Published in final edited form as:

Placenta. 2020 September 15; 99: 27–34. doi:10.1016/j.placenta.2020.07.013.

Stretch, Scratch, and Stress: Suppressors and Supporters of Senescence in Human Fetal Membranes

Lauren S. Richardson, PhD¹, Enkhtuya Radnaa, PhD¹, Rheanna Urrabaz-Garza, B.A.¹, Narmada Lavu, MD, MS¹, Ramkumar Menon, PhD^{1,*}

¹Department of Obstetrics & Gynecology, Division of Maternal-Fetal Medicine & Perinatal Research, The University of Texas Medical Branch at Galveston, 301 University Blvd., Galveston, TX 77555-1062, USA

Abstract

Introduction: Throughout gestation, amnion membranes undergo mechanical and or physiological stretch, scratch, or stress which is withstood by repairing and remodeling processes to protect the growing fetus. At term, increased oxidative stress (OS) activates p38MAPK, induces senescence, and inflammation contributing to membrane dysfunction to promote labor. However, the signaling initiated by stretch and scratch is still unclear. This study compares the induction of p38MAPK mediated senescence by stretch, scratch, and stress in human amnion epithelial cells (AECs).

Methods: Primary AECs from term, not-in-labor, fetal membranes were cultured using the following conditions (N=3); 1) CellFlex chambers with or without 20% biaxial stretch, 2) 8-well coverslips with or without scratch, and 3) cells exposed to cigarette smoke extract (CSE) inducing OS. p38MAPK (western blot or immunocytochemistry), senescence activation, and inflammation (matrix metalloproteinases 9 [MMP9] activity-ELISA) were determined in cells exposed to various conditions. T-test and One-Way ANOVA was used to assess significance.

Results: Biological membrane extension, mimicked by 20% biaxial stretch of AEC, maintained an epithelial morphology and activated P-p38MAPK ($P=0.02$) compared to the non-stretch controls, but did not induce senescence or MMP9 activation. AEC scratches were healed within 40-hrs, which included proliferation, migration, and cellular transitions aided by p38MAPK activation but not senescence. CSE induced OS increased p38MAPK ($P=0.018$) activation, senescence ($P=0.019$), and MMP9 ($P=0.02$).

*Corresponding author: Ramkumar Menon, PhD, Associate Professor, The University of Texas Medical Branch at Galveston, Department of Obstetrics & Gynecology, 301 University Blvd., Galveston, TX 77555-1062, USA, ram.menon@utmb.edu Telephone: 409-772-7596.

Author contributions: LR, ER, RU-G, NL conducted experiments. LR performed data analysis and drafted the manuscript. RU-G helped run western blots. RM conceived the project, designed experiments, provided funding and helped with data analysis and interpretation, and prepared the manuscript.

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Data availability: Data will be made available upon request.

Conflict of interest statement: The authors state no conflicts of interest regarding this study.

Conclusion: Physiologic stretch and scratch experienced during gestation can cause p38MAPK activation without causing senescence or inflammation. This may be indicative of p38MAPK's role in tissue remodeling during pregnancy. Overwhelming OS, experienced at term, results in P-p38MAPK mediated senescence and inflammation to disrupt membrane remodeling.

Keywords

Amniotic epithelial cells; in vivo stretch; wound formation; oxidative stress; fetal membranes

INTRODUCTION

Human fetal membranes (amniochorionic or placental membranes) perform many critical functions to maintain pregnancy^{1–6}. As the innermost lining of the amniotic cavity, membranes perform immune, structural, and mechanical functions that protect the fetus from microbes and other external factors that may compromise healthy pregnancy^{6–15}. Membranes are composed of two critical epithelial cellular layers, the amnion and chorion membranes, joined by a collagen-rich extracellular matrix (ECM), which contains mesenchymal cells^{1,3,16–20}. Similar to their unique cellular composition, both components contribute distinct mechanical properties to the full fetal membranes *in utero* (i.e., amnion membrane is highly elastic, whereas chorion is more mechanically stable)^{7–10,21–24}.

The amnion membrane, which is constantly hydrated by the amniotic fluid, is highly elastic and can withstand pressure without undergoing any rupture during normal pregnancy. This pressure and stretching on the amnion membrane *in utero* are caused by the growing fetus and an increase in amniotic fluid volume as gestation progresses^{23,24}. Almost four decades ago, Lavery and Miller described the ability of the amnion membrane to withstand *in utero* pressure due to the membranes recoverable (i.e., elastic extension) and nonrecoverable components (i.e., creep extension)²⁵. The amnion ECM is comprised of tropoelastins, elastin cross-linking enzymes, lysyl oxidase, and lysyl oxidase-like (LOXL) enzymes that contribute to the amnions mechanical function^{16,26–28}. Changes to these enzymes can predispose membrane to premature rupture resulting in preterm delivery²⁶. Thus, the amnion side of the fetal membrane plays a critical role in maintaining the membranes' structural integrity during gestation.

The cellular remodeling theory that is mediated by localized inflammation has been investigated further. Amnion membrane cells, specifically AECs, undergo constant turnover during pregnancy^{20,29,30}. AECs are shed throughout gestation and develop gaps and distorted areas²⁰. Further examination revealed that specific gaps lead to the development of microfractures in the membranes^{2,31,32}. These microfractures are biological features exhibiting degraded collagen with cells in transit between the epithelial layer or ECM. Microfractures are likely active sites of membrane remodeling^{2,31,33}. Microfractures are higher in number and do not heal at term as well as in preterm prelabor rupture membranes (pPROM)^{31,32}. In both these conditions, rupture is due to increased oxidative stress (OS), which limits AECs healing capacity and predisposes them to weaken. Fetal surgeries are often associated with pPROM due to a lack of wound healing^{34–36}. Although several theories are proposed in such conditions, the exact mechanism that contributes to pPROM

membrane pathology is still unclear. Understanding this mechanism may help to improve pregnancy outcomes after fetal surgeries which are complicated with membrane rupture.

Although amnion cells proliferate and remodel throughout pregnancy, they also undergo replicative senescence (aging) that peaks at term prior to parturition^{1,5,30,37–39}. This process is accelerated at term due to intrauterine OS. Additionally, stress causes activation of secondary signalers such as p38 mitogen activated kinase (MAPK) that can induce cell cycle arrest, inhibit cellular proliferation, and force senescence^{6,38,40–45}. Senescence features localized inflammation (i.e., pro-inflammatory cytokines, matrix metalloproteinases (MMPs), damage associated molecular patterns [DAMPs]) of cells that can further damage membrane architecture^{40,46–49}. Senescence associated inflammatory mediators are chemotactic and recruit immune cells that continue to enhance the inflammatory load contributing to membrane weakening^{6,50–52}. Premature senescence activation in response to various pregnancy risks factors can lead to pPROM and preterm births^{1,32,53–56}.

Thus, stretch, scratch (gaps due to cell shedding and development of microfractures), and stress are inevitable mechano-physical and physiological components of the intrauterine environment during pregnancy^{7,20,22,23,31,38,57,58}. In a normal state, all these factors may contribute in tandem to remodel the amnion membrane. However, their aberrant activities can cause adverse pregnancy outcomes. Therefore, understanding the mechanistic mediators of stretch, scratch, and stress can be beneficial to better understand how they contribute to pathologies. We have already reported that increased OS induces p38MAPK mediated senescence and inflammation leading to labor. However, the contributions of stretch and scratch are still unclear. The objective of this study was to compare the induction of p38MAPK mediated senescence and inflammation by stretch, scratch, and stress in human AECs.

METHODS

IRB approval:

This study protocol was approved by the Institutional Review Board at the University of Texas Medical Branch (UTMB) at Galveston, TX, as an exempt protocol to use discarded placenta after normal term cesarean deliveries (UTMB 11-251). No subject recruitment or consenting was done for this study and no identifiers were collected.

Clinical samples:

Samples we collected from the discarded placentas of term, not in labor, caesarian deliveries. Fetal membranes were dissected from the placenta, washed three times in normal saline, and cleansed of blood clots using cotton gauze. We then proceeded with the amnion cell isolation as described below. *Term sample criteria:* Placentas from women (18-40 years old) undergoing elective repeat cesarean delivery (between 37 and 41 weeks of gestation) prior to the onset of labor were included in the study. Women with a prior history of preterm labor and delivery, preterm premature rupture of the membranes, preeclampsia, placental abruption, intrauterine growth restriction, and gestational diabetes were excluded. Patients that were group B *Streptococcus* carriers, who were treated for urinary tract infection,

sexually transmitted diseases, chronic infections like HIV, hepatitis, and women who smoked cigarettes or reported drug and alcohol abuse were also excluded from this experiment.

Isolation and culture of human AECs:

All reagents and media were warmed to 37°C prior to use. The amnion membrane was manually peeled from normal, term, not in labor cesarean section placentas, then rinsed in saline and transferred to a Petri dish that contained Hanks Balanced Salt Solution (HBSS) (Mediatech Inc., Manassas, VA). The amnion membrane was then cut into 2 cm x 2 cm pieces. They were digested twice in 0.25% trypsin and 0.125% collagenase A (Sigma-Aldrich, St Louis, MO) in HBSS for 35 minutes at 37°C. After each digestion, the tissue was filtered through a 70µm strainer (Fisher Scientific, Waltham, MA) cell and trypsin was inactivated using complete Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 media (DMEM/F12) (Mediatech Inc.) supplemented with 15% fetal bovine serum (FBS) (Sigma-Aldrich), 10% penicillin/streptomycin, 10% amphotericin B (Mediatech Inc.), and 50µg/mL epidermal growth factor (EGF) (Sigma-Aldrich) The collected filtrate was centrifuged for 10 minutes at 3000 g. The cell pellet was re-suspended in 5 mL complete DMEM/F12. The cells were then counted using a hemocytometer. Once cells were counted, approximately 3-5 million cells per flask were cultured in T75 flasks containing complete DMEM/F12 media at 37°C, 5% CO₂, and 95% air humidity until they were 80-90% confluent.

AEC treatment:

Once the cells were 80-90% confluent, they were passed into a variety of culture plates depending on the assay. Cells were cultured under the following conditions for up to 48-hours (N=3 each): 1) control - standard cell culture conditions in a T25 flask, 2) collagen control - standard cell culture conditions in a Matrigel (1:25) coated T25 flask, 3) stretch - CellFlex chambers with or without 20% biaxial stretch, 4) scratch - AECs grown on 8-well coverslip for scratch induction, and 5) OS – treatment with cigarette smoke extract (CSE 1:50) to mimic conditions experienced at term.

Stretch: CellFlex membranes were precoated with Matrigel (1:25 in media) to mimic the basement membrane and incubated at 37°C with 5% CO₂ for 4 hours. CellFlex membranes were gently rinsed with warm media twice before passage 1 AECs were seeded at approximately 80% confluence and incubated at 37°C with 5% CO₂ for 24 hours. The next day, AECs were then serum-starved for 1 hour, and CellFlex membranes were placed inside the manual stretching device to an increased bi-axle stretch of 20% and incubated at 37°C with 5% CO₂ for 1 hour or 48 hours.

Scratch: Passage 1 AECs were seeded at approximately 80% confluence in 8-well coverslips and incubated at 37°C with 5% CO₂ for 24 hours. AECs were then serum-starved for 1 hour, rinsed with sterile 1× phosphate-buffered saline (PBS), and then scratched evenly down the middle of the well, in a straight line, with a 200-µL pipet tip to induce a uniform wound. Cells were washed with sterile 1× PBS four times to remove any cell debris. Scratch

assays were conducted to study wound healing and p38MAPK activation after 1, 25, and 40 hours.

Microscopy

Brightfield microscopy: Brightfield microscopy images were captured using a Nikon Eclipse TS100 microscope (10x and 20x) (Nikon, Melville, NY, USA). Three regions of interest per condition were used to determine the overall cell morphology.

Western blot analysis:

The protein samples were separated using precast gels and transferred to the membrane using an iBlot1 gel transfer device (Thermo Fisher Scientific, Waltham, MA, USA). Membranes were blocked in 5% blotting grade blocker made with 1x Tris buffered saline-Tween 20 (TBS-T) buffer for 1 hour. The primary antibody was added and the membrane was left to rock overnight at 4°C. The membrane was incubated with a secondary antibody. For membranes that were stripped, restore western blot stripping buffer was used; none of the membranes in this study were stripped more than three times. The following non-human antibodies were used: P-p38MAPK (1:300, Cell Signaling, T180/Y182), p38MAPK (1:1000, Cell Signaling), actin (Sigma-Aldrich, A5441).

Immunohistochemistry to localize P-p38MAPK:

After 1, 25, or 40 hours from wound induction, scratch assay cells were fixed in 4% PFA, then washed with PBS and 0.5% Triton-X. The cells were blocked using 3% BSA, and primary antibodies were added: P-p38MAPK (1:300, Cell Signaling, T180/Y182). Cells were then washed with PBS and DAPI was added. The images were then captured using fluorescent microscopy at 20x.

AEC senescence detection by flowcytometry:

After 48 hours the medium was removed from stretch or OS treated cells. Cells were treated with diluted bafilomycin (cat# BML-CM110-0100, Enzo Life Sciences, Farmingdale, NY, USA) and the negative control was treated with DMSO. Cells were incubated at 37°C for 1 hour. C12FDG (cat#7188 Setareh Biotech, Eugene, OR, USA) was added to the cells, except for the negative control. Cells were collected by adding trypsin, then trypsinization was stopped using complete medium. Cell were then transferred to a conical tube and centrifuged at 3,000 g for 10 minutes. The medium was removed and the pellet was re-suspended in 300 µL of annexin buffer treated with propidium iodine. The cells were run on a flow cytometer using a standard senescence-associated-β-galactosidase template (SA-β-Gal).

Matrix metalloproteinases 9 (MMP9) assay:

AEC-conditioned media was collected from stretch or OS treatments after 48 hours and diluted to a 1:10 ratio with calibrator diluent RD5-24. A human Active MMP-9 Fluorescent Assay (R&D Systems, F9M00) was conducted following the manufacturer's instructions. Standard curves were developed with p-aminophenylmercuric acetate (APMA) samples of known quantities. Sample concentrations were determined by correlating the samples

absorbance to the standard curve by linear regression analysis. This assay detected active, TIMP free form, of MMP9.

Statistical analysis:

Statistical analysis for normally distributed data was performed using a One-Way ANOVA with Tukey's multiple comparisons test and t-tests. Statistical values were calculated using GraphPad Prism; P values less than 0.05 were considered significant. Additionally, fold changes were calculated based on data means. Data are represented as mean $\pm$ SEM. All data were analyzed using GraphPad Prism 8.

RESULTS

Amnion stretch induces p38MAPK activation but not senescence or MMP9

Throughout gestation and at term, the amnion, which bares tensile strength for the fetal membranes, undergoes extreme mechano-physical stress as it stretches to accommodate fetal growth^{19,23,24,59,60}. To test if stretch can cause changes similar to that observed under stress conditions, specifically, stretch-induced p38MAPK activation, senescence, and inflammation, AECs were placed on top of CellFlex membranes that underwent a 20% biaxial stretch for up to 48 hours (Figure 1A). Membranes were coated with Type IV collagen matrigel to mimic the basement membrane of the amnion^{1,20,61}. Brightfield microscopy showed AECs maintained an epithelial morphology and did not transition under control, non-stretch, or stretch conditions (Figure 1B). Biaxial stretch induced p38MAPK activation (Figure 1C; $P=0.02$), but not senescence (Figure 1D) or MMP9 activation (Figure 1E) compared to non-stretch and culture controls. This suggests, mechano-physical stress such as biaxial stretch, may not lead to senescence and senescence associated inflammation during gestation.

Amnion cell scratch induces p38MAPK activation but does not inhibit wound healing

Previously, we have shown that AEC wounds exposed to OS prevented their closure, and underwent senescence⁵⁷. However, the role p38MAPK in this process is unclear. AECs under standard cell culture conditions were scratched to produce a wound and monitored for 40 hours (Figure 2). Fluorescent microscopy localized activated p38MAPK (Phosphorylated [P]-p38MAPK) in the cytoplasm and nucleus of cells lining the scratch edges after 1 hour (Figure 2 top). During active cell migration supported by epithelial to mesenchymal transition (EMT), AECs maintain P-p38MAPK expression, which localized to the nucleus of cells forming the leading edge at 25 hours (Figure 2 middle). Wound healing was associated with down regulation of P-p38MAPK and cells undergoing mesenchymal to epithelial transition (MET) at 40 hours (Figure 2 bottom). P-p38MAPK role in the natural wound healing process suggests its role in tissue remodeling during gestation. However, p38MAPK activation is regulated to avoid cellular senescence and inflammation. The factors that may regulate p38MAPK function during healing process (or during normal gestation) is not investigated for this report.

Oxidative stress induced amnion cell p38MAPK activation, cellular transition, senescence, and MMP9 activation

AEC senescence is associated with inflammation^{1,30,62,63}. As a comparison to the mechano-physical stressors tested above, OS was induced on AECs to understand its effect on p38MAPK activation, senescence and inflammation. AECs were treated with CSE, a potent OS inducer, for 48 hours to mimic physiological stress at term. OS treatment transitioned AEC into mesenchymal cell morphology indicative of EMT (Figure 3A) along with increased p38MAPK activation (Figure 3B; $P=0.018$), senescence (Figure 3C; $P=0.019$), and MMP9 activation (Figure 3D; $P=0.02$) compared to control cells. These results showed that CSE induced changes in AEC that mimicked physiological OS associated features term labor amnion membranes.

DISCUSSION

The ability of biochemical and biophysical stressors experienced during pregnancy to induce senescence and senescence-associated fetal membrane weakening is hardly studied. Recent investigations highlighting the importance of OS-induced amnion derived pro-labor signals has driven a broader understanding of which various *in utero* stressors contribute to the onset of term or preterm parturition^{6-8,44,64-68}. In this report, we tested the hypothesis that bio-physical/chemical factors experienced during gestation (i.e., stretch, scratch, and stress) would induce one of the stress signaler p38MAPK mediated senescence and inflammation in human AECs. Our study reports the following: 1) Stretch of AECs activates p38MAPK but does not induce cellular transition, senescence, or inflammation (MMP9 activation), 2) Scratch (or wound) in AEC can cause p38MAPK activation and assist wound healing, and 3) Stress, specifically OS, promotes p38MAPK activation, senescence, and inflammation as seen *in utero* at term. OS at term or preterm, potentially due to infectious or noninfectious (sterile) inflammation, has the potential to activate p38MAPK, which can interrupt the normal functions of the membrane by inducing senescence and inflammation.

The fetal membrane undergoes mechanical stretching as it grows and develops alongside the fetus and distends further at term with the defacement of the uterine wall^{8,69}. This has been validated in classical studies utilizing an *in vitro* model of amnion tissue stretch mimicking fetal descent^{7,22,23,70-72} and a balloon inflation approach to mimic a situation like polyhydramnios in non-human primates, both of which showed stretching induced inflammation²⁴. Over distension of membranes at term or preterm is also mimicked *in vitro* by cyclic stretching of the amnion^{7,8,22,23}. Cyclic stretch has been shown to increase cellular stress and pro-inflammatory cytokine production indicative of a laboring phenotype^{22,23}. However persistent stretch as tested here, corresponds with reports by *Kendal-Wright et al.* which demonstrated physical static strain placed on the amnion cells does not induce pro-labor chemokines like Interlukine-8 (IL-8)⁷. Additionally, static stretch has shown to protect amnion cells from apoptosis and may provide them with extra energy for cytoskeletal re-organization²². Static stretch in our system activated stress signaler p38MAPK and may contribute to the cytoskeletal remodeling as noted in reports by *Kendal-Wright et al.* We have reported senescence inducing properties of p38MAPK under extreme OS conditions; however, p38MAPK activation and lack of senescence with stretch suggest that a regulated

p38MAPK activation during pregnancy is likely a contributor of tissue remodeling. Further, these data suggest that mechanical stretch throughout gestation is a physiological factor that contributes to amnion remodeling and growth but does not contribute to labor associated changes.

In addition to mechanical stretch, at the cellular level, the amnion undergoes cyclic cellular transitions (i.e., EMT $\leftarrow \rightarrow$ MET) to maintain membrane integrity throughout pregnancy^{31,33,57}. These processes are critical for the healing of injury-induced wounds or cell turnover induced gaps in the amnion monolayer (i.e., microfractures)^{20,31}. Previous studies have reported the contribution of cellular transitions in amnion wound healing^{57,73}. *In vitro* scratch assays showed AECs could proliferate, transition into mesenchymal cells (i.e., EMT), migrate, transition back to epithelial cells (i.e., MET), to facilitate wound healing⁵⁷. This study also determined that amniotic fluid heals the AEC wounds at a much faster rate than AECs grown under standard culture conditions⁵⁷. This supports the nurturing mechanisms provided by various factors in the amniotic fluid throughout gestation^{74,75}. Herein, we add to this knowledge by validating p38MAPKs' role in amnion wound healing. Active p38MAPK in cells at the leading edge of healing process suggests its potential role in tissue regeneration^{33,38}. Additionally, the decrease in P-p38MAPK after wound healing supports that p38MAPK activation is also well-regulated during gestation⁴³. These data suggest that amnion cells can transition, undergo repair, and remodel to maintain structural and functional membrane integrity through regulated p38MAPK function. Factors that regulate p38MAPK function under normal gestation needs further investigation.

Unlike physical and mechanical strains and stressors that are present throughout gestation, intrauterine OS increases at term prior to labor or due to risk factors of preterm birth^{42,66–68}. Our results correspond with previously published work showing OS induces cellular aging^{5,30,38,42,45}. Senescent amnion cells produce pro-inflammatory cytokines as well as DAMPs and package them into exosomes that can be propagated through the fetal-maternal interface to cause labor related changes on the maternal tissues^{46,76}. Our data further show that biochemical OS, like that of a physiologic OS seen at term, but not biophysical or biomechanical stressors such as stretch or scratch, has the ability to induce AEC inflammation that can contribute to labor associated pathways.

Using multiple *in vitro* techniques, we determined the ability of various mechanical, biophysical (stretch and scratch), and biochemical factors (stress) in generating pathways that can lead to membrane weakening. Physiologic stretch and scratch experienced during gestation can cause p38MAPK activation without causing senescence or inflammation. On the contrary, overwhelming OS alone can cause p38MAPK activation leading to senescence and inflammation that can weaken the amnion layer of the fetal membrane. This knowledge will benefit future studies by differentiating biological and physical stressors that could contribute to term and preterm membrane weakening.

Acknowledgments

Funding: This study is supported by funding from NIH/NICHD 1R03HD098469-01 to R Menon and R01 HD100729 to both Dr. Menon and Dr. Han.

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HIGHLIGHTS

- Physiologic stretch and scratch experienced during gestation can cause p38MAPK activation without leading to senescence or inflammation.
- Oxidative stress, but not physiologic stretch or scratch, is an accelerator of senescence and inflammation in amnion cells.
- Knowledge of interplay between stretch, scratch and stress in response to pregnancy risk factors may benefit in delineating preterm parturition pathways.

Figure 1: Amnion stretch induces p38MAPK activation but does not cause senescence or MMP9
A) Images of non-stretch and stretch membranes with the yellow region highlighting the area of 20% biaxial stretch. **B)** Brightfield microscopy showing AECs maintaining epithelial morphology under control, collagen, non-stretch, and stretch conditions. **C)** Densitometry of western blot analysis showed P-p38MAPK was significantly activated in stretch compared to non-stretch after 1 hour ($P=0.02$). **D-E)** Flow cytometry did not show an increase in senescence-associated β -Galactosidase (SA- β -Gal+) positive AECs after 48 hours. **F)** MMP9 activation did not increase after 48 hours of stretch and remained similar to that of culture control and non-stretch conditions.

Figure 2: Amnion scratch induced p38MAPK mediated wound healing

P-p38MAPK localization during wound healing. Fluorescent microscopy images showing P-p38MAPK (green) and DAPI (blue) localized in AEC during wound healing. At the scratch sites, P-p38MAPK is located in the cytoplasm and nucleus of AECs within 1 hr. After 25 hours, P-p38MAPK can be seen at the leading edge of migration. Once the wound heals in 40 hrs, P-p38MAPK expression goes away throughout the cell population.

Figure 3: Amnion stress, induces p38MAPK activation cellular transitions, senescence, and MMP9 activation

A) Images of control and oxidative stress (OS) treated AECs. Brightfield microscopy showing OS treatment induced an elongated mesenchymal cell morphology indicative of an epithelial-to-mesenchymal (EMT) cellular transition. **B)** Densitometry of western blot analysis showed significant activation (P)-p38MAPK after 1 hour of OS treatment ($P=0.018$). **C)** 48-hour OS treatment increased senescence-associated-β-Galactosidase (SA-β-Gal) positive AECs documented by flow cytometry ($P=0.019$). **D)** MMP9 activation increased after 48-hour OS treatment compared to controls ($P=0.02$).