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Research Article

Role of different types of spleen tyrosine kinase (SYK) inhibitors on reducing inflammation in human umbilical vein endothelial cell (HUVECs) previously induced by repetitive oxidative stress

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Abstract

Senescent cells are considered to be a state of cell cycle arrest after exceeding the ultimate number of cell division, however they still secreting the senescence associated secretory phenotype (SASP) as proinflammatory factors as a sign of metabolically activity. a certain type of senescence that can be induced by oxidative stress, DNA damage and inflammation this can be called Stress-Induced Premature Senescence (SIPS). Several studies proved that cellular senescence has a massive impact in vivo in aging and aging-related pathophysiology. The main reasons of this phenomenon, which can lead to vascular disorders in endothelial cells, include chronic inflammation, oxidative stress, and diminished nitric oxide bioavailability. Oxidative stress is often linked to many diseases through cell deterioration. It refers to a status where the bioavailability of oxidative stress is much more than the available amount of antioxidant. oxidative stress (ROS) produced by ageing can activate NF-kB and enhance vascular inflammation. To investigate the impact of oxidative stress on endothelial ageing and the assessment of the inflammatory response this study was performed to test the intercellular adhesion molecule (ICAM-1) and the possible correlation with ROS production over a relatively long period of time. Since oxidative stress and premature senescence are correlated and have a major role in endothelial dysfunction this study was performed to investigate the effects of intermittent exposure of HUVECs to H_2O_2 (50µM) on the induction of senescence over a longer period of fifteen days.

Keywords: Senescent cells, ROS, ICAM-1, SIPS, senescence associated secretory phenotype, SYK

Introduction

Aging, as a leading cause of CVD, is caused by vascular cell dysfunction, which alters normal vascular tone and leads to vascular disorders[1]. Vascular homeostasis can be maintained by modulating vascular tone, integrity and remodeling of Vascular endothelial cells (VECs)[2]. Endothelial senescence has been observed in individuals with atherosclerosis, hyperlipidemia, diabetes, hypertension, ageing, and obesity as their regeneration potential declines with age[3]. It was proven that there is a link between aging and chronic, low-grade inflammation, by noticing an increases in circulating acute phase proteins (such as C-reactive protein [CRP]) and pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α)[4],[5] and interleukin (IL)-6[6].

The pro-inflammatory cytokines were used to activate and enhance endothelial dysfunction in primary endothelial cells or isolated arteries, evidence that inflammation plays a vital role in endothelial dysfunction.[7]. Increased nuclear

factor κB (NFκB) activity is linked to this age-related pro-inflammatory arterial profile. NFκB.[8] I kappa B kinase beta (IKKβ) is activated in response to inflammatory stimuli[9] or reactive oxygen species (ROS)[10], and then it phosphorylates IκB-α, removing its inhibitory effect and allowing NFκB to translocate into the nucleus, where it can initiate pro-inflammatory cytokines gene transcription[11]. As a result, it was clear the importance of oxidative stress in the activation of the NF-KB responsive protein intracellular adhesion molecules-1 (ICAM-1) as well as the potential crosstalk with reactive oxygen species (ROS) following excessive exposure of endothelial cells to H_2O_2, which could be used as endothelial senescence markers. Evidence that pharmacological suppression of NFκB signaling greatly decrees-ses cytokines in elderly mice and humans supports the crucial role of NFκB in age-related inflammation-dependent endothelial dysfunction[12].

Inflammatory signaling promotes O_2 production and oxidative stress and vice versa as well as exacerbating inflammation down-stream of NFκB transcription of pro-inflammatory cytokines through different mechanisms[13]. Increased NFκB-mediated transcription of redox-sensitive genes, such as those encoding NADPH oxidase subunits[14], increases ROS bioactivity and activates IKK-NFκB signalling. so, NFκB has a major role in exacer-bating oxidative stress and inflammation[15].

Because oxidative stress and premature sense-cence are linked and play a role in endothelial dysfunction, we set out to explore the effects of intermittent HUVEC exposure to H_2O_2 (50 µM) on senescence induction over a fifteen-day period. We investigated the production of ROS and NO, as well as I-CAM 1 as an SASP, during senescence induction, as well as the protective/reversing role of the synthetic spleen tyrosine kinase inhibitor R046 (SYK) and plant-derived inhibitors thymoquinone (TQ) and eupatoriopicrin (EP). By counting the number of cells, we were able to see if chronic H_2O_2 exposure inhibited cell proliferation.

Material and reagents

Fetal bovine serum (FBS), goat serum, Hydro-gen peroxide (H_2O_2), medium M199, fetal bovine serum (FBS), goat serum, gelatin, Dimethyl Sulphoxide (DMSO), para-formaldehyde (PFA), thymoquinone (TQ), Sulfonamide, Naphthyl ethylenediamine dihydrochloride (NED), vanadium chloride (III) (VCl3) was purchased from Sigma-Aldrich (Vienna, Austria).

fungizone, and penicillin, streptomycin, Hepes Buffer, Trypsin-EDTA were obtained from LONZA (Vienna, Austria).

Endothelial cell growth supplement (ECGS) with heparin was obtained from Promo Cell (Heidelberg, Germany). 2´,7´-dichlorodihydroflurescein diacetate (H_2-DCF) was purchased from ThermoFischer Scientific (Vienna, Austria).

A potent SYK inhibitor (R406) obtained from Selleckchem. Eupatoriopicrin (EP) was isolated from *Eupatorium cannabinum* L.

Methods

1. Isolation and culturing of Primary endothelial cells

Umbilical cord was used to isolate HUVECs as mentioned before[16]. first, we used HBSS (hank's blank salt solution) to keep away any blood then was injected collagenase in both artery and vein for (20 to25 min) finally in turn washed with CMFH was used to leakage endothelial cell. We cultured subconfluently HUVECs cells in a CO_2 incubator in presence of 95% humidity, at 37°C and M199 complete medium that contain 20% FBS, 3 mg/ml ECGS and 22.5 mg/ml heparin, 2mM glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin, and 0.25µg/ml fungizone (full growth medium). we used a cells from passages three to five for the experiment.

2. Freezing of the cells

We would also freeze another aliquot of the EC and use them when needed for other experiments. We took that aliquot in a 50ml Tube and washed it with 5% medium. Then we sucked out the supernatant by centrifugation and resuspend the pellet in 2 ml 5% DMSO in full media. At the end we transferred it into a cryovial and stored in liquid nitrogen.

3. Induction of senescence in HUVECs exposed to intermittent repeated low concentration of H_2O_2 and treatment with inhibitors

We coated the 96-well plates with gelatin. Then we seeded the cell in complete medium and incubated for 24hours. HUVECs were subjected to H_2O_2 (concentration 50µM) for one hour omitting the cells for our negative control. After that H_2O_2 was exchanged for either only medium or with TQ (10 µM), EP (6µM) or SYK inhibitor (0,5 µM). it was repeated after two, four, six and eight days. At the ninth day nine the cells were cultivated in complete growth medium only. Then, Cells were let to grow in complete medium till day fifteen.

4. Measuring the cell number as a way to evaluate the cellular proliferation

It is important to determine the cell number to define the cell proliferation. It was done by seeding the cells in full medium and incubate them for 24 hours. HUVECs were exposed to H_2O_2 (concentration 50µM) for one hour omitting the cells for our negative control. After that the H_2O_2 was exchanged for either only medium or with TQ (10 µM), EP (6µM) or SYK inhibitor (0,5 µM). After 10 and 60 minutes, one day, three, five, seven, nine, eleven, thirteen and fifteen days we fixed the cells by using 4% PFA for 10 minutes, then we washed it twice by PBS, stained with a fluorescent dye 4', 6-diamidino-2-phenylindole (DAPI) that can stain nuclear DNA after staining, cells were washed again with PBS. Afterwards we used the microscope, took pictures of every well. With the help of a certain program, we could count the bright blue dyed nuclei.

5. Measurement of intracellular ROS production in HUVECs

The fluorescent H_2-DCF was used to measure the intracellular ROS[4] adapted to our experiment. The supernatant was removed (collected for later NO assessment) and replaced with DCF diluted in PBS supplemented with 5% FBS and (10µM). After incubation in the dark for 30 minutes at 37°C in the CO_2-incubator the ROS production was then determined by measuring the fluorescence at (excitation: 490 nm, emission: 525 nm) using reader Synergy 3 (Bio Tek). After that, we fixed the cells by using 4% PFA for 10 min following by washing twice with PBS for I-CAM-1 measurement.

6. Detection of ICAM-1 by cell ELISA

The adhesion molecule ICAM-1 was measured in HUVECs fixed at the time points indicated above by using modified cell ELISA [17]. Here we used 4% PFA to fix cells after exposure to H_2O_2 (50µM) for one hour. After that the H_2O_2 was exchanged for either only medium or with TQ (10 µM), EP (6µM) or SYK inhibitor (0,5 µM).

7. Determination of NO in HUVECs

After much reading and testing of various methods we have developed our own protocol according to which we proceeded. First the supernatant, that we had collected at the indicated time points, was deproteinized with Ethanol (EtOH) at room temperature for 1 hour followed by centrifugation (10 000 RPM for 10 min at 4°C). we took 50µl from the supernatant into a 96-well plate with 50µl VCl3 and 50µl of the Griess mixture (sulfonamide and NED) and incubated for 30 Min at 4°C while shaking. Afterwards the absorbance was measured at 540nm.

Statistical analysis

All experiments were performed at least in triplicate. We performed the statistical analysis by using the unpaired two-tailed t-test with the

help of Microsoft Excel, while considering it significant when p-values < 0.05 and it is very highly significant when it is <0.001.

Result

Cell cycle arrest in HUVECs was induced by intermittent exposure to H_2O_2. HUVECs were exposed to a concentration of 50 µM H_2O_2 for one hour, followed by culturing them in full growth medium without H_2O_2. The exposure was repeated on day two, four, six and day eight after which cells were grown till day fifteen in complete growth medium for the subsequent assessment of cellular senescence in these cultures. As a control, cells were exposed to medium only, omitting the addition of H_2O_2. To evaluate the senescence associated cell cycle arrest, we recorded growth curves.

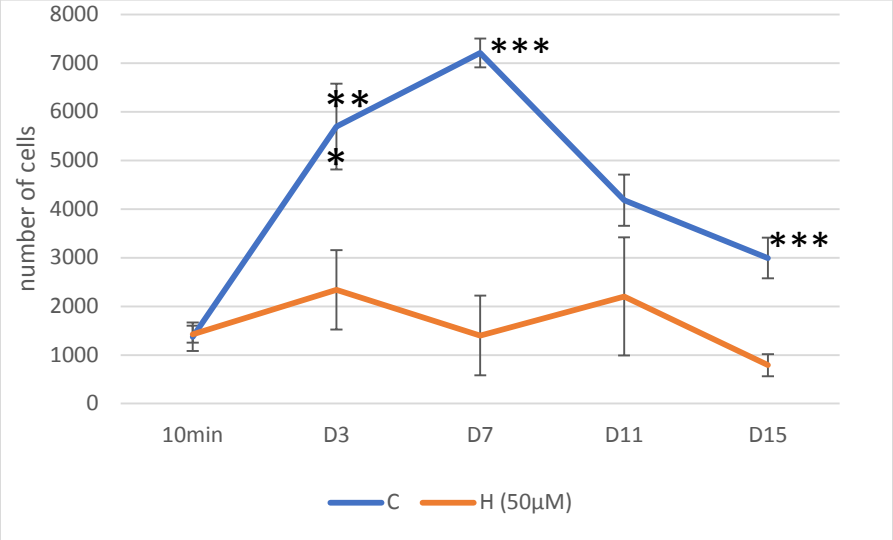


Figure 1: Growth curve of HUVECs on a complete growth medium as a control and after exposure to 50 µM H_2O_2 every two days for 1 hour (on day 0, day two, day four, day six and day eight)

The intermittent exposure of HUVECs (P5) to 50 µM H_2O_2 every two days for 1 hour (on day 0, day two, day four, day six and day eight), significantly reduced endothelial growth, as seen by the lower cell numbers compared to control (non-treated) cultures observed from day3 (D3) until day 15 (D15). . Since the number of cells stressed with H_2O_2 did not change significantly, we could assume that the cells could not recover due to repeated stress. After day 11, even though they have not been treated, they become fewer. Our data show peak point at day 7 where control reached maximum confluency. From day 7 on the number of control cells begins to decline, but the difference between untreated and H_2O_2 treated cells remains significant(p=0.005).

These data showed that when HUVECs were exposed repetively to H_2O_2 (50 µM) it lead to reduction in proliferation which is a strong indication that cell arrest became permanent meaning cells became senescent. To strengthen the notion of senescence induction in response to intermittent exposure to H_2O_2, we then evaluated ICAM-1 as a sign of senescence.

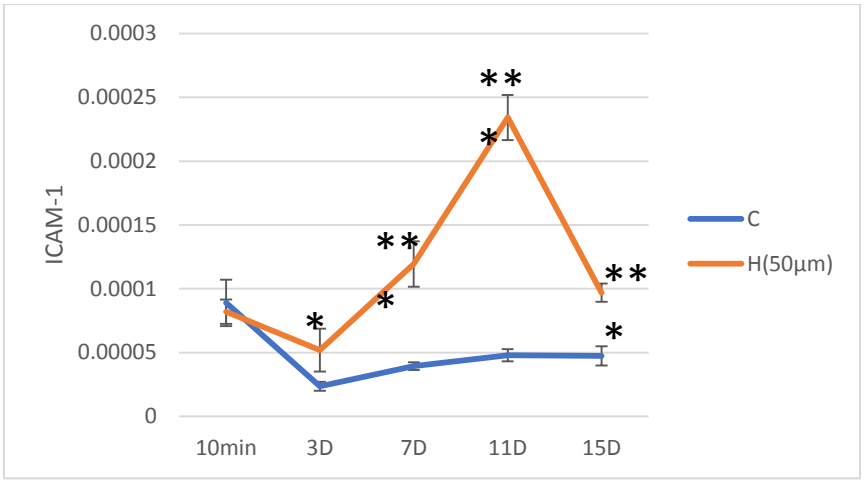


Figure 2: Repetive exposure of HUVECs to H_2O_2 stimulates ICAM-1 expression

As mentioned above, that intermittent exposure to H_2O_2 (50 µM) induced cell cycle arrest, thus, we assessed the adhesion molecule ICAM-1 as SASP proteins, to strengthen the notion that the cells might have developed senescence. SASP proteins are noticed to be elevated in replicative and oxidative stress induced senescence.

In HUVECs, we observed a statistically significant increase in ICAM-1 levels compared to non-treated cells after 60 min exposure to H_2O_2. Compared to our control, (From day 7 on) By days 7, 11 and 15 we found a statistically significant ($p<0.001$) increased expression of this adhesion molecule in the cells exposed to 50 µM H_2O_2 for one hour every other day . We observed an increased level of ICAM-1, followed by a reduction on day three and then again an increased level over the two days,before a second reduction on day 15 with the one hour stimulation with H_2O_2. Whrereas with the control cells we experienced a redution by day three and then a more or less constitent expression level of ICAM-1 til the end.

Our data has shown that intermittent exposure of HUVECs to H_2O_2 has not only slown down proliferation in these cells but has also upregulated the investigated SASP protein ICAM 1. Both of these features would be compatible with the interpretation that the employed treatment by oxidative stress might have lead to the induction of senescence in these cells.

Treatment with H_2O_2 led to higher NO production

NO is considered a regulating parameter for vascular tone and homeostasis, and it was a representative tool for endothelium derived relaxing factor (EDRF). Therefore, we assessed NO since it plays a crucial role in the endothelial health and its loss is always liked to enothelial dysfuntion. we need to evaluate NO values as it has an important role in vasodilation, inflammation and oxidative stress mostly through the formation of reactive oxygen species (ROS) and studies suggested changes in NO levels measured in stressed endothelial cells.

The NO level was significantly increased on day three in the stressed group compared to our control cells because in contrast to the control here the value does not decrease. the significant decrease in production of NO was observed in our control cells at day three compared to the stressed group.

The H_2O_2 treated cells showed the highest level by day seven. For our control cells the NO level did not change much after day three. On day 11 however, when the cells were no longer

stimulated with H_2O_2, the NO level dropped significantly. Our data has shown that intermittent exposure of HUVECs to H_2O_2 has upregulated the NO production significantly. This can be explained as NO expression was induced by H_2O_2 as a way to a self-protective mechanism of the cells.

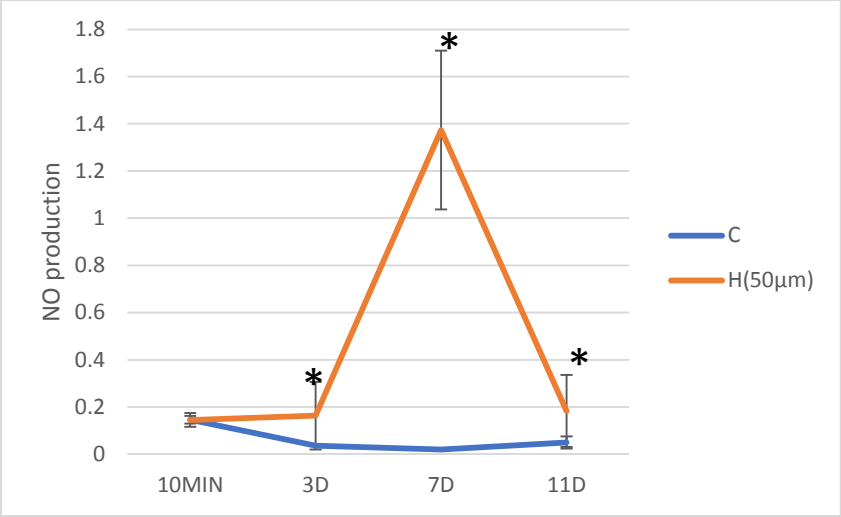


Figure 3: intermittent exposure of HUVECs to H_2O_2 upregulate significantly NO production comparing with control

Role of the R406, EP and TQ in prevention H_2O_2-induced inhibition of cell proliferation in HUVECs

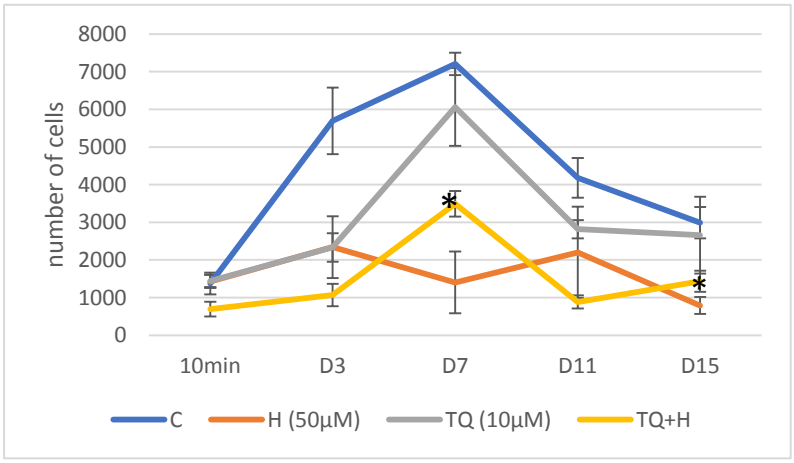
Here we want to assess whether our substances can prevent the H_2O_2 (50 µM) induced reduced proliferation in HUVECs. For this purpose, we added the potent SYK inhibitor R406 (0,5 µM), during the first eight days to the medium, each time after exposing the cells to H_2O_2. In parallel, we did the same with the two other test substances, TQ (10 µM) and EP (6 µM). also, we added these inhibitors to the normal growth medium each time after removal of H_2O_2.

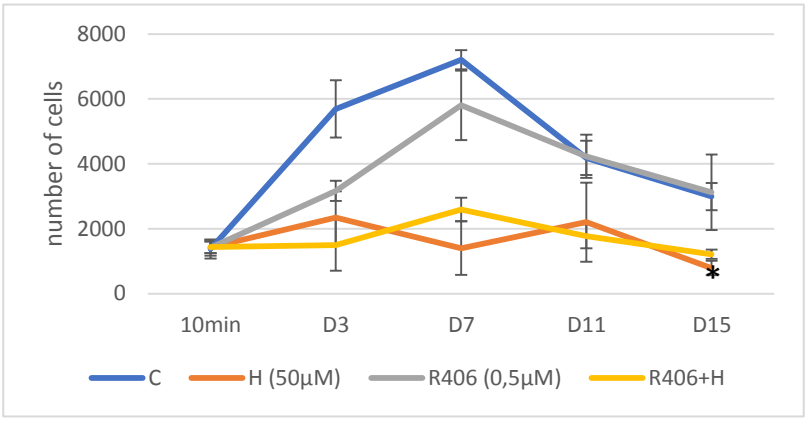
A delay in the effect of H_2O_2 (50 µM) on cell proliferation could be achieved by using R406 L as well as TQ. In contrast to the cells that have been exposed to H_2O_2 (50 µM) without subsequent treatment using an inhibitor, those that are additionally treated with R406 grow up to day seven. By day eleven it came to a drop in cell number.

While TQ result showed between day 11 and day fifteen cells post-treated with TQ seemed to still be able to proliferate, while the treatment of the cells with R406 resulted in a decrease but they still elevated and statistically significant ($p < 0.05$) compared to the H_2O_2 (50 µM) treated cells. These data indicate that the reduction in proliferation in HUVECs due to intermittent exposure to H_2O_2 (50 µM) can in effect be lessened by treating with R406 and TQ directly after applying the stress.

In the group with the cells that were stimulated with H_2O_2 (50 µM) and then treated with EP, we started with a lower cell count from the beginning, which became even lower by day three. The count reached its maximum on day seven and even exceeded the stressed cells. The growth curve then showed a decrease in cell number, so that they were again fewer than the cells treated with only H_2O_2 (50 µM) and during the "recovery" period, the number of cells rose slightly again.

These data indicate that the reduction in proliferation in HUVECs due to intermittent exposure to H_2O_2 (50 µM) could be prevented by using R406 and TQ. Hence, to find further evidence for protective properties of these inhibitors against the effects of intermittent stress by H_2O_2 - such as the resulting drop in cell proliferation - we proceeded to investigate if use of these inhibitors might be able to counteract the stress-mediated induction of the so-called SASP (the senescence associated secretory phenotype). Part of this phenotype is the induced expression of the inflammation related adhesion molecule ICAM-1 and therefore we tested if our substances could prevent the associated pro-inflammatory secretory phenotype as well.





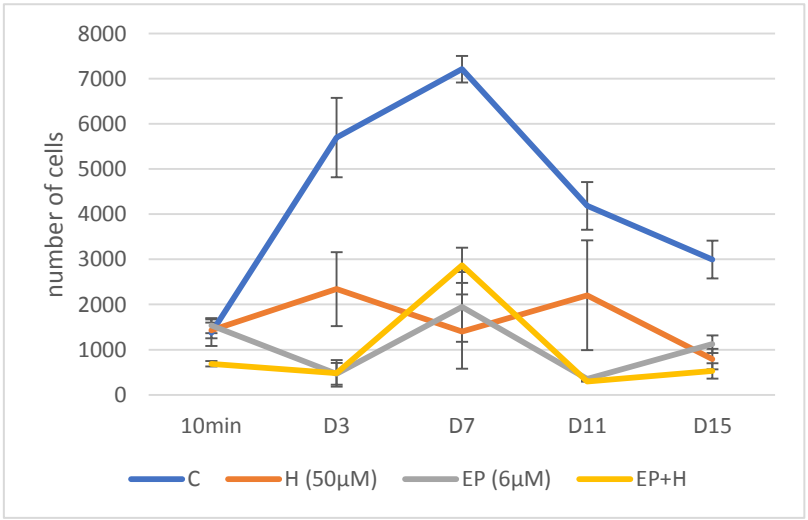


Figure 4: Inhibition of H_2O_2-induced cell-cycle arrest in HUVECs by R406, TQ, and EP: Growth curves of HUVECs after treatment with H_2O_2 (50 µM) ± inhibitors in comparison to control HUVECs for determined time points. Values are presented as mean ± SD of sextuplicates for the untreated and the H2O2-treated groups (C and H) and of triplicates for the inhibitor treated groups. (*p<0.05, **p<0.01, ***p<0.001).

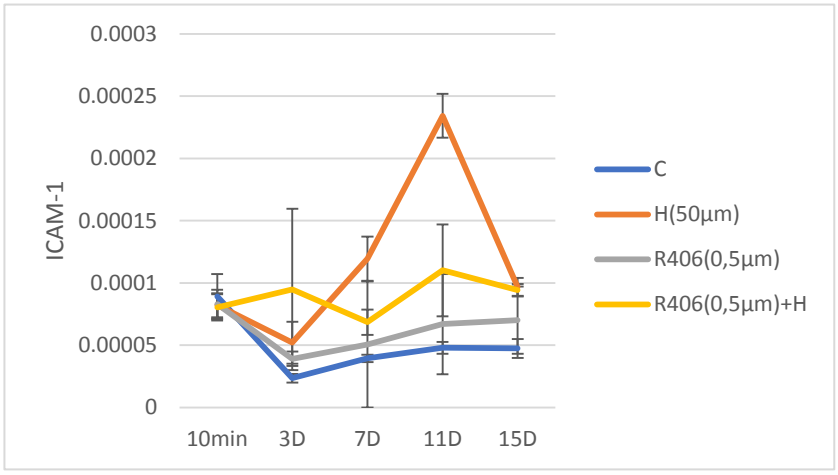
R406, TQ and EP show hints to be able to prevent the H2O2 stimulated senescence associated secretory phenotype (SASP) in HUVECs

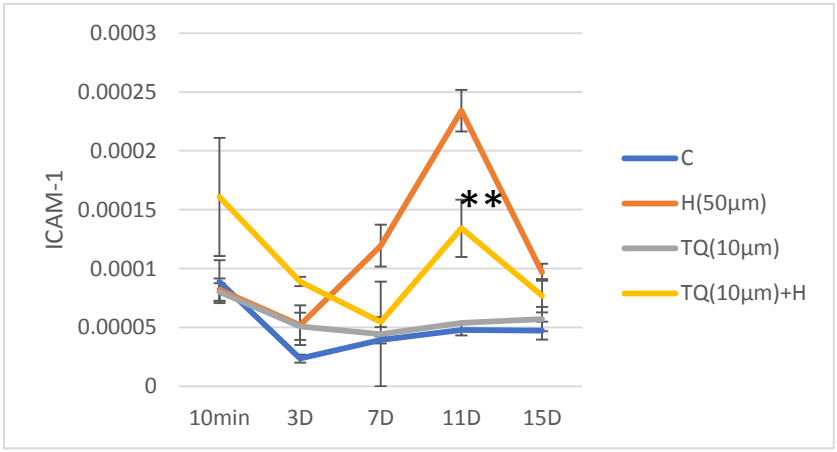
We tested if R406, TQ and EP could prevent the associated pro-inflammatory secretory phenotype as well. For this purpose, we assessed the surface expression of ICAM-1, as selected representative SASP protein, upon the sequential use of an inhibitor in the cells exposed to H_2O_2 (50 µM) every two days for one hour. The cell ELISA revealed that the treatment of H_2O_2 stressed HUVECs not actually simultaneously with R406 could prevented the upregulation ICAM-1 by day seven. TQ also showed a low degree of upregulation in these SASP protein in H_2O_2 treated cells on day seven, which became statically significant on day eleven ($p = 0.00814$), whereas EP did not seem to have a preventive effect.

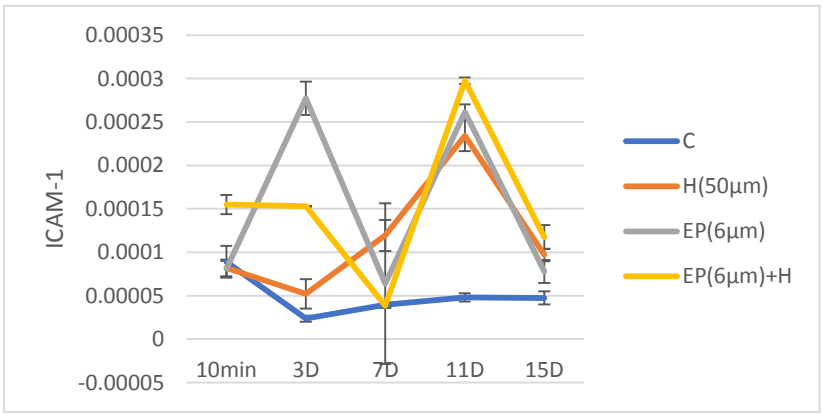
Therefore, our data revealed that R406 could possibly not only prevent the induction of cell cycle arrest in HUVECs intermittently exposed to H_2O_2 but has also substantially reduced the upregulation of the SASP protein ICAM-1.

TQ displayed a similar preventive effect regarding cell cycle arrest and SASP. However, ICAM-1 expression under H_2O_2/TQ greatly exceeds (cannot prevent but instead even increases during the first three days than with H_2O_2 alone. Noteworthy here is periodic fluctuation, an alternation between increasing and decreasing: I-CAM expression was found increased already at the 60 min time point, dropped by day three and rose again by day eleven. This trend was observed not only in the H_2O_2/TQ condition but also when TQ was applied alone.

EP causes a higher ICAM-1 expression compared to the control in a time span between day three and day fifteen. On day three and day eleven it even exceeds the curve for H_2O_2 (50 µM) on its own. With EP I-CAM increased at 60 min time point, dropped at day seven, increased again at day eleven, dropped at day fifteen. The ICAM-1 expression is much higher at the early times until day three with H_2O_2 /EP than with H_2O_2 by itself. This increase drops off at day seven and reaches its maximum on day eleven and falls again on day fifteen.







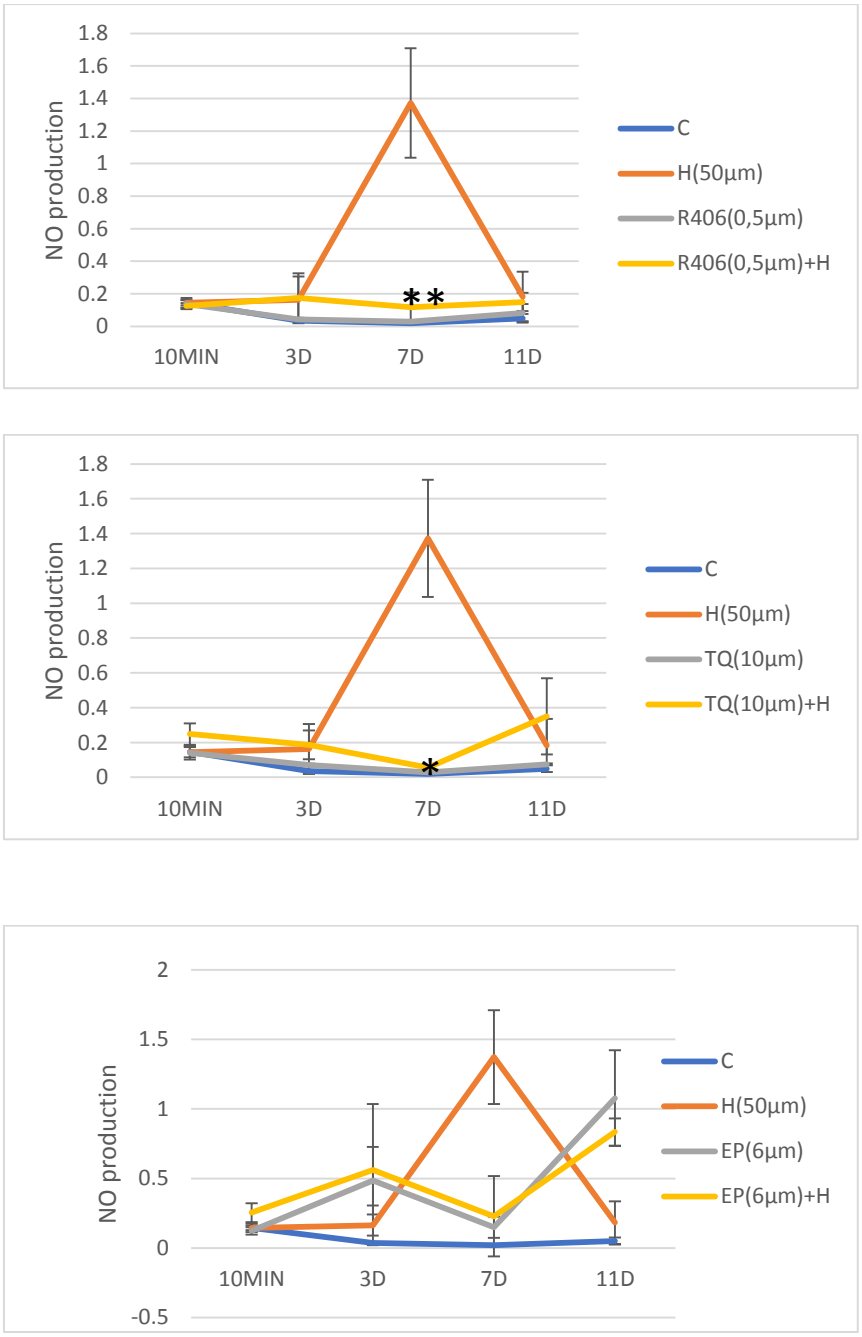
R406 and TQ could prevent induction of SASP in HUVECs whereas EP did not seem to have a preventive effect. Surface expression ICAM-1 quantified by cell ELISA of HUVECs grown in the presence of intermittent concentration of H_2O_2 (50 µM) ± inhibitors in comparison to control HUVECs ± inhibitors for the indicated time points. Values are presented as mean ± SD of sextuplicates for the untreated and the H_2O_2-treated groups (C and H) and of triplicates for the inhibitor treated groups. (*p<0.05, **p<0.01, ***p<0.001).

R406 and TQ could be able to normalize the elevated NO production in H_2O_2-stressed HUVECs in the long term

Next, we evaluated the production of NO upon the succedaneous use of the inhibitors in the cell intermittently exposed to H_2O_2 (50 µM) for one hour. The treatment of H_2O_2 treated HUVECs succedaneous with R406 increased the upregulation of NO on day three. TQ also showed a upregulation in this NO generation in H_2O_2 treated cells from the early time point (10 minutes), whereas cells treated with EP show a very high NO production in the EP treated cells effect on day eleven.

The lowest NO production can be observed in the control cells and a similar trend can be observed with those with R406. H_2O_2 /R406 remain highest until day three and then decrease, initially comparable with H_2O_2 (50 µM) until day three and then finally become lower than H_2O_2 alone till day eleven. The NO production with TQ alone does not differ a lot from our control throughout the whole experiment. With the cells treated with the combination of H_2O_2 (50 µM) /TQ and the NO level is higher over the early time points until day three compared to our stimulated cells. Thereafter it dropped at day seven and rose again on day eleven.

At the early time point of 10 minutes and day three, the highest NO production is measurable in the cells with the combination of H_2O_2 (50 µM) and EP, followed by EP and then those stimulated with H_2O_2 (50 µM). The lowest NO release was seen in the control cells. On day seven the NO level was elevated in the H_2O_2 (50 µM) treated cells and dropped for the cells treated with EP and those with H_2O_2 (50 µM)/EP. However, this sequence changes by a very highly significant NO production in the EP treated cells, followed by H_2O_2 (50 µM)/EP on day eleven.



Prevention of H_2O_2-induced NO production in HUVECs by R406, TQ, and EP.
Quantification NO production in HUVECs stressed with H_2O_2 (50 µM) ± inhibitors in comparison to control HUVECs ± inhibitors for the indicated time points using our developed Griess protocol. Values are presented as mean ± SD of sex tuplicates for the untreated and the H_2O_2-treated groups (C and H) and of triplicates for the inhibitor treated groups. (*p<0.05, **p<0.01, ***p<0.001).

R406 and TQ could possibly quench the H_2O_2 induced ROS generation

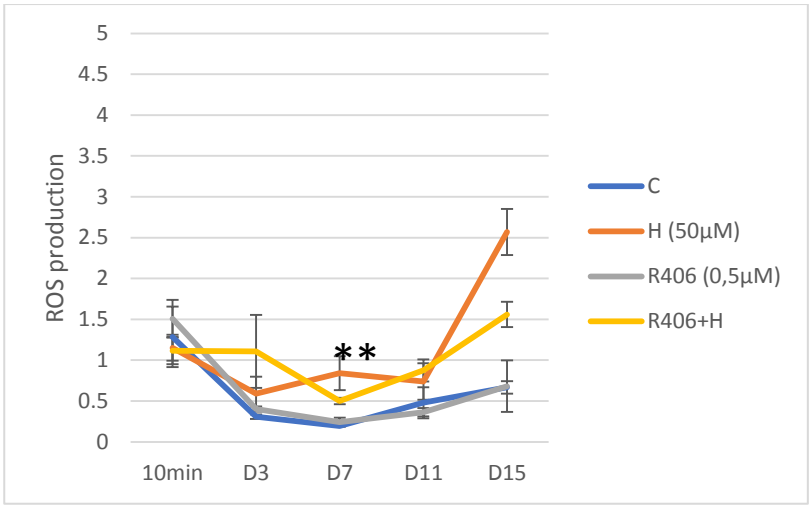
We then analyzed the ROS production over a period of nine days in cells intermittently exposed to of H_2O_2 for one hour on every other day. There was high production of ROS at the 60 minutes time point which decreased on day three but was still elevated. The ROS generation though gradually increased over the span between day 3 and day eleven.

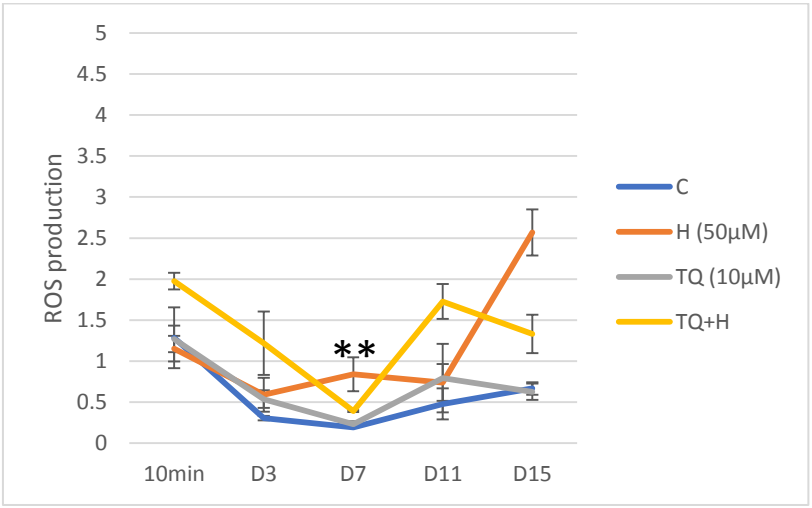
These data revealed the increased ROS generation in HUVECs upon their exposure to H_2O_2 (50 µM) for one hour, and therefore it suggests that this exposure might have led to increase in cell cycle arrest and reduced proliferation in HUVECs through the generation of ROS.

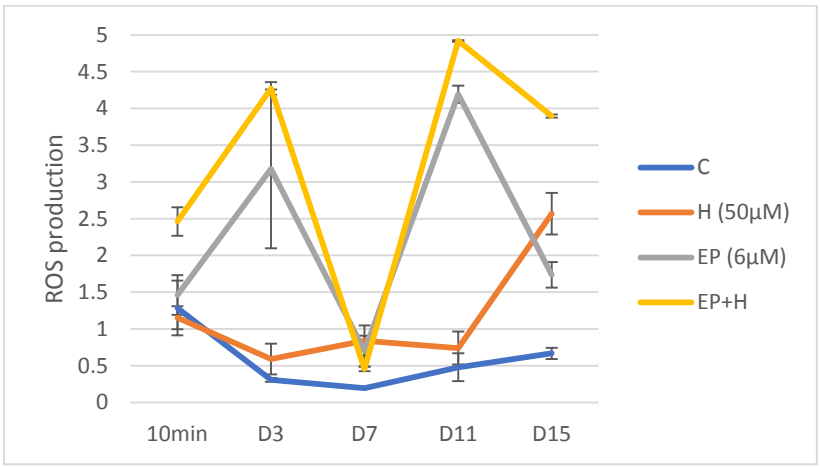
Here we want to assess whether one of our substances could also prevent the H_2O_2 (50 µM) induced ROS production in HUVECs. For this purpose, we used R406 (0,5 µM) after removing the H_2O_2 on the first eight days. Similarly, we also used the plant derived EP (6 µM) and the antioxidant thymoquinone (10 µM) and these inhibitors were added again after removal of H_2O_2 like that of R406.

We noticed that the cells treated with H_2O_2 and treated by using the inhibitors R406 and TQ, lead to statistically significantly reduction in (for both inhibitors) ROS production when compared with those, treated with H_2O_2 alone at the studied time point (day 7 and day 15).

However, EP did not prevent ROS production in H_2O_2 treated cells. Both R406 and TQ were more efficient in preventing the ROS generation in H_2O_2 treated cells than EP. At the 10minute time point the SYK inhibitor alone has a slightly higher generation ROS than H_2O_2, afterwards it falls off and is comparable to the control. The reason for the drop could be the 1:1 dilution before the stimulation on D2, as we noticed under the microscope that the initially chosen concentration had a toxic effect on the cells. On day 15, ROS level is significantly declined by SYK-inhibition. The combination of H_2O_2/R406 even surpasses H_2O_2 alone in ROS production up to day three and falls from day 7 on.







prevention of the H_2O_2-induced increase in ROS levels in HUVECs after treatment with R406, TQ, and EP. Measuring of ROS levels in HUVECs exposed to H_2O_2 (50 µM) ± inhibitors in comparison to control HUVECs ± inhibitors for indicated time points, by using fluorophore H_2-DCF (10 µM). Values are presented as mean ± SD of sextuplicates for the untreated and the H_2O_2-treated groups (C and H) and of triplicates for the inhibitor treated groups. (*p<0.05, **p<0.01, ***p<0.001).

Discussion

Intermittent exposure of HUVECs to H_2O_2 reduces number of cells

Different groups have used H_2O_2 to induce cellular senescence in HUVECs, however the protocols vary in terms of concentration and time of induction[18,19,20,21]. In this study, HUVECs were exposed to H_2O_2 (50µM) for one hour, followed by culturing them in full medium plus or minus inhibitors (R406, TQ, EP) and without H_2O_2. The exposure was repeated on day two, four, six and eight. Cells were then grown until day fifteen in complete medium. The cells were only given complete medium as a negative control.

Cell number, cell morphology, reactive oxygen species (ROS) production, the intracellular adhesion molecule (ICAM-1), and the endothelium-derived relaxing factor (EDRF, NO) were measured to assess the senescence associated phenotype. All these measurements were always done one day after the induction to let the cells to respond to the stimulus as well the inhibitor to avoid the acute response read out. This means that the induction took place on day 0, 2, 4, 6 and 8 while all measurements were assessed at 10 min and on Day 3, 7, 11 and 15. So the cells had time to recover and to develop an actual defense mechanism.

The periodic exposure of HUVECs to H_2O_2 (50 µM) rises slightly the number of cells after 60 min and falls again on 15 compared to negative control. This is due to the fact that stimulating cells with exogenous ROS or growth factors and cytokines stimulates several signalling pathways that promote cell growth and differentiation while simultaneously causing DNA damage, cell senescence, and apoptosis[22]. The mitogenic signals are fast and transient causing little or no damage to cells. Long-term exposure to low levels of ROS, as well as short-term exposure to high levels of ROS, can cause protein oxidation and DNA damage, leading to the initiation of senescence and/or apoptosis programmes.[22].

Our data show peak point at day 7 where control reached maximum confluency. From day 11 on the number of control cells begins to decline, but the difference between untreated and H_2O_2 treated cells remains significant. This peak point at day 7 was reported by (Oda, Shiramizu et al., 2018)[23] who has followed up the cells in their model for only 9 days. The number of cells increased significantly at day 3 for all conditions, which might be because of two times media changes during the stimulation at D0 and D2. While the control cells exhibit further an increase reaching the maximum numbers (confluency) at D7. R406 treated cells alone showed slightly increase at D3 reaching the maximum at D7 as well. Both control and SYK-treated cells failed down after D7 probably due to confluency on one hand, and on the other hand because of long time they have been in culture which could lead a portion of cells to become senescent so that attenuation proliferation. However, the H_2O_2 –treated cells continuously decreased in number reaching the minimum record at D15. This reduction could be rescued partially by SYK in some later time points. We observed a slightly higher ROS production in HUVECs exposed to H_2O_2 after D3, which became very highly significant from day 7 until day 15. These data revealed that repeated exposure of HUVECs to H_2O_2 (50µM) resulted in an increase of ROS production and reduced number of cells.

Treatment with H_2O_2 led to generation of ROS

Following ligand-receptor interactions, reactive oxygen species (ROS) are produced and serve as particular second messengers in signaling cascades involved in cell proliferation and differentiation[22]. the endothelial cell production of ROS is always linked to endothelial dysfunction[24]

Endothelial cells were exposed to H_2O_2 (50µM) for one hour, followed by culturing them in full medium plus or minus inhibitors (TQ, EP and R406). The exposure was repeated on day two, four, six and eight. Cells were then grown till day fifteen in complete medium. Over the full period of fifteen days, we measured the production of ROS using 2',7' – dichlorofluorescin diacetate (DCFDA, also known as H2DCFDA)[22]. Even after 3 days we see a slight increase in ROS production in H_2O_2 treated cells in comparison to the negative control, which became statistically significant after 7 days. The first peak was reached on 7. The ROS production decreased on D11 but was still significantly higher than the ROS measured in the untreated cells. That difference persisted till 15 days and even increased so that we have an increase of almost 4 times on day 15. When analyzing the ROS production of non-treated cells on their own we see a slight elevation from D7 on, the reason could be: we have on day 7the highest cell number and because of the crowd some of them got detached from the gelatin and died and/or some of cells showed a senescence phenotype because of the long culturing period which can also contribute to such ROS augmentation. This data revealed that the exposure of HUVECs to H_2O_2 led to statically significant higher ROS generation while reduced proliferation.

Intermittent exposure of HUVECs to H_2O_2 prompted the senescence associated secretory phenotypes

Besides the permanent cell cycle arrest of senescent cells, there are changes in the gene expression of senescence-associated secretory phenotype proteins (SASP) as inflammatory cytokines and chemokines (e.g., IL-6, −7, and −8), growth factors (e.g., GRO, HGF, and IGFBPs), and the expression of adhesion molecules[25]. For this purpose, we assessed the adhesion molecule ICAM-1, which is one of the SASP proteins known to be expressed excessively in induced senescence, by modified cell ELISA[22]. The data showed significant increase in surface expression of ICAM-1 of H_2O_2-treated cells at one-hour time point on till day15 compared to control non-treated cells. Our data has shown that intermittent exposure of HUVECs to H_2O_2 has not only induced cycle arrest and ROS production in these cells but has also augmented ICAM-1 cell surface expression as one of the SASP proteins together indicating induction of cellular senescence[26].

NO production upon H_2O_2 exposure in HUVECs

Nitric oxide (NO) is the most important autacoids that is produced by the endothelium which responsible for vasodilation, inflammation, and oxidative stress mainly through the production of reactive oxygen species (ROS). In our investigation, HUVECs were exposed to H_2O_2 and, interestingly, the H_2O_2-treated group had higher NO levels than the control group. This could be due to H_2O_2 inducing NO synthesis as part of the cells' self-protective process.

Because The H_2O_2 level that employed in this study was not lethal to HUVEC, the cells were still able to boost their endogenous NO production when challenged. Also, H_2O_2 cause oxidative degradation of the generated NO, this can explain why the increasing level of NO in the H_2O_2-treated group was not as great as the other groups as rutin and the combined rutin and H_2O_2 groups (Figure 4).

H_2O_2-upregulated eNOS expression considered endothelial cells' self-protective method to preserve NO bioactivity under conditions of increased oxidative stress. H_2O_2 also boosts eNOS activity through causing changes in the enzyme's phosphorylation state.

Conclusion

A permanent cell-cycle arrest and a pro-inflammatory secretory phenotype are considered a sign of cellular senescence. This can be stimulated by different ways such ionizing

radiation, oxidative stress, and inflammation. In endothelial cells, this phenomenon could be the reason for vascular disease. Plasma levels of the inflammatory cytokines could be an endothelial senescence marker. It was known that H_2O_2 is an activator of NF-κB (central inflammatory mediator) and stimulate production of intracellular oxidative stress the, however the mechanism has not been well explained. So, we tested the effect of intermittent exposure on of endothelial cells to 50 μM H_2O_2 and found that such treatment induced premature senescence. This stimulated endothelial senescence could be prevented by the protective/reversing role of the synthetic spleen tyrosine kinase inhibitor R046 (SYK) and plant-derived inhibitors thymoquinone (TQ) and eupatoriopicrin (EP), inhibitors of the NF-κB pathway. Our findings suggested that intermittent exposure could harm endothelial cells by generating senescence, and that chronically elevated H_2O_2levels could play a role in the pathogenesis of chronic inflammatory disorders by causing premature endothelial senescence.

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