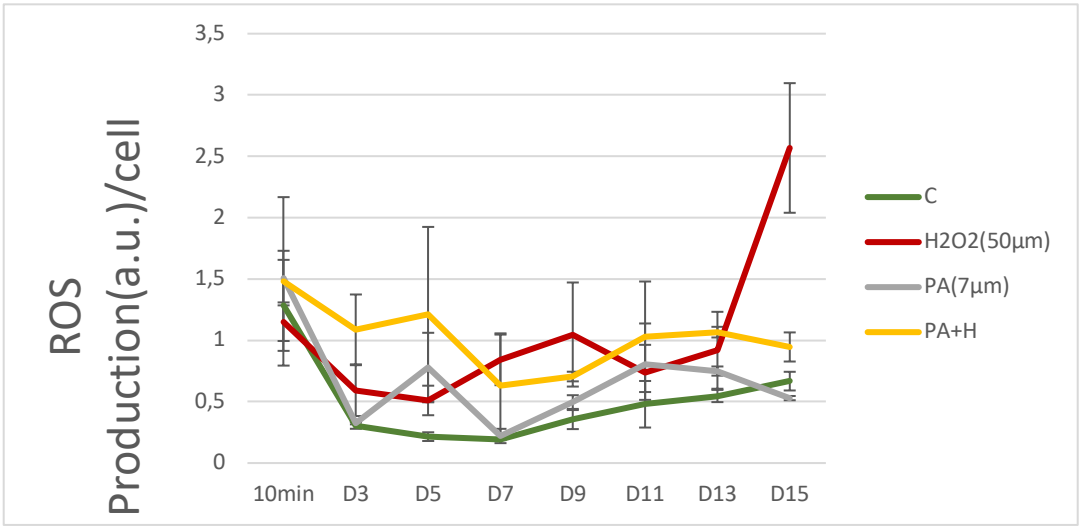
The use of DPI at a concentration of 10µM lead to significantly increased ICAM-1 expression from day 7 until day 11 and decreased thereafter until day 15. Our data showed that DPI+H as well as DPI alone the ICAM-1 expression is much higher than H_2O_2 -stressed cells by themselves, especially at later time points (until day 11). DPI couldn´t counteract the upregulated ICAM-1 expression by H_2O_2 and also here support our previous results that DPI at the used concentration was toxic for these cells.



4.2.3 Treatment of H_2O_2-stressed HUVEC with BHT reduced the H_2O_2-induced the generation of ROS

Here we wanted to assess if used inhibitors could prevent the H_2O_2 induced (50µM) ROS production in HUVECs. For this proposal, we used, during the first nine days, BHT (40µM), PA (7µM) and DPI (10µM) after exposing the cells to H_2O_2. We measured the ROS production one day after exposure to H_2O_2.

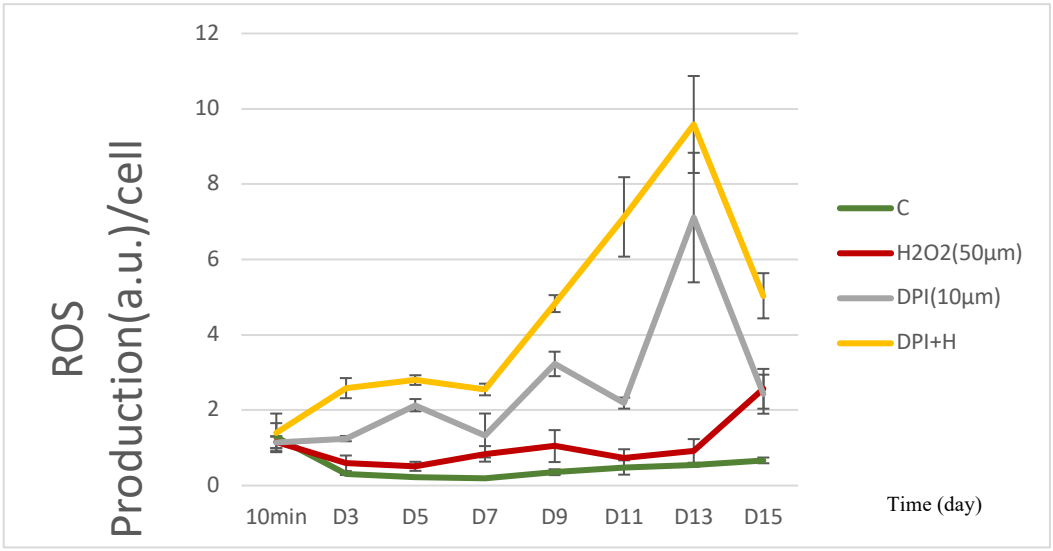




We found that use of BHT prevented a strong response (ROS production) to the H_2O_2-exposure. Beginning with day 9, and until day 13, the ROS production values are lower than in the positive control and by day 15 the difference became very pronounced.

Perlatolic acid seemed to show an effect only by day 15.

We observed that in the cells stressed with H_2O_2, the use of BHT and PA led to decrease in ROS level on day 15. The ROS production was increased in BHT+H from day 11 after the treatment were stopped. Also, the preventive effect of our reagent was clear on day 15.

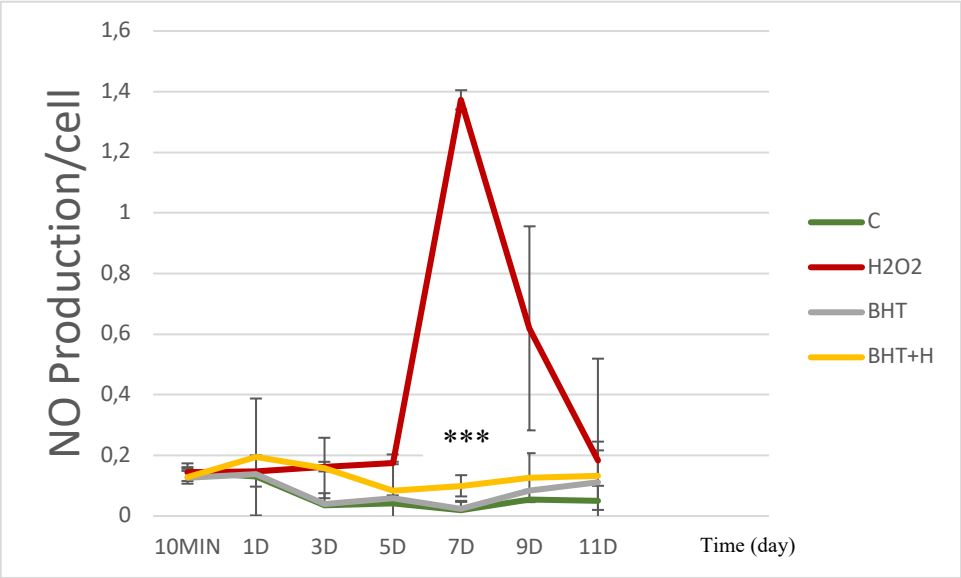


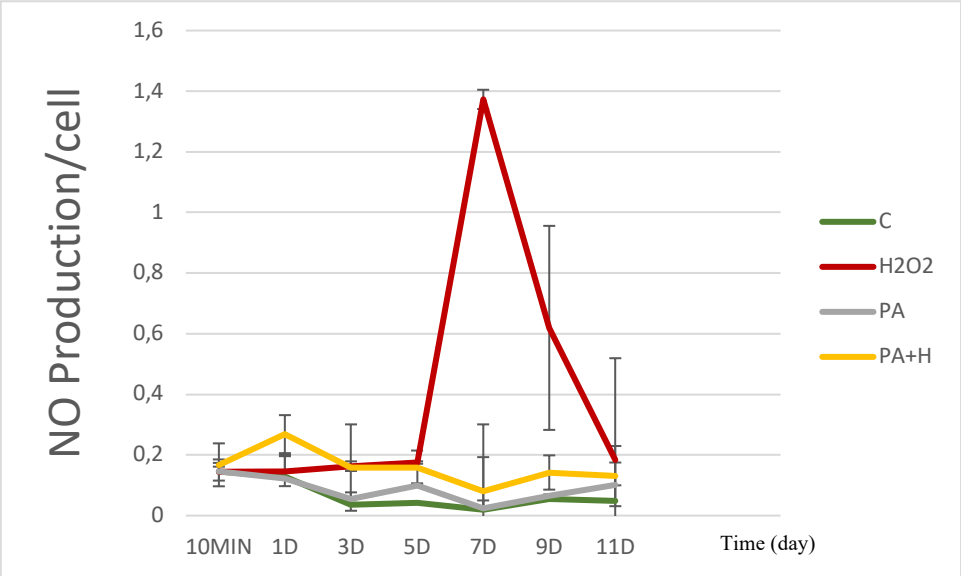
Our data showed that by DPI+H and DPI alone the ROS production was higher than H_2O_2 during the whole experiment. Although DPI, as an NADPH oxidase inhibitor, should be expected to decrease the cellular generation of ROS, our results showed the opposite; by DPI the ROS production was increased.

Overall, our results show that both, BHT and PA reduced ROS production until day 13 only minimally. However, both inhibitors markedly reduced the ROS production on day 15. DPI increased ROS production during the whole experiment due to its toxic concentration.

4.2.4 BHT and PA partially prevented NO production in response to H_2O_2

After having shown that BHT and PA are efficient in preventing the induction of DNA damage and in preventing the expression of ICAM-1 in HUVECs we measured their preventive effect on NO production upon H_2O_2 -exposure.

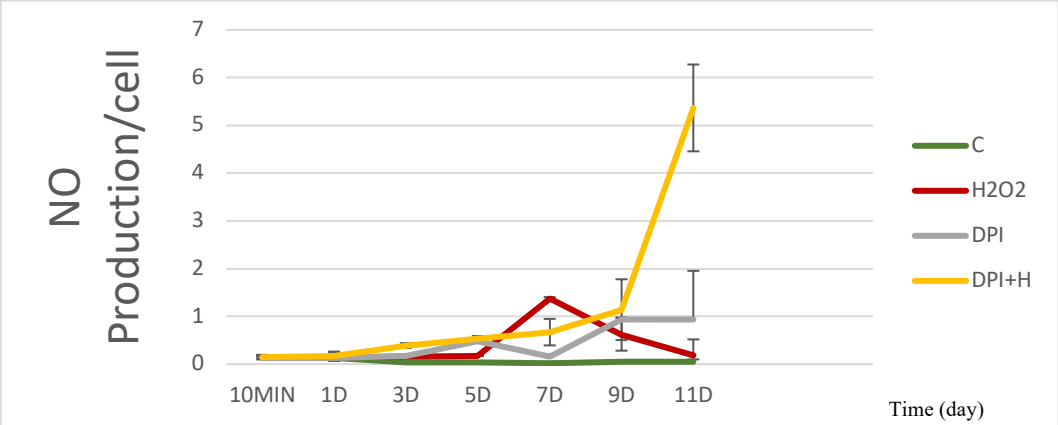




In stressed cells, by days seven and nine, the oxidative stress led to an obvious increase in cellular NO production. This effect could be blocked by application of either antioxidant BHT or PA. In the case of DPI, the low number of surviving cells prohibited assessment of its inhibitory capacity.

The lowest NO output can be seen in control cells, and close is BHT alone.

By post treated stressed cells with BHT the NO production increased on day 1 then decreased gradually until day 5 and from day 5 the NO production hasn´t changed much until day 11. Compared to stressed cells the NO production was significantly reduced on day 7 and 9. We noticed the same by PA+H. By PA alone the development of NO is somewhat analogous to control cells. In stressed cells treated with PA the NO production slightly increased on day 1 then decreased gradually until day 11.



The NO production by stressed cells treated with DPI reached the maximum at day 11 and contrary to other inhibitors NO expression is at most of the time point higher than H_2O_2 alone.

Taken all results together cell number, Ki67, γ-H2AX, ICAM-1, ROS and NO, the post treatment with BHT and PA seems to have prevented to some extent the induction of oxidative stress in endothelial cells.

The results are summarized as the mean ± SD of sextuplicate for the untreated and H_2O_2-treated groups (C and H), as well as triplicates for the inhibitor-treated groups

Here I present the summary of our results.

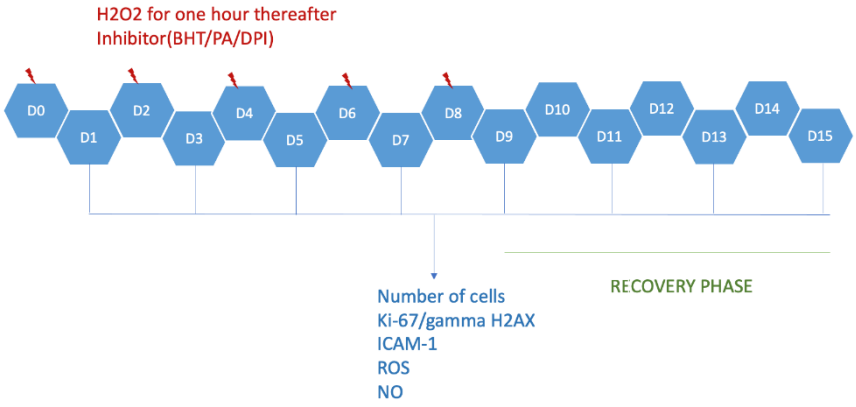
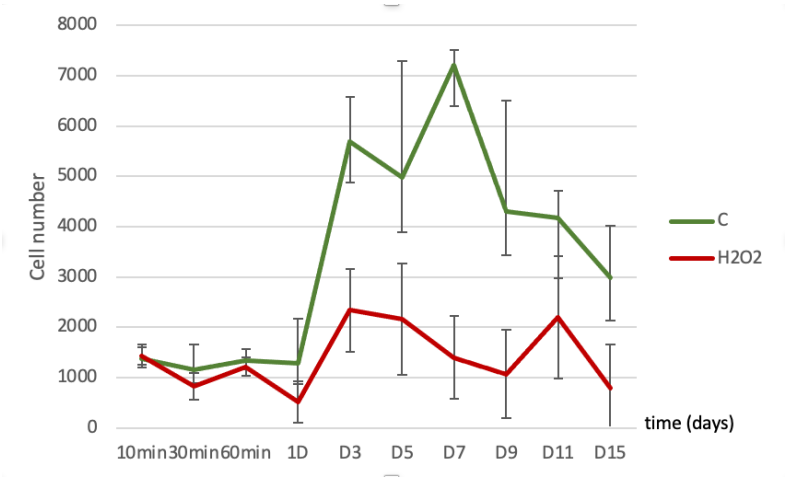


Figure 1. Experiment design.
Gelatin was applied to the 96-well plates. Endothelial cells (P5) were then seeded in EC growth medium for 24 hours before being exposed to H_2O_2 (50µM) for one hour, except for the cells used as a negative control. The H_2O_2 was then replaced with either EC growth medium only or containing BHT (40µM), PA(7µM) or DPI (10µM). for the next days
On days two, four, six, and eight, the exposure was repeated. The cells were grown in EC growth medium only after day ten (recovery phase). The cells were then grown in EC growth medium until day fifteen. Assessments took place always 24 hours after the respective exposure to H_2O_2, on days three, five, seven, nine, eleven, thirteen and fifteen.

Figure 2.

a)



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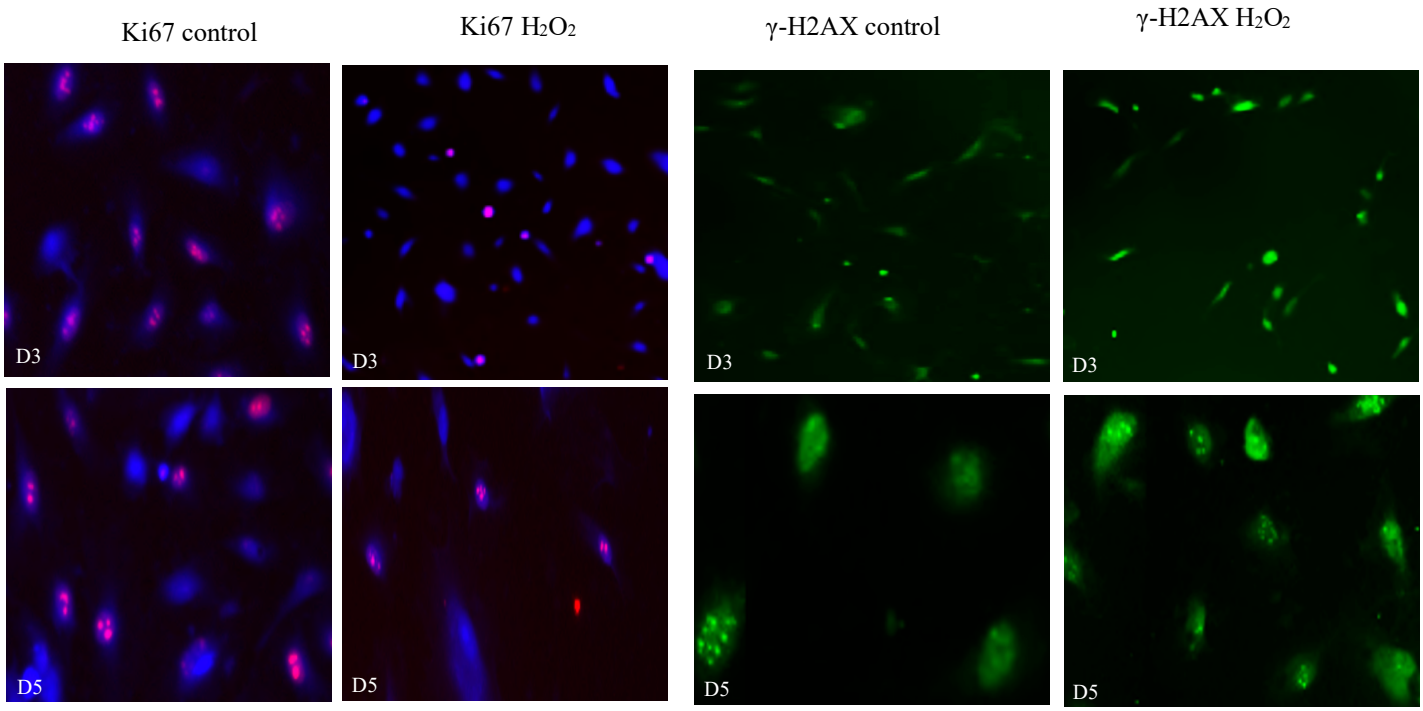

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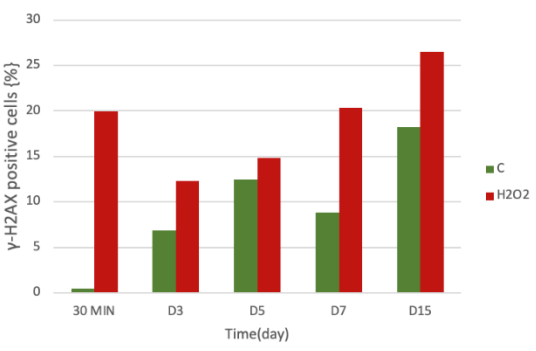

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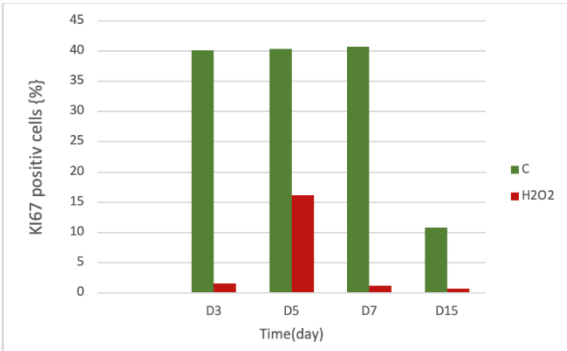

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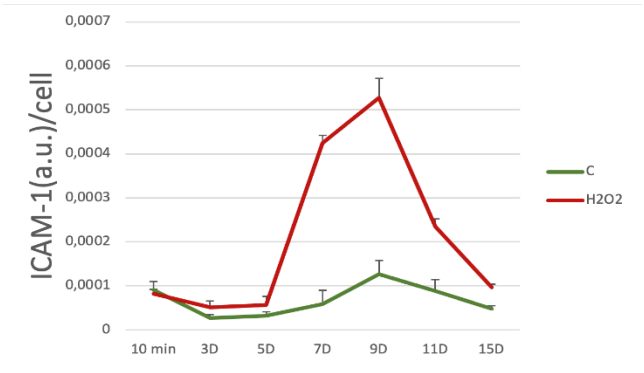

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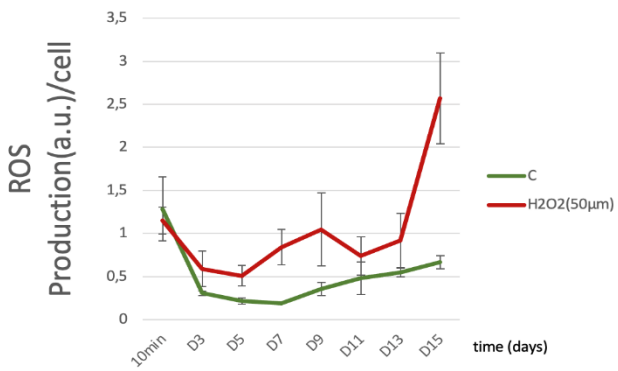

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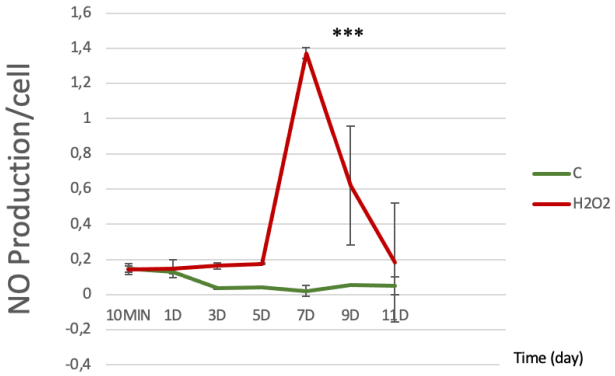
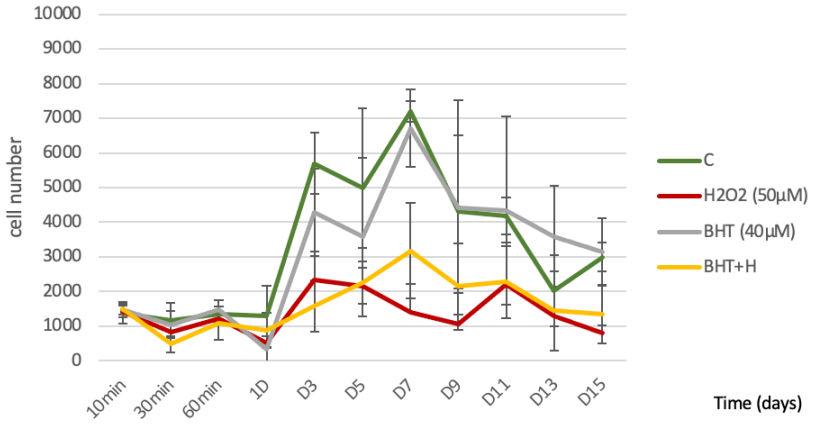


Figure 2. Effects of eight days of cultivation of HUVECs exposed to intermittent repeated low concentrations of H_2O_2 (50 µM) followed by a seven-day recovery phase. a) Retardation of growth in H_2O_2 stressed cells. b) representative images of immune-fluorescent detection Ki67 and γ-H2AX in HUVECs. c) Quantification of the changes in the percentage of Ki67 positive cells in cultures of H_2O_2 -stressed HUVEC. d) Percentage of γ-H2AX positive cells.
e) Surface expression of ICAM-1 determined by ELISA. f) Time course of ROS production.
g) NO production.

The results are summarized as the mean ± SD of sextriplicates for the untreated and H_2O_2 -treated groups (C and H), as well as triplicates for the inhibitor-treated groups except Ki67 and γ-H2AX the outcome is a result of single well.

Figure 3.

a)



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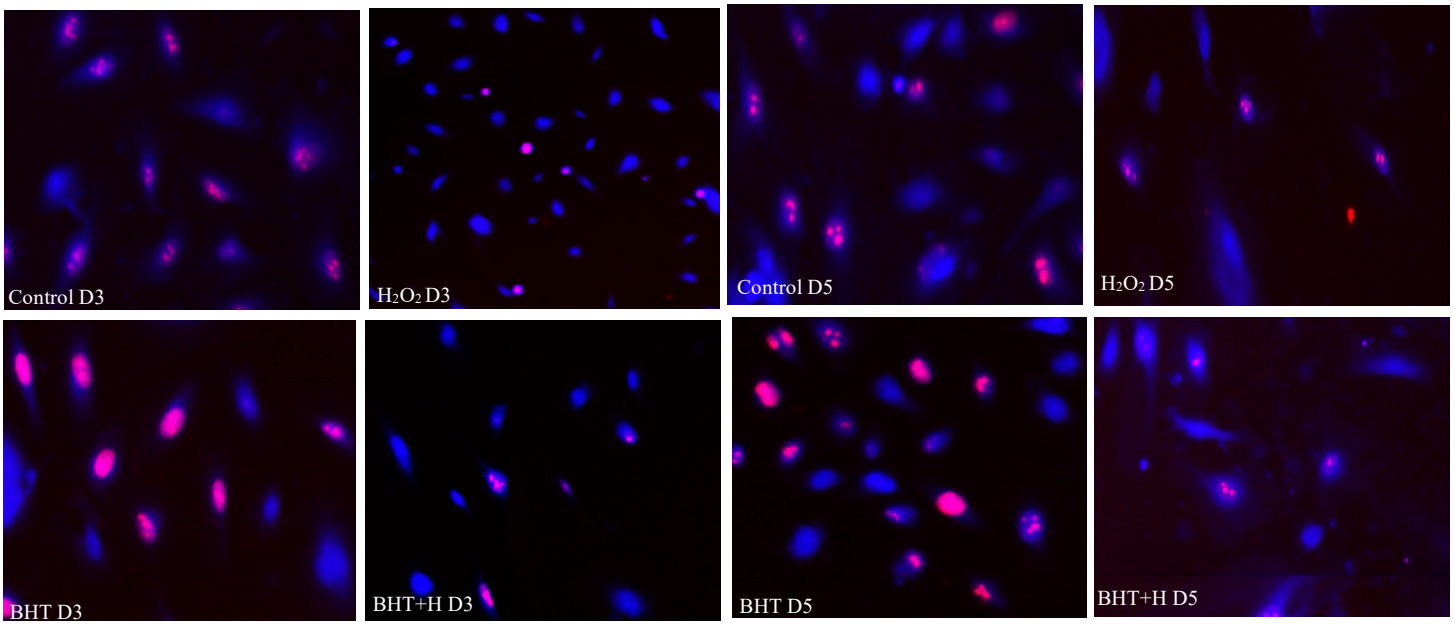
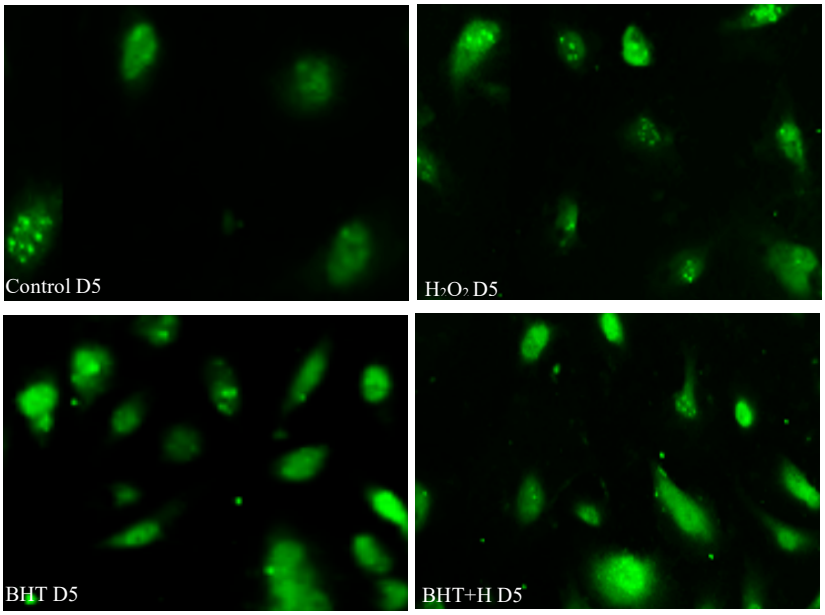
Ki67



γ-H2AX



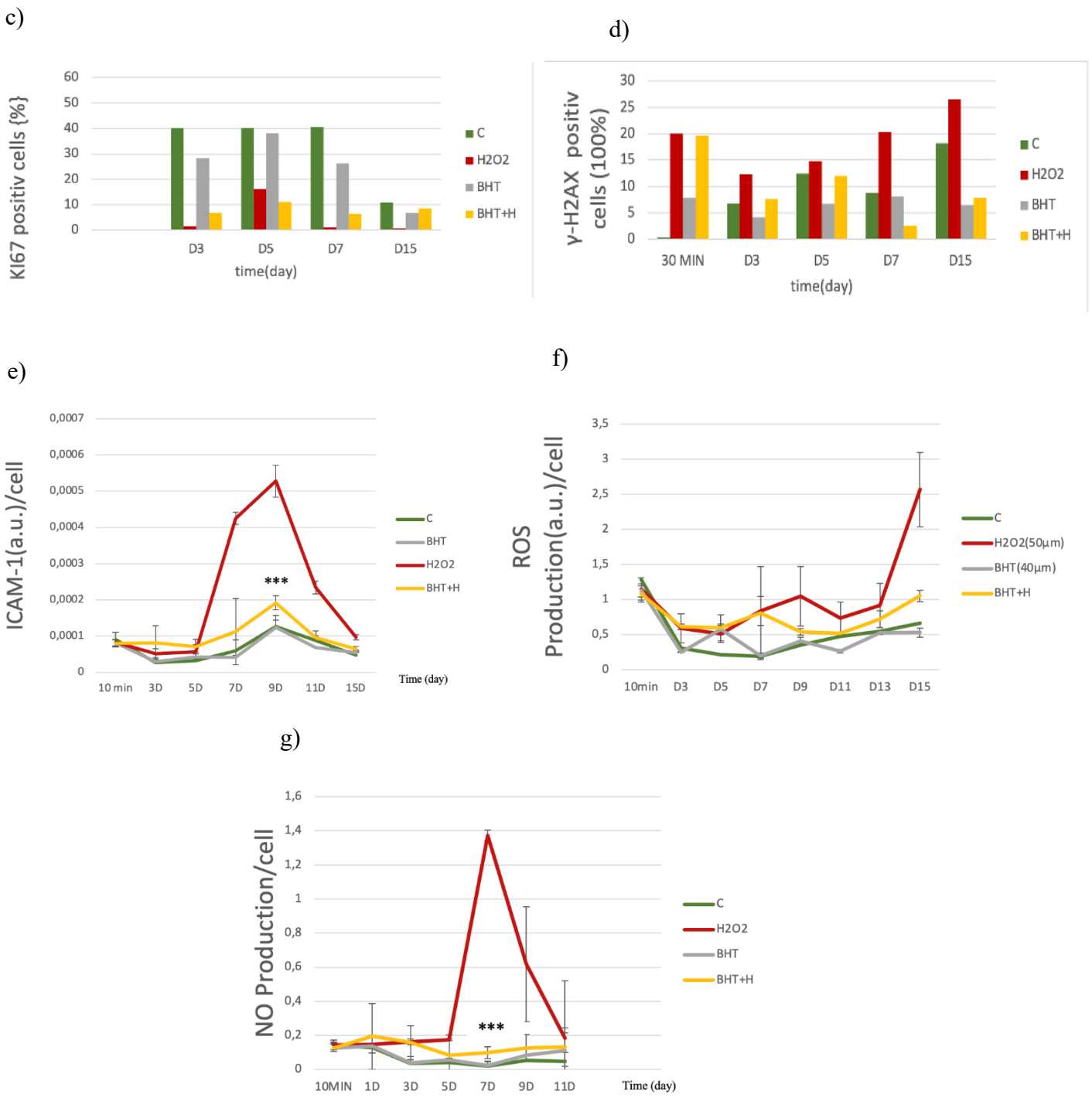
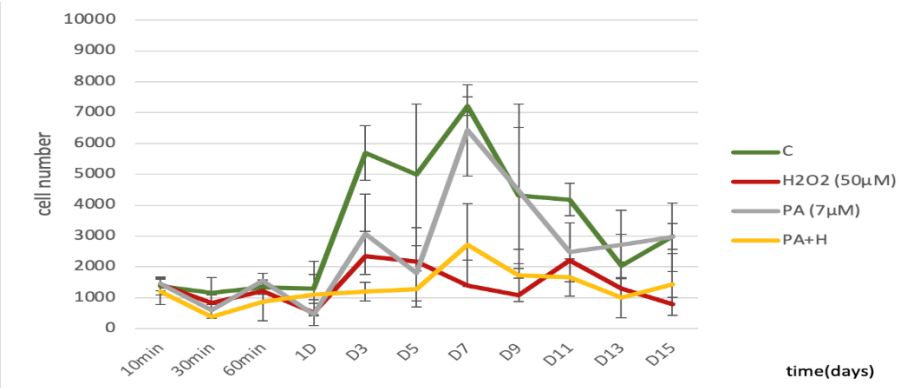


Figure 3. Preventive effect of BHT in H_2O_2 stressed cells. HUVECs cells were stressed with H_2O_2 for one hour every 48h. After that, cells were incubated with EC growth medium only, or medium containing BHT. a) Retardation of growth in H_2O_2 stressed cells. b) representative images of immune-fluorescent detection Ki67 and γ-H2AX in HUVECs. c) Quantification of the changes in the percentage of Ki67 positive cells in cultures of H_2O_2 -stressed HUVEC. d) Percentage of γ-H2AX positive cells. e) Surface expression of ICAM-1 determined by ELISA. f) Time course of ROS production. g) NO production.

The results are summarized as the mean ± SD of sextriplicate for the untreated and H_2O_2 -treated groups (C and H), as well as triplicates for the inhibitor-treated groups except Ki67 and γ-H2AX the outcome is a result of sing

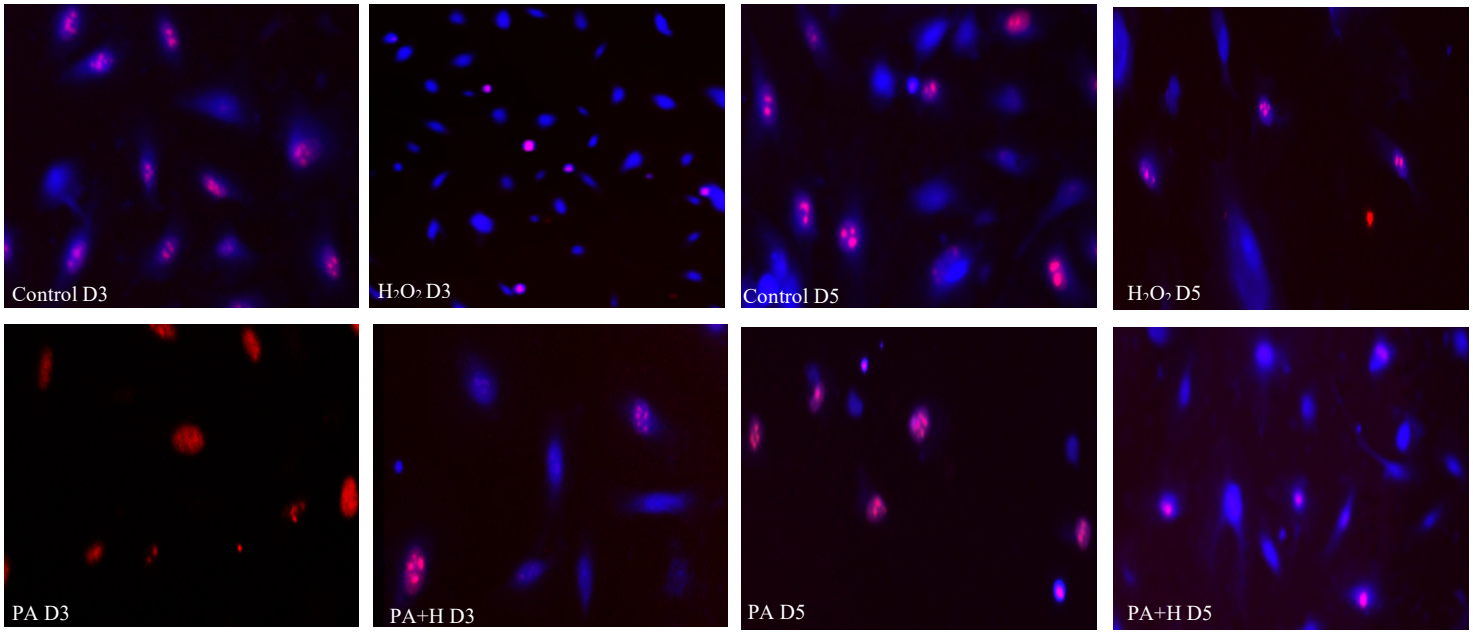
Figure 4.

a)

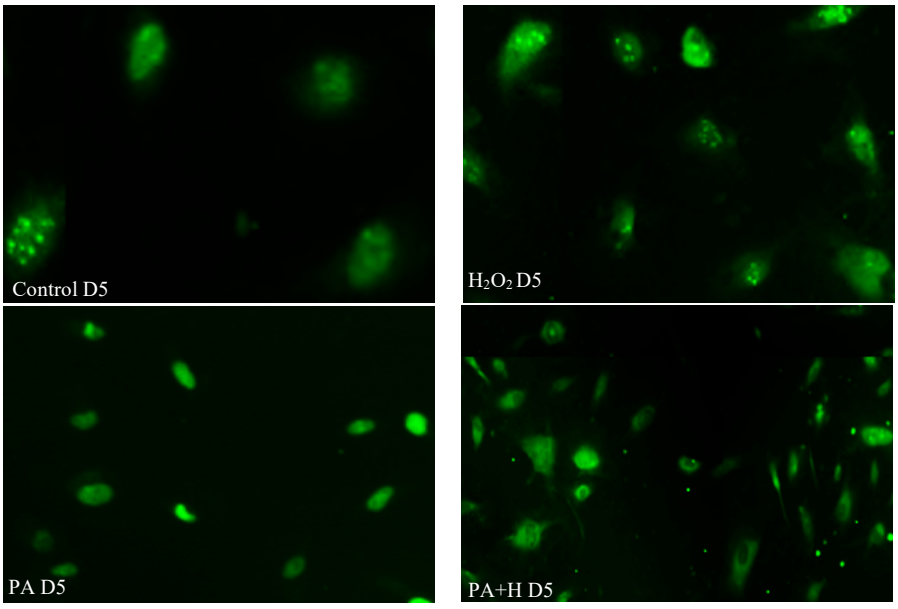


b)

Ki67



γ-H2AX day 5



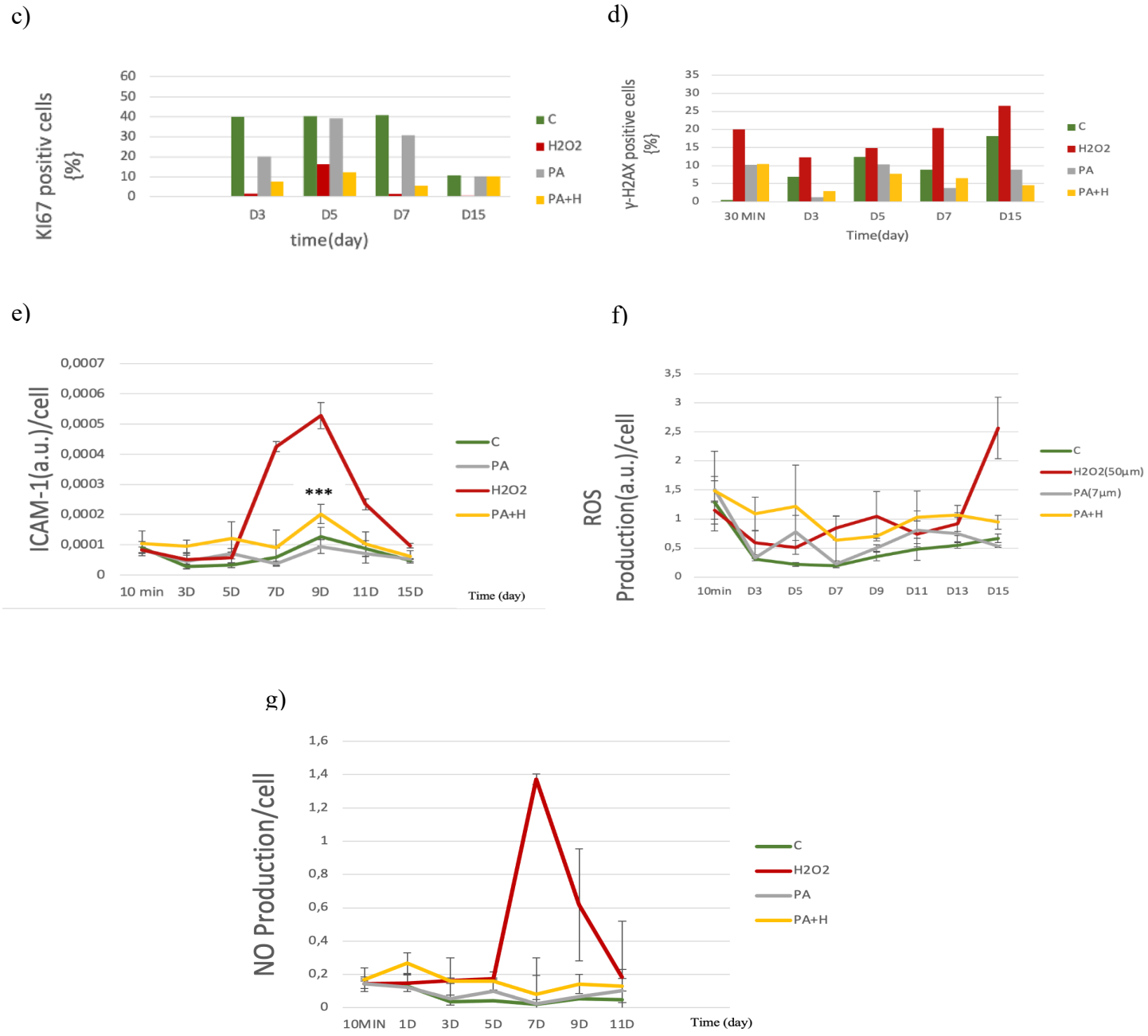
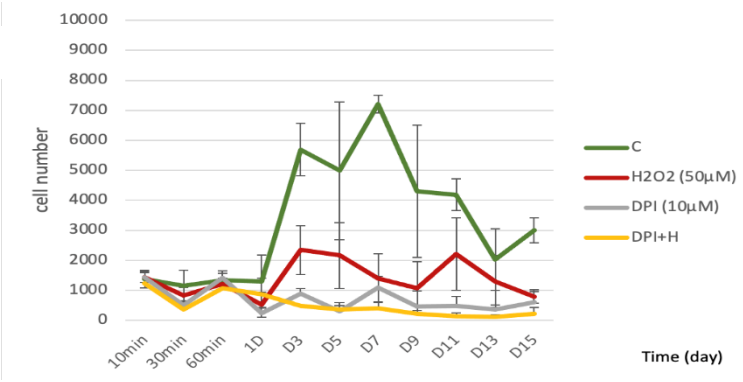


Figure 4. Preventive effect of PA in H_2O_2 stressed cells. HUVECs cells were stressed with H_2O_2 for one hour every 48h. After that, cells were incubated with EC growth medium only, or medium containing PA a) Retardation of growth in H_2O_2 stressed cells. b) representative images of immune-fluorescent detection Ki67 and γ-H2AX in HUVECs. c) Quantification of the changes in the percentage of Ki67 positive cells in cultures of H_2O_2 -stressed HUVEC. d) Percentage of γ-H2AX positive cells. e) Surface expression of ICAM-1 determined by ELISA. f) Time course of ROS production. g) NO production.

The results are summarized as the mean ± SD of sextriplicate for the untreated and H_2O_2 -treated groups (C and H), as well as triplicates for the inhibitor-treated groups except Ki67 and γ-H2AX the outcome is a result of single we

Figure 5.

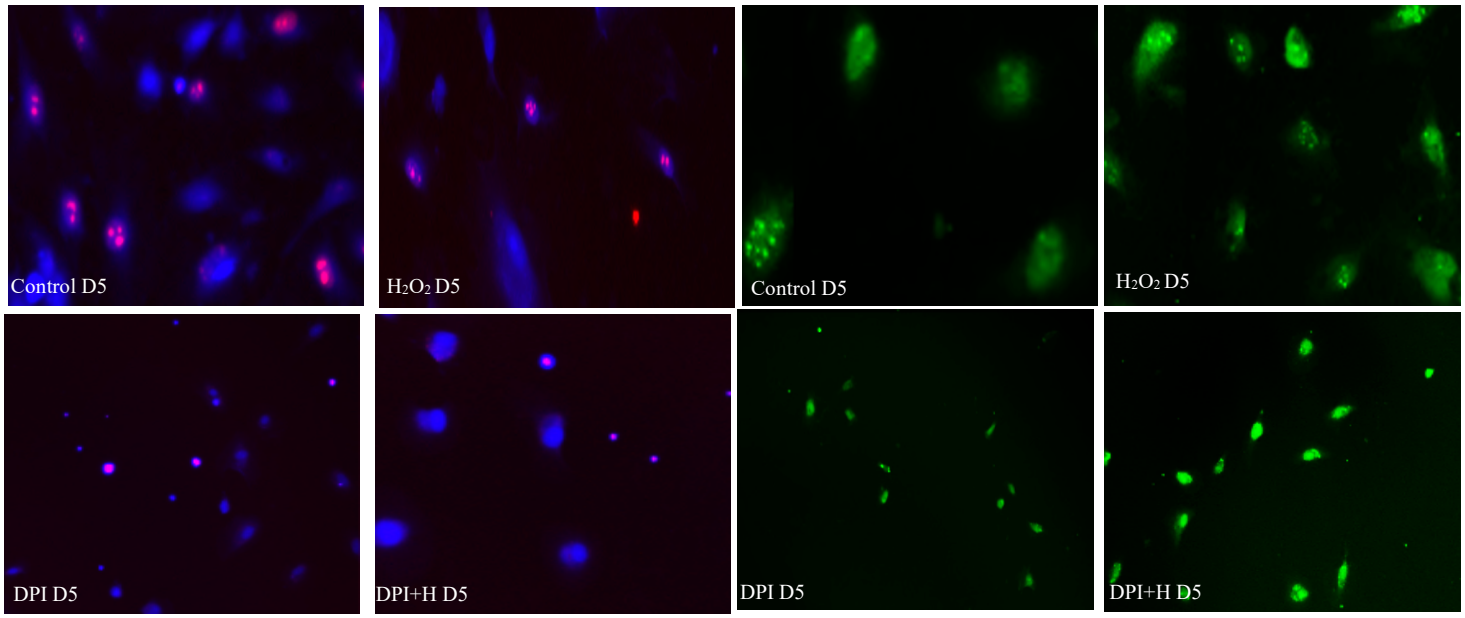
a)



b)

Ki67 day5

γ-H2AX D5



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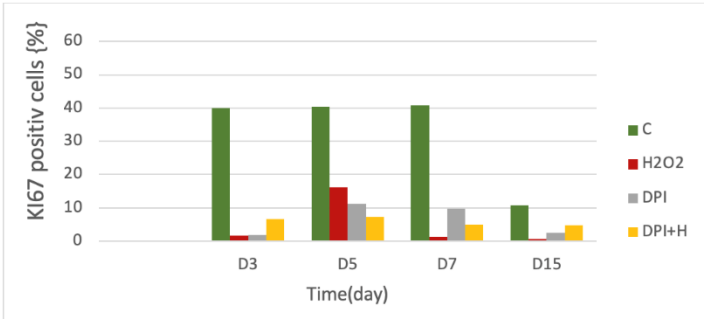

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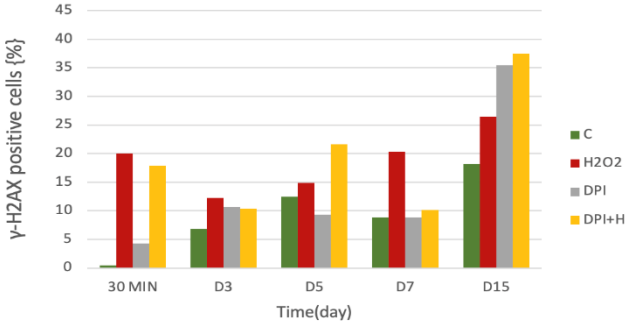

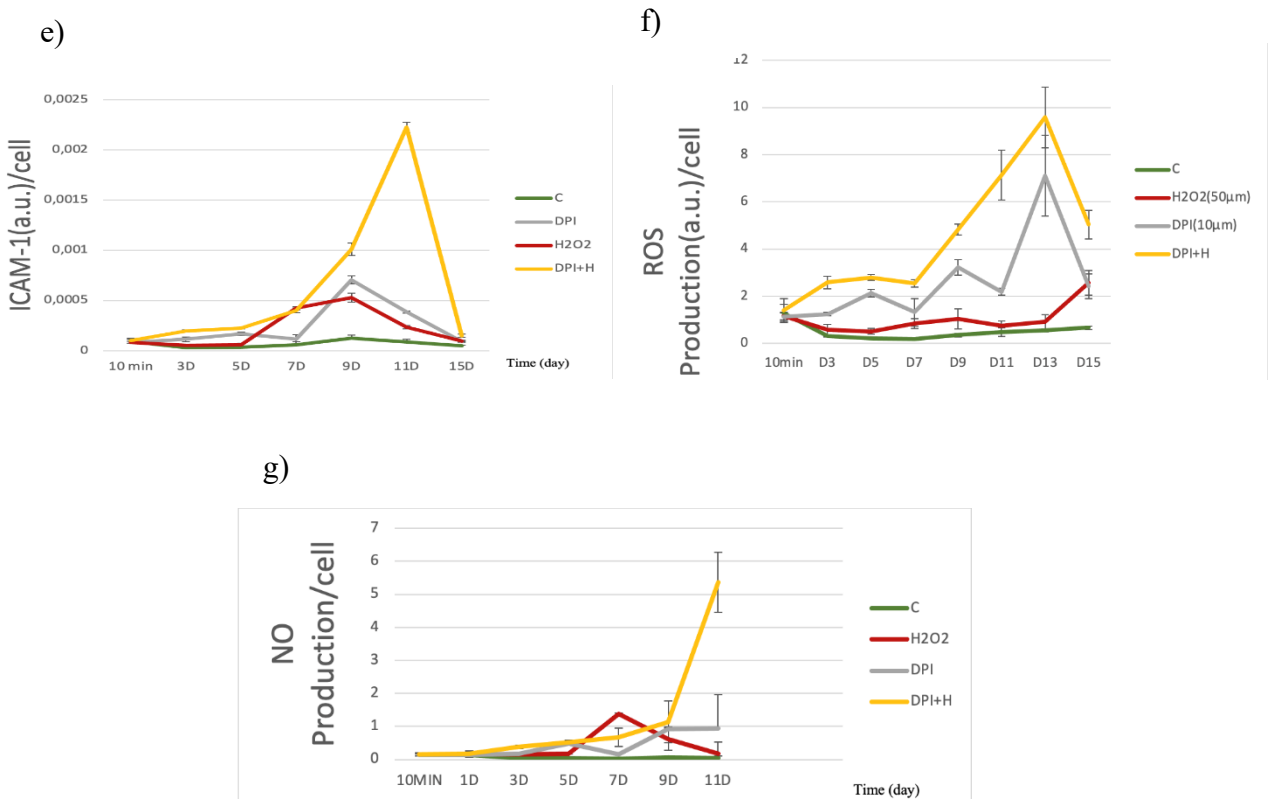

figure 5. DPI toxicity at concentration of 10µM. HUVECs cells were stressed with H_2O_2 for one hour every 48h after that cell were incubated with EC growth medium only or containing DPI. a) Retardation of growth in H_2O_2 stressed cells. b) representative images of immune-fluorescent detection Ki67 and γ-H2AX in HUVECs. c) decrease in Ki67(proliferation marker) expression in DPI treated cells
d) increase γ-H2AX expression in cells exposed to DPI e) DPI enhanced ICAM-1 expression in HUVECs. f) increase ROS production by treatment with DPI
g) increase NO production by DPI treatment
Values are presented as mean ± SD of sextuplicate for the untreated and the H_2O_2 -treated groups (C and H) and of triplicates for the inhibitor treated groups except Ki67 and γ-H2AX the outcome is a result of single well.

5 Discussion

Endothelial cells are important for maintenance of blood fluidity, control of vascular tone, modulation of proinflammatory molecule activity, proinflammatory immune responses, and neovascularization, all are essential physiological functions of endothelial cells in vascular homeostasis [110]. There is strong proof that endothelial dysfunction resulting from elevated oxidative stress substantially leads to both compromised coronary arteries dilation [137] and promotes myocardial ischemia and neurovascular uncoupling [148,149]. Endothelial dysfunction is a pathophysiological signature of coronary disease development. Studies have linked vascular impairment with nitric oxide declines, endothelial progenitor cell dysfunction, and vascular inflammation to vascular aging, and together they promote vascular disease [113–115]. Heart disease, stroke and hypertension are all conditions which are known to be partly caused by arterial disease [150, 161].

H_2O_2 has been used in many studies to examine the effects of oxidative stress or stress-induced senescence on endothelial cells in vitro. In some studies, H_2O_2 is applied at a high dose, often in excess of 250 µM for about 12 h [180]. This H_2O_2 dosage is almost eight times higher than the H_2O_2 peak in plasma concentrations [181] and led to reduction in cell proliferation as well as changes in cell morphology and junction staining. Other studies have used H_2O_2 in lower concentration and different exposure time and they reported that 30–100 mmol/L of H_2O_2 was efficient in inducing senescence in HUVECs [84, 182, 183]. In this study they used acute as well as prolonged low doses of H_2O_2 (10 µM) the prolonged exposure of human diploid fibroblasts took place every 3days followed by 3 days of recovery this repeated H_2O_2 exposure led to irreversible cell arrest after 2 weeks and the cells developed the senescent morphology after 3-5 days [184]. In another study they observed an increase in the percentage of SA-β-gal positively stained cells as well as a reduction in the percentage of BrdU (Bromodeoxyuridine) incorporation after four consecutive exposures of nucleus pulposus cells to H_2O_2 (500 µM) [185].Here, in the presented study, intermittent exposure to H_2O_2 was used to mimic chronic oxidative stress and its senescence-inducing effect on endothelial cells. In this experiment HUVECs were exposed to (50 µM) H_2O_2 for 60 minutes every other day for one hour until day 8 followed by cell culturing in EC growth medium only. In this study H_2O_2 was used to create the senescence cell phenotype, which we found to correlate strongly with induction of cell cycle arrest and DNA damage. Generally, it is postulated that antioxidants block or impede the process of oxidative damage to cellular membranes, slowing or blocking oxidative chain reactions and researchers believe that consuming antioxidants and free radical scavengers helps to prevent and cure different cardiovascular diseases [170]. We used the antioxidant BHT

(40μM) and DPI (10μM) and anti-inflammatory PA(7μM), after the one-hour exposure to H_2O_2 to test if our antioxidant or anti-inflammatory substances can attenuate the induction of premature senescence to determine the senescent induction caused by H_2O_2 we counted the cell number, measured Ki67 (proliferation marker), γ-H2AX (DNA damage marker) and ICAM-1 surface expression as well as the ROS and NO production. All measurement were done one day after exposure to H_2O_2 to give cells time to readjust after the acute stress, and thus aimed to address more specifically the lasting changes of stress exposure.

The intermittent exposure of HUVECs to H_2O_2 caused a marked decrease in culture growth compared to negative control. Non-stressed control cultures reach the highest cell density at day 7, after which the cell number even in these confluent control cultures decreased again. The drop in the growth curve from day 7 until the end may be because of fact that confluent ECs become quiescent due to contact inhibition of growth. Additionally, the long period of incubation of cells at confluency for 8 days may also induce in a portion of cells replicative senescence, thus requiring more space and thus leading to elimination of "excess" cells. The increase in cell size is shown in the phase contrast photo from a control cell on day 15 (figure 1b) and the data on control cells provided here demonstrate the decrease in Ki67 and increase of γ-H2AX expression on day 15.

H_2O_2 exposure significantly decreased the expression of proliferation marker Ki67 and increased the DNA damaged marker γ-H2AX. The decrease of ki-67 expression and increase γ-H2AX upon H_2O_2 exposure support the notion of induction of senescence in stressed cells. Also, the exogenous ROS (which is H_2O_2) might induced DNA damage, senescence and apoptosis. Our results are in agreement with previous reports [186–189] that exposure of cells like Human Mesenchymal Stem Cells, Human Bone Marrow Mesenchymal Stromal/Stem Cells to H_2O_2 led to reduction in Ki67-expressing cells as well as to accumulation of γ-H2AX in human spermatozoa and Human aortic endothelial cells (HAECs) [186–189].

Cellular senescence is associated with the induction of a proinflammatory phenotype that cause chronic inflammation through secretion of proinflammatory cytokines, chemokines and proteases one of this protein named ICAM-1 as one of the senescence associated secretory phenotype (SASP) proteins. Also, SASP proteins have the potential to attract immune cells and can cause chronic inflammation [31–33]. The cell adhesion molecule ICAM1 has long been recognized as a marker of endothelial cell activation induced by TNF via NFB translocation [190] . In our results cells exposed to H_2O_2 for one hour have increased the ICAM-1 expression significantly from day 5 until day 15. Also, intermittent exposure of cells to H_2O_2 has not only

induced DNA damage, degradation of Ki67 (impairment in cell proliferation) and augmented ROS production but has also increased the ICAM-1 expression as one of the SASP proteins. All these are a hallmark of senescence, which strengthen the notion that these cells became senescent.

The occurrence of senescence is associated with a variety of stresses, such as oxidative stress [84, 85]. Oxidative stress occurs when the accumulation of reactive oxygen species (ROS) overwhelms endogenous antioxidant systems and/or when endogenous antioxidant systems are damaged. ROS are a class of oxygen-based molecules that have a high chemical reactivity [86, 87]. ROS are composed of both free radicals such as superoxide (O2−) and hydroxyl radicals (OH·), as well as nonradical species such as hydrogen peroxide (H_2O_2) [86, 87]. Also, oxidative stress is one of the factors that cause senescence to develop [85, 94] but the mechanisms of stress induced senescence occurring by oxidative stress are not completely clear. ROS has dual effects, on one hand ROS induced cell cycle progression for instance in cancer cells on the other hand ROS caused cell cycle arrest in normal cell. These opposing effects of ROS dependet on the amount and duration of ROS exposure. Short exposure to relatively small doses results an enhanced cell proliferation through cell cycle progression while long exposure or use high ROS concentration contribute to impairment the cell proliferation [191, 192]. The phenomenon of the contradictory effects of ROS exposure depending on the ROS concentration is a typical hormetic effect. Molecular damage resulting from both stress and aging causes many diseases and mortality. While high levels of a stressor can contribute to a cellular damage, low levels can be an external stimulus that leads to increases in somatic maintenance and resistance to stress and this response can also cause some resistance to aging [193].

When ROS levels rise, the p53/p21CIP1 axis is activated through the p38 MAPK (mitogen activated protein kinase [95]. This temporary growth arrest in cell senescence progresses through the upregulation of p16INK4A in the stressed cells [1]. Upregulation of p16INK4A and p21CIP1 results in phosphorylated RB1 which in turn prevents the cell cycle from proceeding to the next round of cell replication and stops at G1 phase [17]. Also, p53 is activated by elevated ROS levels leading to cell cycle arrest by upregulating p21, which inhibits the cell cycle regulators cdk4 and cdk2 [194, 195]. In this study, we used 50 μm H_2O_2 every 48 hours for 8 days, which resulted in loss of apparent proliferation. This is clearly illustrated by the compatibility of proliferation observed parameters represented as the number of cells and portion of Ki67 positive cells with ROS production (Figure 2; a, c, f). This simultaneous

increasing of ROS production and reduction of proliferation might be explained by the mentioned above mechanisms that "when ROS levels rise, the p53/p21CIP1 axis is activated through the p38 MAPK (mitogen activated protein kinase [95] leading to cell cycle arrest. Similarity here the cell number of stressed cells ceased to increase, and the ROS production increased (Figure 2f). This loss in cell proliferation and evaluated of ROS production is compatible with above mentation study.

Since NF-κB is a redox sensitive transcription factor, many studies reported the correlation between NF-κB and ROS claiming that the release of H_2O_2 in the cytoplasm of aging cells activated the NF-κB. Although NF-κB appears to be the key transcriptional factor mediating H_2O_2-induced vascular-cell response [169] by oxidative stress the cells trying to prevent further oxidative damage and maintain cell survival. This occurs via modulation of response to ROS by NF-κB resulting in an increase of the expression of antioxidant proteins such as MnSOD [196, 197]. However, the prolonged cell exposure to H_2O_2 might lead to an overload of this defense mechanism resulting in high ROS production. Cells exposed to overwhelming oxidative stress might succumb to senescence and in this state of senescence also present with higher endogenous level of ROS.

NO is one of the most essential molecules for maintaining vascular functions and haemostasis. It is a vasodilator for control the vascular tone. Oxidative stress can affect several aspects of vascular function by oxidation essential proteins, or by redox sensitive transcription factors, but the inactivation of endothelium-based nitric oxide is one of its more powerful results (NO). The failure of endothelial to trigger an adequate vasodilatory response because of inadequate nitric oxide (NO) bioavailability is one of the hallmarks of endothelial dysfunction [151] which ultimately lead to cardiovascular disorders like coronary artery disease and myocardial ischemia. The increase in NO as a cell response because of oxidative stress was observed in some studies they suggested that upregulation of eNOS may be a part of compensatory response to a reduction in NO availability [198]For instance, the induction of oxidative stress by addition of H_2O_2 in “Human Coronary Artery Endothelial Cells (HCAECs)” led to increase of eNOS mRNA and protein [199]. Previous researches have shown that S-glutathionylation of eNOS causes superoxide rather than NO under oxidative stress [200]. Our stressed HUVECs cells with H_2O_2 revealed excessive amount of NO production from day 3 until day 7 compared to non-stressed cells (negative control) and decreased again until day 15. It has been reported that short-term H_2O_2 exposure (within one hour at 50–500μM) of cultured

ECs has been shown to activate eNOS and increase NO level [201–203]. These findings indicate that NO regulates eNOS expression in endothelial cells via a negative feedback process at both the transcriptional and translational stages. However, in our study, the NO of HUVECs-treated with H_2O_2 exhibited an upregulation of NO starting after one hour until day eleven compared to negative control which would agree with the previous studies. Moreover, in our study and for the first time, we covered a long-time scope and measuring the NO in the resting days to avoid the acute effects of H_2O_2 exposure. This would mean that the upregulation of NO is the real NO synthesis via eNOS as reported by the previous study [201–203]. It has been reported that NO may be scavenged by excess ROS and lead to transformation to $ONOO^-$. Peroxynitrite is a highly reactive oxidant which causes oxidizing endogenous antioxidant and contribute to an increased oxidative stress [128]. This might explain why both NO and ROS were simultaneously increased on day 7.

Our data in agreement with study Zadeh et al. (2000) who reported that exposure of HUVECs to different concentration of H_2O_2 (50, 100, 200 μM) stimulate iNOS directly and 100 μM H_2O_2 enhanced synthesis of NO_2^- and NO_3^- at 12 and 36 h [204] . Superoxide radicals and NO induce antioxidant defenses in cells by acting as signaling molecules [16,21]. Although lower concentration of NO mediated many of the physiological process such as smooth muscle relaxation and platelet inhibition, higher concentrations of NO especially in combination with increased production of oxidants, caused a tissue damage and inflammation by the production of $ONOO^-$ [205]. Interestingly enough that ICAM-1 upregulation is mediated by NO in brain and retinal endothelial cells induced by VEGF which attributed to eNOS expression [206]. Additionally, it has been reported that iNOS was increased in retinal endothelial cells concomitantly with high ICAM-1 expression [207]. This might explain, in our study, the simultaneous upregulation of ICAM-1 and NO from day five until day 11. This would confirm both our “hypothesis and conclusion” that ICAM-1, ROS and NO are tightly linked factors to investigate the endothelial cell dysfunction in vascular disorders especially in vascular age-related diseases. In other words, one might claim that the approach in this thesis targets both inflammatory and oxidative cascade in senescence associated vascular diseases.

The observed lack of increase of cell number over the course of this experiment likely reflects the lack of proliferation – but could –theoretically – also come about by balanced amounts of proliferation and cell death. Likely though, in our culture the lack of increase in cell number during the 7 days of culture in EC growth medium, is indeed caused by senescence, since we did not observe an increase in the number of dead cells. Additional support for our claim of senescence comes from the fact also the number of $Ki67^+$ cells decreased throughout the

experiment and was very low in the late culture (day 15). We analyzed the ROS production in cells that had previously been exposed to H_2O_2 and found that at the end of our experimental period the cells exposed to H_2O_2 had a much higher level of ROS production than the cells not exposed to stress. Similarly the production of NO was found to be higher at day 15 (data not shown) in cells that have been exposed to H_2O_2 . Together with the upregulated ICAM-1 expression which was taken as a marker for development of the SASP of senescence cells all the analyzed parameters would be compatible with the interpretation that our cells indeed after this treatment regimen became senescent. Then we utilized this model of senescence induction to analyze our substances for their capacity to prevent the induction of senescence by H_2O_2.

As stated, before the intermittent exposure of HUVECs to H_2O_2 caused a significantly reduction in cell number compared to control. H_2O_2 exposure reduced the expression of proliferation marker Ki67 and increased the DNA damage marker γ-H2AX. The decrease of ki-67 expression and increase γ-H2AX upon H_2O_2 exposure support the notion of induction of senescence of H_2O_2 stressed cells. Next, we used 3 antioxidant substances (BHT, PA, DPI) to investigate there capacity to prevent the induction of senescence by H_2O_2.

BHT is a lipophilic compound widely used as antioxidant to scavenge reactive oxygen (ROS) species. It works by donating a labile hydrogen to oxygen radicals, leaving an oxidized phenolic ion stabilized by the inherent resonance of the benzene ring [208]. In our study the treatment with BHT after exposure to H_2O_2 resulted in slightly higher cell number at day 7, 9 and 15 and increase the Ki67 positivity on day 7 and 15 and at the same time attenuated γ-H2AX expression especially at later time points (day 7, day 15). This reduction of γ-H2AX expression was also observed in this study, which investigated the genotoxic response of CHO (Chinese hamster ovary) cell to 7-ketocholesterol as lipid peroxidation. They used 25 μM 7-ketocholesterol for 48 h after concurrent treatment with 20 μM BHT [209].
In our experiment, BHT significantly reduced the ICAM-1 expression from day 7 until day 15.

Perlatolic acide has been identified as anti-inflammatory plant derived substance. The anti-inflammatory activity of PA described through the inhibition of three major pro-

inflammatory targets (mPGES, 5-LO, NF-κB) [177]. PA prevented the loss of cell proliferation caused by H_2O_2 on day 7 and 15. PA like BHT has significantly decreased the ICAM-1 expression from day 7 until day 15. Adding to its anti-inflammatory effect PA has efficiently impeded ROS production in cells exposure to H_2O_2 on day 15 but failed to prevent ROS generation initially. This failure in preventing ROS generation at early time points might be due to inhibiting the NF-κB and therefore prevent the expression of antioxidant protein.

DPI inhibits the activity of NADPH oxidases (NOXes) and dual oxidases (DUOXes) two enzymes contributing to H_2O_2 generation. Even though DPI can be expected to decrease cell ROS generation as an NADPH oxidase inhibitor recent evidence on DPI intracellular ROS regulation is controversial. Both stimulation and inhibition of ROS generation have been reported [210, 211]. For example, DPI induced intracellular ROS generation in immortalized cell line of human retinal pigment epithelium (ARPE-19) at concentration of (0.1, 1, and 10 μM) after 24 hours and decreased the cell proliferation [212] this would be in agreement with our result since the 10μM caused both reduction of number of cells and high ROS production. We could support these changes with Ki67 a proliferation marker which showed a dramatical decrease at different time points of DPI-treatment compared to control cell at several time points (figure 5c). This would suggest that the 10μM of DPI is toxic for endothelial cells, therefore, the other parameters like ICAM-1 and NO showed the same tendencies toward the severe inflammation and NO deficiency as shown in figure (5g). All the above described changes of parameters have been one in line with the upregulation of the surrogate DNA damage marker (γ-H2AX) as shown in Figure (5d). This would be another mechanistic factor explaining the toxic, pro-oxidant, and anti-proliferative effects of such high doses of DPI. Our results might explain, at least partially, the toxicity of DPI usages. One can also add that the cell context and the experimental design could play roles since we have used endothelial cells which might be more sensitive than others, and used a long-time exposure of the reagents which, led to cell death by treating the cells with this high DPI dosage. Our results also might be in agreement with Park et al.2007 [212] who measured DNA fragmentation using TUNEL assay to evaluate the apoptotic response of the control and cells treated with DPI, for 24 and 48 h showing that 10 μM DPI for 48 h led to 50% of cells TUNEL-positive.

5.1 Limitation of experimental work

This study has certain limitations. Most importantly, we have performed only one experiment (albeit with replicates) because of time limitations. Thus, for significant statistical evaluation

this experiment will have to be repeated. Another problem was encountered due to the fact that the sensitivities of the assays for ROS production and ICAM-1 expression would be calling for higher initial seeding densities to allow for more reliable assay readout. Additionally, the frequently required changes of culture media connected to the H_2O_2-exposures might in some cases have affected the intactness of the cell layers and thus the recorded cell numbers. A difficulty for the subsequent assessments was getting enough surviving senescent cells upon stressing them with H_2O_2 (50µM), since many cells are going into apoptosis after H_2O_2 treatment.
Finally, in the case of DPI, the evaluation of its preventive capacity to suppress the induction of senescence, was hampered by the fact that it turned out to be toxic for ECs at the used concentration.

5.2 Conclusion

In summary, we have implemented a modified procedure for modeling the induction of premature senescence by chronic oxidative stress. The range of markers to support the claim of having induced cellular senescence in the stressed endothelial cells included cell growth curve, proliferation marker Ki67, DNA damage marker γ-H2AX, ROS and NO production as well as ICAM-1 expression

The assessment of perlatolic acid and BHT for their preventive/reverting capacity regarding oxidative stress-induced changes in exposed ECs, indicated, that PA and BHT might have a beneficial effect on the extent of several stress-induced cellular changes. They failed to prevent the loss of proliferative capacity, the core feature of cellular senescence.

6 References

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