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Comparison of the effects of adipose tissue mesenchymal stromal cell-derived exosomes with conditioned media on neutrophil function and apoptosis

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ABSTRACT

Background: Neutrophils are short-lived cells of the innate immune system that have an important role in defending against pathogens by producing reactive oxygen species (ROS). Therefore, effective strategies for increasing neutrophil's viability and function may be beneficial, especially in many conditions such as infections and immunodeficiency diseases. Some studies suggest using multipotent mesenchymal stromal cells (MSCs) and MSC-conditioned media (MSC-CM) for this aim. But, there is no study on using MSC-derived exosomes for improving neutrophil's viability and function. So, we examined the effects of MSC-exosomes and also MSC-CM on neutrophil's function and survival and compared them with each other.

Methods: Exosomes and CM were isolated from human adipose tissue MSCs. Exosomes were characterized, and the protein content of them was determined. Neutrophils were isolated from five healthy donors, and the effects of the two independent treatments (exosomes and conditioned media) on neutrophil's apoptosis were measured by Annexin V-PI method, then neutrophil's function was evaluated using NBT and phagocytosis assays.

Results: It was recognized that exosomes decreased neutrophils apoptosis and increased their phagocytosis capacity. The conditioned media augmented neutrophil's phagocytosis and reactive oxygen species (ROS) production, but it couldn't decrease neutrophil's apoptosis.

Discussion: Briefly, we concluded that MSC-exosomes augment neutrophil's viability more than their function while MSC-CM increase neutrophil's function more than the survival. This report showed that the use of MSC-exosomes and CM might be useful for increasing immunity by improving neutrophil's function and survival.

1. Introduction

Neutrophils, also known as PMN (polymorphonuclear) leukocytes, are the components of the innate immune system that are usually described as “short-lived cells,” as compared with other circulating cells or other phagocytic cells such as macrophages [1]. They are responsible for the first line of defense against pathogens by generating high amounts of reactive oxygen species (ROS) to create a toxic environment for pathogens. The ROS and their derivatives mediate many of the microbicidal and inflammatory activities of this cell [2,3]. They also have an important role in the activation of innate and acquired immune systems [4]. Therefore, effective strategies for increasing neutrophil's viability and function, especially in patients with immunodeficiency or infectious diseases, are necessary [5–7]. According to various studies, there are some suggestions for increasing neutrophil's viability and function, such as anoxia condition, glucose supplementation [1] and multipotent mesenchymal stromal cells (MSCs). Different studies reported that MSCs and their conditioned media (CM) could improve the function of neutrophils by increasing ROS production, chemotaxis, phagocytosis and they also have anti-apoptotic effects on these cells by increasing expression of MCL-1, as an anti-apoptotic protein, and decreasing Bax, as a pro-apoptotic protein [8–11].

MSC-CM contains many factors produced by MSCs such as; extracellular vesicles (EVs) that account for paracrine effects of MSCs [12].

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EVs are secreted by many cells and involved in intercellular communication. They are classified into exosomes, microparticles, or microvesicles (MVs) and apoptotic bodies that are characterized by their biophysical properties and biogenesis [13–15]. The use of exosomes has several advantages over MSCs; for example, in contrast to MSCs, that are very large, the small size of exosomes, allow easy circulation of them through capillaries. These vesicles have more safety because the damaged DNA that may be present in cells are not transferred by them, and the proliferation issue is not a concern about these nanoparticles. In addition to this, their function can be maintained during the long storage periods. So, it can be concluded that MSC-exosomes display the same effects of MSCs on cells, but with lower side effects and they can be used in conditions that requiring cell-free therapies [16–18]. Until now, there is no study on the evaluation of the effects of MSC-exosomes on neutrophil's apoptosis and function.

Therefore, in this research, we explored and compared the effects of MSC-exosomes with MSC-CM on the apoptosis and function of neutrophils, which would be used for increasing innate immunity by improving neutrophil's function or survival.

2. Materials and methods

2.1. Isolation, culture, and expansion of Adipose tissue MSCs (AD-MSCs)

The adipose tissues from healthy donors that became available as a waste product during liposuction procedures was collected after obtaining written informed consent as approved by the National Institute For Medical Research Development (IR.NIMAD.REC.1397.472) and Ethical Committee of the Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.MSP.REC.1397.539). First, 10× collagenase type I (Gibco, Invitrogen, USA) was diluted in Dulbecco's Modified Eagle's Medium (DMEM) at a ratio of 1:10. Then, the samples were washed in phosphate buffered saline (PBS) and separated from connective tissue in a sterile glass petri dish. These separated adipose tissues were cut into smaller pieces and put into the diluted collagenase solution, that was previously prepared. The mixture was incubated for 30 min at 37 °C. During the incubation period, the tubes containing adipose tissues were inverted for several times, every 5 min. After that, the tubes were centrifuged at 500*g* for 10 min. The upper fat layer and the supernatants were discarded. The pellet was resuspended with DMEM containing 15% fetal bovine serum (FBS), glutamine, penicillin/streptomycin (all from Invitrogen, USA) and cultured in 25 cm^2 cell culture flasks. They were incubated under a humidified air at 37 °C. After about 24 h, the flasks were washed with PBS. The proliferation of the cells was monitored and when they reach about 80–90% confluence, they were passaged in 75 cm^2 cell culture flasks.

2.2. Flow cytometric analysis of AD-MSCs

To evaluate cell-surface marker expression through flow cytometry, AD-MSCs from the second passage were stained with anti-human antibodies, including Anti-CD90, anti-CD45, anti-CD73, anti-CD14 and anti-CD105 (all antibodies were from eBioscience) and a FACSCalibur flow cytometer (BD Biosciences, USA) and FlowJo software were used to analyze the samples.

2.3. The differentiation capacity of AD-MSCs

To examine the differentiation capacity of isolated MSCs to osteocytes and adipocytes, 3×10^4 cells per well were cultured in 4 well plates. The media containing glycerol phosphate (10 mM), dexamethasone (100 mM), and ascorbic acid-2 phosphate (5 g/ml) were added to one well of the plate for osteogenic differentiation and complete media supplemented with indomethacin (100 mM), 3-isobutylmethylxanthine (0.5 mM), dexamethasone (250 mM), and insulin (5 mM) (all from Sigma-Aldrich, Missouri, USA) were added to another well of the plate, for adipogenic differentiation. The two other wells were considered as controls and DMEM with 10% FBS was added to them. This examination was performed in three plates to confirm the results. The plates were monitored for about three weeks. Media were changed every three days. After 21 days, cells were stained with Alizarin Red-S (ARS) and Oil Red-O (ORO) to confirm their differentiation into osteocyte and adipocyte lineages, respectively. The plates were seen using an inverted microscope, and images were taken from them.

2.4. Preparation of multipotent mesenchymal stromal cells conditioned media

After reaching 80–90% confluency of the MSCs from the second passage, the medium of them was replaced with the medium containing less FBS, every three days. Finally, FBS-free supernatants of the cultured MSCs were collected. After filtering by 0.22 μm filters, the supernatants were stored at −70 °C. These media were used to treat some neutrophils and also to isolate exosomes.

2.5. MSC-exosome isolation

Exosomes were isolated from FBS-free CM based on the protocol of Exocib kit (Cib Biotech Co.). Briefly, the filtered supernatants were mixed with the exosome precipitation solution and incubated overnight at 4 °C. The samples were centrifuged for 40 min at 3000 rpm. Then, the supernatants were discarded, and the pellet was resuspended with PBS. Finally, the purified exosome samples were stored at −70 °C. The protein content of the MSC-exosomes was determined using a Bicinchoninic Acid (BCA) protein assay kit (Sigma-Aldrich, Missouri, USA).

2.6. Scanning electron microscopy of MSC-exosomes

Scanning electron microscopy (SEM) (MIRA3 TESCAN) was used for characterization of the isolated exosomes; for this method, they were first fixed with glutaraldehyde (Sigma–Aldrich GmbH, Taufkirchen, Germany) for about 15 min. Then, the fixed samples were washed with PBS, and ascending sequence of ethanol was used for dehydrating the samples. Ethanol was evaporated, and then, the samples were kept at room temperature and allowed for 24 h to dry. Finally, they were analyzed by SEM after gold–palladium sputtering [19].

2.7. Dynamic light scattering of MSC-exosomes

The size distribution of the particles that have the diameter ranging from 1 nm to 6 μm can be measured using dynamic light scattering (DLS). The particles are illuminated with a laser beam. So, all vesicles present in the beam will disseminate light. The intensity fluctuations of the disseminated light will be measured, and a mathematical model derived from Brownian motion, and light scattering theory will be applied. So, the size distribution of these vesicles is assessed [20]. For this aim, samples were diluted to 1 μg/ml in PBS, and 0.05% Tween-20, and the size of them was evaluated by DLS Zetasizer Nano ZS (Malvern Instruments, UK).

2.8. Isolation and culture of neutrophils

After obtaining informed consent from 5 healthy donors, neutrophils were isolated from heparinized venous blood. For this aim, 3% dextran was added to the samples at a ratio of 1:1 and mixed throughly. After sedimentation, the samples were centrifuged (400*g*, 30 min) on a Ficoll-Hypaque density gradient. Residual erythrocytes were discarded by adding lysis buffer and PBS, respectively. Then, neutrophils were resuspended in RPMI-1640 (Gibco, NY, USA) with 10% heat-inactivated FBS, 1% glutamine, and penicillin/streptomycin. The viability and

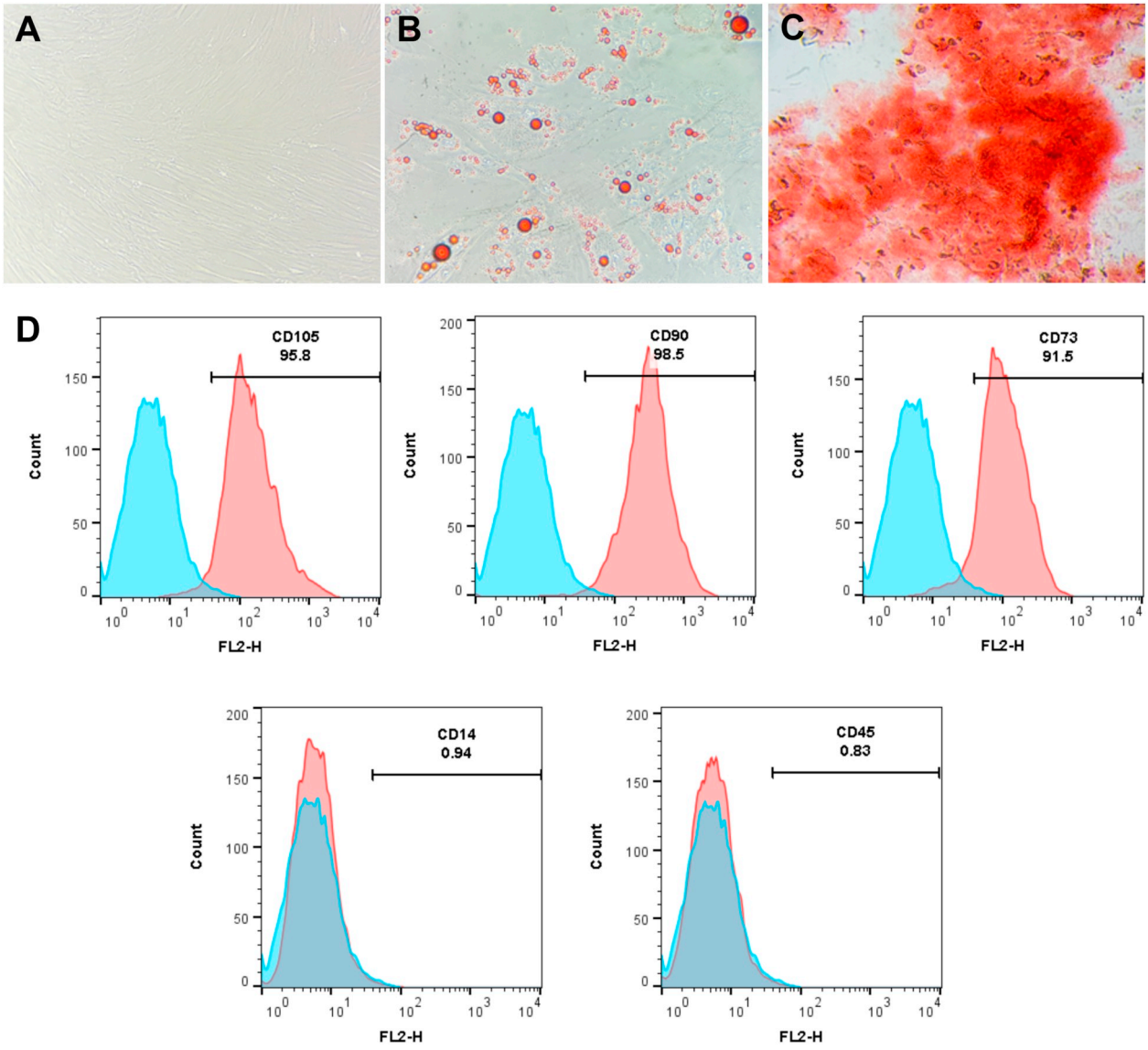

Fig. 1. Characterization and differentiation of adipose tissue MSCs. (A) Morphology of AD-MSCs. (B) Oil Red O staining of the differentiated MSCs into adipocytes. (C) Alizarin Red S staining of AD-MSCs that were differentiated into osteocytes. (D) AD-MSC surface marker expression, including CD73, CD90, and CD105, CD45 and CD14.

purity of isolated neutrophils were determined by trypan blue dye exclusion and Giemsa staining, respectively. Neutrophil suspensions were cultured in triplicate with medium (as control groups), MSC-exosomes (100 μg/ml) or MSC-CM (1:1 ratio) in tubes (for phagocytosis) or 96-well flat bottom plates (for other experiments) at 37 °C, for 12 h.

2.9. Exosomes uptake by neutrophils

The PKH67 dye (Sigma-Aldrich, Missouri, USA) was used for labeling the MSC-exosomes based on the protocol of the kit. Briefly, 0.1 mg of exosomes were mixed and incubated with 2 μl PKH67 dyes for 5 min. For blocking the reaction, exosome-free FBS was added to the mixture. Exosome isolation was done to remove free dyes. Then, 2×10^6 neutrophils were treated with these purified exosomes in 4 well culture plate containing RPMI 1640 medium to assess the transfer of exosomes into neutrophils. They were incubated for six h at 37 °C, to allow incorporating the exosomes into neutrophils. Finally, PKH-positive cells were identified by fluorescence microscopy.

2.10. Neutrophil's apoptosis assay

Isolated neutrophils were cultured with medium, MSC-exosomes, or MSC-CM, at a density of 3×10^5 cells per well, in 96-well plates. For each treatment, two wells were considered, one well for unstained and another for the annexin V-PI stained cells. After 12 h of treatment, the cells were transferred to flow cytometry tubes and then neutrophils were washed with PBS and resuspended in 500 μl isotonic-binding buffer. Then, annexin V and propidium iodide (PI) were added to the test tubes according to the guidelines of the kit (Annexin V-FITC apoptosis detection kit; eBioscience), and all of the unstained and test tubes were analyzed using a FACSCalibur flow cytometer (BD Biosciences, USA) and FlowJo software.

2.11. Measurement of respiratory burst

Neutrophils were cultured with medium, MSC-exosomes or MSC-CM in 96-well flat bottom plates for 12 h. At the end of the culture, the release of ROS by neutrophils was measured by colorimetric NBT (Nitroblue Tetrazolium) assay. NBT solution (1.25 mg NBT, 1 μg of PMA

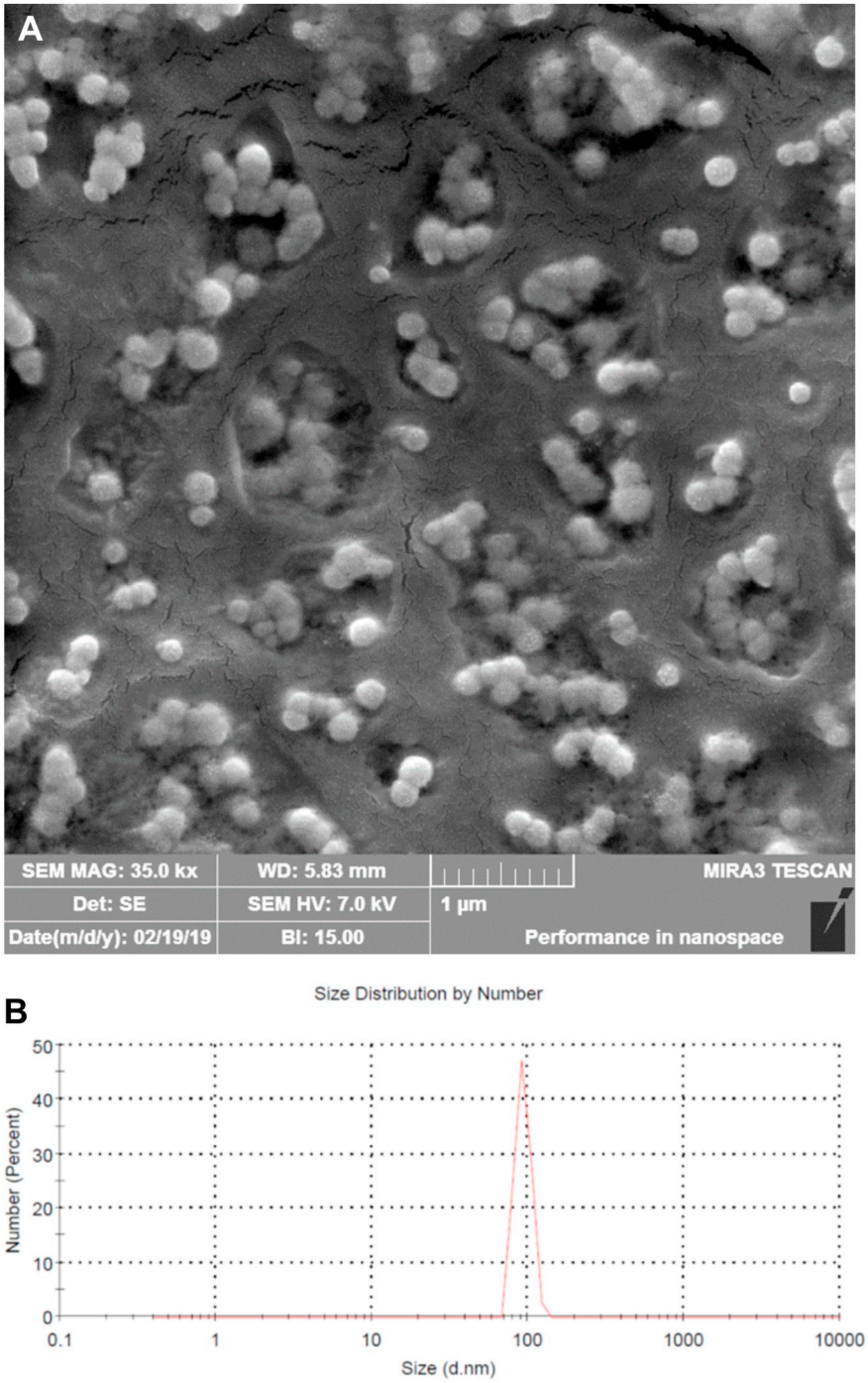

Fig. 2. Characterization of MSC-exosomes. (A) Scanning electron microscopy of the MSC-exosomes to visualize the shape and size of these vesicles. (B) Dynamic light scattering of MSC-exosomes for measuring the mean size of these nanoparticles.

and 17.5 mg of bovine serum albumin, in 1 ml normal saline [21], all from Sigma-Aldrich, Missouri, USA) was added to each well. The plates were kept at a 37 °C incubator for 40 min. Cells were pelleted on the bottom of the wells by 10 min centrifuging the plates at 400 g. The supernatant was removed. The insoluble formazans were solubilized by adding 120 µl/well 2 M KOH, and then 140 µl/well dimethyl sulphoxide (DMSO). To complete solubilization, the contents of the wells were then mixed. The optical density (OD) of each well was measured using an ELISA plate reader at 630 nm [22].

2.12. Neutrophil phagocytosis assay

For the phagocytosis assay of neutrophils, they were cultured with medium, MSC-exosomes or MSC-CM in tubes for 12 h. Heat-killed baker's yeast particles were washed with PBS and resuspended in RPMI-1640 medium. Then, they were added to the cultured neutrophils at a 8:1 ratio. The tubes were kept at 37°C for 60 min to monitor the phagocytosis capacity of the neutrophils. Smears were prepared on slides and fixed in methanol. After Giemsa staining for 10 min, 400 neutrophils from five or more fields were analyzed using a microscope. Then, the percentage of phagocytosis (The percentage of neutrophils that phagocyted at least one yeast), mean number of yeasts ingested by

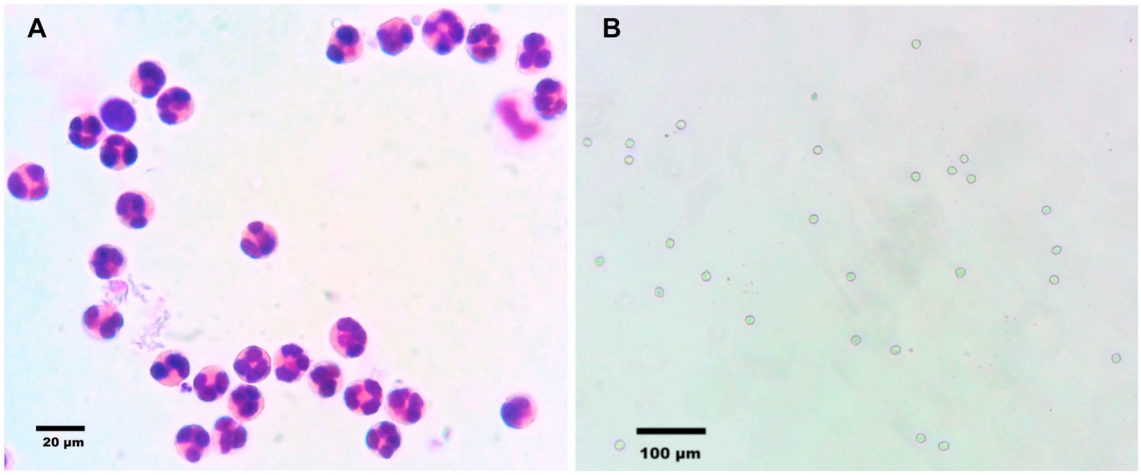

Fig. 3. The purity and viability of neutrophils. (A) The purity of neutrophils was > 95%, as determined by Giemsa staining. (B) Neutrophil's viability was > 97%, as determined by trypan blue dye exclusion.

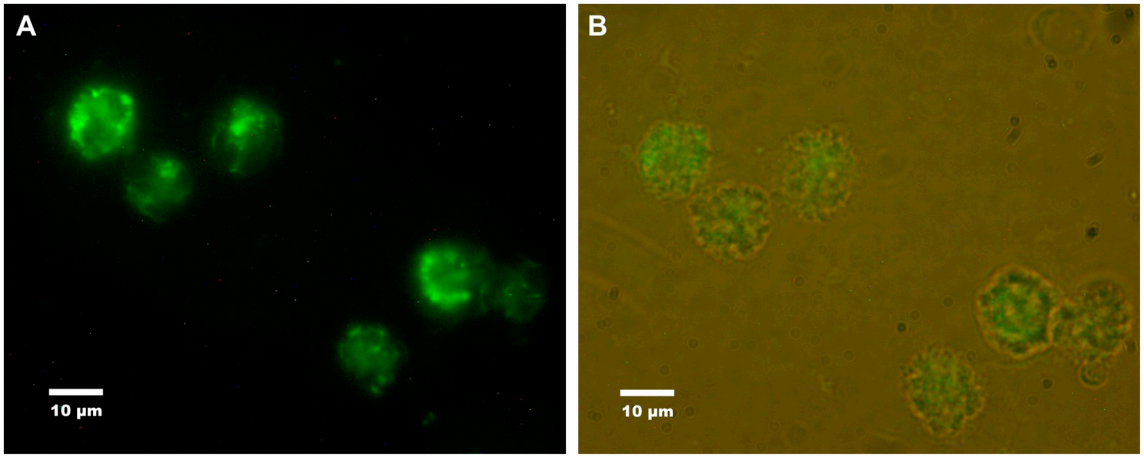

Fig. 4. PKH labeling of exosomes uptaken by neutrophils. (A) Fluorescent microscopy (B) Merge of Fluorescent with phase contrast microscopy. The intensity of cells fluorescent indicates the rate of exosomes uptake by neutrophils.

neutrophils, and phagocytosis index (Phi) [23] (the percentage of neutrophils with evidence of phagocytosis (*i.e.*, > 1 yeast internalized) multiplied by the average number of ingested yeasts) were measured after analysis of samples.

2.13. Statistical analysis

The data of this research were presented as the mean ± standard deviation (SD). Statistical differences were analyzed by analysis of variance (ANOVA) using GraphPad Prism 8 software. A p-value < 0.05 was considered statistically significant. All experiments were performed in triplicate.

3. Results

3.1. Characterization and differentiation potential of AD-MSCs

Flow cytometric analysis of AD-MSCs demonstrated that the majority of MSCs expressed high levels of the CD73, CD90, and CD105 markers, whereas CD14 and CD45 markers were relatively absent. These results are shown in Fig. 1. After three weeks, according to the Alizarin Red-S and Oil Red-O staining, it was recognized that AD-MSCs had the ability to differentiate into osteocytes and adipocytes.

3.2. Exosomes characterization

Characterization of the shape and size of the isolated exosomes was carried out by SEM and DLS, respectively. Among the EVs, the size of the exosomes is between 50 and 150 nm [24] and as we expected, the mean size of isolated exosomes in our study was 93.36 (Fig. 2).

3.3. Purity and viability of isolated neutrophils

The viability of neutrophils was > 97%, as evaluated by trypan blue dye exclusion, and their purity was about 95%, as determined by Giemsa staining (Fig. 3).

3.4. Exosomes uptake by neutrophils

After labaling the MSC-exosomes with a fluorescent PKH67 dye, they were added to the cultured neutrophils. After 6 h, neutrophils became fluorescent. Fig. 4 indicates that the intensity of cells fluorescent shows the rate of exosomes uptake by neutrophils. Our data show that exosomes were uptaken by neutrophils.

3.5. The effects of MSC-exosomes or MSC-CM on neutrophil's apoptosis

After 12 h of treatment with MSC-exosomes or MSC-CM, flow cytometric analysis was performed to assess neutrophil's apoptosis. The

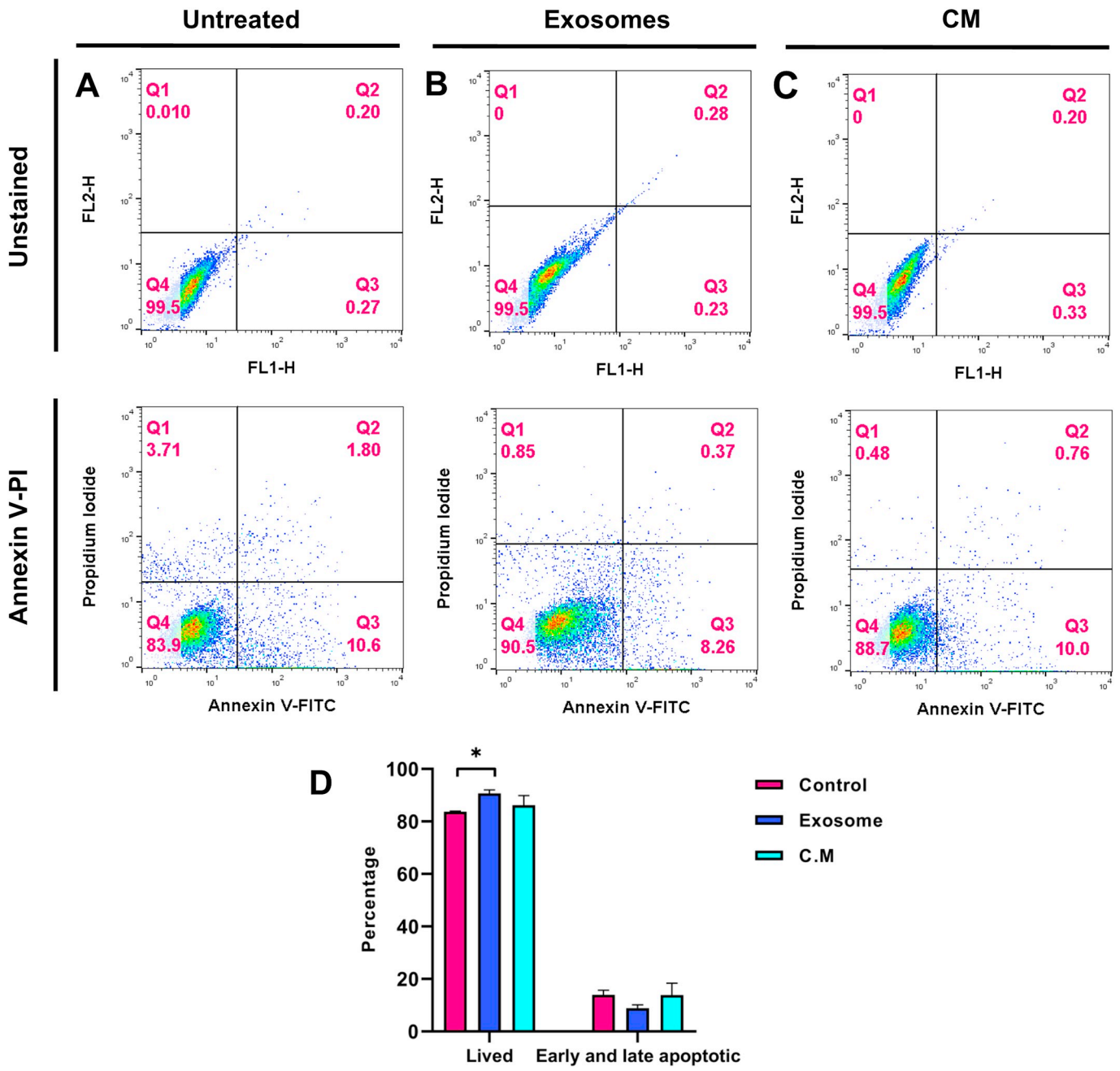

Fig. 5. Apoptosis assay of neutrophils cultured in medium (the control group), MSC-exosomes and MSC-conditioned media (in 1:1 ratio) by flow cytometry. (A, B & C) The percentages of cells in each group within the gated areas are shown. In the gated areas, the up-right quadrants show the populations corresponding to late apoptotic cells (both Annexin V and PI positive) and the lower-right quadrants are for the early apoptotic cells (Annexin V positive and PI negative). The up-left quadrants indicate necrotic cells (Annexin V negative and PI positive) and the low-left quadrants show viable cells (both Annexin V and PI negative). Unstained cells were used for gating the cells. (A) The unstained (upper panels) and double stained (lower panels) cells of the control group (untreated), (B) MSC-exosome treated group, and (C) MSC-CM treated group are demonstrated. (D) The left bars show the percentage of viable neutrophils and the right bars show the percentages of neutrophil's apoptosis. CM: Conditioned Media.

data demonstrated that neutrophil's apoptosis was significantly decreased by MSC-exosomes, but MSC-CM didn't have any significant effect on neutrophil's apoptosis (Fig. 5).

3.6. The effects of MSC-exosomes or MSC-CM on neutrophil's respiratory burst

The ROS production by neutrophils was measured by colorimetric NBT assay. As shown in Fig. 6, MSC-exosomes couldn't increase neutrophil's ability to release ROS, while MSC-CM significantly augmented ROS production by neutrophils.

3.7. The effects of MSC-exosomes or MSC-CM on neutrophil's phagocytosis

To assess the effects of MSC-exosomes or MSC-CM on the phagocytic potential of yeast particles by neutrophils, after 12 h, neutrophils were allowed for 1 h to phagocyte the yeast particles. The results show that both MSC-exosomes and MSC-CM could significantly increase phagocytosis percentage, phagocytosis index, and the mean number of yeasts ingested by neutrophils as compared with controls (Fig. 7).

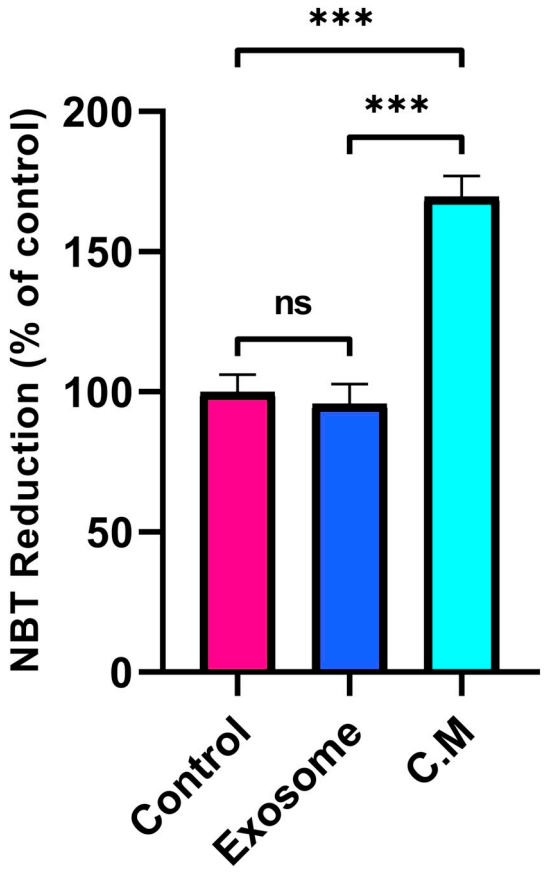

Fig. 6. ROS production of neutrophils in control, MSC-exosome treated, and MSC-CM treated groups as measured by NBT assay. The experiments were carried out in triplicate. CM: Conditioned Media.

4. Discussion

Neutrophils are the first cells involved in defending against infections. The main functions of them, include ROS production, phagocytosis, and neutrophil extracellular traps (NETs) formation. They usually described as "short-lived cells," as compared with other circulating cells or other phagocytic cells [25]. Due to the important roles of these cells, tuning their function and survival is beneficial in many conditions such as infections and immunodeficiency diseases [5–7]. Our report suggested that some treatments such as MSC-exosomes or MSC-CM can enhance their lifespan and even function *in vitro*.

Multiple pieces of evidence have demonstrated that, in addition to cell-cell contact, MSCs also affect other cells through the releasing of EVs and soluble factors. EVs are classified into three main groups, including exosomes, microvesicles, and apoptotic bodies [13]. Various studies have shown that MSC-exosomes and MSC-CM also have the same effects of MSCs [26–28]. Exosomes transfer their contents after interacting with recipient cells and cause some changes in target cells. Since MSC-exosomes and MSC-CM are involved in intercellular communication between MSCs and target cells, they might be a beneficial treatment for several diseases such as neutropenia and chronic granulomatous disease (CGD) in which low neutrophil count and impaired function can cause severe infections, respectively, without common side effects that are associated with MSC therapy [17,29,30]. So, these treatments may recover the innate immunity of these patients and reduce the complications that are related to the lack of sufficient neutrophil count or function. Although there are several concerns about the use of exosomes and CM. For example, based on the different exosome isolation methods, the yield of exosomes from 1 ml of MSC culture supernatants, is approximately lower than 1 μg and sometimes the purity of isolated exosomes is low and should be optimized. One of the concerns about both exosomes and CM is that they are static and restricted to the dose of injection, and no more production is expected [16,17,31]. So, for the therapeutic use of these products, all of the mentioned challenges should be solved.

In this report, we explored the supportive roles of exosomes and supernatants from AD-MSCs cultured on apoptosis and function of neutrophils. Exosomes and conditioned media were isolated from human adipose tissue MSCs. Then neutrophils were isolated from five healthy donors. The effects of exosomes and conditioned media on neutrophil's apoptosis, phagocytosis, and respiratory burst were measured *in vitro*. It is recognized that after 12 h of treatment, exosomes significantly increased the percentage of viable neutrophils and their phagocytosis percentage and index, but no effect on ROS production by neutrophils was observed (Fig. 6). The conditioned media significantly augmented neutrophil's phagocytosis and ROS production, but it couldn't increase neutrophil's viability. These observations can be due to the presence of any soluble factors such as granulocyte-colony stimulating factor (G-CSF), interleukin-8 (IL-8) interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interferon-α (IFN-α) and interleukin-6 (IL-6) [9,11,32,33]. The exosomes may also contain many mRNAs and miRNAs that may affect the function or viability of neutrophils. For example, it was shown that MSC-derived exosomes contain the let-7 family of miRNAs. A member of this family, calling let-7a target caspase-3 and subsequently inhibits apoptosis. In addition to this, IL-6 mRNA may also present in MSC-exosomes and transferred to neutrophils cytosol. So, the production of IL-6 may have autocrine effects on neutrophils and prolongs the survival and improve the inflammatory responses of these cells [34–36]. To identify the main factors that are responsible for these effects, more studies should be done in the future.

For the identification of the possible factors, a study conducted in 2017, the researchers evaluated the effects of periodontal ligament stem cells (PDLSCs) on the apoptosis of neutrophils under cell-cell contact culture and transwell system, with or without anti IL-6 antibody [33]. This study demonstrated that PDLSCs reduced neutrophil's apoptosis, whether under transwell culture or cell-cell contact. By adding anti-IL-6 antibody, an increase in apoptosis rate was seen. As MSCs are one of the cells secreting IL-6 [37], and IL-6 has stimulatory effects on inflammatory cells such as neutrophils, IL-6 could be one of the main mediators for the effects of MSCs on neutrophils [38]. So, the effects that were seen in our report may be due to the presence of IL-6 in MSC-exosomes and CM, as a soluble factor. Some molecules derived from pathogens such as lipopolysaccharide (LPS) bind to the toll-like receptor 4 (TLR4) that expressed by MSCs and stimulated them. Following the stimulation, MSCs secrete large amounts of pro-inflammatory cytokines such as IL-6 that increase the function and survival of neutrophils [38]. Marco A. Cassatella et al. in 2011 investigated the effects of TLR4-activated MSCs on neutrophil's survival and function [9]. Their results showed that primed-MSCs secreted higher amounts of IL-6, as compared with controls, so they have more supportive effects on neutrophil's viability. These results suggest that our study can be developed using LPS-treated MSCs and doing all of our experiments on exosomes and CM derived from these MSCs.

For the different results that were observed about the effects of the exosomes or CM on ROS production and viability of neutrophils, we can reference to this fact that: "lower amounts of ROS lead to cell survival, while higher amounts of it, activate apoptosis" [39]. Because CM that could increase the ROS production, could not augment the viability of cells. Whereas exosome-treated groups had more viability, but less ROS production. In addition to this, some toxic materials that may produce during the metabolism of MSCs and released into MSC-CM can account for the lower survival of CM-treated groups in comparison with exosome-treated groups.

Up to now, studies addressing the effects of MSCs on neutrophils were mostly restricted to cell-cell contact and *in vitro* experiments [4,8,32]. In Yoon Shin Park et al. study the effects of adipose tissue-

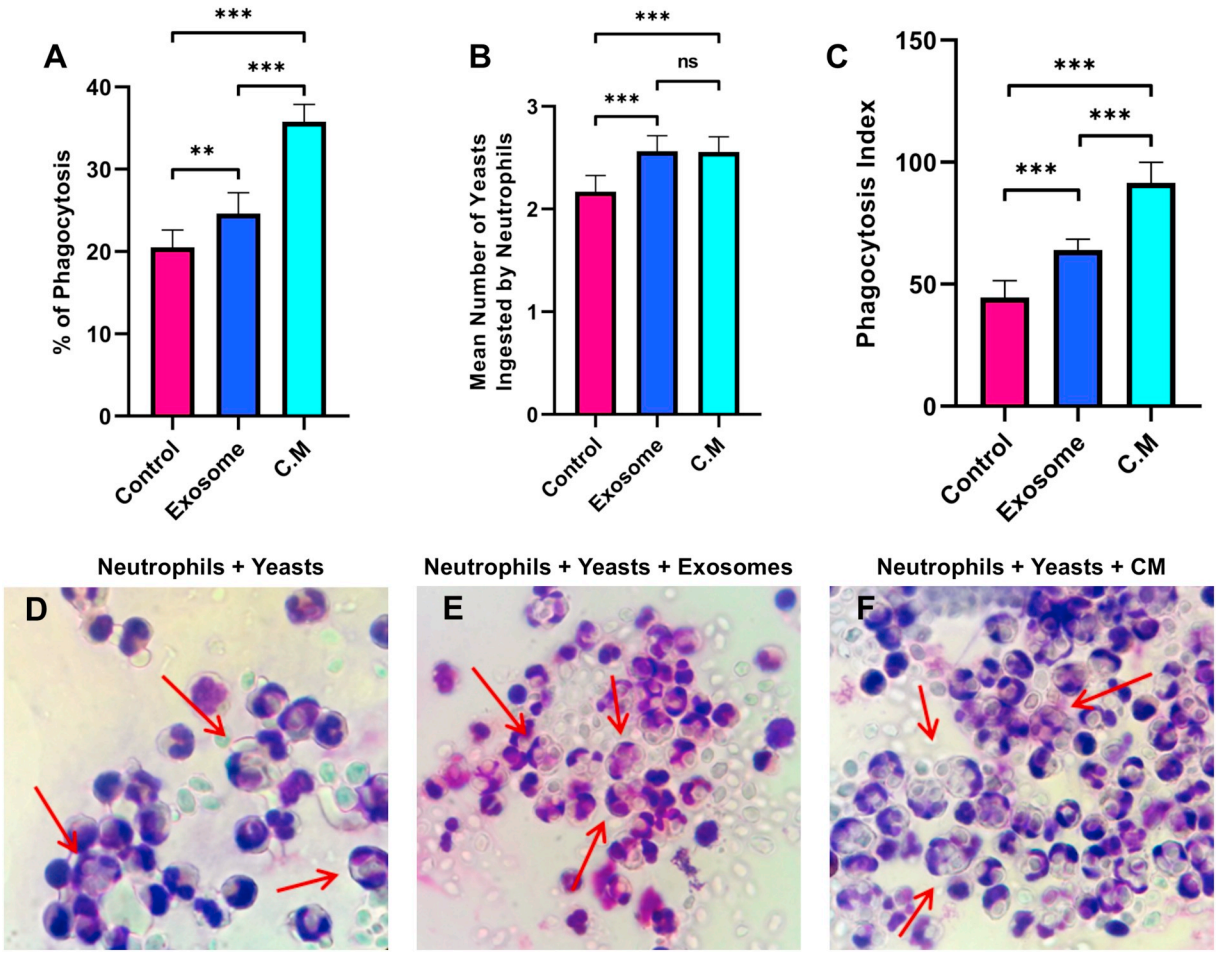

Fig. 7. Phagocytosis assay of neutrophils. (A) The Percentages of neutrophils that phagocyted at least one yeast, (B) The mean number of yeasts ingested by neutrophils, and (C) Phagocytosis index (Phi) (the percentage of neutrophils with evidence of phagocytosis multiplied by the average number of ingested yeasts) were measured after analysis of samples. (D) Representative results for phagocytosis of yeast particles by untreated neutrophils, (E) exosome treated and (F) MSC-CM treated neutrophils. CM: Conditioned Media.

derived MSCs on the neutrophil function and survival were evaluated and among the functional and survival assays for neutrophils, ROS production, apoptosis and viability assays were reported [4]. AD-MSCs increased neutrophil viability and decreased neutrophil apoptosis, similar to our results about MSC-exosome treated groups and enhanced respiratory burst similar to our results about MSC-CM treated groups.

Briefly, we concluded that MSC-exosomes increase neutrophil's survival more than their function while MSC-CM increase neutrophil's function more than their viability. Our results demonstrate a novel MSC-exosome or CM dependent activation of neutrophils in defending against pathogens. These observations should be considered in the future to improve the therapeutic use of neutrophils, especially in patients with immunodeficiency or infectious diseases. However, the relevance of our observations has to be confirmed in *in vivo* models.

Declaration of Competing Interest

None.

Acknowledgments

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Author contributions

Mohammad Mahmoudi and Mahsa Taghavi-Farahabadi performed the experiments and data analysis. Seyed Mahmoud Hashemi and Nima Rezaei designed experiments. All authors prepared the manuscript. Mohammad Mahmoudi and Mahsa Taghavi-Farahabadi wrote the paper.

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