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Human Umbilical Vein Endothelial Cells (HUVEC) Derived Exosomes as a Novel Modulator of Neutrophil Function

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Abstract

Neutrophils, essential cells of the innate immune system, play dual roles in the defense of host and disease pathogenesis. Neutrophils crosstalk with endothelial cells to maintain homeostasis via mechanisms such as phagocytosis, neutrophil extracellular trap (NET), and reactive oxygen species (ROS) generation, excessive neutrophil activity contributes to inflammation and tissue damage in various diseases, including autoimmune disorders and cancer. This study investigates the effects of HUVEC-derived exosomes (H-Exo) on neutrophil function. H-Exo were isolated, and characterized using TEM, DLS, and flow cytometry, and their effects on neutrophils were evaluated. Our results demonstrate that H-Exo significantly reduces neutrophil ROS production, as confirmed by the Nitroblue Tetrazolium (NBT) test, and decreases neutrophil yeast-killing activity. These findings highlight the potential of H-Exo in managing diseases associated with excessive neutrophil activation and oxidative stress. In conclusion, H-Exo represents a promising therapeutic avenue for conditions characterized by neutrophil-induced inflammation.

Keywords: Neutrophil, NBT, Yeast, Exosome, HUVEC.

1. Introduction:

The human innate immune system comprises diverse immune cells, including neutrophils (1). Neutrophils, as polymorphonuclear (PMN) leukocytes, are the most prevalent type of white blood cells and have a short half-life in the bloodstream, ranging from 8 to 20 hours (2). The bone marrow produces neutrophils at a rate of 1×10^{11} cells per day (3). These cells play a crucial role in initiating inflammatory responses. They are rapidly attracted from the bloodstream to area of tissue trauma or infection (4). Neutrophils detect microorganisms using pattern recognition receptors (PRRs) and subsequently attack and eliminate them (5). In fact, neutrophils eliminate pathogens through the release of web-like structures called neutrophil extracellular traps (NETs), generating of reactive oxygen species (ROS), in addition to degranulation and phagocytosis (6, 7).

Beyond their role in host defense, neutrophils also contribute to the pathogenesis of several diseases, including autoimmune and cancer disorders. Tumor-associated neutrophils (TANs) can be further categorized into two subgroups: N1 and N2. N1 neutrophils can inhibit cancer

progression by killing tumor cells, activating T cells, and enhancing antibody-dependent cellular cytotoxicity (ADCC). On the other hand, N2 neutrophils can accelerate cancer development by promoting tumor growth, carcinogenesis, metastasis, angiogenesis, and suppressing the immune system. Neutrophils are also involved in autoimmune disorders directly or indirectly. Typically, they contribute to disease progression through various mechanisms, including the release of inflammatory substances, the activation of other immune cells, and the facilitation of autoantibody production (8).

Therefore, neutrophils have multifaceted roles. By targeting and manipulating neutrophil function, we can potentially develop novel therapies for a wide range of conditions. For instance, in individuals with weakened immune systems, enhancing neutrophil function can support their defense against microbial infections. Conversely, in cancer and autoimmune diseases, suppressing neutrophil activity may help mitigate disease progression.

Several studies have shown that cell-derived exosomes impacts neutrophils function and vitality (9-11). Exosomes are lipid-enclosed vesicles containing a variety of components, including lipids, metabolites, RNA, DNA, and diverse proteins (12). Exosomes from specific immune cells can influence acquired immune responses to tumors and pathogens (13). The specialized activities of exosomes rely on their parent cells (14). Some studies indicate that exosomes derived from parent cells have effects comparable to those of whole-cell therapy, with the added benefit of increased safety. Exosomes can be isolated from various body fluids or even from the culture medium of cells (15). Exosomes have diameters of approximately 40–160 nm and can be sterilized through filtration. They can also be stored long-term in a freezer (16).

Endothelial cells, which have shared ancestry with immune cells, exhibit immunomodulatory properties (17). Endothelial cells, like human umbilical vein endothelial cells (HUVECs), are known to secrete exosomes (18). HUVECs have been useful to study a diverse range of diseases and biological processes, including cardiovascular disease, inflammation, preeclampsia, apoptosis, cancer, and regenerative medicine (19). To date, no research has examined the impact of HUVEC-derived exosomes (H-Exo) on neutrophils.
This study investigates the potential of H-Exo to influence neutrophil function. By elucidating the mechanisms underlying these interactions, we aim to uncover novel therapeutic strategies targeting neutrophils to mitigate disease progression.

2. Material and methods:

2.1. Culture of HUVECs:

HUVEC cells were obtained from GenIran Company (Iran) and immediately cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Capricorn, Germany), supplemented with 10% fetal bovine serum (FBS; Anacell, Iran) and penicillin-streptomycin (Biosera, France). After the third passage, the cells were seeded in a T-75 flask at a density of 10^6 cells. When the cells reached 70% to 80% confluence, the medium was carefully removed. The cells were then washed with phosphate-buffered saline (PBS) and incubated

again with FBS-free medium. The conditioned media (CM) was gathered after 48 hours, centrifuged, and stored at -20°C for subsequent exosome isolation.

2.2. Isolation of Exosomes:

Exosomes were isolated from HUVECs-derived CM. The Exocib kit (Cib Biotech Co., Iran, Cat number: 3603-450) was used to isolate exosomes from the CM. To remove particles and debris, the CM was first centrifuged at 3000 RPM for 20 minutes. Preheated reagent A was then added to the CM at a 1:5 ratios and vortexed for 5 minutes until a cloudy solution appeared. The tubes containing the solution was incubated overnight at 4°C, with shaking every hour. After incubation, the tubes were vortexed again and centrifuged for 40 minutes at 3000 RPM at 4°C. The remaining supernatant was removed, and the pellet was resuspended in 200 µl of Reagent B. The isolated exosomes were stored at -20°C for future use.

2.3. Transmission electron microscopy (TEM):

To assess the size and shape of the H-Exo, TEM (Zeiss-Leo 906, Germany) was used. Initially, 3% glutaraldehyde was mixed with the suspended exosomes at a 1:1 ratio to fix the structures. Two µl of the suspension were then transferred on Formvar-carbon coated grids. The grids were placed in the specimen holder of the TEM, and images were taken.

2.4. Dynamic Light Scattering (DLS):

The dynamic light scattering (DLS) method was used to measure the size distribution of the exosomes. To prepare the exosome solution for DLS analysis, the exosome suspension was first diluted at a 1:100 ratios with particle free PBS. The diluted exosome solution was then passed through a filter to eliminate any larger particles or contaminants that could interfere with the DLS readings. After filtration, aliquots of the exosome solution were carefully loaded into disposable and clean cuvettes. The cuvettes were then placed into the Zetasizer Nano ZS (Malvern Instruments, UK).

2.5. Flow cytometry:

To confirm the endosomal origin of exosomes, the presence of the CD63 marker on them was evaluated using flow cytometry. The exosome samples were diluted and labeled with fluorescently labeled CD63-FITC antibodies (BioLegend, Cat number: 353005), which are specific markers commonly used to identify exosomes. After labeling, the exosome samples were analyzed by means of a BD FACSCalibur flow cytometer (BD biosiences, San Jose, CA, USA). The data was also evaluated by flow cytometer’s software.

2.6. Bradford Protein Kit

To estimate the concentration of H-Exo, the protein content was measured using the Bradford assay kit (PROTOCIB, CIB Biotech Co., Iran, Catalog No. 3501-200), following the manufacturer's protocol. This method involves the use of Bradford reagent, which binds to proteins, resulting in a color change proportional to the protein concentration. The absorbance of the solutions was measured at a wavelength of 595 nm.

2.7. Neutrophil isolation:

Neutrophils were isolated from fresh whole blood samples of non-smoking and healthy individuals. Blood samples were collected in heparinized tubes (10 U/ml) and mixed with an equal volume of 3% dextran. After a 45-minute incubation at room temperature, the upper layer was centrifuged on a Ficoll-Hypaque density gradient at 400g for 30 minutes. The resulting neutrophil pellet was resuspended in DMEM. To assess neutrophil purity, Giemsa staining will be performed on cell smears. The thin smears were fixed with methanol and stained with Giemsa solution. Additionally, trypan blue exclusion was used to determine cell viability (11).

2.8. Pretreatment of neutrophils:

Neutrophils were transferred to sterile microtubes and pretreated with 100 µg/ml of H-Exo or medium (as a control). The incubation of all samples were done at 37°C for two hours.

2.9. Effects of H-Exo on vitality of neutrophils:

To evaluate neutrophil vitality, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) dye was employed. After a 2-hour pretreatment, 100 µl of the cell suspension was transferred to each well of a 96-well microplate, followed by the addition of 20 µl of MTT solution. The microplate was then centrifuged at 3000 RPM for 10 minutes using a microplate centrifuge (Eppendorf, France). After centrifugation, 100 µl of dimethylsulfoxide (DMSO; Merck, Germany) was added to each well to dissolve the formed formazan crystals. Absorbance was then measured at 570 nm, with each test performed in triplicate independently to ensure accuracy.

2.10. Nitroblue Tetrazolium (NBT) test

NBT is a dye that is sensitive to superoxide (O_2^-) produced by neutrophils. NBT is reduced in the presence of oxygen radicals and forms blue formazan crystals (20). To initiate the assay, untreated and pretreated cells (500 µl) with exosomes were combined with 500 µl of fresh NBT dye (0.01%) and 500 µl of an opsonized yeast suspension. The microtubes containing

the mixture were incubated for 30 minutes, and then centrifuged. A mixture of Potassium hydroxide (KOH, 1M) and DMSO was added to each microtube, and the tubes were vortexed thoroughly. From each suspension, 100 µl was transferred to a flat-bottom 96-well microplate, and the absorbance was measured at 540 nm to quantify the results.

2.11. Yeast Killing Assay

A total of 100 µL of blood serum was added to microtubes, followed by the addition of 100 µL of pretreated neutrophils (5×10^6 cells/mL). The microtubes were incubated at 37°C for 5 minutes to activate the neutrophils. Microtubes containing serum and DMEM, without neutrophils, served as the control group. Subsequently, 100 µL of a yeast suspension, containing an equal number of yeast cells to neutrophils, was added to each microtube. The microtubes were incubated at 37°C for 90 minutes to allow neutrophil-yeast interactions. After the incubation, distilled water was added to lyse the neutrophils. The samples were then centrifuged at 3000 RPM for 10 minutes, and the resulting supernatants were transferred to microplate wells. To each well, 20 µL of MTT solution was added, and the mixture was incubated for 2 hours at 37°C. After incubation, 100 µL of DMSO was added to dissolve the formazan crystals, and the OD was measured at 490 nm.

2.12. Statistical analysis

Results are presented as means ± standard deviation (SD). Statistical analysis was conducted using GraphPad Prism software, version 8. The Shapiro–Wilk and Kolmogorov–Smirnov tests were conducted to assess the normality of the data distribution, and an unpaired t-test was used for group comparisons. A p-value of < 0.05 was considered statistically significant.

3. Results:

3.1. TEM analysis

The TEM analysis confirmed that the particles have a spherical morphology, with a size of 118 nanometers. The TEM images showed that the particles exhibited a uniform and consistent shape, characteristic of exosomes. These size measurements are consistent with the typical size range for exosomes, supporting their identification. Additionally, the images indicated that the H-Exo were successfully isolated and remained undamaged (Figure 1).

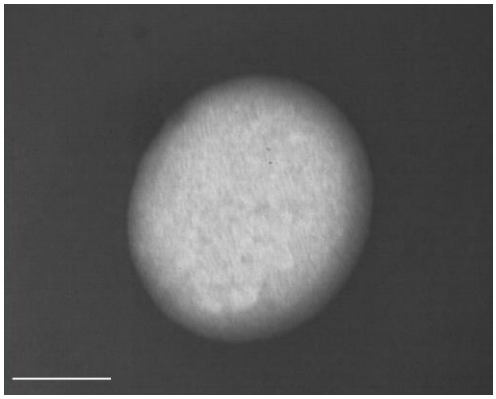


Figure 1. Transmission Electron Microscopy (TEM) image of an HUVEC-derived exosome sample, showcasing the characteristic spherical morphology and nanoscale dimensions of extracellular vesicles (scale bar: 50 nm, exosome diameter: 118 nm)

3.2. DLS

DLS analysis revealed an asymmetric size distribution for the exosomes, with an average diameter of approximately 115.5 nanometers (Mean: 115.5 nm, S.D.: 12.3 nm, Mode: 113.2 nm) (Figure 2). The distribution showed a predominant population of exosomes within a narrow size range. The 90° scattering angle was chosen for its ability to effectively capture light scattered by smaller particles, ensuring precise and reliable size measurements.

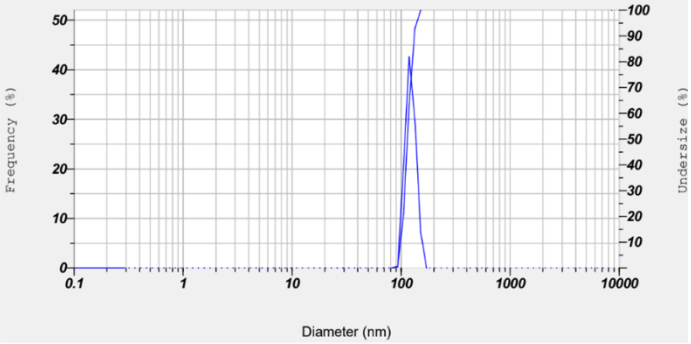


Figure 2. Size distribution of HUVEC-derived exosomes analyzed using dynamic light scattering (DLS).

3.3. Flow cytometry analysis

To confirm the endosomal origin of exosomes, CD63-FITC antibodies were used. Flow cytometry analysis (Figure 3) confirmed the presence of CD63, a tetraspanin protein that is commonly found on exosomes and is widely used as a marker for their identification (21).

3.4. Protein Concentration:

The Bradford assay was performed to determine the concentration of H-Exo protein content. This assay involves the use of Coomassie Brilliant Blue dye, which binds to proteins in the sample. When the dye interacts with the protein, it undergoes a color change, shifting from brown to blue. Based on the standard curve generated from known protein concentrations, the concentration of proteins was estimated to be 2 mg/ml.

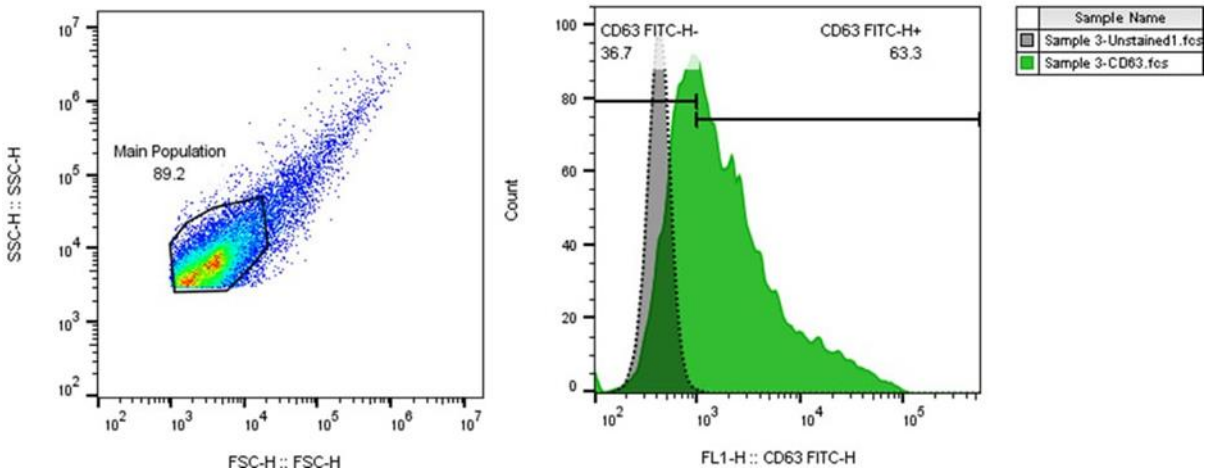
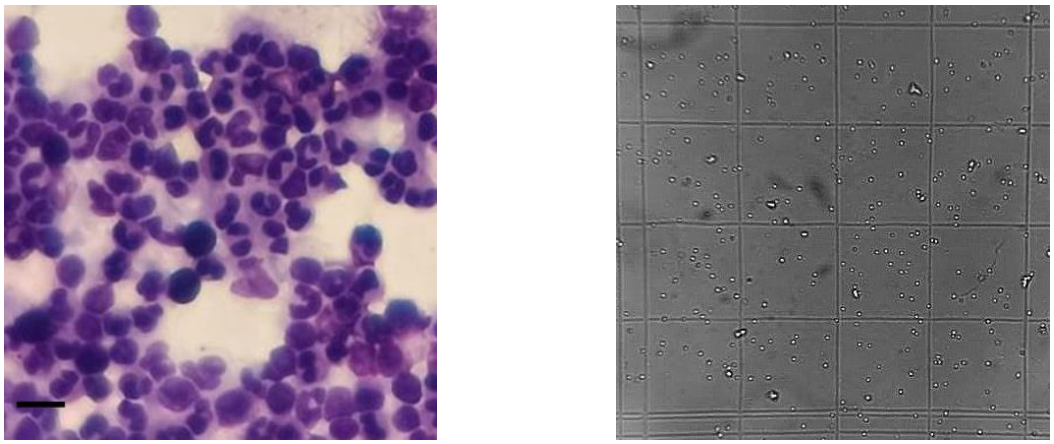


Figure 3. Flow cytometry analysis demonstrating the presence of CD63 exosomal marker.

3.5. The purity and viability of isolated neutrophils

Giemsa staining (Figure 4-A) and trypan blue exclusion (Figure 4-B) confirmed the purity and viability of the neutrophils to be greater than 95%.



a b

Figure 4 Isolated Neutrophils. a) The purity of isolated neutrophils. The neutrophil purity exceeded 95%, as assessed through Giemsa staining (scale bar: 20 µm). b) microscopic image of isolated neutrophils stained with trypan blue, observed using a Neubauer chamber. The image highlights live and dead cells for viability assessment.

3.6. 2.9. Effects of H-Exo on vitality of neutrophils

To rule out the potential impact of compound toxicity on neutrophil, the cytotoxicity of H-Exo was assessed by means of an MTT assay after a 2-hour incubation with neutrophils. As shown in Figure 5, pretreatment with H-Exo affected neutrophil survival. A comparison of the results revealed reduced cell vitality (15.29% compared to the controls) in the exosome-treated group compared to the untreated (negative control) group (figure 5).

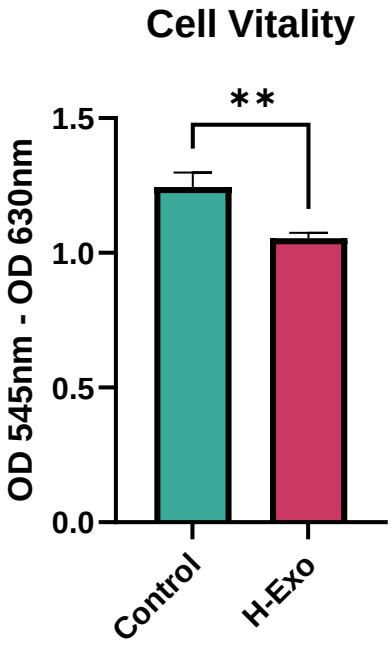


Figure 5: Effect of H-Ex on Neutrophil Vitality (MTT Assay). Treatment with H-Ex significantly reduced cell viability, as measured by MTT assay, by 15.29% compared to the control (**p < 0.01). Data are presented as mean ± SD. H-Exo refers to neutrophils treated with HUVEC-derived exosomes.

3.7. Nitroblue Tetrazolium (NBT) test:

H-Exo could significantly decrease NBT reduction by neutrophils (18.35% compared to the controls) (figure 6).

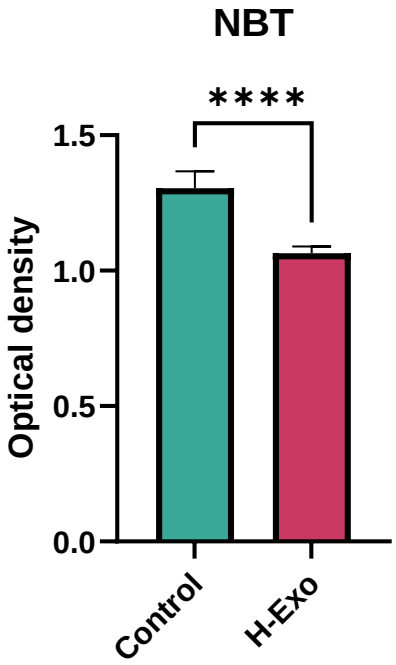


Figure 6. Respiratory burst in the control and H-Exo-treated groups of neutrophils, as measured by the NBT assay. Data are presented as the mean ± SD of three independent experiments. ****P < 0.0001.

3.8. Yeast Killing Assay

To assess the effects of H-Exo on the potential of yeast Killing by neutrophils, the neutrophils with yeasts were incubated at 37°C for 90 minutes to allow neutrophil-yeast interactions. As shown in figure 7, H-Exo could significantly (42.98%) decrease yeast Killing by neutrophils as compared with controls (figure 7).

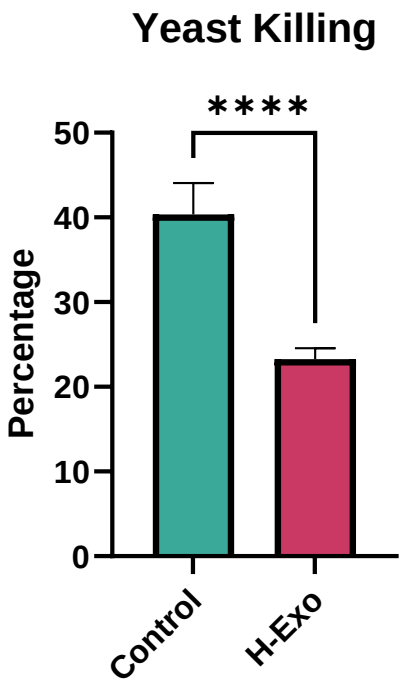


Figure 7. Yeast-killing ability of neutrophils in the presence of H-Ex compared to the control group. Data are presented as the mean ± SD of three independent experiments ****P < 0.001.

4. Discussion

Neutrophils play a critical role in the innate immune system (22). Neutrophils participate in the defense against viruses, bacteria, and fungi (23). They also play a variety of roles in the pathogenesis of several diseases, including autoimmune and cancer disorders (8). Neutrophils can also release proteases and generate excessive ROS at the injury site, leading to tissue damage, increased susceptibility to infection, and worsening chronic inflammation. This pathological effect has been frequently observed in various infectious and pulmonary diseases, including severe cases of COVID-19 (23). Excessive ROS levels within cells can trigger oxidative stress, leading to cellular damage, pathological changes, and the progression of diseases like cancer, inflammation, Parkinson's disease, and Alzheimer's disease (24).

It is well-established that certain healthy cells, including mesenchymal stem cells (MSCs), astrocytes, and neural progenitor cells (NPCs) are resistant to oxidative damage. However, their use in clinical applications is restricted due to concerns about safety, ethical considerations, and national regulation. Interestingly, exosomes derived from these cells exhibit the same efficacy as the parent cells (25).

Exosomes are small vesicles that carry a variety of molecules, including DNA, proteins, RNA, and lipids. They pass various materials between cells, thereby influence their functions. Exosomes have important roles in immune responses, particularly due to the presence of major histocompatibility complex (MHC) molecules on their surface. Additionally, exosomes express various proteins that can influence the immune system through non-antigenic mechanisms. Immune cell-derived exosomes exhibit both immunostimulatory and immunosuppressive properties (26). Prior studies have shown that exosomes can modulate neutrophil functions, including ROS production, phagocytosis, apoptosis, and lifespan (9-11, 27). For example, exosomes derived from Wharton's Jelly MSCs and adipose tissue mesenchymal stromal cells demonstrated no increase in ROS production (9, 10), whereas another MSC-derived exosomes enhanced ROS generation under certain conditions (11). This variability underscores the importance of investigating exosome effects in specific cellular and experimental contexts.

In the present study, we investigated the effects of H-Exo on neutrophil activity, focusing on ROS production and yeast-killing function. Our findings revealed that H-Exo significantly reduced ROS production. Additionally, we observed a decrease in neutrophil yeast-killing activity, which may further underscore the immunomodulatory properties of H-Exo. These results suggest a potential therapeutic role for H-Exo in conditions characterized by excessive oxidative stress and neutrophil overactivation.

Exosome treatment induced a 15.29% decrease in neutrophil viability. However, the effect on neutrophil-mediated yeast killing was considerably more substantial. Reductions of 18.35% and a remarkable 42.98% were observed, indicating a profound impact of exosomes on this crucial aspect of neutrophil function.

The mechanisms underlying the effects of H-Exo on neutrophils function remain unclear but may involve the delivery of bioactive molecules such as microRNAs (miRNAs), proteins, and antioxidants. These components are known to regulate ROS production through various pathways implicated in previous studies. Healthy cells-derived exosomes with antioxidant capabilities help reduce oxidative stress in recipient cells via different mechanisms. They transmit cellular signals by delivering bioactive molecules contained within them, thereby reducing intracellular oxidative stress through various mechanisms. Several studies have shown that the antioxidant effects of exosomes depend on the encapsulated proteins, micro RNAs (miRNAs), messenger RNAs (mRNAs), and circular RNAs (21). For example, Jiang et al. demonstrated that plasma exosomes can transfer exosomal 70 kDa heat shock protein (HSP70), effectively reducing ROS production (28). Another research by Wang et al., suggested that bone marrow-derived MSCs release microRNA-214-containing exosomes to overwhelm oxidative stress injury in cardiac stem cells by silencing calmodulin-dependent protein kinase II (CaMKII) (29). Additionally, exosomes from adipose tissue MSCs have been demonstrated to reduce inflammation and oxidative stress in macrophages through regulating the nuclear factor erythroid 2-related factor 2 (NRF2)/Heme oxygenase-1 (HO-1) axis in macrophages (30). It is plausible that H-Exo in our study employ similar mechanisms to suppress ROS production in neutrophils.

While MSC-derived exosomes have been widely studied, this is the first investigation of H-Exo effects on neutrophil function. Our results align with findings from other exosome studies that demonstrated antioxidant and immunomodulatory properties. However, the observed reduction in ROS production and yeast-killing activity highlights the need for further exploration of H-Exo's unique molecular cargo and its downstream effects on neutrophil functions. Future studies should evaluate other key neutrophil activities, such as phagocytosis, apoptosis, and lifespan, to provide a more comprehensive understanding of the immunomodulatory role of H-Exo.

5. Conclusion

In conclusion, our study demonstrates that H-Exo can effectively modulate neutrophil function by attenuating ROS production and reducing yeast-killing activity. We observed a 15.29% decrease in neutrophil viability following H-Exo treatment. Moreover, H-Exo significantly attenuated neutrophil-mediated yeast killing, with reductions of 18.35% and, in a particularly pronounced instance, 42.98%. These findings have significant implications to management inflammatory diseases where excessive neutrophil activity and oxidative stress contribute to disease progression. Future studies should investigate the molecular mechanisms underlying these effects.

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