Online Data Supplement

Quercetin Eliminates Senescent Fibroblasts and Diminishes Pulmonary Fibrosis in Aged Mice.

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Methods

Isolation of primary pulmonary fibroblast lines

IPF lung fibroblasts were cultured from surgical lung biopsies acquired from clinically classified IPF patients with rapid or stable progressing disease. Normal lung fibroblasts were cultured from lung samples obtained from normal subjects. The lung tissues were finely minced and the dispersed tissue pieces were placed into 150-cm^2 cell culture flasks with complete media. Cells were serially passaged a total of five times to yield pure populations of lung fibroblasts.

Cell counting

Quantification of number of fibroblasts was performed on MACSQuant 10 (Miltenyi Biotech) flow cytometer and data were analyzed using Flowjo software (Treestar Inc.).

Caspase-3 activity

Caspase-3 activity was measured using the Caspase-3 Fluorometric Assay Kit (BioVision). First, the 96-well plates were centrifuged at 400 x *g,* conditioned supernatant was removed from the wells, and the cells were lysed with Cell Lysis Buffer for 30 min on ice. Next, 2x Reaction Buffer containing 10 mM DTT and 50 µM DEVD-AFC substrate were added to lysed samples and incubated at 37°C for 1 h. Samples were read in a

fluorometer equipped with a 400 nm excitation filter and 505 nm emission filter and results were expressed as relative fluorescence units (RFU) normalized to β-actin.

Cell viability

For cell viability assay, the supernatant was removed from the wells containing the cultured fibroblasts were washed twice with DPBS. The TetraZ solution provided in the TetraZ Cell Counting Kit (Biolegend) was added to the wells and incubated for 2 h at 37ºC and 10% CO_2. The absorbance at 450 nm was directly proportional to the number of viable cells.

LDH release

The release of LDH by dying cells was assessed in the conditioned supernatant following the manufacturer's instructions (Pierce LDH Cytotoxicity Assay Kit, Life technologies). Briefly, the fibroblast-conditioned supernatant was transferred to a new 96-well plate containing the kit Reaction Mixture. Samples were incubated at room temperature for 30 min and adding Stop Solution stopped reactions. The absorbance at 490 nm and 680 nm was measured using a plate-reading spectrophotometer to determine LDH activity.

Hydroxyproline Assay

Hydroxyproline content in whole mouse lungs was used to quantify lung collagen content and was measured colorimetrically. At the time of killing, all lobes of lung were removed and the extrapulmonary airways and blood vessels excised and discarded. The lung parenchyma was homogenized in 0.5 mL of ultra-pure water, after which 25 µL of sample was transferred to another tube. Next, 0.580 mL of 12 N HCl was added, and the samples were hydrolyzed at 120°C overnight. 50 µL of each sample were transferred into uncapped microtubes and incubated at 100ºC for 2 h to promote evaporation. First, 50 µL of citrate-acetate buffer (5% citric acid, 1.2% glacial acetic acid, 7.25% sodium acetate, and 3.4% sodium hydroxide) was added. Next, 1 mL100/80 of chloramine-T solution (1.4% chloramine-T, 10% N-propanol, and 80% citrate-acetate buffer) was added, and the mixture was incubated for 20 min at room temperature. Ehrlich's solution was added and the samples were incubated at 65°C for 20 min. Absorbance was measured at 550 nm. A standard curve was generated for each experiment using reagent hydroxyproline (Sigma-Aldrich) as a standard. Results were expressed as µg of hydroxyproline/mg of protein.

Histologic Analysis

For histologic analysis of the lungs, the whole left lobe was formalin-fixed and paraffin-embedded. Lung sections (5 µm) were stained with Masson’s trichrome stain to visualize collagen deposition and representative images at 100x magnification were presented.

Reactive oxygen species (ROS) levels

Lung fibroblasts were treated with quercetin (50 µM) or vehicle (0.05% DMSO) for 24 hours and total intracellular ROS and hydrogen peroxide (H_2O_2) release was determined. Intracellular ROS levels were measured by flow cytometry using the CellROX Green Flow Cytometry Assay Kit (Life Technologies). Geometric mean fluorescence intensity (GMFI) was acquired using Flowjo (Treestar inc.). H_2O_2 levels were measured using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (ThermoFisher Scientific).

Western Blot antibodies

Anti-p-AKT (Ser473) and -AKT (pan) (Cell Signaling Technology), -Caveolin-1 (ThermoScientific), -β-actin (Santa Cruz Biotechnology), -p21 (abcam), and -p19 ARF (abcam).

Table E1. Human TaqMan Primers and probes ID for qPCR

Genes	**ID**
18S	Hs99999901_s1
ACTA1	Hs00559403_m1
AXL	Hs01064444_m1
BMP4	Hs03676628_s1
CCND3	Hs01017690_g1
CDKN1A	Hs00355782_m1
CDKN2A	Hs00923894_m1
COL1A1	Hs00164004_m1
COL3A1	Hs00943809_m1
CTGF	Hs01026927_g1
CXCL12	Hs03676656_mH
FASLG	Hs00181225_m1
FGF10	Hs00610298_m1
FN1	Hs00365052_m1
GAS6	Hs01090305_m1
GPX3	Hs01078668_m1

GSR	Hs00167317_m1
GSS	Hs01047959_m1
HIF1A	Hs00153153_m1
HMOX1	Hs01110250_m1
IFNA1	Hs00855471_g1
IFNB1	Hs01077958_s1
IGF1	Hs01547656_m1
IGF1R	Hs00609566_m1
IL13RA2	Hs00152924_m1
IL17RA	Hs01064648_m1
IL1A	Hs00174092_m1
IL1B	Hs01555410_m1
IL33	Hs00369211_m1
IL6	Hs00985639_m1
IL8	Hs00174103_m1
KIT	Hs00174029_m1
KITLG	Hs00241497_m1
KRT14	Hs00265033_m1

KRT5	Hs00361185_m1
LGALS3	Hs00173587_m1
MMP28	Hs01020031_m1
NOX4	Hs00418356_m1
PDGFB	Hs00966522_m1
PGF	Hs00182176_m1
RAC2	Hs01036635_s1
SERPINE1	Hs01126606_m1
SOD2	Hs00167309_m1
TGFB1	Hs00998133_m1
TNF	Hs01113624_g1
TNFSF10	Hs00921974_m1
TWIST2	Hs02379973_s1
TXN	Hs01555214_g1
WNT5A	Hs00998537_m1

Table E2. Human Primer sequence for qPCR

Genes	Sequence
18S	Forward: 5’-AACCCGTTGAACCCCATT-3’ Reverse: 5’-CCATCCAATGCGTAGTAGCG-3’
CAV1	Forward: 5’-CAGGGAAACCTCCTCACAG-3’ Reverse: 5’-TGTAGAGATGTCCCTCCGA-3'
DR4	Forward: 5'-CAGAACGTCCTGGAGCCTGTAAC-3' Reverse: 5'-ATGTCCATTGCCTGATTCTTT GTG-3'
DR5	Forward: 5'-GCGAAGAAGATTCTCCTGAGATGTG-3' Reverse: 5'-ACATTGTCCTCAGCCCCAGGTCG -3'
FAS	Forward: 5'-GGTTCTTACGTCTGTTGCT-3' Reverse: 5'-CATGTTCACATCTGGAGGAC-3'
HO1	Forward: 5'-GCCAGGTGCTCAAAAAGATT3’ Reverse: 5’-CCTGCAACTCCTCAAAAGAGC3’
IL6	Forward: 5'-ATGTAGCCGCACACAGA-3' Reverse: 5'-ATTTGCCGAAGAGCCCCTCAG-3'

IL8	Forward: 5'-AGAGTCATTGAGAGTGGACC-3' Reverse: 5'-ACTTCTCCACAACCCTCTG-3'
NOX4	Forward: 5'-GGAGAGCCAGATGAACAGG-3’ Reverse: 5'-CTCAGTCTTTGACCCTCGG3-’
NRF2	Forward: 5’-GAGAGCCCAGTCTTCATTGC-3’ Reverse: 5’-TTGGCTTCTGGACTTGGAAC-3’

Table E3. Mouse Primer sequence for qPCR

Genes	Sequence
Gapdh	Forward: 5'- CATACCAGGAAATGAGCTTG-3' Reverse: 5'- ATGACATCAAGAAGGTGGTG-3'
Fasl	Forward: 5'- ATCCCTCTGGAATGGGAAGA -3' Reverse: 5'- CCATATCTGTCCAGTAGTGC -3'
Trail	Forward: 5'- GAAGACCTCAGAAAGTGGC-3' Reverse: 5'- GACCAGCTCTCCATTCCTA-3'
Cdkn2a	Forward: 5'- CGGGGACATCAAGACATCGT -3' Reverse: 5'- GCCGGATTTAGCTCTGCTCT -3'
Cdkn1a	Forward: 5'- ACATCTCAGGGCCGAAAACG -3' Reverse: 5'- AAGACACACAGAGTGAGGGC -3'
Cdkn2d	Forward: 5'- CTGGAAGAAGTCTGCGTCGG -3' Reverse: 5'- GTCTTGCCAAAGCGGTTCAG -3'
Mmp12	Forward: 5'- TGTACCCCACCTACAGATACCTTA -3' Reverse: 5'- CCATAGAGGGACTGAATGTTACGT -3'
	Forward: 5'- CCAACTCTCACTGAAGCCAGCTCT -3'

Mcp1	Reverse: 5'- TCAGCACAGACCTCTCTCTTGAGC -3'
Il6	Forward: 5'- GAGGATACCACTCCCAACAGACC -3' Reverse: 5'- AAGTGCATCATCGTTGTTCATACA -3'

Figure legends

Figure E1. Serial passage leads to replicative senescence in human lung fibroblasts. (*A*) Number of passages necessary for fibroblasts to become senescent, (*B*) representative phase-contrast images of proliferating age and senescent fibroblasts at late passage, and (*C*) *CDKN1A*, *CDKN2A*, *IL6*, and *IL8* mRNA expression in senescent fibroblasts. Images were taken at 10X and 40X magnification. Data are presented as means ± SEM, n = 3 - 4 per group. **P* ≤ 0.05, ***P* ≤ 0.01, and ****P* ≤ 0.001 as indicated by the bars.

Figure E2. Quercetin alone does not induce apoptosis in senescent primary lung fibroblasts. Caspase-3 activity, cell viability, and lactate dehydrogenase (LDH) release by senescent lung fibroblasts from normal patients (NL) and patients with stable (or rapidly progressing IPF (stable IPF and rapid IPF, respectively) following 24 hours treatment with quercetin (50 or 100 μM) or vehicle (0.05%DMSO). Caspase-3 activity was expressed as relative fluorescence units (RFU) normalized to β-actin levels, cell viability

as OD values at 450 nm, and LDH release as the difference in the absorbance values at 490 and 680 nm. Data are presented as means ± SEM, n = 3 - 4 per group.

Figure E3. Representative phase-contrast images of senescent human lung fibroblasts derived from normal patients (NL) (*A* - *D*) and patients with stable (*E* - *H*) or rapidly progressing (*I* - *L*) IPF (stable IPF and rapid IPF, respectively) treated with: vehicle, quercetin (50 μM), FasL (75 ng/mL), quercetin + FasL for 24 hours. Images were taken at 10x magnification and n = 3 per group.

Figure E4. Quercetin increases the susceptibility to TRAIL-induced apoptosis in senescent human lung fibroblasts. Caspase-3 activity, cell viability, and lactate dehydrogenase (LDH) release by senescent fibroblasts from normal lungs (NL) (A-C) and the lungs of patients with stable (D-F) or rapidly progressing (G-I) IPF (stable IPF and rapid IPF, respectively) following 24 hours treatment with vehicle (0.05%DMSO), quercetin (50 μM), TRAIL (100 ng/mL), or quercetin + TRAIL. Caspase-3 activity was expressed as relative fluorescence units (RFU) normalized to β-actin levels, cell viability as OD values at 450 nm, and LDH release as the difference in the absorbance values at 490 and 680 nm. Data are presented as means ± SEM, n = 3-4 per group. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and ****$P \leq 0.0001$ as indicated by the bars.

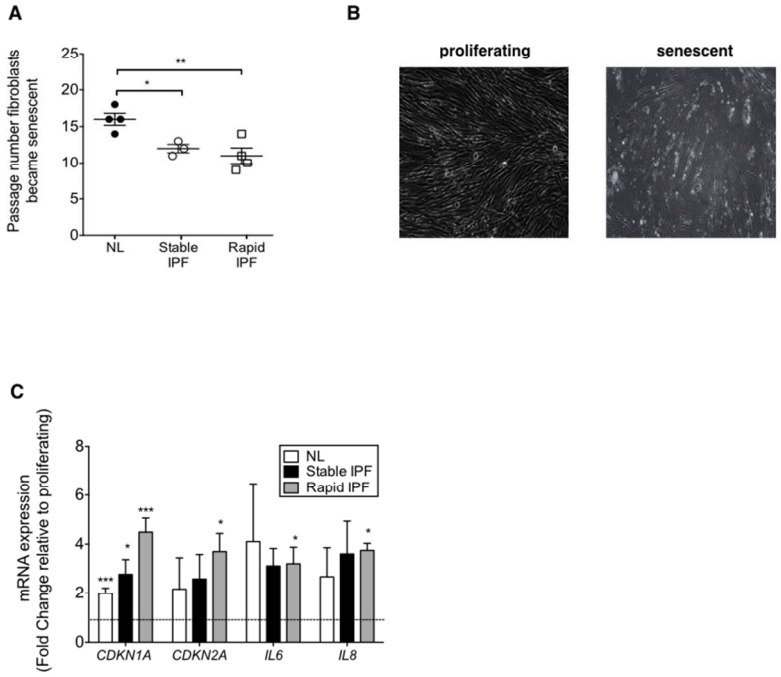
Figure E5. Representative phase-contrast images of senescent human lung fibroblasts derived from normal patients (NL) (A – D) and patients with stable (E – H) or rapidly progressing (I – L) IPF (stable IPF and rapid IPF, respectively) treated with: vehicle, quercetin (50 µM), cross-linked TRAIL (TRAIL) (100 ng/mL), quercetin + TRAIL for 24 hours. Images were taken at 10x magnification and n = 3 per group.

Figure E6. Quercetin's effect is independent of the oxidative status in senescent human lung fibroblasts. Effect of quercetin (50 µM, 24 hours) on (A) total intracellular reactive oxygen species (ROS) levels, (B) hydrogen peroxide levels in the conditioned supernatant, and (C) *NOX4* and (D) *NRF2* mRNA expressional in senescent lung fibroblasts isolated from normal patients (NL) and patients with stable or rapidly progressing IPF (stable IPF and rapid IPF, respectively). Data for ROS levels were expressed as geometric mean fluorescence intensity (GMFI) and hydrogen peroxide levels as relative fluorescence units (RFU). Data are presented as means ± SEM, n = 3-4 per group.

Figure E7. Whole lung (A) FasL and (B) TRAIL mRNA expression 7, 14, and 28 days post bleomycin-induced injury. Data are presented as means ± SEM, n = 7 mice per group. *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$ as indicated by the bars.

Figure E8. Masson's trichrome blue staining for collagen in the lungs of aged mice. Aged C57BL/6 mice received intratracheal (i.tr.) instillation of bleomycin (1.25 U/kg) or saline (equal volume). (A) Masson´s trichrome staining for collagen at day 7 (100x magnification). n = 7-10 mice per group.

Figure E1



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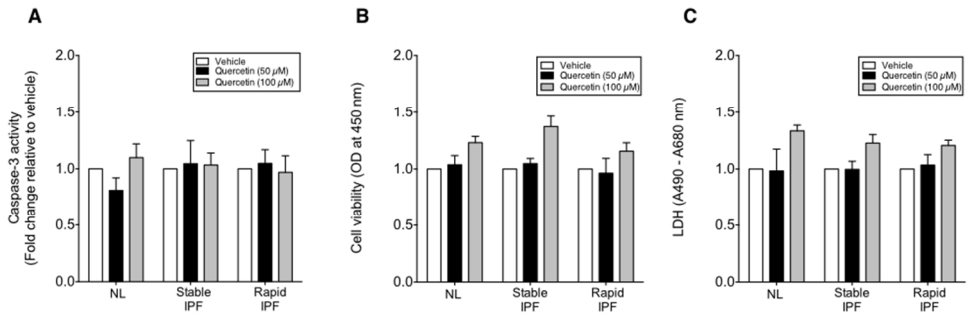

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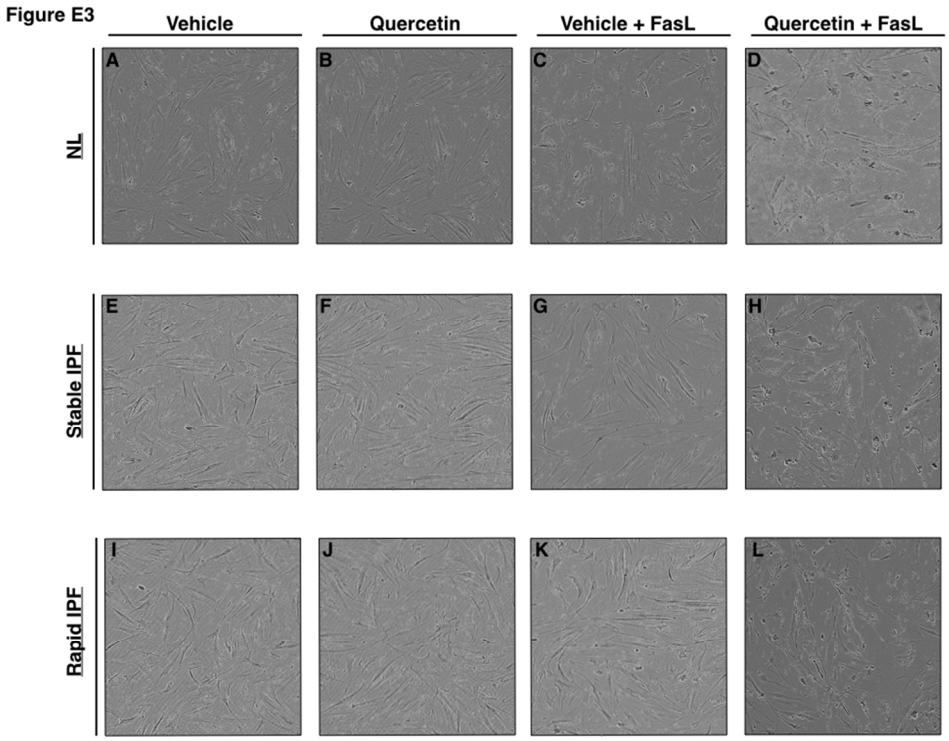

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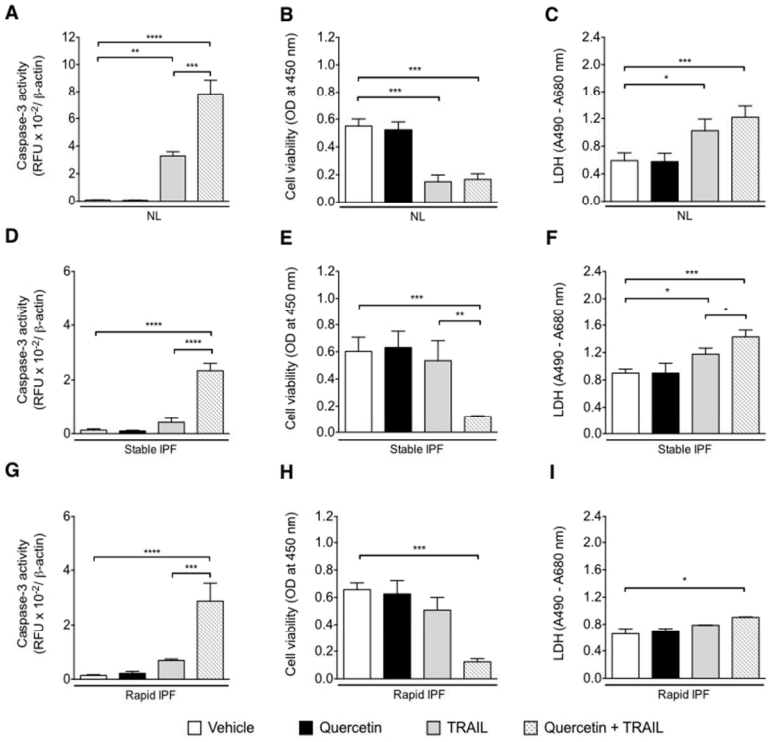

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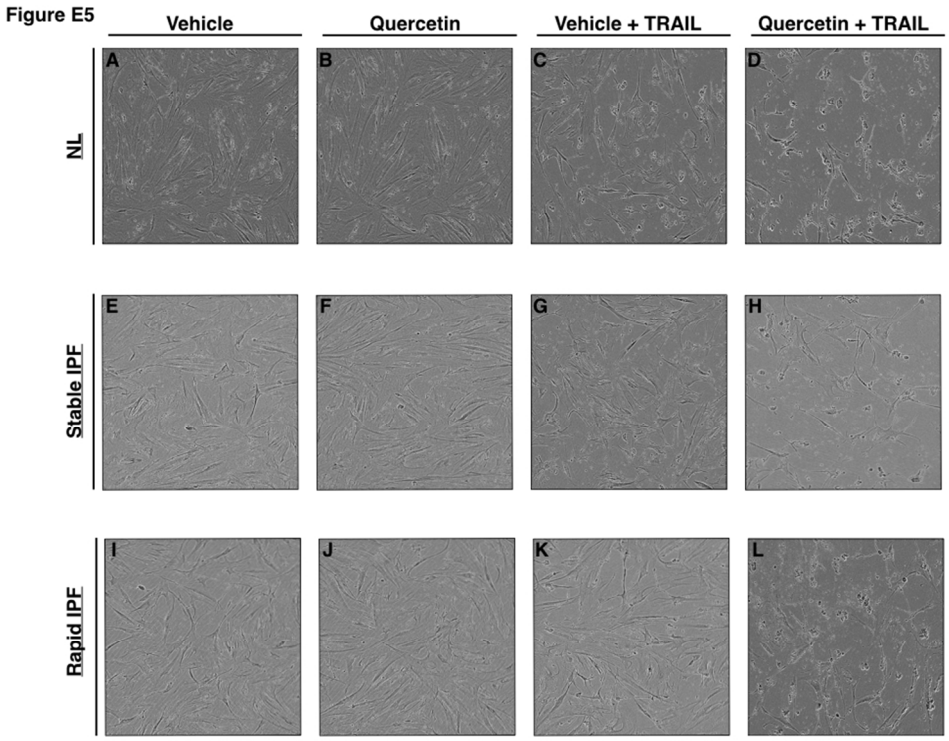

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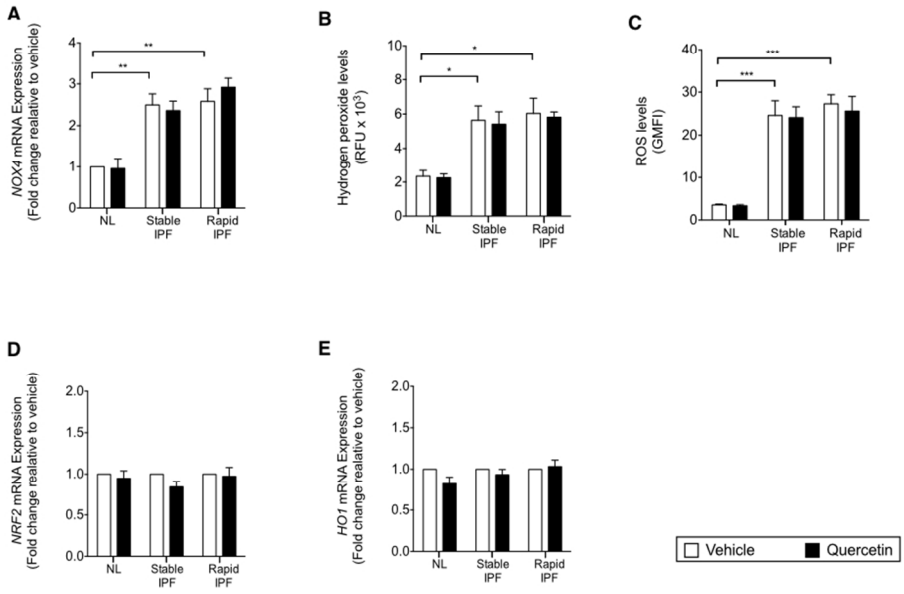

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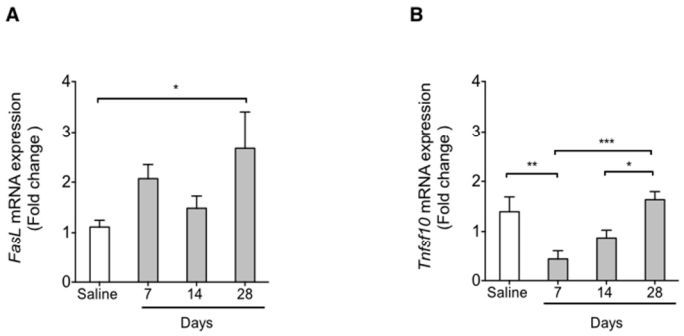

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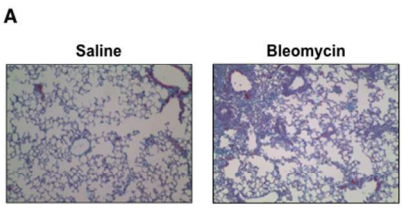

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