Human Immunology 80 (2019) 990–998

Contents lists available at ScienceDirect

Human Immunology

journal homepage: www.elsevier.com/locate/humimm

Exosomes derived from mesenchymal stem cells improved function and survival of neutrophils from severe congenital neutropenia patients *in vitro*

Mohammad Mahmoudi[a,1], Mahsa Taghavi-Farahabadi[b,1], Saeed Namaki[a], Kaveh Baghaei[c], Elham Rayzan[d], Nima Rezaei[b,e,f,*], Seyed Mahmoud Hashemi[a,g,h,*]

[a] Department of Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[b] Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[c] Basic and Molecular Epidemiology of Gastrointestinal Disorder Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Science, Tehran, Iran
[d] International Hematology/Oncology of Pediatrics Experts, Universal Scientific Education and Research Network, Tehran University of Medical Sciences, Iran
[e] Research Center for Immunodeficiencies, Children's Medical Center, Tehran University of Medical Sciences, Tehran, Iran
[f] Cancer Immunology Project (CIP), Universal Scientific Education and Research Network (USERN), Sheffield, UK
[g] Urogenital Stem Cell Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[h] Department of Tissue Engineering and Applied Cell Sciences, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Keywords:
Severe congenital neutropenia
Exosomes
Mesenchymal stem cells
Neutrophils
Apoptosis

ABSTRACT

Severe congenital neutropenia (SCN) is described by the absolute neutrophil counts less than 500 cells/mm^3, bacterial infections, and an arrest of neutrophil differentiation. So, effective strategies for improving the function and lifespan of the existing neutrophils in these patients are necessary. Mesenchymal stem cells (MSCs) have supportive effects on neutrophils. Recently, it was determined that MSCs exert their effects, mostly by secreting soluble factors and exosomes. So, in this study, neutrophils were isolated from the bloodstream of healthy donors and SCN patients and cultured with medium, MSC-exosomes or MSC-conditioned media (MSC-CM). Then, the effects of the two treatments on neutrophil respiratory burst, apoptosis and phagocytosis percentage were assessed using nitro blue tetrazolium (NBT) assay, annexin V-propidium iodide (PI) and Giemsa staining, respectively. Both treatments could significantly augment respiratory burst of neutrophils from SCN patients and healthy donors. But, only CM could significantly enhance phagocytosis index. About the lifespan of neutrophils, only exosomes could significantly enhance it in both groups. Based on these results, both exosomes and CM derived from MSCs could be attractive candidates for rescuing SCN patients from serious infections.

1. Introduction

Severe congenital neutropenia (SCN) is a genetic disorder that is identified by the absolute neutrophil counts less than 500 cells/mm^3, bacterial infections, and an arrest of neutrophil differentiation [1]. This syndrome is genetically heterogeneous. The autosomal dominant mode of inheritance has been frequently due to heterozygous mutations in ELANE [2]. Recently, it was recognized that, the patients with autosomal recessive inheritance demonstrate homozygous mutations in HAX1 [3].

Neutrophils are the major population of the innate immunity that have multiple proteases, antimicrobial peptides, and reactive oxygen species (ROS) for killing pathogens [4]. So, the low level of neutrophils, that is the main hallmark of SCN patients, can lead to severe and recurrent infections [5]. Therefore, effective strategies for increasing the population of neutrophils or even improving the function and lifespan of the existing neutrophils in these patients are necessary. One of the common treatments for them, is granulocyte-colony stimulating factor (G-CSF) administration [5], but there are some limitations associated with this treatment, for example, it was reported that myelodysplasia (MDS) and acute myeloid leukemia (AML) are the complications that were observed in some patients receiving it [6]. To overcome these limitations, it is beneficial to find other strategies for rescuing these patients from severe infections. Multiple studies demonstrated that,

* Corresponding authors at: Research Center for Immunodeficiencies, Children's Medical Center, Dr Qarib St, Keshavarz Blvd, 14194 Tehran, Iran (N. Rezaei). Department of Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Department of Applied Cell Sciences, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran (S.M. Hashemi).
E-mail address: smmhashemi@sbmu.ac.ir (S.M. Hashemi).
[1] Author 1 and 2 contributed equally and should be considered co-first authors.

https://doi.org/10.1016/j.humimm.2019.10.006
Received 5 August 2019; Received in revised form 28 September 2019; Accepted 28 October 2019
Available online 06 November 2019

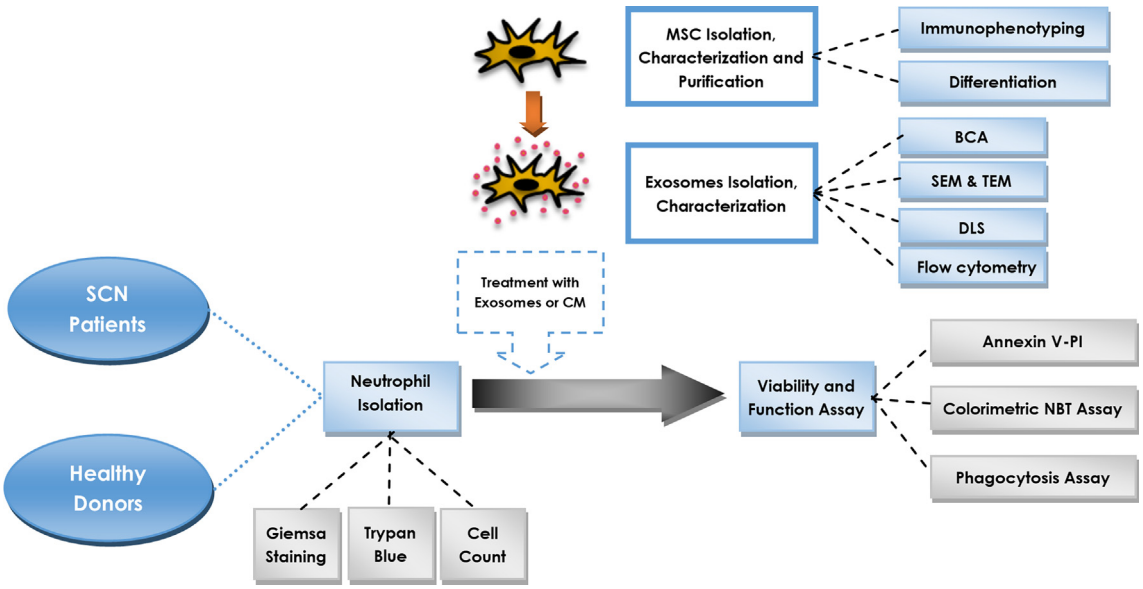

Fig. 1. Study design.

mesenchymal stem cells (MSCs) have supportive effects on neutrophil function and lifespan [7–14].

Recently, it was recognized that, secreting soluble factors and exosomes that are a type of extracellular vesicles (EVs), is one of the main mechanisms that MSCs used to affect other cells [15]. Up to now, there is no study about evaluating the effects of MSC-derived exosomes on the function and lifespan of neutrophils whether in healthy donors or SCN patients. So, we designed this study to investigate how MSC-exosomes and also conditioned media (MSC-CM) affect the function and apoptosis of neutrophils that were isolated from SCN patients and healthy donors (Fig. 1). After that, we compared their effects with each other.

2. Materials and methods

2.1. Adipose tissue MSCs (AD-MSCs) isolation

For isolation of MSCs, adipose tissues were obtained from abdominal subcutaneous fat of healthy adult donors. After separating the adipose tissues from connective tissues, the adipose tissues were cut and digested by incubating with 0.1% type I collagenase (Gibco, Invitrogen, USA) in Dulbecco's modified Eagle's medium (DMEM) for 30 min at 37° C. After that, digested tissues were centrifuged at 500*g* for 10 min. Then, the pellet was resuspended and cultured in DMEM containing 15% fetal bovine serum (FBS) supplemented with 2 mM L-glutamine, streptomycin and penicillin (all from Invitrogen, USA). When MSCs reached 80–90% confluency, they were passaged.

2.2. Characterization of AD-MSCs

The differentiation capacity of AD-MSCs into adipogenic and osteogenic lineages were assessed *in vitro*. For this aim, 3×10^4 cells were cultured in each well of 4-well tissue culture plates. Then, osteogenic and adipogenic induction medium was added to two wells, separately. Another wells were considered as controls. The differentiation of the cultured MSCs was monitored and after about 3 weeks, it was confirmed by Alizarin red-S and Oil red-O staining for osteogenic and adipogenic differentiation, respectively.

The surface antigenic profile of AD-MSCs was evaluated using a flow cytometer FACSCalibur (BD Biosciences, USA). Five antibodies against human-MSC Markers were used for phenotyping of AD-MSCs, including CD14, CD90, CD73, CD45 and CD105 (all from eBioscience).

2.3. Preparation of MSC-conditioned media and isolation of exosomes

After the culture of AD-MSCs, the medium was changed about twice a week until reaching about 80% confluence. Then, the cells were passaged. Approximately every 3 days, the medium of MSCs from passage 2, was replaced with the medium containing lower FBS. MSCs were gradually adapted to FBS-free medium. Finally, FBS-free supernatants were collected and filtered by 0.22 μm filters.

Exosomes were isolated from the collected supernatants according to the manufacturer's guidelines of Exocib kit (Cib Biotech Co.). Based on the protocol of this kit, first the reagent A of the kit was added to each tube in a ratio of 1:5 (1 reagent A: 5 MSC-CM). This reagent is a polymer that causes the precipitation of the exosomes. After mixing the tubes, they were incubated an overnight at 4 °C. Then, they were centrifuged at 1000*g* for 45 min. The upper layer was removed. Finally, the reagent B was added to exosome pellet for resuspending it. For evaluating the concentration of isolated exosomes, a bicinchoninic acid (BCA) protein assay kit (DNAbiotech Co.) was used.

2.4. Characterization of MSC-exosomes

Scanning electron microscopy (SEM) was performed to visualize the vesicles. For this aim, isolated exosomes were fixed in glutaraldehyde. Samples were coated with gold by sputter coating and images were taken by scanning electron microscope (SEM) (MIRA3 TESCAN) [16]. In addition to this, transmission electron microscopy (TEM) (Zeiss-EM10C) was done to confirm the shape of the isolated exosomes and before preparing the images, MSC-exosomes were fixed in paraformaldehyde and glutaraldehyde.

The isolated exosomes were characterized using 0.1 μm polystyrene beads to adjust the instrument voltages and then they were labeled with the anti-human antibodies, including anti-CD81 and anti-CD63 antibodies, for flow cytometric analysis (both antibodies were purchased from eBioscience). The analysis was carried out, using a FACSCalibur flow cytometer. To analyze the size distribution of the exosomes, they were diluted in PBS and Tween-20. The size of them was measured using dynamic light scattering (DLS) Zetasizer Nano ZS (Malvern Instruments, UK).

2.5. Neutrophil isolation

After collecting informed consents, fresh heparinized blood (10 U/

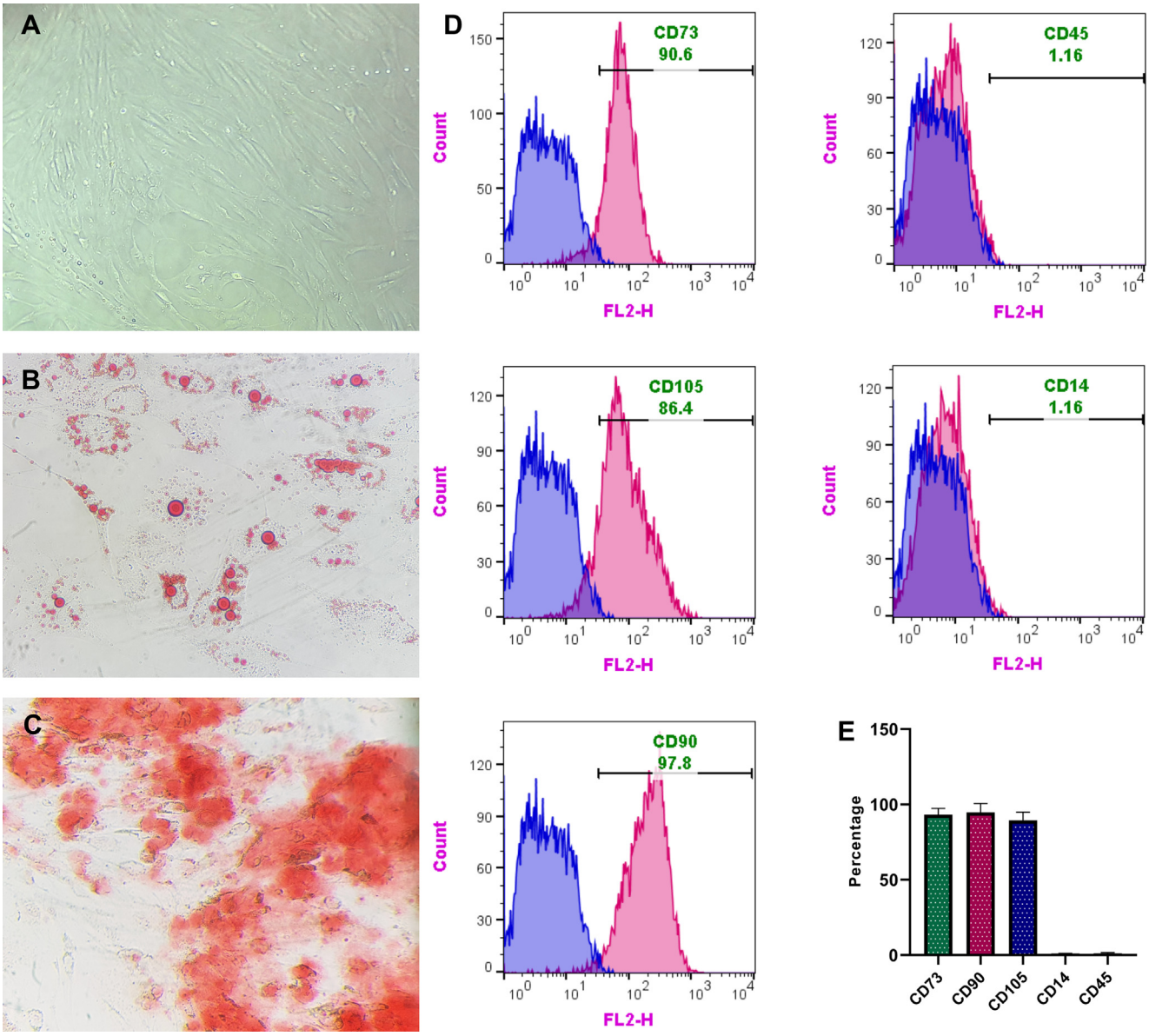

Fig. 2. *In vitro* characterization of AD-MSCs. (A) Fibroblast-like shape of AD-MSCs as evaluated by light microscopy. (B) Oil droplets that were stained with Oil red O to make sure that MSCs were differentiated into adipogenic lineages, and (C) calcium phosphate accumulation that was seen after Alizarin red S staining to be certain about the MSCs osteogenic differentiation. (D) The majority of MSCs were positive for surface markers including CD73, CD90, and CD105 and negative for CD14 and CD45 as determined by flow cytometry. (E) The bars indicate the percentage of MSCs that are positive for each surface marker. The figures show one representative result from three independent experiments.

ml heparin) was obtained from 5 SCN patients and 5 healthy donors. First, an equal volume of 3% dextran was added to each blood. After about 45 min, the upper layer was centrifuged at 400*g* for 30 min on a density gradient with Ficoll-Hypaque. Hypotonic lysis was performed and for preparing cell suspension, RPMI-1640 (Gibco, NY, USA) were added to isolated neutrophils. One droplet of cell suspension was put on the glass and Giemsa staining was done to evaluate the purity of the cells. Another droplet were mixed with trypan blue and microscopic analysis was performed to measure cell viability. After seeding the neutrophils in 96 well plates, some wells were treated with 100 μg/ml exosomes, some other wells were treated with CM at a ratio of 1:1. Finally the only medium was added to control group wells. The plates were incubated for 18 h.

2.6. PKH staining

One of the approaches to investigate the exosome uptake is labeling the isolated exosomes with a fluorescent dye which should be incorporated into the membrane of exosomes. So, isolated exosomes were stained with PKH67 dye in Diluent C (Sigma-Aldrich, Missouri, USA) and then blocked with exosome-depleted FBS, according to manufacturer's protocol. Neutrophils were treated with PKH67-stained exosomes to monitor the transfer of exosomes into neutrophils. After about 6 h of treatments, the cell suspension was put on a slide and the exosome uptake by these cells was observed by fluorescence microscopy.

2.7. Apoptosis assay

The apoptosis percentage of neutrophils was measured using annexin V-FITC apoptosis detection kit (eBioscience). Briefly, after washing the cells with PBS, they were labelled with FITC-conjugated annexin V and propidium iodide (PI) for 15 min at room temperature and analyzed by two-colour flow cytometry using a FACSCalibur flow cytometer.

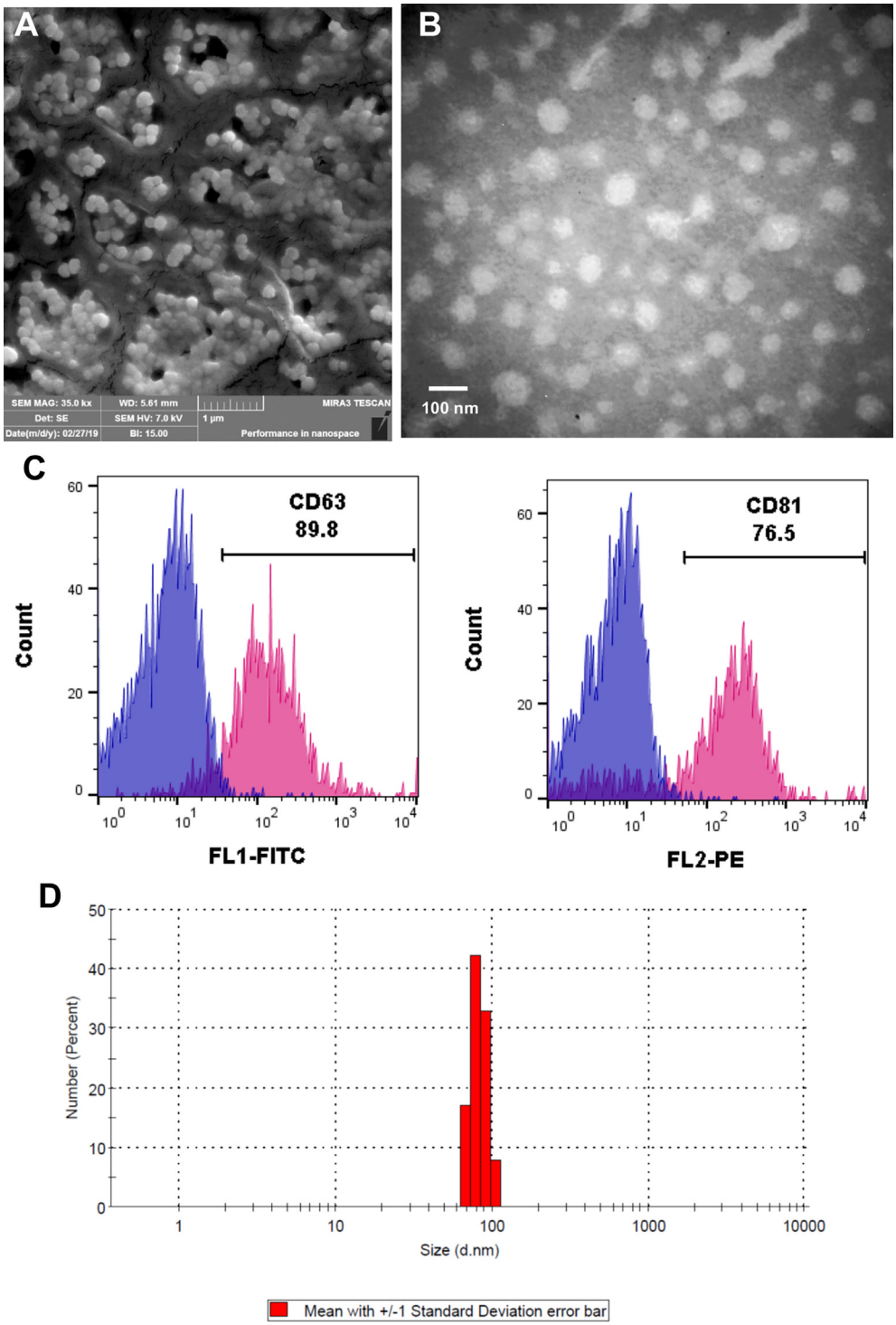

Fig. 3. Characterization of exosomes released from AD-MSCs. (A) The shape of MSC-exosomes was evaluated by scanning electron microscopy and (B) transmission electron microscopy. (C) Cell surface marker expression of isolated exosomes by flow cytometry including CD63 and CD81. (D) The size distribution of them was monitored by dynamic light scattering. The figures show one representative result from three independent experiments.

2.8. Colorimetric NBT assay

First NBT solution (Sigma-Aldrich, Missouri, USA) was freshly prepared. For this aim, 2.5 mg of NBT powder were mixed with 35 mg of bovine serum albumin (BSA) and this mixture was resuspended in 2 ml normal saline. Finally, 4 µg phorbol myristate acetate (PMA) was added to this solution. At the end of the treatment period, 100 µl of the final NBT solution was added to each well of the plate containing the treated or untreated groups. The cells were incubated for 40 min at 37 °C. The plates were centrifuged at 400*g* for 10 min. The formazans were dissolved by adding 2 M KOH (120 µl/well) followed by dimethyl sulphoxide (DMSO) (140 µl/well). Optical density (OD) of the blue solution was read on an ELISA reader at 630 nm [17].

2.9. Phagocytosis assay

After the treatment period, yeast particles that were previously killed by heat, suspended in RPMI-1640 and they were counted. Then,

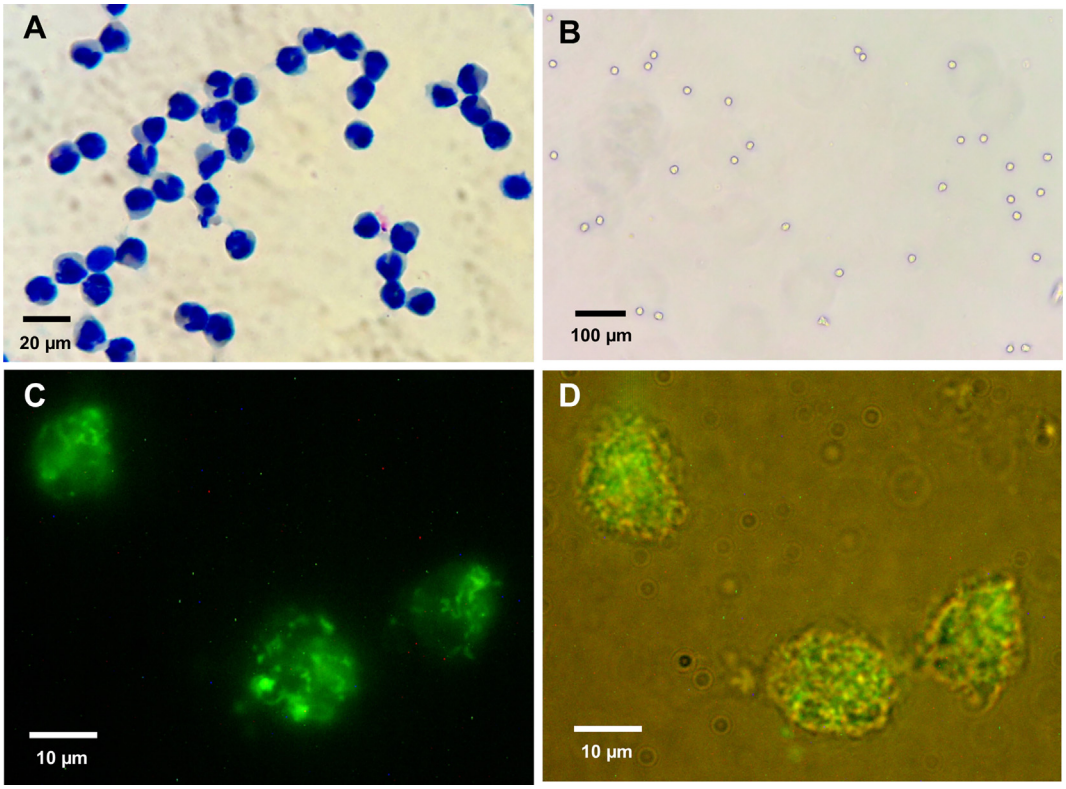

Fig. 4. The purity and viability of neutrophils, and exosomes uptake by neutrophils using PKH67 dye. (A) The purity of neutrophils was more than 95%, according to Giemsa staining. (B) Neutrophil viability was more than 97%, as determined by trypan blue dye exclusion. (C) Exosomes, purified from MSC-CM, were labeled with PKH67 dye and added to cultured neutrophils. PKH-positive neutrophils that were assessed by fluorescent microscopy, (D) Overlay of phase contrast and fluorescent microscopy. The figures show one representative result from three independent experiments.

this solution was added to the neutrophil suspension at a ratio of 1:10 and incubated at 37 °C. After 60 min, smears were prepared from the suspensions on the slides and left to dry. Methanol was added to smears to fix them. Finally, Giemsa staining was carried out and the smears were analyzed using a light microscopy. Three parameters were reported after analysis. First, the percentage of neutrophils that performed phagocytosis. Second, all yeasts that the analyzed neutrophils were phagocyted, divided into the number of neutrophils that performed phagocytosis, and the last parameter, that is called phagocytosis index (Phi), was obtained by multiplying the two mentioned parameters.

2.10. Statistical analysis

All experiments in this study were done at least in triplicate. The results of this report are expressed as means ± standard deviation (SD). Statistical analysis was performed using Graphpad Prism 8 software. For checking the normality of the distribution, Shapiro–Wilk test was used and analysis of variance (ANOVA) test was used to analyzed the statistical differences. P values < 0.05 were considered statistically significant.

3. Results

3.1. Characterization of AD-MSCs

Morphological analysis of MSCs by light microscopy indicated that they have a fibroblast-like shape (Fig. 2A). The differentiation capacity of AD-MSCs into adipocytes and osteocytes were assessed *in vitro*. After about 3 weeks, they differentiated into adipogenic (Fig. 2B) and osteogenic (Fig. 2C) lineages as monitored by Oil red O staining of oil droplets and Alizarin red S staining of calcium phosphate accumulation, respectively. The surface antigenic profile analysis of AD-MSCs identified that the most of the cells were positive for CD105, CD90, and CD73, as MSC-specific markers, and negative for CD14 and CD45 (Fig. 2D and E).

3.2. Characterization of MSC-derived exosomes

To confirm the shape of the exosomes, SEM and TEM were performed and visualized the vesicles (Fig. 3A and B). As shown in Fig. 3C, the isolated exosomes were positive for CD81 and CD63, as exosomes-specific markers, and the size distribution of them was determined using DLS (Fig. 3D).

3.3. Exosomes uptake by neutrophils

The purity and viability of neutrophils were more than 95%, according to Giemsa staining and trypan blue dye exclusion, respectively (Fig. 4A and B). Treatment of neutrophils with labeled exosomes was performed about 6 h. After that, the neutrophil suspension was washed with PBS to remove the exosomes that were not uptake by neutrophils. Then, the labeled-exosome uptake by neutrophils were observed by fluorescence microscopy (Fig. 4C and D).

3.4. MSC – exosomes reduced neutrophil apoptosis

As shown in Fig. 5A-G, in both SCN patients and healthy donors, only exosomes could significantly increase the percentage of viable neutrophils (SCN patients: 72.6 ± 1.75; healthy donors: 93.2 ± 0.4) ($P < 0.01$), but MSC-CM did not affect neutrophil lifespan, significantly. After the comparison of the results between neutrophils isolated from healthy donors and SCN patients, it was shown that, the percentage of lived cells in untreated and both of the treated groups,

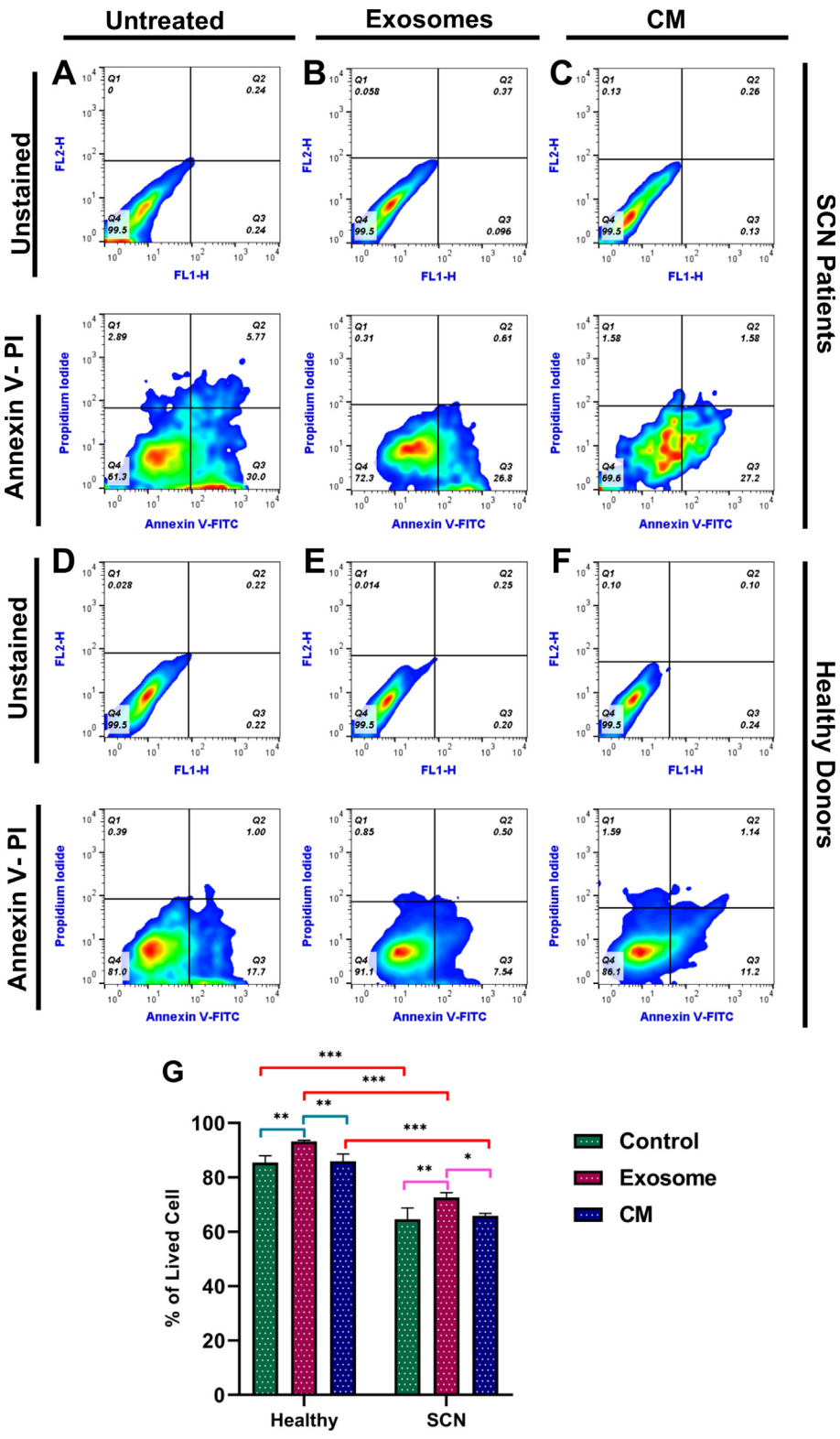

Fig. 5. Evaluation of neutrophil apoptosis in SCN patients and healthy donors by flow cytometry. (A, B & C) The upper panels are unstained and the lower panels are annexin V, PI-stained neutrophils of SCN patients. In the lower panels, the "annexin V and PI positive" quadrants show the population of late apoptotic cells and the "annexin V positive, PI negative" quadrants are related to cells in early stages of apoptosis. "Annexin V negative, PI positive" quadrants are associated with necrotic cells and "annexin V and PI negative" quadrants indicate viable cells. (A) Representative data of untreated group, (B) exosome-treated group and (C) CM-treated group of SCN patients is demonstrated. (D, E & F) The upper panels are unstained and the lower panels are annexin V, PI-stained neutrophils of healthy donors. (D) Representative data of untreated group, (E) exosome-treated group and (F) CM-treated group of healthy donors is indicated. (G) The left and right bars show the percentages of viable neutrophils in SCN patients and healthy donors, respectively. Data are the mean ± SD (n = 5) of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (two-way ANOVA).

were significantly higher in healthy donors (all $P < 0.001$).

3.5. MSC–exosomes and MSC-CM augment ROS production of neutrophils

As we expected, both CM and exosomes derived from MSCs could significantly enhance NBT reduction by neutrophils from SCN patients (exosome: 141.47 ± 0.24; CM: 119.26 ± 0.3) ($P < 0.001$), and healthy donors (exosome: 146.43 ± 4.27; CM: 112.32 ± 2.99) ($P < 0.05$) (Fig. 6).

3.6. MSC–CM increased neutrophil phagocytosis

As shown in Fig. 7, in SCN patients, CM treatment significantly increased phagocytosis percentage (41 ± 6.48; $P < 0.05$), mean number of yeasts ingested by neutrophils (3 ± 0.13; $P < 0.05$) and phagocytosis index (123.75 ± 22.55; $P < 0.01$). But exosome

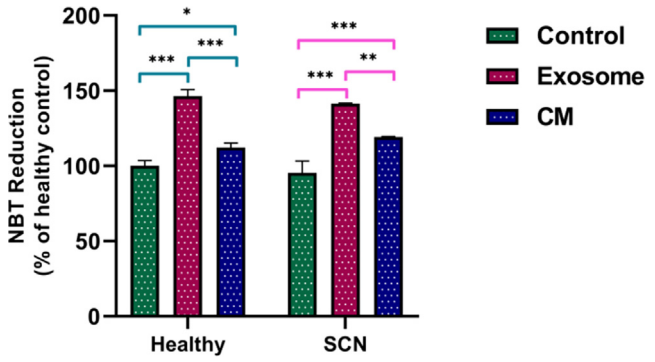

Fig. 6. NBT reduction by neutrophils in untreated, exosome-treated and CM-treated groups. The left and right bars show NBT reduction by neutrophils of SCN patients and healthy donors, respectively. Data are the mean ± SD (n = 5) of three independent experiments. *$P < 0.05$, **$P < 0.01$,***$P < 0.001$ (two-way ANOVA).

treatment only enhanced the average number of ingested yeasts (3.32 ± 0.36; $P < 0.05$), significantly. In healthy neutrophils, CM treatment significantly increased phagocytosis percentage (41.66 ± 7.23; $P < 0.05$) and phagocytosis index (118.66 ± 32.25; $P < 0.05$), but exosome treatment only augmented phagocytosis percentage of neutrophils (47 ± 5.29; $P < 0.01$), as compared with controls. After the comparison of the results between neutrophils isolated from healthy donors and SCN patients, it was recognized that in exosome-treated group, the "phagocytosis percentage" was significantly higher ($P < 0.05$), but "the mean number of yeasts ingested by neutrophils" was significantly lower in healthy neutrophils ($P < 0.05$).

4. Discussion

One of the common treatments for SCN patients is G-CSF therapy that has some supportive effects on neutrophils [5]. Nonetheless, high risk of MDS or AML is a major concern about the patients receiving G-CSF. So, other new strategies for improving the neutrophils in these patients may be beneficial. In the present study, we first explored the effects of MSC-exosomes and MSC-CM on neutrophils that were isolated from healthy donors and SCN patients. It was shown that both MSC mediators can augment the function and viability of their neutrophils. So, these mediators may be useful treatments for SCN patients.

It was observed that both treatments significantly augmented ROS production in SCN patients and healthy donors. In SCN patients, MSC-CM significantly enhanced phagocytosis percentage, the average number of ingested yeasts and Phi. But exosomes only increased the mean number of yeasts ingested by neutrophils, significantly. In healthy neutrophils, CM treatment significantly augmented phagocytosis percentage and Phi, but exosomes only improved phagocytosis percentage of neutrophils. About the lifespan of neutrophils, only exosome treatment could significantly increase the percentage of viable neutrophils

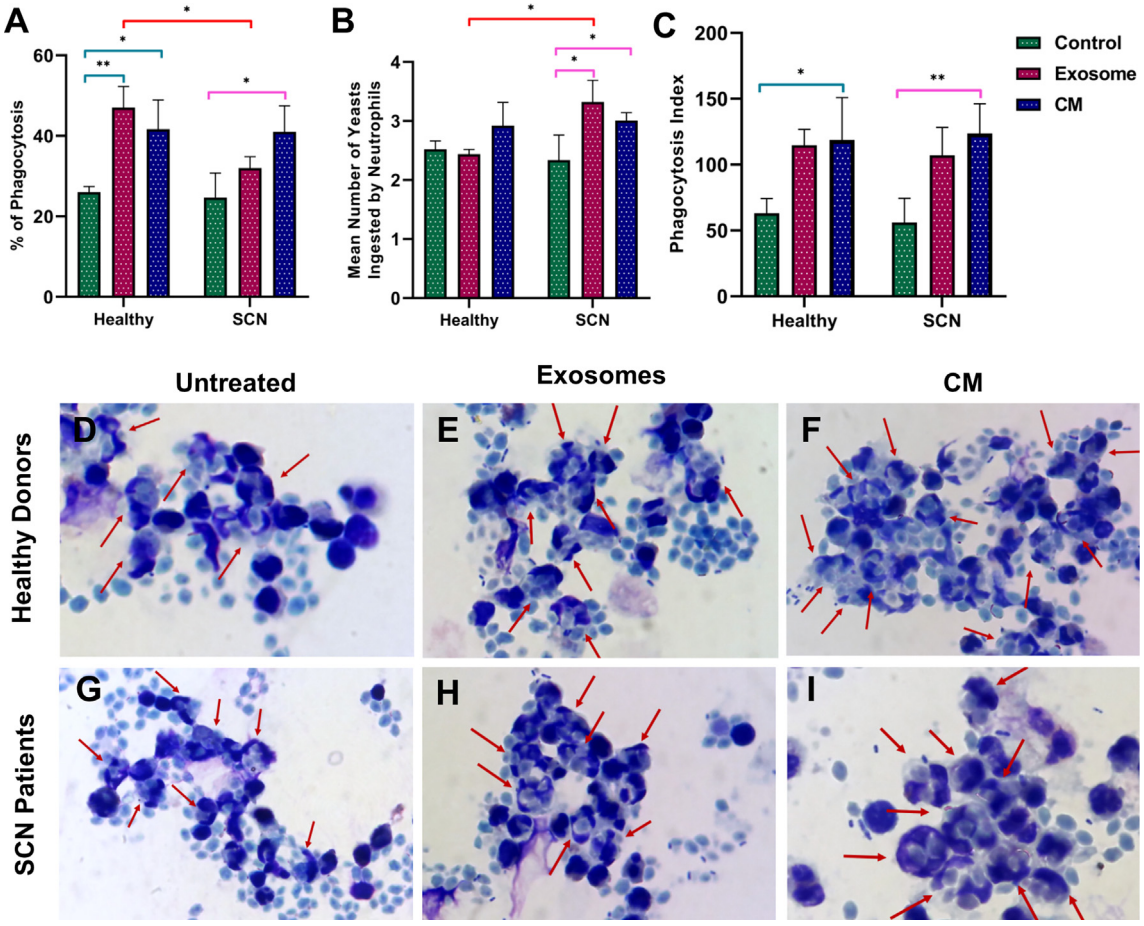

Fig. 7. Neutrophil phagocytosis assay. (A) The phagocytosis percentage of neutrophils, (B) The mean number of yeasts ingested by neutrophils, and (C) Phagocytosis index (Phi) of neutrophils from SCN patients (left bars) and healthy donors (right bars) is indicated. (D, E & F) Representative data for phagocytosis by (D) untreated neutrophils, (E) exosome-treated neutrophils, and (F) CM-treated neutrophils of healthy donors. (G, H & I) Representative data for phagocytosis by (G) untreated neutrophils, (H) exosome-treated neutrophils, and (I) CM-treated neutrophils of SCN patients. The arrows show the neutrophils that ingested the yeasts. Data are the mean ± SD (n = 5) of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (two-way ANOVA).

in both groups.

There are many factors that can account for the effects of MSC-exosomes or MSC-CM on neutrophils. For example, there are several mRNAs or miRNAs in exosomes that can cause cell survival or better function. In a study performed in 2017, the anti-apoptotic effects of MSC-exosomes on nucleus pulposus cell were investigated. They showed that MSC-exosomes contain high level of miR-21. This miRNA can mediate cell survival by targeting many pro-apoptotic genes such as programmed cell death protein 4 (PDCD4), Apaf-1, PTEN and DR-5. This report indicated that the main target of miR-21 is PTEN that affects cells by inhibiting the PI3K/Akt pathway. In addition to PI3K/Akt pathway activation, it was suggested that PTEN inhibition has also resulted in the activation of Bcl-2 and downregulation of Bad, caspase-3 and Bax molecules [18]. These roles of miR-21 were also reported in Ji wei Li et al. study that assessed the anti-apoptotic effects of miR-21 on pulmonary cell apoptosis by suppressing PDCD4 and PTEN [19].

It was recognized that exosomes that were isolated from amniotic fluid stem cells contain miR-10a. The miR-10a is targets Bim and inhibits apoptosis [20]. In addition to miRNAs, exosomes can transfer multiple mRNAs into recipient cells. For example, the mRNA of interleukin-6 (IL-6) might be transferred from MSC-exosomes into neutrophils and translated to IL-6 protein. The autocrine effect of IL-6 will result in neutrophil survival and better function [21].

Pre treatment or manipulation of the cell source of exosomes can affect the contents of secreted vesicles or CM. For instance, in Bin Yu et al. study, the exosomes derived from the GATA4-transduced MSCs were contained high levels of miR-19 that inhibits PTEN and BIM [22]. A study that performed in 2010, reported that LPS pre-treatment of MSCs caused inflammatory cytokine secretion by them and enhanced neutrophil lifespan [8]. So, providing an inflammatory environment containing LPS or poly I:C for MSCs might be beneficial for increasing the effects of MSC mediators on neutrophils [8,9,13].

MSC–CM also contains multiple factors such as several pro-inflammatory cytokines that can mediate the effects of MSC-CM on neutrophils [9]. This hypothesis can be confirmed by using tranwell system and antibodies against the candidate factors. Some studies performed these experiments to identify the main candidate factors and according to these reports IL-6 was the main mediator of MSCs paracrine effects [7–10,13,14].

Up to now, the related studies investigated the effects of MSCs or MSC-CM on different neutrophil sources. For example, in Imteyaz Khan et al. study, the sources of neutrophils were cord and adult blood. The neutrophils were co-cultured with Wharton's jelly-MSCs (WJ-MSCs). Then, apoptosis percentage and NADPH oxidase-1 (NOX-1) expression were measured. They reported that WJ-MSCs prolonged neutrophil lifespan and decreased gene expression of NOX-1 in both sources of neutrophils [11]. In another study, that published in 2012 the source of neutrophil was HL-60 cell. These neutrophils were co-cultured with AD-MSCs. Then, the function, viability and proliferation of cultured neutrophils were evaluated. They mentioned that this co-culture delayed the apoptosis of neutrophils up to 72 h and increased respiratory burst of them. The authors suggested that these effects of AD-MSCs on neutrophils may be due to the higher levels of some cytokines such as interferon-α, G-CSF and transforming growth factor-β in AD-MSCs [12]. Also, in a study that performed in 2011, it was reported that IL-6, interferon-β, and GM-CSF and endogenous soluble factors can be mediators of these protective effects [9]. In all of the mentioned studies, the neutrophils from healthy donors or cell line were used for experiments, but in our study neutrophils from both healthy donor and SCN patient groups were used and the results of these groups were compared. Moreover, all of the them explored the effects of MSCs or MSC-CM, but another aspect of novelty in our study was evaluating the effects of MSC-exosomes and even MSC-CM on neutrophils. In line with this study, we investigated the same experiments on neutrophils isolated from chronic granulomatous disease (CGD) patients and observed satisfactory results about the neutrophil phagocytosis and NBT reduction. In addition to this, we performed the same study with Wharton's jelly MSCs at different treatment periods and evaluated the effects of MSC mediators on neutrophils isolated from healthy donors. We recently published a study showing that the mediators of the AD-MSCs increased neutrophil survival and function after 12 h of treatment [23]. Some of the results of that study were different from the current one. It should be mentioned that all of the five healthy donors in this study was thoroughly independent and different from the prior publication (in terms of age and sex). In addition to this, the main difference between these projects, is the treatment period that was 12 h and 18 h for the prior and current studies, respectively. The treatment period can be the main factor that is responsible for the differences that were seen. For example, about the NBT reduction, different results were observed in the exosome-treated groups. In the current study, the exosome could significantly increase NBT reduction, but in prior study it couldn't. It might be related to the exosome cargo. Because the exosomes contain mRNAs and miRNAs that apply their effects in longer treatment periods in comparison with the proteins that exist in CM. In addition to this, the concentration of the PMA that was used for the neutrophil stimulation was different in two projects. It was 1 μg/ml for the prior and 2 μg/ml for the current manuscript. About the different concentrations, it should be expressed that, a related study showed that the neutrophils of SCN patients may have functional deficiencies [24]. So, in the current study it was decided to use a higher concentration of PMA for better observation of the differences between the groups.

Briefly, in this study after the comparison of exosome-treated and CM-treated groups with control groups, it was observed that both treatments had approximately better effects on neutrophil function and even lifespan. Between MSC-exosomes and MSC-CM, MSC-exosomes was better candidate for improving neutrophil lifespan.

In summary, we concluded that, mediators of MSCs have supportive effects on neutrophil function and lifespan in SCN patients and healthy donors and they can be attractive candidates for treating SCN patients. However, all of our experiments have to be confirmed in *in vivo* models and further studies are needed to determine whether these results can be generalized to all SCN patients.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethical Committee of the Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.MSP.REC.1398.263).

Informed consent

Informed consent was obtained from all individual participants included in the study.

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.

Acknowledgments

The present article is financially supported by "Research Department of the School of Medicine Shahid Beheshti University of Medical Sciences" (Grant No. 14008).

References

[1] K. Welte, C. Zeidler, D.C. Dale, Severe congenital neutropenia, Semin. Hematol. 43 (2006) 189–195, https://doi.org/10.1053/j.seminhematol.2006.04.004.
[2] A.A. Schäffer, C. Klein, Genetic heterogeneity in severe congenital neutropenia:

how many aberrant pathways can kill a neutrophil? Curr. Opin. Allergy Clin. Immunol. 7 (2007) 481–494, https://doi.org/10.1097/ACI.0b013e3282f1d690.
[3] C. Klein, M. Grudzien, G. Appaswamy, M. Germeshausen, I. Sandrock, A.A. Schäffer, C. Rathinam, K. Boztug, B. Schwinzer, N. Rezaei, G. Bohn, M. Melin, G. Carlsson, B. Fadeel, N. Dahl, J. Palmblad, J.-I. Henter, C. Zeidler, B. Grimbacher, K. Welte, HAX1 deficiency causes autosomal recessive severe congenital neutropenia (Kostmann disease), Nat. Genet. 39 (2007) 86–92, https://doi.org/10.1038/ng1940.
[4] A.W. Segal, How neutrophils kill microbes, Annu. Rev. Immunol. 23 (2005) 197–223, https://doi.org/10.1146/annurev.immunol.23.021704.115653.
[5] R. Badolato, S. Fontana, L.D. Notarangelo, G. Savoldi, Congenital neutropenia: advances in diagnosis and treatment, Curr. Opin. Allergy Clin. Immunol. 4 (2004) 513–521 http://www.ncbi.nlm.nih.gov/pubmed/15640692 (accessed May 31, 2019).
[6] M. Horwitz, F.-Q. Li, D. Albani, Z. Duan, R.E. Person, K. Meade-White, K.F. Benson, Leukemia in severe congenital neutropenia: defective proteolysis suggests new pathways to malignancy and opportunities for therapy, Cancer Invest. 21 (2003) 579–587 http://www.ncbi.nlm.nih.gov/pubmed/14533448 (accessed May 31, 2019).
[7] S. Brandau, M. Jakob, K. Bruderek, F. Bootz, B. Giebel, S. Radtke, K. Mauel, M. Jäger, S.B. Flohé, S. Lang, Mesenchymal stem cells augment the anti-bacterial activity of neutrophil granulocytes, PLoS One 9 (2014) e106903, , https://doi.org/10.1371/journal.pone.0106903.
[8] S. Brandau, M. Jakob, H. Hemeda, K. Bruderek, S. Janeschik, F. Bootz, S. Lang, Tissue-resident mesenchymal stem cells attract peripheral blood neutrophils and enhance their inflammatory activity in response to microbial challenge, J. Leukoc. Biol. 88 (2010) 1005–1015, https://doi.org/10.1189/jlb.0410207.
[9] M.A. Cassatella, F. Mosna, A. Micheletti, V. Lisi, N. Tamassia, C. Cont, F. Calzetti, M. Pelletier, G. Pizzolo, M. Krampera, Toll-like receptor-3-activated human mesenchymal stromal cells significantly prolong the survival and function of neutrophils, Stem Cells 29 (2011) 1001–1011, https://doi.org/10.1002/stem.651.
[10] C.P. Chen, Y.Y. Chen, J.P. Huang, Y.H. Wu, The effect of conditioned medium derived from human placental multipotent mesenchymal stromal cells on neutrophils: possible implications for placental infection, Mol. Hum. Reprod. 20 (2014) 1117–1125, https://doi.org/10.1093/molehr/gau062.
[11] I. Khan, L. Zhang, M. Mohammed, F.E. Archer, J. Abukharmah, Z. Yuan, S. Saif Rizvi, M.G. Melek, A.B. Rabson, Y. Shi, B. Weinberger, A.M. Vetrano, Effects of Wharton's jelly-derived mesenchymal stem cells on neonatal neutrophils, J. Inflamm. Res. 8 (2014) 1, https://doi.org/10.2147/JIR.S71987.
[12] Y.S. Park, G.W. Lim, K.A. Cho, S.Y. Woo, M. Shin, E.S. Yoo, J. Chan Ra, K.H. Ryu, Improved viability and activity of neutrophils differentiated from HL-60 cells by co-culture with adipose tissue-derived mesenchymal stem cells, Biochem. Biophys. Res. Commun. 423 (2012) 19–25, https://doi.org/10.1016/j.bbrc.2012.05.049.
[13] L. Raffaghello, G. Bianchi, M. Bertolotto, F. Montecucco, A. Busca, F. Dallegri, L. Ottonello, V. Pistoia, Human mesenchymal stem cells inhibit neutrophil apoptosis: a model for neutrophil preservation in the bone marrow niche, Stem Cells 26 (2008) 151–162, https://doi.org/10.1634/stemcells.2007-0416.
[14] Q. Wang, G. Ding, X. Xu, Periodontal ligament stem cells regulate apoptosis of neutrophils, Open Med. 12 (2017) 19–23, https://doi.org/10.1515/med-2017-0004.
[15] M. Mahmoudi, M. Taghavi Farahabadi, S.M. Hashemi, Exosomes: Mediators of Immune Regulation, Immunoregulation. 1 (2019) 121–126. doi: 10.32598/immunoregulation.1.3.121.
[16] V. Sokolova, A.-K. Ludwig, S. Hornung, O. Rotan, P.A. Horn, M. Epple, B. Giebel, Characterisation of exosomes derived from human cells by nanoparticle tracking analysis and scanning electron microscopy, Colloids Surfaces B Biointerfaces 87 (2011) 146–150, https://doi.org/10.1016/j.colsurfb.2011.05.013.
[17] G.A.W. Rook, J. Steele, S. Umar, H.M. Dockrell, A simple method for the solubilisation of reduced NBT, and its use as a colorimetric assay for activation of human macrophages by γ-interferon, J. Immunol. Methods 82 (1985) 161–167, https://doi.org/10.1016/0022-1759(85)90235-2.
[18] X. Cheng, G. Zhang, L. Zhang, Y. Hu, K. Zhang, X. Sun, C. Zhao, H. Li, Y.M. Li, J. Zhao, Mesenchymal stem cells deliver exogenous miR-21 via exosomes to inhibit nucleus pulposus cell apoptosis and reduce intervertebral disc degeneration, J. Cell. Mol. Med. 22 (2018) 261–276, https://doi.org/10.1111/jcmm.13316.
[19] J. Wei Li, L. Wei, Z. Han, Z. Chen, Mesenchymal stromal cells-derived exosomes alleviate ischemia/reperfusion injury in mouse lung by transporting anti-apoptotic miR-21-5p, Eur. J. Pharmacol. (2019) 68–76, https://doi.org/10.1016/j.ejphar.2019.01.022.
[20] G.Y. Xiao, C.C. Cheng, Y.S. Chiang, W.T.K. Cheng, I.H. Liu, S.C. Wu, Exosomal miR-10a derived from amniotic fluid stem cells preserves ovarian follicles after chemotherapy, Sci. Rep. 6 (2016) 1–12, https://doi.org/10.1038/srep23120.
[21] S.G. Ericson, Y. Zhao, H. Gao, K.L. Miller, L.F. Gibson, J.P. Lynch, K.S. Landreth, Interleukin-6 production by human neutrophils after Fc-receptor cross-linking or exposure to granulocyte colony-stimulating factor, Blood 91 (1998) 2099–2107 http://www.ncbi.nlm.nih.gov/pubmed/9490696 (accessed May 31, 2019).
[22] B. Yu, H.W. Kim, M. Gong, J. Wang, R.W. Millard, Y. Wang, M. Ashraf, M. Xu, Exosomes secreted from GATA-4 overexpressing mesenchymal stem cells serve as a reservoir of anti-apoptotic microRNAs for cardioprotection, Int. J. Cardiol. 182 (2015) 349–360, https://doi.org/10.1016/j.ijcard.2014.12.043.
[23] M. Mahmoudi, M. Taghavi-Farahabadi, N. Rezaei, S.M. Hashemi, Comparison of the effects of adipose tissue mesenchymal stromal cell-derived exosomes with conditioned media on neutrophil function and apoptosis, Int. Immunopharmacol. 74 (2019) 105689, , https://doi.org/10.1016/j.intimp.2019.105689.
[24] M. Donini, S. Fontana, G. Savoldi, W. Vermi, L. Tassone, F. Gentili, E. Zenaro, D. Ferrari, L.D. Notarangelo, F. Porta, F. Facchetti, L.D. Notarangelo, S. Dusi, R. Badolato, G-CSF treatment of severe congenital neutropenia reverses neutropenia but does not correct the underlying functional deficiency of the neutrophil in defending against microorganisms, Blood 109 (2007) 4716–4723, https://doi.org/10.1182/blood-2006-09-045427.