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Protective role of mesenchymal stem cells and mesenchymal stem cell-derived exosomes in cigarette smoke-induced mitochondrial dysfunction in mice

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Abstract

Background: Cigarette smoke (CS)-induced lung inflammation and Chronic Obstructive Pulmonary disease (COPD) involves mitochondrial dysfunction. Mesenchymal stem cells (MSC) and MSC-derived exosomes (EXO) are reported to show therapeutic effects in many animal models of inflammation and injury. In the present study, we determined the role of MSC and EXO intervention in CS-induced lung inflammation with focus on mitochondrial dysfunction.

Methods: EXO were characterized using Western blot for exosomal markers, tunable resistive pulse sensing by qNano and transmission electron microscopy (TEM). Mitochondrial reporter mice (Mt-Keima and mito-QC) were exposed to air or CS for 10 days. mt-Keima mice were treated with intraperitoneal injections of MSC or EXO or MSC and EXO (MSC+EXO) for 10 days. Total cell counts, differential cell counts were performed using automated cell counter and flow cytometry respectively. Further, the levels of pro-inflammatory mediators in bronchoalveolar lavage (BAL) fluid were measured using ELISA. Western blot analysis, quantitative PCR, confocal microscopy were used in the current study to determine the effects in the lungs of CS exposed mice. Seahorse flux analyzer was used to measure the oxidative-phosphorylation (OXPHOS) in the BEAS2B cells and BEAS2B+mMSC co-culture experiments.

Results: CS exposure increased the inflammatory cellular infiltrations in the lungs of the mt-Keima mice. MSC+EXO treatment showed protection compared to individual treatments (MSC or EXO alone). There were no changes in the mitophagy proteins like Pink1 and Parkin, which was

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Author contributions

KPM, IKS and IR designed and interpreted the experimental results. KPM and IKS performed all the experiments, analyzed the data and wrote the manuscript. IKS and IR improvised and approved the final version of the manuscript.

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Conflict of interests

The authors declare no conflict of interest.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

also found using the mitoQC mice. CS exposure led to significant increase in the mitochondrial fission protein DRP1 and other DAMPs pathway mediators like S100A4 and S100A8, HMGB1, RAGE and AGE. MSC+EXO treatment increased the gene expression of (fusion genes) like *mfn1*, *mfn2* and *opa1*. Additionally, the *thot1* gene expression was increased in MSC+EXO treatment group compared to Air- and CS exposed groups. BEAS2B-MSC co-cultures showed protective response against the CSE-altered mitochondrial respiration parameters, confirming the beneficial effect of MSC towards human bronchial lung epithelial cells.

Conclusion: CS affects some of early mitochondrial genes involved in the fission/fusion process, enhancing the damage response along with altered cytokine levels. MSC+EXO combination treatment showed their protective effects. MSC+EXO combination treatment may act against these early events caused by CS exposure owing to its anti-inflammatory and other mitochondrial transfer mechanisms.

Keywords

Mitochondria; Cellular Senescence; Mesenchymal stem cells; Exosomes; COPD

1. Introduction

Cigarette smoke (CS) is the main etiological risk factor involved in the pathogenesis of Chronic Obstructive Pulmonary Disease (COPD) [1]. CS contains several free radicals and chemicals, which cause lung inflammation [2, 3]. Earlier, we have characterized that acute CS exposure causes lung inflammation and changes in some of the early cytokine responses inducing lung damage [4], and CS extract caused mitochondrial dysfunction in lung epithelial cells [5]. Mesenchymal stem cells (MSC) and MSC-derived exosomes (EXO) are gaining wide attention as therapeutic intervention in various inflammatory models of disease [6–9]. MSC showed its beneficial effect in several acute lung injury models [10]. Further, MSC shows its therapeutic benefits in multitude ways in which transfer of mitochondria is also reported as one of the potential mechanisms [11]. MSCs also exert paracrine effect by releasing the vesicles-like exosomes and other mediators which carry the information and signaling for the cellular protection [12]. However their role in the acute CS-induced lung inflammation and injury is not known. On the other hand exosomal cargo includes but not limited to DNA, mRNA, miRNA, lncRNA and proteins, which play a crucial role in the cell signaling and response [13]. Exosomes were also reported to carry the functional mitochondrial elements and communicate [14].

The role of MSC and exosomes were shown in several cellular and animal models of lung injury [9, 10, 15, 16]. Recently, we have shown that plasma-derived extracellular vesicles (EVs) contain distinct protein signature among smoker and COPD subjects, compared to never smoking group [17]. Both MSC and EXO were reported to have anti-inflammatory effect in several animal models [18]. Recently, it was reported that intraperitoneal injection of EXO prevented bronchopulmonary dysplasia (BPD) in rats [19]. Porzionato *et al.*, found that intratracheal injection of EVs showed better activity compared to MSC in a rat model of BPD [20]. Recently it was shown that mesenchymal stromal cell exosomes prevent the severity of pulmonary fibrosis induced by bleomycin in mice [21]. Previously, it was reported that CS exposure induces lung inflammation, along with the altered levels of

cytokines in the BAL fluid of different mouse strains [3, 4]. Keeping in view the beneficial effects of both MSC and EXO, here we have investigated the combination effects of bone marrow-derived MSC and EXO (MSC+EXO) in acute CS-induced (10 days) lung inflammation model in mitochondrial reporter mt-Keima mice. We hypothesized that exosomes given along with the MSC may have better beneficial outcomes compared to individual treatments (MSC or EXO alone) in acute CS-induced lung inflammation. We have determined the effect of this treatment on 1) inflammatory cytokines/related pathways, 2) key mitochondrial proteins involved in biogenesis, dynamics (fusion and fission process) and mitophagy, and 3) damage-associated molecular patterns (DAMPs).

2. Materials and methods

All the key biological and/or chemical resources that are used in this study were validated and authenticated (methods and resources) and are of scientific standard from commercial sources.

Ethical approval

All the animal protocols and procedures described in this study were approved by the University Committee on Animal Resources of the University of Rochester, Rochester, NY.

2.1. Animals and treatment—mt-Keima mice (4-7 months) was used in the present investigation to observe the effects of MSC and EXO in the lung inflammation induced by acute CS exposure as previously reported [4]. The mice were sacrificed 2 hours after the last day of the CS exposure. The mice (n=6/group) were divided into the following, group-1; air exposed group (Air), in which the animals were air exposed and given intraperitoneal dose of saline, group-2; cigarette smoke group (CS), mice under this group were exposed to 10 days of cigarette smoke, group-3,4 and 5 were similar to the group-2 in terms of CS exposure, but received MSC (~1x10⁶ cells, one day prior to CS and on 6th day of CS exposure, designated as MSC), EXO (~15 µg, were given daily through intraperitoneal route after the CS exposure, designated as EXO) or both MSC and EXO (designated as MSC +EXO). mito-QC (heterozygous, 6-7 months) were also used in this study and were exposed to either air or CS for 10 days, as described above to determine the mitochondrial/mitophagy parameters. In the current experiment intraperitoneal route was selected based on its advantages as reported earlier [22] and since there are multiple or daily treatments with MSC and EXO, this route was chosen over intravenous route. Further, the dose of EXO was selected based on prior reports that showed beneficial effects *in vivo* in mouse [19,23].

2.2. MSC procurement and culture—Mouse MSC were purchased from Cyagen (#MUBMX-01001) received with passage number 6 and used within passage 10 (mostly 7-8) as per the manufacturer's instructions. The MSC are positive for CD34, CD44, Sca-1 and negative for CD117 by flow cytometry analysis. Mouse MSC were grown in the Mesencult expansion medium (Stem Cell Technologies, 05513) and later maintained in Iscove's Modified Dulbecco's Medium (Gibco, 12440) with 10% exosome-free FBS (Fetal Bovine Serum, exosome-depleted, One Shot, A2720803).

2.3. Exosome isolation and characterization—Exosomes from mouse MSC conditioned media was isolated as per the manufacturer's instructions (Norgen Biotek kit for cell culture media-exosomes-midi preparation # 60500). The isolated EXO were characterized for the presence of markers like CD63, flotillin and alix and the purity using anti-calnexin antibody. EXO were quantified using tunable resistive pulse sensing by qNano (Izon). TEM imaging was performed to observe the size and morphology of the isolated EXO. EXO protein was quantified using micro BCA kit (ThermoFisher).

2.4. Cell count analysis and pro-inflammatory mediators in the BAL—Total and differential cell counts in BAL fluid was performed as described earlier [24], except the detection was done using Guava easyCyte (Millipore). ELISA and Mouse cytokine array (R&D systems) analysis were performed to detect different cytokines in the BAL fluid [24].

2.5. BEAS2B and mMSC co-culture and measurement of OXPHOS—Seahorse XFp Cell Mito Stress Test kit (103010-100) was used to determine the effect of cigarette smoke extract (CSE) on BEAS2B, mMSC and mMSC-BEAS2B (bronchial epithelial cell) co-culture in XFp plate using Seahorse XFp analyzer. Cells (BEAS2B alone, mMSC alone and BEAS2B+mMSC co-culture) were treated for 24 hours using 0.5% CSE prepared as described previously [25]. Various OXPHOS parameters were evaluated using the WAVE software version 2.6.0 (Agilent).

2.6. Protein quantification in whole lung homogenate and mitochondrial fraction using Western blotting—Lung tissue homogenates were used to quantify various levels of mitochondrial, inflammatory and DAMPs markers across all the groups. Based on the initial observations in Air, CS and MSC+EXO groups, the study was expanded to determine other protein markers in rest of the groups. The following antibodies were procured from abcam parkin (ab15954, 1:1000), PGC1 α (ab72230, 1:1000), AGE (ab23722, 1:1000), S100A4 (ab27957, 1:500), OXPHOS for rodent (MS604-300, 1:500), MMP9 (ab38898, 1:1000), MMP12 (ab39876, 1:1000), flotillin (ab1927, 1:250) and β -actin (ab20272, 1:1000); Cell signaling technologies pink1 (6946S, 1:500), OPA1 (80471S, 1:1000), pP65-Nf κ B (3036S, 1:1000), HMGB1 (6893S, 1:1000); Santa Cruz NF- κ B p50 (SC-114, 1:1000), RAGE (SC-365154, 1:500), CD63 (SC-15363, 1:500), Aik (SC-11397, 1:250), Calnexin (SC-53540, 1:250); DRP1 (Novus biological, NB110-55288,1:1000), Tfam (EMD Millipore, ABE483, 1:1000), TSG6 (Sigma, SAB4502987, 1:1000) to determine the relative expression of various proteins as described earlier [4].

The mitochondrial fraction from lungs of mice were isolated according to the manufacturer's protocol (ThermoFisher, kit #89801) and used to check for the protein expressions as described above.

2.7. Quantitative real-time PCR—Total RNA was extracted from the lungs using Direct-zol RNA Kits (Zymo Research, #R2072) and this was subsequently used to prepare cDNA, and following this gene expression analysis were performed as described previously [25]. The following primers were used in the current study *rhof1* F-TGGCAGCACTGATAGAATAGA, R- GCAAAGACCGTAGCACCAAAG; *mfn1* F-CCTACTGCTCCTTCTAACCCA, R- AGGGACGCCAATCCTGTGA; *mfn2* F-

CCAAGTGTCCGCTC, R- GTCCAGCTCCGTGGTAACATC; *opa1* F-TGGAAAATGGTTCGAGAGTCAG, R- ATTCCGTCTCTAGGTTAAAGCG; *fis1* F-GCCCTGCTACTGGACCAT, R- CCCTGAAAGCCTCACACTAAGG and *gapdh* F-TTCCCGTTCAGCTCTGGG, R- CCCTGCATCCACTGGTGC. All the primers were obtained from integrated DNA technologies.

2.8. Confocal imaging—Mitochondria reporter (mito-QC) mouse lungs were OCT fixed, cryo-sectioned and kept in PBS for two intervals of 5 min each and mounted with the help of prolong anti-fade containing DAPI. These sections were observed for GFP, mCherry and DAPI under suitable wavelengths using FV1000 Olympus confocal microscope with a 100x oil immersion objective.

2.9. Statistical analysis—Two-tail unpaired student's t-test or One-way analysis of variance (ANOVA) with Tukey's *post hoc* analysis were used to compare the differences in the mean across various groups using GraphPad Prism 7. All the values are represented as mean \pm SEM and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Isolation and characterization of MSC-derived exosomes

Exosomes were isolated using the Norgen Biotek kit based method and immunoblot analysis was performed to observe for various exosomal markers. The mMSC exosomes were found to be positive for CD63 (using slot-blot analysis), flotillin and Alix as shown in Fig. 1A. The isolated exosomes were not contaminated by the endoplasmic reticulum as the calnexin bands were not detected but showed positive for the presence of the complex V (mitochondrial OXPHOS component) protein (data not shown). The isolated exosomes were observed for their size and morphology using TEM as shown in the Fig. 1A. Tunable resistive pulse sensing (TRPS) by qNano was used to determine the particle size, distribution and count/concentration of the isolated exosomes. The mean diameter was found to be around 115 ± 5 nm as determined using the NP250 membrane and CPC100 calibration beads.

3.2. Effect of different treatments on the cell counts in BAL of CS exposed mice

CS exposure led to a significant increase in the levels of total cell counts. Further, differential cell count analysis showed increase in the macrophage, neutrophil and CD4⁺ lymphocyte counts in the BAL fluid. Treatment with MSC, EXO and MSC+EXO significantly decreased the levels of the cell counts, macrophage, neutrophils and CD4⁺ cell population in the BAL as shown in the Fig. 1B implying some protective role during the acute CS-induced lung inflammation.

3.3. Effect of MSC and EXO treatment on various cytokine levels in BAL of CS exposed mice

CS exposure for 10 days significantly increased the levels of KC and S100A8, among the estimated inflammatory mediators using ELISA. However, the MSC and EXO showed no effect in decreasing these levels. Among other estimated mediators IL-6, MCP-1 and RAGE

levels were unchanged across all the groups compared to air exposed control (Fig. 1C). While cytokine profiler has showed increased levels of Adiponectin, KC, MPO, BAFF, MPO, IGFBP-2, RAGE, CCL6/C10 and CCL17 in the CS exposed mice. MSC+EXO treatment was found to be beneficial in restoring these changes as shown in the Fig. 2.

3.4. Effect of different treatments on the gene expression and protein levels in lungs of CS exposed mice

Initial gene expression analysis showed increase in the *rho1* (plays crucial role in the transfer of mitochondria and mitophagy) levels in MSC+EXO combination. Further, the expression levels of mitochondrial fusion genes *mfn1*, *mfn2* and *opa1* were significantly decreased in the CS group and MSC+EXO combination significantly improved these levels. However, the *fts1* expression levels remain unaltered (Fig. 3). Protein expressions of PINK1 (mitophagy related), TFAM (mitochondria biogenesis and damage related), BECN1 (autophagy marker) remain unaltered, while PGC1 α (mitochondria biogenesis) was alone found to be decreased in MSC+EXO group compared to air alone (Fig. 4). The protein expression analysis of DAMPs markers revealed that MSC+EXO significantly reduced the levels of S100A4 as compared to CS group, while RAGE and AGE remain unaltered (Fig. 4). Furthermore, protein analysis among all the groups showed that CS exposure increased the levels of DRP1 (mitochondrial fission related) with no changes in OPA1 (fusion) and parkin (mitophagy) in all the treatment groups (Fig. 5). Significant increase was observed in the levels of other inflammatory and DAMPs markers like MMP9 and HMGB1 in the CS group, whereas MSC+EXO significantly decreased these levels. However, no changes were observed in the MMP12 (data not shown) and TSG6 levels (Fig. 5). MSC treatment alone showed changes in the p65 NF- κ B levels compared to MSC+EXO treatment group (Fig. 5). The protein expressions of parkin, OPA1 and TFAM remain unaltered in the mitochondrial protein fractions analyzed by Western blotting (Fig. 6). OXPHOS immunoblot analysis for various mitochondrial OXPHOS complexes remains unaltered in the current study (Fig. 7).

3.5. Co-culture of MSC with lung epithelial cells protects from the mitochondrial stress

BEAS2B cells when exposed to 0.5% CSE for 24 hrs decreased many OXPHOS parameters like basal respiration, spare respiratory capacity, mitochondrial ATP production and maximal respiration. On the other hand, mMSC when exposed to 0.5% CSE for 24 hrs increased the maximal respiration and spare respiratory capacity. BEAS2B+ mMSC (at 1:1 ratio) co-culture when exposed to 0.5 % CSE for 24 hrs has no change in any of the parameters measured when compared their respective untreated controls (Fig. 8).

3.6. Effect of CS exposure on mitophagy in the lungs of mitoQC mice

mito-QC mice were exposed to either air or acute CS exposure for 10 days, similar to that of mt-Keima mice as mentioned earlier. In mito-QC mice, the mitochondrial fluorescence both red and green under basal/steady condition, but under mitophagy conditions, the mCherry fluorescence remains stable with diminished GFP signal, which can be seen as the puncta in the merged images and are indicative of mitophagy. Air group has basal mitophagy as evident by the presence of both GFP and mCherry signals. CS exposure has a slight increase in the number of mCherry puncta (Fig. 9A). Protein analysis revealed no significant changes

in the whole lung homogenates of the mito-QC mice, affirming the results as observed in the mt-Keima mice (Fig. 9B).

4. Discussion

CS exposure induced mitochondrial gene alterations, structure and function in several types of lung cells *in vitro* and animal models *in vivo* as reported previously [26–30]. Several of the mitochondrial targeting compounds show beneficial effect in CS-induced lung/cellular dysfunction [31–33]. In the present study, we used the Seahorse XFp flux analyzer to determine the mitochondrial bio-energetics in control and CSE treated human bronchial epithelial cells and MSC. Our data showed that CSE-treated BEAS2B and mMSC co-cultures showed protective response compared to their individual CSE exposure profiles, implying that MSC has potential to protect against CS-induced lung epithelial damage. Exosomal transfers of whole mitochondrial genome as well as functional mitochondria are reported, emphasizing on the importance of novel cellular communication mediated through exosomes [14, 34]. Mitochondrial reporter, mt-Keima mice exposed to CS were treated to determine the possible ameliorative effects of MSC, EXO and MSC+EXO on CS-induced lung inflammation, with emphasis on the mitochondrial damage responses. CS exposure increased cellular infiltrations in the BAL fluid of mt-Keima mice. MSC, EXO and MSC +EXO treatment significantly restored these levels to some extent, implying a plausible protective role towards early damage responses evoked by the inflammatory cells. The increase in the neutrophil levels was reflected in the increased levels of chemokine KC in the BAL of the mice exposed to CS. Along with this S100A8 was also significantly increased by CS treatment indicating the involvement of DAMPs in CS-induced lung inflammation as previously reported in several CS exposure models [35, 36]. Furthermore, cytokine protein profiler showed increase in various pro-inflammatory markers which were earlier reported to be increased by CS exposure in various organ systems implying the CS-induced inflammatory effects on multiple organ markers [36].

The role of mitochondria accounting for the lung damage in the pathogenesis of lung diseases is gaining wide importance for therapeutic interventions [37]. Lung epithelial cells are rich in mitochondria, owing to their function in maintaining various physiological activities [38]. Mitochondria maintain their dynamics using various processes among which the balance between fission and fusion are widely studied. Various cellular responses including but not limited to CS were reported to cause changes in these process, by mainly altering the crucial proteins involved in either fusion process like MFN1, MFN2 and OPA1 or fission like DRP1 and FIS1. We and others have reported the role of CS in causing changes in these proteins, which ultimately lead to the cellular damage responses/cell death or mitophagy [39, 40]. On the other hand, mitophagy process also plays an important role in counteracting various cellular insults by recruiting/processing the damaged mitochondria to lysosomes and prevent from further cell damage [39]. PINK1 and parkin plays an important role in initiating the mitophagy process. CS was reported to have varying effects in inducing the mitophagy depending upon the duration of the exposure [31]. Here we have found no change in these protein levels, among all the groups compared to air exposed mice. mito-QC and mt-Keima mice were developed to easily monitor/visualize the process of mitophagy in various organs. Mitochondrial reporter mice mito-QC has advantage with respect to tissue

fixation, giving stable signals as compared to the pH-dependent mt-Keima mice, which cannot be fixed and needs live imaging of tissues immediately after the experiments. For the first time, we have used mito-QC (heterozygous) mice to observe the changes caused by acute CS exposure in the lung mitophagy. CS exposure has no much effect in inducing the mitophagy changes as visualized by GFP/mCherry signals (although there was a non-significant increase in the mCherry signal by the CS), which is in accordance with the mt-Keima protein expression data mentioned above. Even though there is no change in the mitophagy initiation markers like PINK1 and parkin, we have found that CS increased the levels of mitochondrial fission protein DRP, indicating the initiation of the mitochondrial dysfunction. Previous studies observed changes in the p-DRP1 levels in the mice lungs exposed to CS for 3 weeks, without any changes in the autophagy related proteins [31], implying that fission/fusion plays an important role in counter acting the acute CS-induced damage response *in vivo*. Further CS exposure decreased the mitochondrial fusion genes like *mfn1*, *mfn2* and *opa1*, implying the initiation of mitochondrial damage responses, which may be later followed by the phenomena of mitophagy. Further the MSC+EXO treatment reversed these changes, indicating its therapeutic potential in counteracting these damage responses. MSC were reported to show their protective action by direct mitochondrial transfer involving some of the crucial genes like *rhot1* (*miro1*) or by secreting vesicles (exosomes) containing mitochondrial elements/protein to confer its protection [41, 42]. We found that MSC+EXO treatment increased the Rhot1 levels compared to air and CS groups. However, further protein analysis and specific Rhot1 related mechanisms need to confirm this initial observation. Studies are in progress to further dissect the exact role of *rhot1* gene in these transfer mechanism, employing cell-specific knockout mice. In parallel with the mitochondrial markers, DAMPs pathway is also taken into account in this study, since DAMPs are reported to connect with the mitochondrial damage process [43]. CS exposure increased some of the DAMPs markers like RAGE, HMGB1, S100A4 and S100A8. MSC +EXO treatment was found to be beneficial in bringing down these levels in some of these markers. Our data was in accordance with some of the observations with respect to increase in markers like MMP9 and S100A8. MSC+EXO decreased MMP9 levels in the lungs, but not S100A8.

In conclusion, intraperitoneal injection of MSC+EXO combination showed protective response against acute CS-induced lung inflammation in mice. This study further emphasizes the beneficial role of MSC+EXO treatment in short-term CS exposure in mice. The CS exposure and treatments targeted the DAMPs and mitochondrial genes, implying the crucial role played by the mitochondria towards the progression of the COPD. Further studies employing different sources/routes of exosomes needs to be characterized to better understand the cell/vesicle based therapeutics in CS-induced models of COPD/emphysema.

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Research Highlights

The protective role of MSC and EXO in CS-induced lung mitochondrial dysfunction was determined.

MSC + EXO treatment showed much protection compared to individual treatments.

CS exposure led to significant increase in the mitochondrial fission protein and DAMPs.

BEAS2B-MSC co-culture showed protection against the CSE-altered mitochondrial respiration.

MSC + EXO treatment protect against CS-mediated against mitochondrial dysfunction.

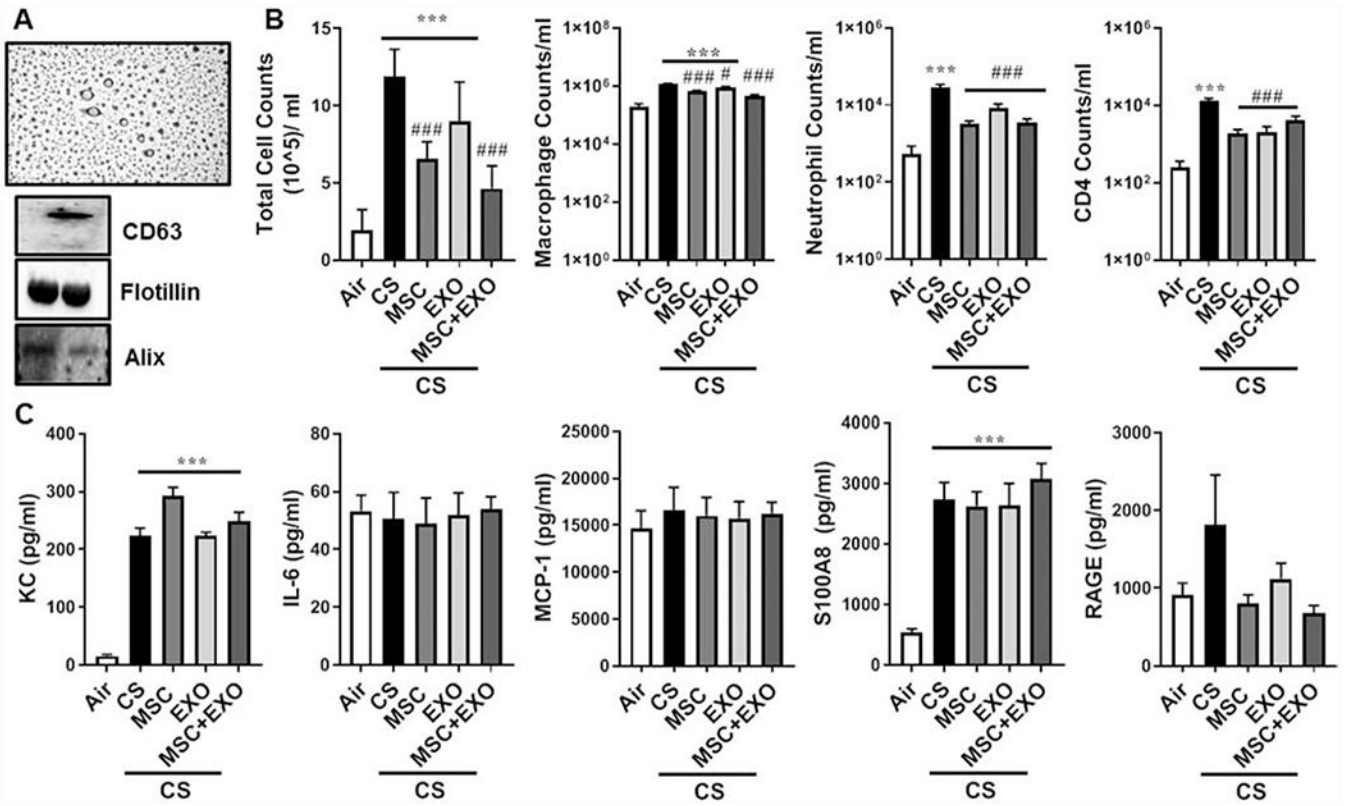


Fig. 1. Characterization of mMSC-derived exosomes and the effect of their combination MSC +EXO on various pro-inflammatory mediators in BAL fluid of acute 10days CS exposed mice. **A)** Isolation and characterization of mMSC-derived EXO using TEM, qNano and Western blotting. **B)** Intraperitoneal injection of MSC and EXO combination decreased lung cell infiltrations in mice exposed to acute CS estimated in the BAL fluid. **C)** Effect of MSC and EXO on different pro-inflammatory mediators in response to acute CS. Data were shown as mean \pm SEM (n=6/group). *** P < 0.001 vs. air; # P < 0.05, ### P < 0.001 vs. CS.

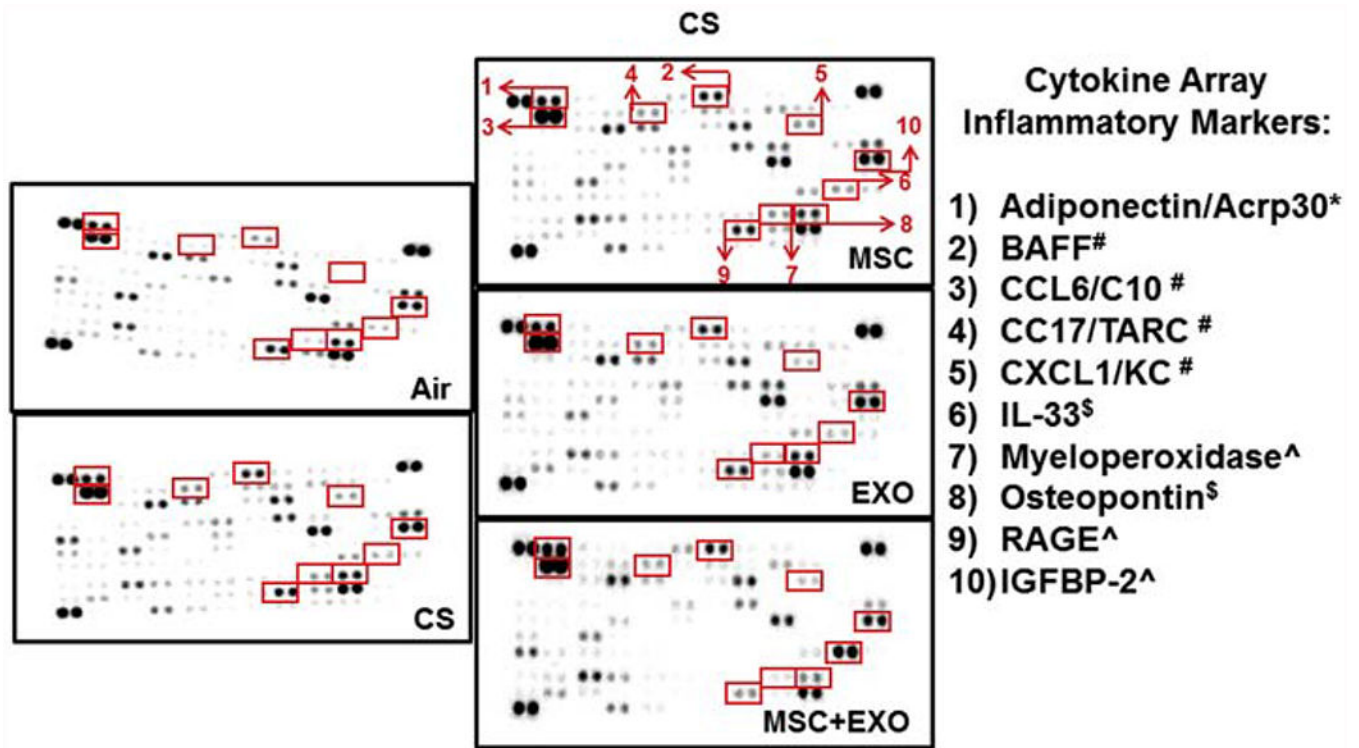


Fig. 2. Estimation of various pro-inflammatory cytokines in the BAL fluid following acute CS exposure by mouse cytokine array. Representative images of the cytokine arrays with the red arrows indicating the position of particular cytokines which was significantly altered among the different treatment groups. The list of significantly altered cytokines along with their original positions in the cytokine array is clearly indicated. * Cytokines increased in all the treatment groups except CS exposed group; # Cytokines increased in all the CS exposed groups (MSC, EXO and MSC+EXO); \$ Cytokines osteopontin was decreased and IL-33 increased only in CS exposed MSC+EXO group; ^ Cytokines increased in all the CS exposed groups (except decreased in MSC+EXO).

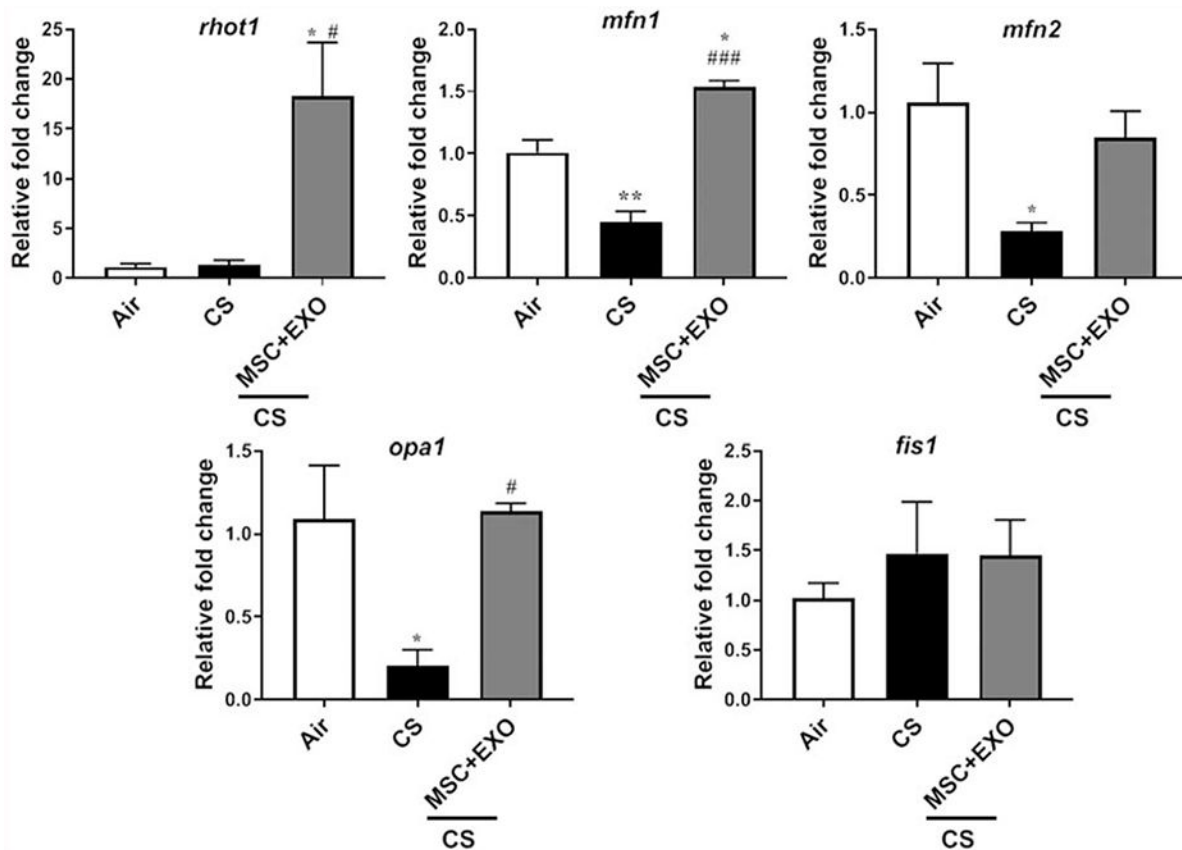


Fig. 3. Effect of intraperitoneal treatment of MSC and EXO combination on different mitochondrial trafficking and dynamics genes assessed by qPCR in acute CS exposed mouse lungs. mt-Keima mice was exposed to acute CS and total RNA isolated from mouse lung were used for gene expression analysis by qPCR. Gene expression of mitochondrial trafficking and dynamics target genes such as *rhot1*, *mfn1*, *mfn2*, *opa1* and *fis1* were qPCR using the 2^{-Ct} method. Bar graphs represent the mean normalized expression of samples in air, CS and CS exposed MSC+EXO treatment groups. Data were normalized using the endogenous housekeeping gene *gapdh* as reference and air as calibrators. Data were shown as mean \pm SEM (n=3/group). * $P < 0.05$ vs. air; # $P < 0.05$, ### $P < 0.001$ vs. CS.

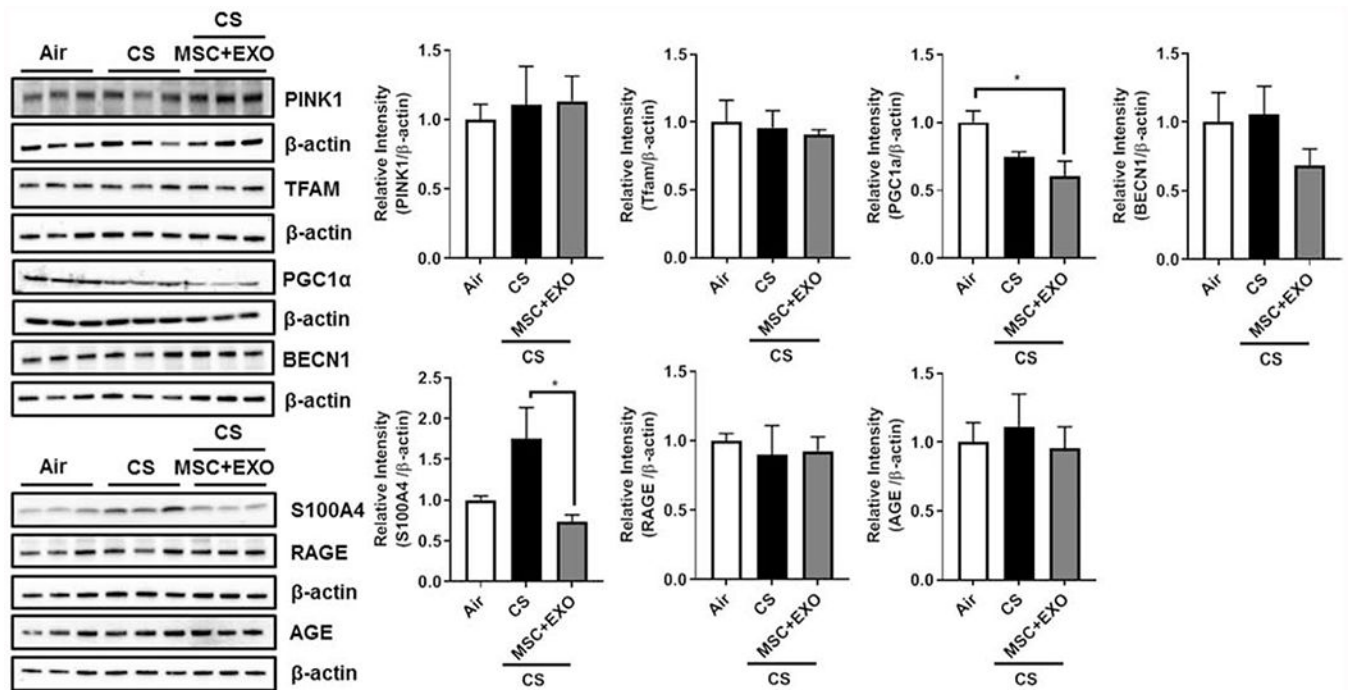


Fig. 4.

Effect of intraperitoneal treatment of MSC and EXO on mitochondrial and DAMPs protein expression in acute CS exposed mouse lung. Whole lung homogenates were used for Western blot analysis against mitochondrial markers using anti-Pink1, anti-TFAM, anti-PGC1 α , and anti-BECN1; DAMPs markers using anti-S100A4, anti-RAGE and anti-AGE antibodies. The protein abundance of mitochondrial and DAMPs markers was measured following acute CS exposure in mouse lungs. The band intensity was measured by densitometry, and data are shown as relative intensity to β -actin as loading control. Data were shown as mean \pm SEM (n=3/group). * represent P value $<$ 0.05.

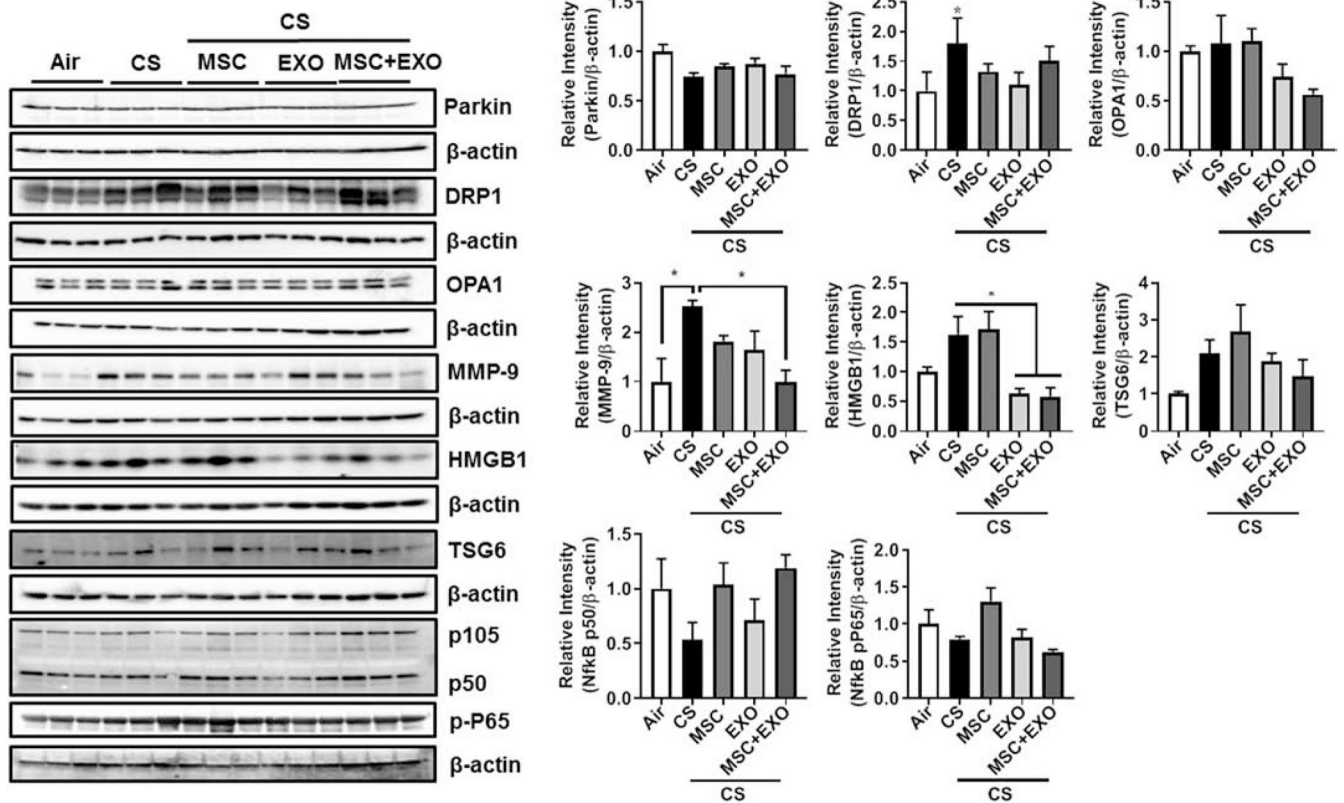


Fig. 5.

Effect of intraperitoneal treatment of MSC and EXO combination on mitochondrial, DAMPs and inflammation protein expression in acute CS exposed mouse lung. Whole lung homogenates were used for Western blot analysis against mitochondrial (anti-parkin, anti-Drp1, and anti-Opa1), DAMPs (anti-MMP9 and anti-HMGB1), and inflammation (anti-TSG6, anti-105, anti-p50 and anti-pP65) markers using antibodies. The protein abundance of mitochondrial, DAMPs and inflammation markers was measured following acute CS exposure in mouse lungs. The band intensity was measured by densitometry, and data are shown as relative intensity to β -actin as loading control. Data were shown as mean \pm SEM (n=3/group). * $P < 0.05$.

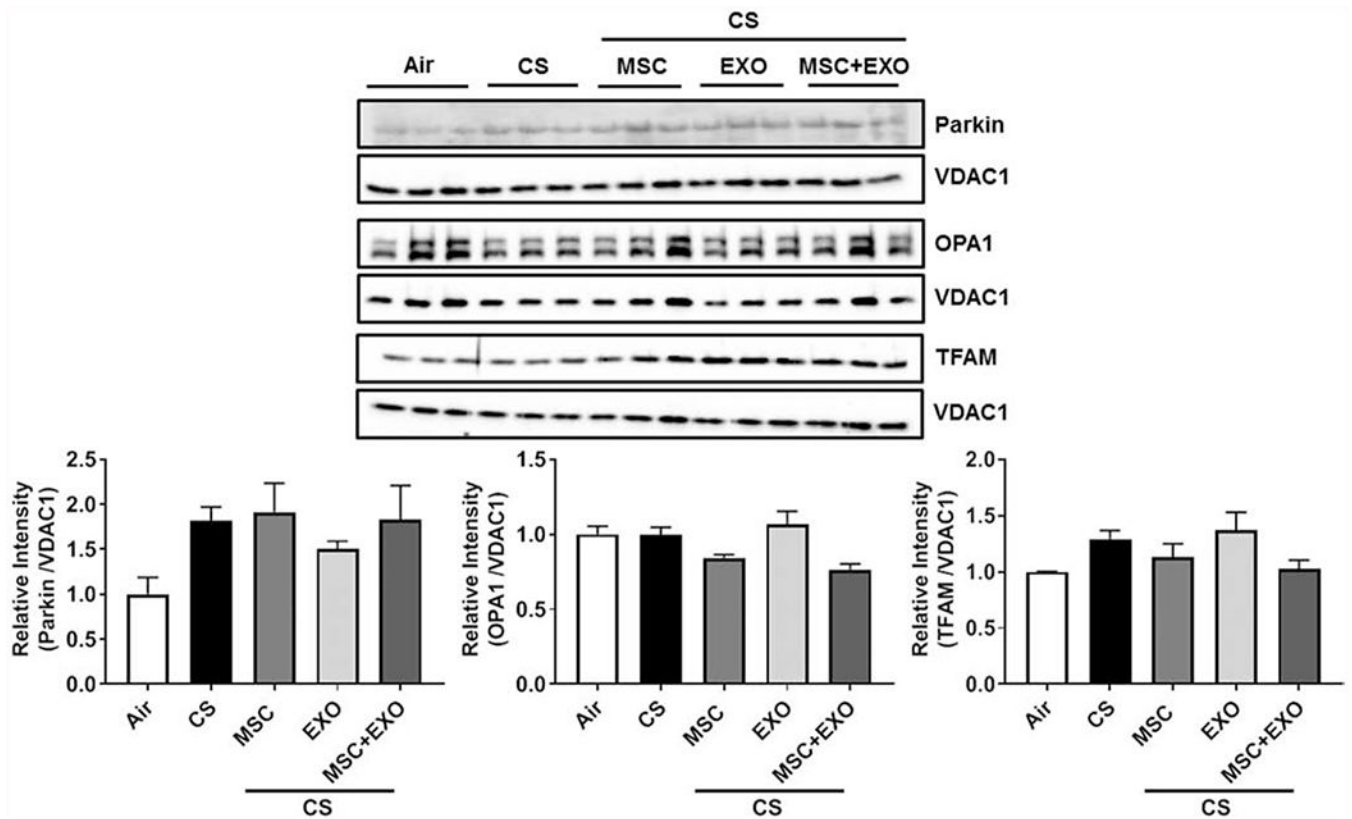


Fig. 6. Effect of intraperitoneal treatment of MSC and EXO combination on the key mitochondrial proteins using the mitochondrial fraction in acute CS exposed mouse lung. Mitochondrial extracts were used for Western blot analysis against mitochondrial markers (anti-parkin, anti-Opa1 and anti-TFAM antibodies) and anti-VDAC1 antibody for mitochondrial loading control. The protein abundance of mitochondrial markers was measured following acute CS exposure using the mitochondrial fraction isolated from the mouse lung. The band intensity was measured by densitometry, and data are shown as relative intensity to VDAC1 as loading control. Data were shown as mean \pm SEM (n=3/group).

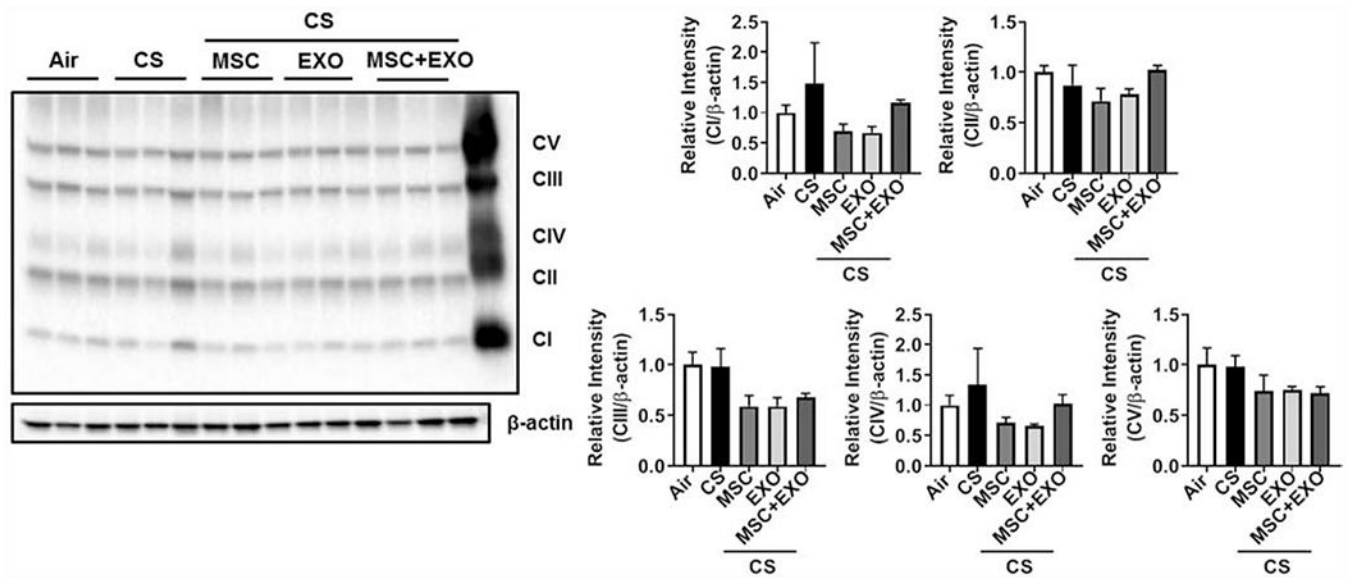


Fig. 7. Effect of intraperitoneal treatment of MSC and EXO combination on mitochondrial OXPHOS related complex proteins in acute CS exposed mouse lung. Representative Western blot for Total OXPHOS showing levels of mitochondrial Complex I (NDUFB8), Complex II (SDHB), Complex III (UQCRC2), Complex IV (MTCO1) and Complex V (ATP5A) in whole lung homogenates. Densitometry of the corresponding mitochondrial complexes (Complexes I–V) was normalized with β -actin as loading control. Last lane in the image represents positive control sample from rat heart mitochondria provided for location and identification of various OXPHOS protein complexes. Data were shown as mean \pm SEM (n=3/group).

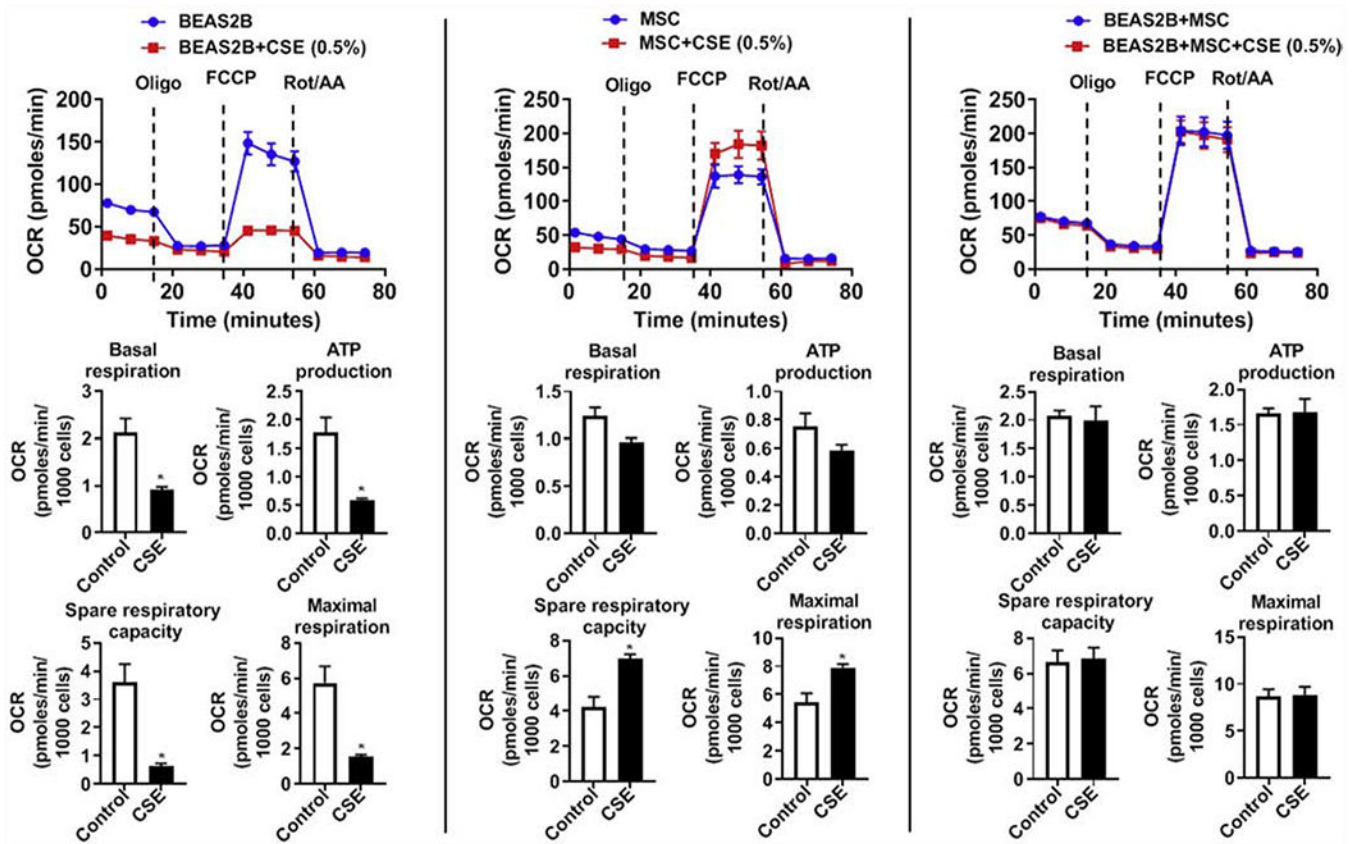


Fig. 8. Effect of CSE treatment on the mitochondrial function in BEAS2B and MSC (1:1) co-culture estimated using Seahorse XFp flux analyzer. Individual BEAS2B or MSC alone or co-culture (BEAS2B+MSC) were treated with 0.5% CSE for 24 hrs and oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using Seahorse XFp Mito Stress Test kit at the indicated time points as shown. CSE treatment induced changes in basal respiration, ATP production, spare respiratory capacity, and maximal respiration were quantified. Data were shown as mean \pm SEM ($n=3$ /group) and were normalized to the total cell count and expressed per 1000 cells. * $P < 0.05$ vs control.

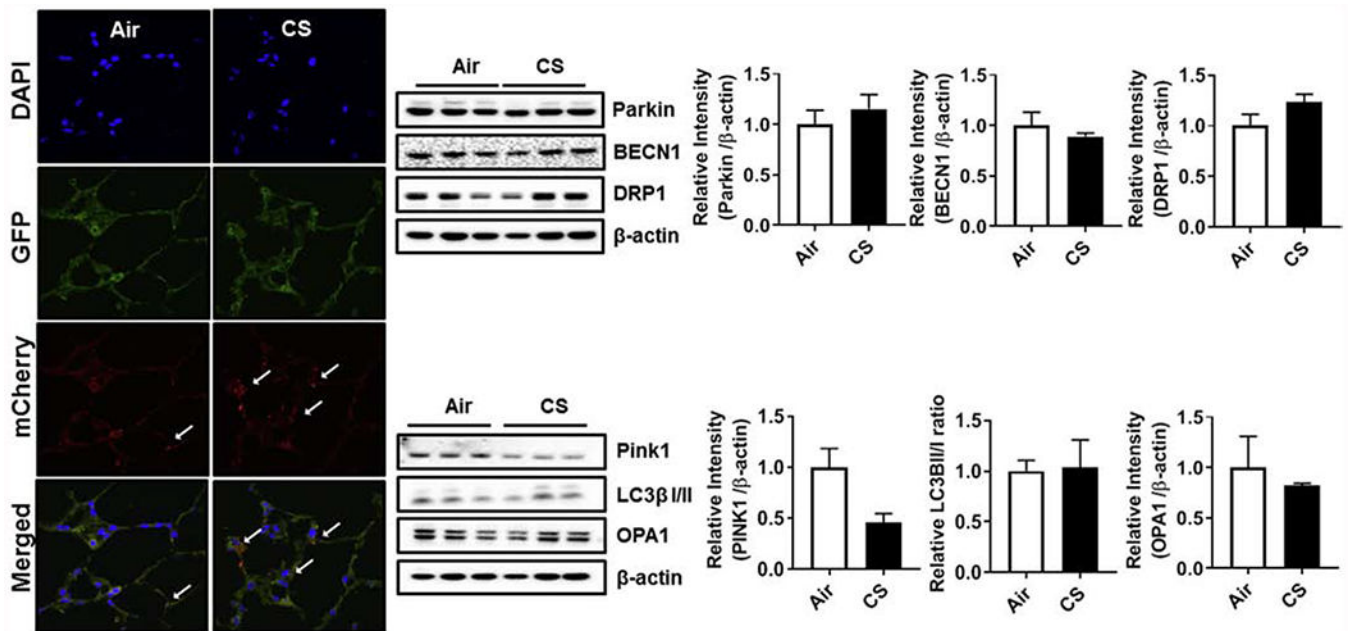


Fig. 9.

Mitophagy and mitochondrial architecture in alveolar lung epithelium and mitochondrial/mitophagy markers in acute CS exposed mitoQC mouse lung. (A) Representative images from cryo-sectioned OCT-fixed mitoQC mouse lung following acute 10 days CS exposure were analyzed by confocal microscopy. mCherry⁺ and GFP⁺ signal indicates no mitophagy and mCherry⁺ and GFP⁻ signal co-localization indicates occurrence of mitophagy. The increased mCherry signals is indicated by white arrows. All the images were captured using a 100 x oil objective. (B) Mitochondrial and mitophagy protein abundance in whole lung homogenates of acute CS exposed mito-QC mice. Western blot analysis against mitochondrial/mitophagy markers (anti-parkin, anti-BECN1, anti-Drp1, anti-Pink1, anti-LC3β-I/II and anti-OPA1 antibodies) was measured in whole lung homogenates. The band intensity was measured by densitometry, and data are shown as relative intensity to β-actin as loading control. Data were shown as mean ± SEM (n=3/group).