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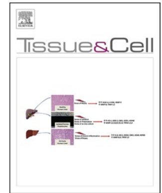

Crosstalk between exosomes signaling pathway and autophagy flux in senescent human endothelial cells

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ABSTRACT

The intracellular endomembrane system contributes to maintaining cell homeostasis. We investigate the dynamic of exosomal and autophagy pathways in Human Umbilical Vein Cells (HUVECs) following incubation with H2O2 in vitro. Cellular senescence was induced to HUVECs using 100 μM H_2O_2. ELISA and AChE assay was used to calculate the number of exosomes. Exosomes were isolated and characterized by dynamic light scattering, flow cytometry, and SEM. Transcript and protein levels of genes involved in the exosomal and autophagy pathways were measured by real-time PCR (Q-PCR) and western blotting. Subcellular distribution of CD63 was monitored by immunofluorescence microscopy. We also measured the expression of miR-182 and miR-155 by qPCR assay. Results showed that secretion of exosomes was increased in treated cells ($p < 0.05$). Exosomes were confirmed by size and positive for CD63 marker. Molecular analysis of the exosomal secretory pathway has revealed a significant induction of CD63, CD81, TSAP6, Rab11, Rab27a, and Rab27b in response to H_2O_2 ($p < 0.05$). The distribution of CD63 was increased inside treated cells. The western blotting technique revealed a significant up-regulation in Becline-1 and P62 and a significant decrease in LC3 II/I ratio in treated cells ($p < 0.05$)s. Concomitant with an up-regulation of common molecules in exosomes biogenesis and autophagy including Atg5, P62 and P53, expression of miR-182 decreased and miR-155 increased in cells incubated with H2O2 ($p < 0.05$). Data suggested an induction in the exosomal secretory pathway coincided with a block in autophagy progress, accelerating senescence, which might be targeted for the treatment of age-related diseases.

1. Introduction

All cells produce bilayer-phospholipid vesicles, collectively known as extracellular vesicles (EVs), in their milieu that participate in cellular and biological processes (Doyle and Wang, 2019). Recent progress in EVs has confirmed their cell-to-cell communication role (Amini et al., 2021). By comprising and transferring different biological molecules, like RNAs, lipids, and proteins, to target cells, EVs communicate favorable, impartial or harmful effects on target cells, including regulating gene expression, inducing cell morphology, affecting signaling pathways and facilitating biological processes (Doyle and Wang, 2019). Exosomes, one subset of EVs, with a size ranging from 30 to 150 nm, have attracted researchers' attention due to their fundamental roles in pathological and physiological conditions (Doyle and Wang, 2019). Exosomes biogenesis occurs inside cells, where various molecules and distinct vesicles called multivesicular bodies (MVBs) begin to load biological cargoes and form intraluminal vesicles (ILVs)(Urbanelli et al., 2013). ILVs are located inside MVBs, which are then called exosomes when secreted into the extracellular matrix (ECM) following fusion with the plasma membrane. In return, ILVs may fuse either with lysosomes or the plasma membrane to degrade or recycle their cargoes, respectively (Rezaie et al., 2021; Urbanelli et al., 2013). Exosomes biogenesis and secretion are complex, however, existing evidence indicates such distinct proteins such as CD63, CD81, Alix, and Rabs as well as complexes located on MVBs may passively or actively medicate exosomes loading, formation, and destination (Patil and Rhee, 2019; Urbanelli et al., 2013). Exosomes can be produced by cardiovascular system-related cells, for example, endothelial cells (ECs), which play pivotal roles in the regeneration of vascular beds, angiogenesis, cell proliferation, and cell migration (Fernandes Ribeiro et al., 2013). Cellular senescence and ECs dysfunction are the major risk factor for many diseases such as cardiovascular disease (CVD), cancer, osteoarthritis, chronic kidney disease, neurodegeneration, osteoporosis, and diabetes, the leading causes of death worldwide (Ahmadi et al., 2018;

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Alamdari et al., 2021; Jia et al., 2019; Vallianou et al., 2019). Age-related impairment of ECs also has significant implications for the efficacy of therapeutic approaches aimed at facilitating cell function in aged individuals with circulatory system diseases (Seals et al., 2011; Xu et al., 2017). Autophagy is a dynamic and strongly structured homeostatic cellular process known for the sequestration of damaged molecules and organelles into autophagic vesicles and the degradation of cargoes by fusion of vesicles with lysosomes (Rezabakhsh et al., 2017, 2022). Regulation of autophagy flux depends on cell status, where different molecules and complexes are involved (Rezabakhsh et al., 2017, 2022). The interplay between exosomes biogenesis and autophagy flux has been shown to regulate cellular homeostasis (Xu et al., 2018). There is a high degree of synergies between autophagy and exosome signaling, which include endocytosis and endosomes, lysosomes, exocytosis and exosomes, autophagosomes, and amphisomes. These transport systems contribute to sustaining cellular homeostasis and function synergically against instabilities in the internal and external environment including during oxidative conditions (Salimi et al., 2020; Tukmechi et al., 2014). A growing body of evidence suggests that senescence can affect the dynamic of exosomes and autophagy signaling pathways, *VIC. Versa* (Cuervo et al., 2005; Xu and Tahara, 2013). Understanding the mechanisms involved in exosomes biogenesis and its cross-talk with autophagy flux are pivotal owing to the potential of the endomembrane system (exosomes and autophagy) in mediating cellular processes in such pathological conditions as senescence (Gudbergsson and Johnsen, 2019). To the best of our knowledge, while some research has been carried out on senescence and exosomes, the mechanism by which cellular senescence may affect the exosome signaling pathway in ECs has not been established. This study, therefore, set out to assess the effect of H2O2, a common mediator of senescence, on the exosomes signaling pathway. We used human umbilical vein cells (HUVECs), a cellular model acquiescent to experimental toxicity essential to address cell signaling.

2. Materials and methods

2.1. Cell culture and treatment

HUVECs were obtained from the Pasture (Iran) and maintained in a DMEM medium supplemented with 10% FBS in a CO2 incubator at 37 °C. To induce senescence, HUVECs cells grown on proper surface dishes were incubated in the presence of 100 μM of hydrogen peroxidase (H_2O_2)(Chowdhary, 2019; Kao et al., 2010). Experiments were carried out 24 h after H2O2 incubation in three sets.

2.2. Exosome purification

Cells were grown in treatment media; then, they were rinsed with PBS and incubated with FBS free media for 48 h. Cell-free media was prepared by centrifugation of supernatants at 3000 RPM for 20 min. Then filtered through 0.22 μm filter to eliminate cell debris. Briefly, the appropriate volume (1/5 ratio) of the exosome isolation kit (EXOCIB, Iran) was added to each sample and kept overnight at 4□C. Following, the solutions were centrifuged at 3000 RPM for 1 h. Exosomes pellets were resuspended in 100 μl PBS and stored at −80 °C.

2.3. Exosomes quantification

To measure the amount of secreted exosomes, we used the acetylcholinesterase assay (AChE activity) according to a kit (Cat no. BXC080, Iran) guidelines‚ as described previously (Savina et al., 2003; Taverna et al., 2012). Briefly, 1 ml A reagent (2 mM potassium hexacyanoferrate + 75 mM pyrophosphate) was added with 100 μl exosome from each sample and left for 5 min at room temperature. Thereafter, 20 μl B reagent (sbutyrylthiocholine iodide) was added and absorbance was recorded at 405 nm at 3 intervals in and microplate reader system (BioTek). The formula: Activity (U/l) = ΔAbs/60 s × 65,800 used to measure AChE activity. In addition, for further confirmation, we designed an Enzyme-linked immunosorbent assay (ELISA) against exosomal CD63 protein as previously described (Soraya et al., 2021). The amount of CD63 in the treated sample was described as the percentage of the control.

2.4. Exosomes characterization

2.4.1. DLS analysis

To measure the size of exosomes samples, DLS analysis was performed using a Zetasizer Nano Z system (Malvern, UK). In brief, 100 μl of exosome suspension was added to 900 μl PBS and introduced to the system at 25 ∘C. The scattered light was examined at 633 nm wavelength and 173°. Results were obtained using Zetasizer software ver. 6.0.

2.4.2. Flow cytometry analysis

To detect exosomal marker CD63, we used it for flow cytometry analysis. In brief, according to the manufacturer's recommendation, the proper amount of primary anti-CD63 antibody (Cat no: sc-5275) was mixed with 200 μl exosome suspension and left for 2 h at 4 °C. Following, IG-FITC antibody (cat no: 406001, Biolegend) was mixed and kept for 1 h at room temperature. Expression of CD63 was detected by BD FACSCalibur system and evaluated by Flow Jo software (version 7.6.1).

2.4.3. Scanning electron microscope (SEM)

For imaging with SEM, 100 μl of exosome sample was laden on grids and then frozen-dried for 1 h. Next, SEM grids were Au-coated and micrographs obtained at 30 kV by an SEM system (Tescan).

2.5. RNA extraction and real-time PCR assay

Total RNA from samples was extracted by Trizol reagent (Merk), according to the standard protocol decried previously (Feghhi et al., 2021). RNA quality and quantity were measured using namedrop instrument spectrophotometrically (Bio Tek). According to the recommendation of the kit, 1 μg of total RNA from each sample was used to produce cDNA using a cDNA synthesis kit (Yektatajhiz, Iran). As well, the same total RNA was used to make miRNA First-Strand cDNA using a kit (BN-0011.17, Iran) according to the manufacturer's protocol. Following, 1 μg of cDNA was amplified using MIC Real-Time PCR System (Swiss) in the presence of SYBR Green PCR Master Mix (Yektatajhiz, Iran) for mRNA analysis and SYBR Green High ROX Master mix (STRC, Iran) for miRNAs analysis and proper primers (Table 1). The sequences

Table 1
List of primers used for qPCR.

Genes	Primers Sequence	Tm
CD63	Forward: TCCTGAGTCAGACCATAATCC Reverse: GATGGCAAACGTGATCATAAG	63
TSAP6	Forward: CCTCTACAGCTTCTGCTTGCC Reverse: TAGATCTCCATCCGCCAGACC	63
Alix	Forward: CTGGAAGGATGCTTTCGATAAAGG Reverse: AGGCTGCACAATTGAACAACAC	63
Rab11	Forward: CCTCAGCCTCTACGAAGCAAA Reverse: CCGGAAGTTGATCTCCTCCTG	59
Rab27b	Forward: GGAACTGGCTGACAAATATGG Reverse: CAGTATCAGGGATTTGTGTCTT	59
Rab27a	Forward: AGAGGAGGAAGCCATAGCAC Reverse: CATGACCATTTGATCGCACCAC	59
ATG5	Forward: GCAGATGGACAGTTGCACACAC Reverse: GAGGTGTTTCCAACATTGGCTCA	60
P62	Forward: CATCGGAGGATCCGAGTGTG Reverse: TTCTTTTCCCTCCGTGCTCC	60
GAPDH	Forward: CAAGTTCAACGGCACAGTCAAG Reverse: ATACTCAGCACCAGCATCACC	60

of primers of miRs are listed as miR-182; forward: TTG GCA ATG GTA GAA CT and for miR-155; forward: TAG GCT AAG CGT GAT AG. Universal reverse primer: ATC ACT GTA AAA CCG TT. In addition, relative expression of mRNAs and miRNAs were measured against GAPDH and Snord-47 as internal genes, respectively. 2 -ΔΔCT method was used to evaluate the relative expression levels of the genes.

2.6. Protein isolation and western blotting analysis

The cells were lysed on ice with 500 ml of RIPA buffer (Sigma) containing 250 mM NaCl, 50 mM NaH_2PO_4, pH 7.6, 10 mM imidazole, 50 mM NaF, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin. The cell lysate was kept on ice for 15 min and centrifuged at 13 000g at 4 °C for 20 min. The protein concentrations in each supernatant of samples were calculated using a Nanodrop system (Bio Tek). Electrophoresis was done on an Electrophoresis System (SDS-PAGE) using 100 mg of protein extract for each lane. Proteins were then transferred to 0.2-μm polyvinyldifluoride (PVDF, Millipore) membranes. Membranes were blocked with 5% skim milk (Gibco) overnight and incubated with the proper dilution of primary antibodies (SANTA CRUZ BIOTECHNOLOGY, INC) for 1 h at room temperature. After three-time washing with 0.1% Tween 20, membranes were incubated with HRP-conjugated secondary antibody for 1 h at room temperature. All proteins bands were identified using Chemiluminescence Reagent (ECL), exposed to X-ray film and quantified using an NIH ImageJ software ver. 1.44p. for normalization, β-actin (SANTA CRUZ BIOTECHNOLOGY, INC) was used as a housekeeping protein.

2.7. Immunofluorescence imaging

The intracellular distribution of CD63 as a consistent exosomal marker in aged cells was examined by immunofluorescence (IF) assay. Briefly, 5×10^4 HUVECs were seeded per well of an 8-well slide chamber (SPL) and treated with H2O2. Then, cells were fixed by cold 4% paraformaldehyde (PFA) solution, permeabilized with 0.1% Triton 100-X (Sigma) solution for 10 min, and blocked with 1% bovine serum albumin (BSA, Sigma) for 30 min. HUVECs were incubated overnight at 4 °C with the primary CD63 antibody (Cat no: sc-5275) and were washed with PBS twice, then incubated with FITC-conjugated anti- mouse IgG (cat no: 406001, Biolegend) for 40 min at 37 °C in the dark. Cells were nuclear stained with 1 μg/ml DAPI solution (Sigma) and were visualized by a fluorescent microscope.

2.8. Statistical analysis

The statistical analysis was done by the GraphPad Prism for windows software set (ver 8.0). The data signify means ± standard deviation (SD). Statistical differences were analyzed using the Student's t-test. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Identification of exosomes by flow cytometery, SEM, and DLS

First, flow cytometery analysis confirmed the expression of an exosomal marker, CD63 on isolated particles (Fig. 1A). Secondary, SEM demonstrated that the exosomes showed rounded and nano-sized structures (Fig. 1B). Thirdly, DLS confirmed the size of isolated exosomes by a kit (Fig. 1C).

3.2. Secretion of exosomes into the supernatant was increased in H2O2-treated cells

AChE activity assay and ELISA were used to monitor the secretion rate of exosomes from cells into the supernatant. We found that AChE activity was increased in treated cells as compared to control cells ($p < 0.01$, Fig. 2A). Simultaneously, we found that level of CD63 in the exosome sample of treated cells was higher than that of control cells ($p < 0.05$, Fig. 2B).

3.3. The expression of genes involved in the exosomal pathway was increased in H2O2-treated cells

Different key molecules contribute to exosomes formation and release from cells. To test whether the above observation could be correlated to genes regulating exosomes biogenesis, we analyzed the expression of genes including CD63, Alix, Rab11, Rab27a, Rab27b, and TSPA6 by *q*-PCR (Fig. 3). According to results, we identified that mRNA

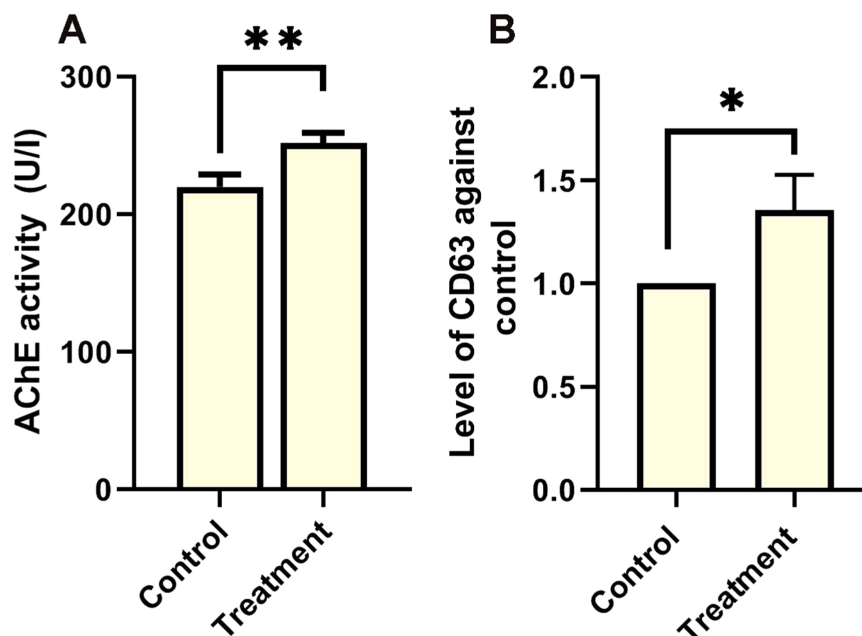

Fig. 1. Quantification of exosomes released into conditioned media by acetylcholinesterase (AChE) assay (A) and ELIZA (B). N = 3; *$p < 0.05$, **$p < 0.01$, n.s = non-significant; Error bars represent standard deviation (SD).

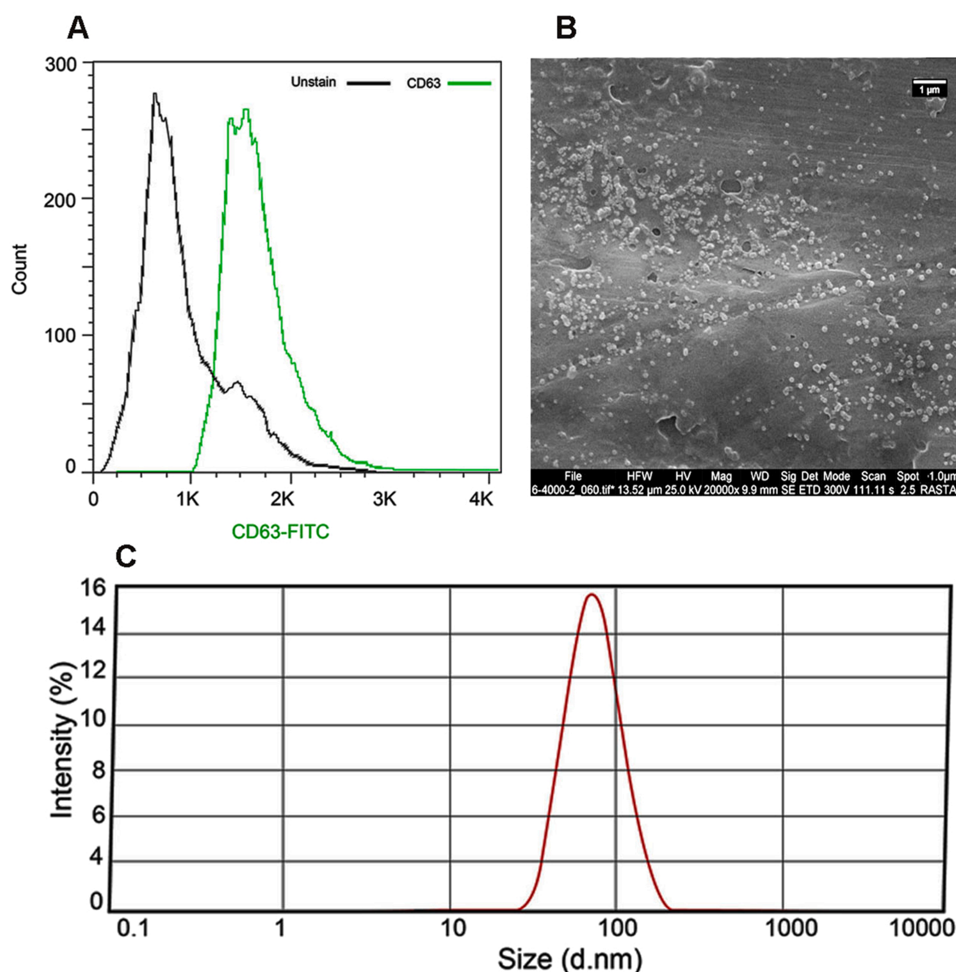

Fig. 2. Characterization of isolated exosomes by a kit using flow cytometry analysis (A), scanning electron microscopy (SEM) (B), and dynamic light scattering (DLS) (C). Scale bars = 1 μm.

levels of CD63 ($p < 0.01$), Rab11 ($p < 0.01$), Rab27a ($p < 0.01$), Rab27b ($p < 0.01$), and TSPA6 ($p < 0.01$) were up-regulated in H2O2-treated cells (Fig. 3). At the same time, we found that despite a somewhat increase in mRNA levels of Alix, it was not significantly changed in cells treated with H2O2 ($p > 0.05$). These results indicate that ECs could respond to H2O2 treatment by generating exosomes.

3.4. *The expression of P62, Atg-5, miR-182, and miR-155 was changed in H2O2-treated cells*

To further investigate, we performed a q-PCR analysis. The results are shown in Fig. 3. The expression of autophagic factors such as P62 and Atg-5 was significantly up-regulated in treated cells compared with control cells ($p < 0.01$). Furthermore, it was found that expression of miR-182 (0.599 ± 0.195-fold change) and miR-155 (1.25 ± 0.13-fold change) decreased and increased respectively in H2O2-treated cells ($p < 0.01$, Fig. 3). Our results showed that autophagy may be modulated in treated cells.

3.5. *The protein level of exosomal markers was increased in H2O2-treated cells*

To further confirm exosomes biogenesis, we performed western blotting analysis for P53, CD63 and CD81. Data analysis showed that protein levels of P53 (0.88 ± 0.068 *v.s* 1.23 ± 0.058, $p < 0.01$), CD63 (1.17 ± 0.057 *v.s* 1.42 ± 0.18, $p < 0.01$), and CD81 (1.16 ± 0.061 v.s 2.21 ± 0.1, $p < 0.05$) were increased in treated cells compared to control cells (Fig. 4A and B). These findings demonstrate the up-regulation of exosomal proteins.

3.6. *The protein level of autophagic markers was altered in H_2O_2-treated cells*

We measured the relative protein level of autophagy-related genes including Becline1, LC3 II/I, and P62 in cells using western blotting analysis. We observed that the relative protein level of Becline-1 ($p < 0.01$) and P62 ($p < 0.05$) was significantly increased and the LC3 II/I ratio decreased ($p < 0.01$) in cells treated with H_2O_2 (Fig. 4A and B). These findings demonstrate a modulated autophagy flux at the basal level in treated cells.

3.7. *Expression of subcellular CD63 was increased in H_2O_2-treated cells*

The higher expression levels of CD63 were further confirmed by immunofluorescence analysis. As shown in Fig. 4C, immunofluorescence microscopy showed that the number of CD63 + compartments inside cells was increased upon incubation with H2O2.

4. Discussion

Despite prolonged research on cellular senescence, the exact molecular mechanism is not clear. Previous studies revealed that ECs can undergo cellular senescence upon exposure to H_2O_2, which may be due to increased oxidative stress (Kiyoshima et al., 2012; Ruan et al., 2014).

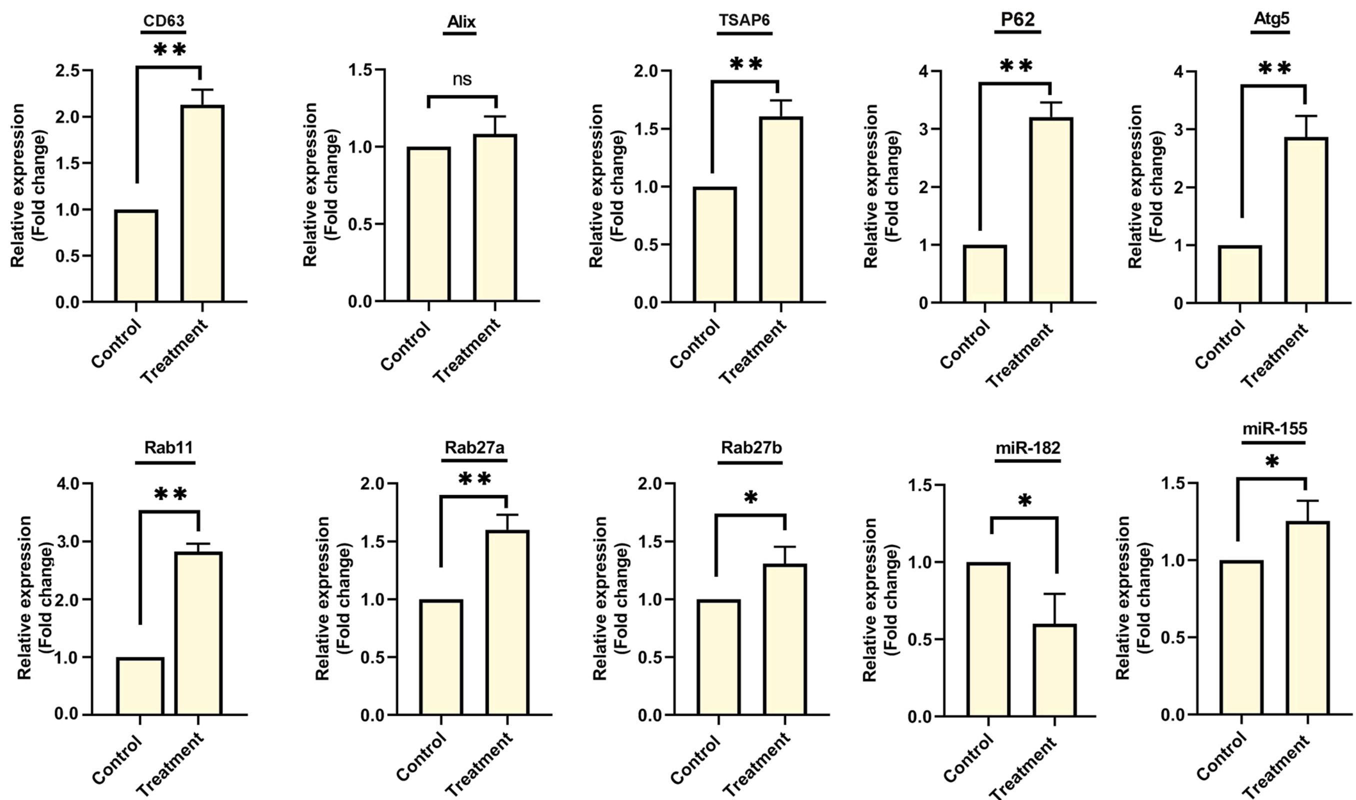

Fig. 3. Comparative analysis of mRNA expression of genes involved in exosomes biogenesis and autophagy as well as expression of miR-182 and miR-155 by quantitative PCR (q-PCR). N = 3; * $p < 0.05$, * * $p < 0.01$, n.s = non-significant; Error bars represent standard deviation (SD).

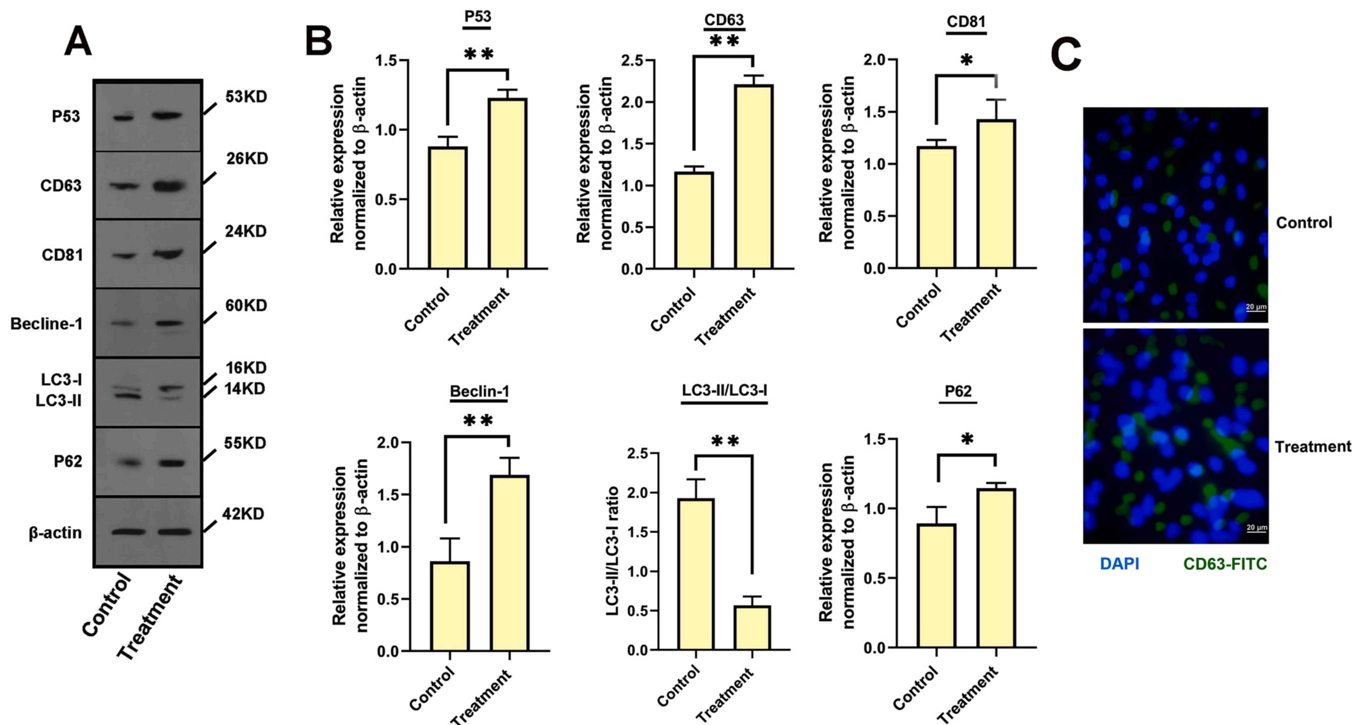

Fig. 4. Descriptive western blotting presenting the relative protein level of P53, CD63, CD81, Becline-1, LC3, and P62 (A and B). Representative immunofluorescence images presenting CD63 staining in control and treatment cells (C). Scale bars = 20 μm. N = 3; * $p < 0.05$, ** $p < 0.01$; Error bars represent standard deviation (SD).

Oxidative stress impairment to cells is one of the main reasons for senescence where ROS overproduction cause impairment of organelles and functional biomolecules (Bachschmid et al., 2013; El Assar et al., 2013; Tukmechi et al., 2014). The senescence of ECs encourages vascular senescence and fluctuations in the molecular dynamics of cells (Abdyazdani et al., 2017; Bachschmid et al., 2013; El Assar et al., 2013).

Vascular senescence is the major risk factor for different diseases, including CVD (Bachschmid et al., 2013; El Assar et al., 2013). Two cellular mechanisms, (i) exosome biogenesis and (ii) autophagy flux, have been proposed as promising approaches for cellular homeostasis (Tian et al., 2019). These two major pathways may coordinate cells responses against stress conditions. To shed a light on the underlying molecular mechanism of H2O2-treatment, and senescence mediator, we monitored the status of exosomes biogenesis and autophagy flux in HUVECs following incubation with H2O2 in the present study.

Senescent cells are thought to secrete many paracrine including chemokine, pro-inflammatory cytokines, proteinases, vesicles, and soluble molecules, as a group termed the senescence-associated secretory phenotype (SASP)(Gonzalez-Meljem et al., 2018). We, firstly, observed that secretion of exosomes from treated cells increased by ELISA and AChE assay. This lends support to previous studies (D'Anca et al., 2019; Soraya et al., 2021).

To explore detailed mechanisms, we directly analyzed the expression of key factors implicated in exosomes biogenesis and secretion and found that mRNA levels of CD63, TSPA6, Rab11, Rab27a, and Rab27b as well as protein levels of CD63 and CD81 up-regulated in treated cells. Various molecules are implemented in the exosomal secretory pathway, of which tetraspans and Rab proteins have frequently been confirmed to mediate exosomes loading, biogenesis, and secretion (Babaei and Rezaie, 2021; Fernandes Ribeiro et al., 2013). CD63 and CD81, tetraspan proteins on MVBs membrane, known widely as exosomes markers have been confirmed to mediate exosomes biogenesis through a mechanism is known as ESCRT-independent pathway (Babaei and Rezaie, 2021; Fernandes Ribeiro et al., 2013). In addition, we found that the expression of Alix, an exosomal key factor, did not alter, which may be elucidated that the function of Alix depends on cell type and/or study condition. Rab proteins belong to GTPase proteins family that mediate the trafficking of vesicles inside cells (Babaei and Rezaie, 2021; Fernandes Ribeiro et al., 2013). Among them, Rab27b facilitates the movement of MVBs toward the cytoplasm cortex and the plasma membrane, however, Rab11 and Rab27a facilitate the fusion of MVBs with the plasma membrane (Babaei and Rezaie, 2021; Fernandes Ribeiro et al., 2013). Another protein that mediates exosomes biogenesis is TSPA6, which its expression increased in such stress conditions (Jabbari et al., 2019). This molecule is a direct P53 target gene that is activated in response to stress conditions (Passer et al., 2003). According to a growing body of evidence, P53 is a multifunctional molecule that participates in a variety of cellular processes for example apoptosis, stress oxidative modulation, autophagy, and exosomes biogenesis (Brooks and Gu, 2010; Lehmann et al., 2008). Previous studies showed that TSPA6/P53 axis induces exosomes biogenesis, which is likely confirmed in our study due to the elevated expression of P53 protein and TSPA6 transcript (Lespagnol et al., 2008). Based on our observation in this panel, therefore, it is likely that exosomes biogenesis and secretion was increased upon incubation with H_2O_2.

In keeping, we explored whether H_2O_2 treatment affects autophagy flux simultaneous with exosomes biogenesis. Under normal conditions, autophagy mediates the removal of waste and impaired organelles and molecules, however, under stress conditions, autophagy supplies energy for cells by recycling the cellular components from an internal source (Salimi et al., 2020). Consistent with previous studies, we found that H_2O_2 could modulate the expression of autophagic factors (Du et al., 2019; Zhang et al., 2009). Our result indicated that expression of ATG5, Becline-1, and P62 up-regulated, while at the same time the LC3II/I ratio reduced, indicating modulation of autophagy at basal level in H2O2-treatment condition (Du et al., 2019; Hassanpour et al., 2020b). A possible explanation for this might be that autophagy progress was decreased (Du et al., 2019; Hassanpour et al., 2020b). Atg5 is known as an autophagic factor that supports Atg12 within the Agt12 conjugation complex, which contributes to the development of the phagophore membrane (Guo et al., 2017; Salimi et al., 2020). This molecule is a bifunctional molecule that regulates the exosomal secretory pathway by dispassion of V 1/V 0 –ATPase (vacuolar proton pumps) from the MVBs membrane, which in turn prevents the acidification of MVB's lumen and facilitates MVBs and the plasma membrane fusion rather than lysosomes-fusion (Guo et al., 2017). To further evidence on exosomes biogenesis and autophagy flux, we explored putative miRNA-182 and miR-155 targets by such databases as miRDB (http://mirdb.org/), Targetscan human software (http://www.targetscan.org/vert_71/), and PicTar (http://pictar.mdc-berlin.de/); and found that miR-182 may target Rab11, Rab27b, and Rab27a, whereas miR-155 target LC3, Atg, and autophagy inhibitors. In our assessment, the observed decrease in miRNA-182 and increase in miR-155 could be attributed to the up-regulated mRNA level of Rab genes and down-regulated autophagy inhibitor genes (Wang et al., 2018, 2013).

It seems possible that these results show exosomes biogenesis and autophagy flux were induced upon treatment with H2O2. The expression of Atg5 and P53 molecules, which can modulate both pathways, are up-regulated in treated cells (Lehmann et al., 2008; Salimi et al., 2020). Treatment with H2O2 possibly changes cellular levels of key autophagic factors including Atg5, Beclin-1, LC3II/I ratio, and P62. An increase in expression of Atg5, Becline-1, and P62 and a decrease in LC3II/I ratio indicates that the progress of autophagy was inhibited and degradation of the dysfunctional molecules was delayed (Du et al., 2019; Murugan and Amaravadi, 2016; Rezabakhsh et al., 2018). It is important to note that the present evidence indicates ECs senescence was associated with a decline in autophagy flux to facilitate senescence. Exosomes biogenesis and autophagy flux share similarities in functional and signaling pathways (Tian et al., 2019). Crosstalk between exosome biogenesis and autophagy flux has been shown to contribute to cellular homeostasis (Salimi et al., 2020). The link between exosomal and autophagy pathways occurs at both the molecular level and membranous vesicles (Salimi et al., 2020). Based on the literature, under certain conditions, the exosomal secretory pathway compensates the autophagy and vice versa (Salimi et al., 2020; Tian et al., 2019). This finding, while preliminary, suggests that modulated exosomal and autophagy pathways contribute to accelerating senescence in cells. At this stage of understanding, one could hypothesize that the progress in degradation, recycling, and secretion was compensated by the exosomal secretory pathway (Hassanpour et al., 2020a; Salimi et al., 2020), however, further scrutiny may elucidate the possible functional/applicable cross-talk between exosomes biogenesis and autophagy in H2O2-treatment situation. It is not clear whether up-regulated paracrine activity coincided with impaired autophagy flux may contribute to sending specific signals to neighboring cells or that is an adaptive mechanism for cells to overcome stress conditions by removing damaged molecules from aged cells (Hassanpour et al., 2020a; Wang et al., 2018). In addition, impaired/decreased autophagy may be implicated in inducing autophagy via overlapping different signaling pathways, however, in our study, we only investigated autophagy flux in senescent-induced cells (Kwon et al., 2017; Young et al., 2009). Discovery in this field is growing, and the experimental data are rather controversial, it was demonstrated that exosomes from senescence cells carry oxidized biomolecules, which cause negative effects on target cells (Larson et al., 2014). On the contrary, these exosomes may transfer antioxidant molecules to target cells and protect them against further damage (Yarana and Clair, 2017). To further our research, we are planning to uncover some cargo and functions of these exosomes in a co-culture system.

5. Conclusion

The evidence from this study implies that H2O2 could modulate exosomes biogenesis and autophagy flux in HUVECs. The more significant findings to emerge from this study is that the activity of the exosomal secretory pathway was synchronized with a block in autophagy progress, which may contribute to accelerated senescence. The present results are significant in at least major two respects: (i) these results prompted us to further discover the mechanism underlying cellular

senescence regarding the interplay between exosomes and autophagy and; (ii) these results may be useful for helping us to improve current therapies against senescence-related disease.

CRediT authorship contribution statement

Shadi Mahbubfam: Data collection and performance. **Jafar Rezaie:** Conceptualization, Methodology, Software, Validation, Formal analysis, Writing – review & editing. **Vahid Nejati:** Conceptualization and Methodology , Software, Validation, Formal analysis, Writing – review & editing.

Declarations

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable – no living animals have been used in this study.

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Declaration of Competing Interest

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.

Data Availability

The data are available upon request to the corresponding author.

Acknowledgements

Not applicable.

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