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Unleashing the Impact of Exosomes Derived from Human Placental Mesenchymal Stem Cells (hPMSCs) on U-266 Myeloma Cell Line

Ayda Baghery Saghchy Khorasani[1], Mina Soufizomorrod[2], Davood Bashash[3]

[1]Department of Hematology, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[2]Department of Applied Cell Sciences, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[3]Department of Hematology and Blood Banking, School of Allied Medical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Corresponding Author: Mina Soufizomorrod, Department of Applied Cell Sciences, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
E-mail: M.soufi@modares.ac.ir

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ABSTRACT

Multiple myeloma (MM) is a malignancy of plasma cells, terminally differentiated B cells, with complications like hypercalcemia, renal failure, anemia, and bone disease, which are also known as CRAB criteria. MM develops from monoclonal gammopathy of unknown significance (MGUS), a pre-malignant plasma cell dyscrasia. Over some time, MGUS has the potential to progress into smoldering multiple myeloma (SMM), which can evolve into MM. MM rarely progresses into plasma cell leukemia (PCL), a condition in which malignant plasma cells no longer stay in the bone marrow niche and circulate in the peripheral blood. In MM, various soluble factors play important roles, and interleukin-6 has different vital roles.

Interleukin-6, an inflammatory cytokine, has significant roles in the growth, survival, angiogenesis, metastasis, and apoptosis resistance in MM. Interleukin-6 is produced and secreted by both autocrine from myeloma cells and paracrine from bone marrow stromal cells. To tackle MM, various therapeutic approaches were applied over many years, and according to the results, most patients with MM can respond well to first-line treatment. However, the majority of patients may relapse as conventional treatment may not be curative. So, there is an urgent need for novel cell-based and cell-free therapeutic strategies, such as mesenchymal stem cell-based therapies and their products to offer new therapeutic strategies for MM.

Materials and Methods: In the present study, we investigated the impacts of exosomes derived from human placental mesenchymal stem cells (hPMSCs) on apoptosis and interleukin-6 expression in a myeloma cell line, U-266, for the first time. hPMSCs were isolated from the human placenta and cultured in a DMEM medium. After characterizing the cells and acknowledging their identity, they underwent several passages and their supernatant was collected to harvest exosomes. The exosomes were isolated by ultracentrifugation and characterized by DLS and TEM, and their concentration was measured by BCA protein assay. U266 cells were treated with different concentrations of exosomes and then MTT and annexin/propidium iodide flow cytometry tests were performed to evaluate cell viability. Afterward, a real-time PCR test was performed to evaluate interleukin-6 gene expression.

Results: According to our findings, treatment of U-266 cells with hPMSCS-derived exosomes led to the preservation of myeloma cells without changes in their cell cycle. Surprisingly, treatments did not hinder the expression of interleukin-6 in the myeloma cells.

Conclusion: In MM patients, interleukin-6 pl
ays different roles, and it is a desirable target to design new therapeutic strategies. To evaluate the effects of new therapeutic strategies, we designed and performed our study to estimate the effects of cell-free therapeutic strategy. In the present study, the impacts of hPMSCS-derived exosomes on the viability of MM cells and interleukin-6 gene expression were evaluated. The results showed that hPMSCS-derived exosomes resulted in the perseverance of myeloma cells without changes in the cell cycle. Furthermore, the interleukin-6 gene expression level showed no significant change.

Keywords: Mesenchymal stem cells; Exosomes; Multiple myeloma; Interleukin-6

INTRODUCTION

Multiple myeloma (MM) is one of the most prevalent hematologic malignancies distinguished by the clonal proliferation of malignant plasma cells (PC), mainly in the bone marrow (BM), producing elevated levels of monoclonal immunoglobulin (Ig), known as M-protein. Several complications are reported to be prevalent in MM, including hypercalcemia, renal failure, anemia, and bone disease, which are also known as CRAB criteria[1,2].

Various successive stages distinguish in MM development. The initial stage is called monoclonal gammopathy of undetermined significance (MGUS), recognized by the presence of a low rate of plasma cell proliferation, low immunoglobulin burden, and no apparent symptoms[26,40]. These patients may develop MM at a rate of 1% per year at 20 years[25]. In some patients, an intermediate stage or smoldering multiple myeloma (SMM) is also identifiable with elevated levels of higher immunoglobulin (Ig) without symptoms[36]. The condition is called MM as long as the symptoms mentioned above happen. There are several treatment strategies to tackle MM, including corticosteroids, alkylating agents, anthracyclines, proteasome inhibitors (PIs), immunomodulatory drugs (IMIDs), histone deacetylase inhibitors (iHDACs), monoclonal antibodies (mAbs) and high-dose chemotherapy rescued by autologous stem cell transplantation (ASCT)[6]. Human interleukin (IL)-6 is a glycoprotein whose gene is located at chromosome 7p21 and consists of 5 exons and 4 introns, and it possesses differentiating and proliferating activities on normal B cells and myeloma cells, respectively[19,21,39]. While BM stromal cells are the primary source of IL-6 in MM , it is well known that several myeloma cells produce their IL-6, as well[22,39]. The signaling cascade of IL-6 contributes to the activation of STAT3, which regulates the transcription of proteins relating to the Bcl-2 family leading to the protection of cells from apoptotic cell death. The IL-6 signaling pathway also promotes proliferation, survival, drug resistance, and migration of MM cells, thereby facilitating disease progression[5,34]. As a result, targeting this cytokine may serve as an effective therapeutic strategy in this malignancy. Noteworthy, one of the primary goals of targeted anticancer therapeutic strategies is to eradicate the cancerous cells by activating the apoptotic cascades and induction of apoptosis. While Fas (CD95) is a member of the tumor necrosis factor (TNF) receptor superfamily capable of triggering an extrinsic apoptotic pathway[11], several cancer cells, including MM cells, acquire resistance and can escape Fas-ligand (FasL)/Fas-mediated apoptosis[27]. Consequently, counteracting tumor escape and induction of Fas-mediated apoptosis can potentiate the vulnerability of MM to therapy.

Mesenchymal stem cells (MSCs) are multipotent progenitor cells possessing self-renewal, differentiation, and immune regulation capabilities. These cells can be obtained from various sources, including bone marrow (BM), umbilical cord blood (UCB), fat tissue, and placenta[12]. Human placenta-derived mesenchymal stem cells (hPMSCs) are multipotent progenitor cells capable of differentiating into several cell lineages. Interestingly, hPMSCs can be isolated and expanded quickly in large quantities in vitro, and they display low immunogenicity owing to their fetal origin. Similar to BM-MSCs, hPMSCs express several surface markers, including CD29, CD44, CD73, CD90, CD105, and CD166; however, they do not express CD 106, CD34, CD45, and HLA-DR. Additionally, positive co-stimulator molecules such as CD80, CD83, CD86, and CD95 and negative co-stimulator molecules such as PD-1 and PD-L2 are not expressed by hPMSCs[13,14,41].

Nowadays, it is well-established that the therapeutic benefits of MSCs are majorly due to the production of extracellular vesicles (EVs) (14-16). EVs are lipid bilayer-closed structures secreted by almost all cell types which are categorized into three different categories compromised of exosomes (30–150 nm), microvesicles (150 nm to 1 μm), and apoptotic bodies (1–5 μm)[9]. Exosomes were initially regarded as cellular mechanisms to excrete cellular waste[18]; however, there are well-recognized to play significant roles in various physiological and pathological conditions, including tissue repair[43], stem cell maintenance[37], central nervous system (CNS) communication[30], cancer[32] and inflammation[8] which strengthening the importance of them.

Several articles evaluated the effects of hPMSCs in various contexts. Firstly, it was reported that hPMSCs could inhibit the proliferation of T lymphocytes as long as they express ample amounts of indolamine 2,3-dioxygenase (IDO) and immunomodulatory cytokines[28]. Secondly, it was reported that the conditioned medium derived from hPMSC could rescue neutrophils from apoptosis via the IL-6/STAT3 signaling cascade. Notably, pre-treatment of conditioned medium with LPS or IL-6 depletion curbed its survival effects on neutrophils[7]. Furthermore, the results from the first trial depicted that intravenous (IV) injection of MSCs derived from prenatal sources, placenta, and the umbilical cord was well tolerated and safe in patients. Besides, the cell therapy improved respiratory symptoms and reduced inflammatory conditions of critically ill COVID-19 patients[17]. In the present study, we hypothesize whether exosomes derived from hPMSCs could modulate both viability and IL-6 expression of the myeloma cell line, U-266, or not.

MATERIALS AND METHODS

Isolation and characterization of hPMSCs

Placentas delivered by ordinary pregnant women were collected immediately following delivery. As long as the placenta is considered medical waste, we did not require consent from the patients. Following the drainage of umbilical cord blood, the harvested pieces of the tissue were extensively washed with ice-cold phosphate-buffered saline (PBS), and then they were mechanically minced and enzymatically digested with 0.01% collagenase type II solution (Gibco; Thermo Fisher Scientific, Inc.) and incubated in 37 degrees Celsius with 5% CO2 for 60 minutes. Following the incubation, the Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (FBS) (DNA Biotech Inc., Iran) was added and centrifuged at 300 g for 15 minutes. Following the centrifugation, the cells were resuspended in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (DNA Biotech Inc.) and seeded in 75 cm2 flasks (SPL Life Sciences Co) and incubated in 37 degrees Celsius with 5% CO2. Every two or three days, the medium was replaced, and approximately 3 weeks later, some colonies consisting of fibroblast-like cells were observed. It is worth mentioning that MSCs after three passages were used in these experiments. The harvested cells were assessed for the expression of CD34, CD73, and CD90 to evaluate their immune phenotype and surface markers. To evaluate the differentiation capability of the cells, they were differentiated into both osteoblastic and adipocyte lineages. For osteoblastic differentiation, the hPMSCs were cultured for three weeks in osteoblastic differentiation media (Bioidea Com., Iran). Moreover, they were then stained with Alizarin (Bioidea Com., Iran). For adipocyte differentiation, the hPMSCs were cultured for two weeks in adipocyte differentiation media (Bioidea Com., Iran). Moreover, they were then stained with S Red Oil (Bioidea Com., Iran).

Isolation and characterization of exosomes

When the cultured hPMSCs reached 70-80% confluency, their medium was replaced with DMEM serum-free medium, and the cells were assessed for their health daily. Then, the culture medium was collected after 48 hours when the cells reached 85%–90% confluency with viability of more than 95%. The collected media were stored at minus 80°C and pooled together. The next step was the ultracentrifugation (UC) (Beckman 60 TI) of the supernatant at 100,000 g and 4°C. Then, the pelleted exosomes were resuspended in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) The exosome protein concentration was measured using the bicinchoninic acid (BCA) Assay Kit (Bio Basic Inc., Canada). The sample was analyzed via DLS to determine the diameter of the particles by the application of Microtrac (Microwave II) and PBS as blank. Then, the sample was examined by Transmission Electron Microscopy (TEM) (LEO 906, Germany) following the negative staining with uranyl acetate.

Preparation of U-266 cells for treatment with exosomes

The myeloma cell line, U-266, was purchased from the Iranian Biological Resource Center (IBRC™, Tehran, Iran) and seeded and cultured in 25 cm^2 flasks (SPL Life Sciences Co) with RPMI-1640 medium

supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

MTT assay

To evaluate the effect of hPMSCs-derived exosomes on the viability index of U-266 cells, a microculture tetrazolium assay (MTT) was performed. The U-266 cells (20000/well) were seeded in 96-well plates and incubated with the indicated concentrations of the agents for up to 24h and 48h. After removing the media, cells were further incubated with MTT solution (Sigma, USA) (5 mg/ml in PBS) at 37 °C for 3 h, and untreated cells were defined as the control group. The resulting formazan was solubilized with DMSO ((DNA Biotech Inc., Iran), and the absorption was measured at 570 nm in an enzyme-linked immunosorbent assay (ELISA) reader (Bio Tek).

Analysis of programmed cell death (apoptosis) by flow cytometry

U-266 cells were subjected to flow cytometry analysis to evaluate their death following the treatment with hPMSCs exosomes. The cells were harvested after 24 h and 48h treatment with the exosomes, washed with PBS, and re-suspended in a total volume of 100 µl of the incubation buffer. Annexin-V Flores (2 µl per sample) was added, cell suspensions were incubated for 20 min in the dark, and then the fluorescence was measured by flow cytometry. Annexin V-positive and PI-negative cells were considered to be in the early apoptotic phase, and cells having positive staining both for annexin-V and PI were deemed to undergo late apoptosis.

Analysis of DNA content by flow cytometry

The cells were treated with exosomes for 48h and their cellular DNA content was ascertained by flow cytometric analysis to assess the effect of hPMSCs-derived exosomes on the cell cycle progression of U-266 cells. Briefly, cells were collected, washed, and fixed with 70% ethanol. Next, to stain DNA and degrade RNA, propidium iodide (PI) and RNase were used, respectively. Finally, the DNA content of the cells was quantified from the peak analysis of flow cytometric histograms, and the data were interpreted using the Windows FlowJo V10 software.

RNA extraction and cDNA synthesis

Total RNA from U-266 cells was extracted after treatment with hPMSCs-derived exosomes for 24h and 48h by TRIzol (GeneAll, Seoul, Republic of Korea) according to the manufacturer procedure. The quantity of RNA samples was assessed spectrophotometrically using Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE). The total RNA was reverse transcribed to cDNA utilizing an easy cDNA Synthesis kit (Parstous Biotechnology, Iran). A 20 µl reaction was composed of the following components, including 10 µl 2X buffer mix, 2 µl enzyme mix, up to 20 µl DEPC-treated water, and 1-5 ng of total RNA. The components were all mixed and vortexed in an RNase-free tube. In order to synthesize the first strand of cDNA, the mixture, as mentioned earlier was incubated on an Eppendorf thermocycler according to the manufacturer protocol (25 °C × 10 min and 60 min at 47 °C × 60 min), and then the reaction was terminated at 85 °C × 5 min, and then chilled on the ice or at 4°C.

Gene expression analysis by quantitative real-time RT-PCR (qRT-PCR)

The real-time PCR was applied to evaluate the Changes in the mRNA expression level of the desired gene. For this purpose, the real-time PCR reaction mix was composed of 2 µl of cDNA, 7 µl of nuclease-free water, 0.5 µl of each sense and antisense primers (10 pmol), and 10 µl of 2X SYBR Green Master mix (Ampliqon, Denmark) in a final volume of 20 µl. The PCR reaction was performed according to the manufacturer protocol as follows: 95 °C × 4 min, (94 °C × 30 s, 57 °C × 30 s, 72 °C × 30 s) × 35 cycles, 72 °C × 5 min on Applied Biosystems. For the verification of the single PCR product of each primer, the melting curves were analyzed. Hypoxanthine phosphoribosyl transferase (HPRT) was amplified as an internal control, and fold change in expression of each target mRNA relative to HPRT was calculated based on a comparative on 2-ΔΔCt relative expression formula.

Statistical analysis

Data are presented as mean ± SD. Paired Student's t-test was used for statistical analysis. The probability

of null hypothesis < 0.05) was considered statistically significant.

RESULTS

Characterization of hPMSCs

hPMSCs possessed a spindle-shaped appearance with extensions in opposite directions from a small cell body. Immunophenotypic analysis was performed on hPMSCs between P3 and P5, which were derived from independent placenta samples. All the studied samples expressed CD73 and CD90 but did not express CD34. The differentiation results acknowledged that the isolated cells were capable of differentiating into specialized cell lineages such as osteoblasts and adipocytes. The Alizarin staining depicted the presence of calcium oxalates on the differentiated hPMSCs to osteoblasts, and intracellular lipid droplets staining using oil red- O proved the adipogenesis of hPMSCs. These observations were absent in the undifferentiated hPMSCs groups. All in all, these findings confirmed the characterization of cells as hPMSCs (Figure 1).

Characterization of hPMSCs- derived exosomes

Exosomes derived from conditioned media (CM) of hPMSCs were isolated by ultracentrifugation. We measured the amount of protein content of the exosome sample by BCA assay, and according to the results, the protein content was 800 µg/mL (Figure 2A). The DLS test was applied to evaluate the size distribution of the particles, and the results indicated the accepted size distribution according to the defined range (30-200nm) for the exosomes[15](Figure 2B). Furthermore, the morphology of exosomes was examined by TEM, and the presence of clearly structured cup-shaped particles of low electron density with a preserved membrane was acknowledged, the exosome is marked with the red circle (Figure 2C).

Insignificant changes in cell viability of the cells by hPMSCS-derived exosomes

While it was discussed before that the hPMSCs are known to express Fas-L and PDL-1 according to previous reports38. It is well-known that the exosomes possess the characteristics of their parental cells, surprisingly the results of the MTT assay depicted that treatment of U-266 cells with various concentrations of hPMSCs- derived exosomes (20,40, and 80 µg/mL) did not induce any significant differences in cell viability following both 24h and 48h in comparison to the control group (Fig 3A, B). By analyzing the results of annexin-V/PI staining, we also found that treatment of the U-266 cells with hPMSCs-derived exosomes did not lead to considerable changes in annexin-V/PI double-positive cells in the treated group as compared with the control group (Figure 3 C, D).

Inconsequential influence of hPMSCS-derived exosomes on DNA content by flow cytometric analysis

It was of great interest to evaluate whether the hPMSCs-derived exosomes were capable of inducing variations in the DNA content of the U-266 cells. In order to assess the impacts of exosomes on the DNA content of the cells, the cells were treated for 48h with exosomes of two different concentrations, and the results depicted no considerable changes in DNA content in the treated group as compared with the control group (Figure 4).

Insignificant changes in interleukin-6 gene expression level by (qRT-PCR)

Although it was reported that the hPMSCs were significantly capable of reducing the serum level of IL-6 in patients[17], treatment of U-266 cells capable of expressing and producing IL-6 with different concentrations of hPMSCs-derived exosomes (20, 40, and 80 µg/mL) for 24 h and 48h did not change the expression level of the IL-6 gene. The differences in gene expression results between treated groups and control were insignificant after incubation times and various concentrations of exosomes (Figure 5).

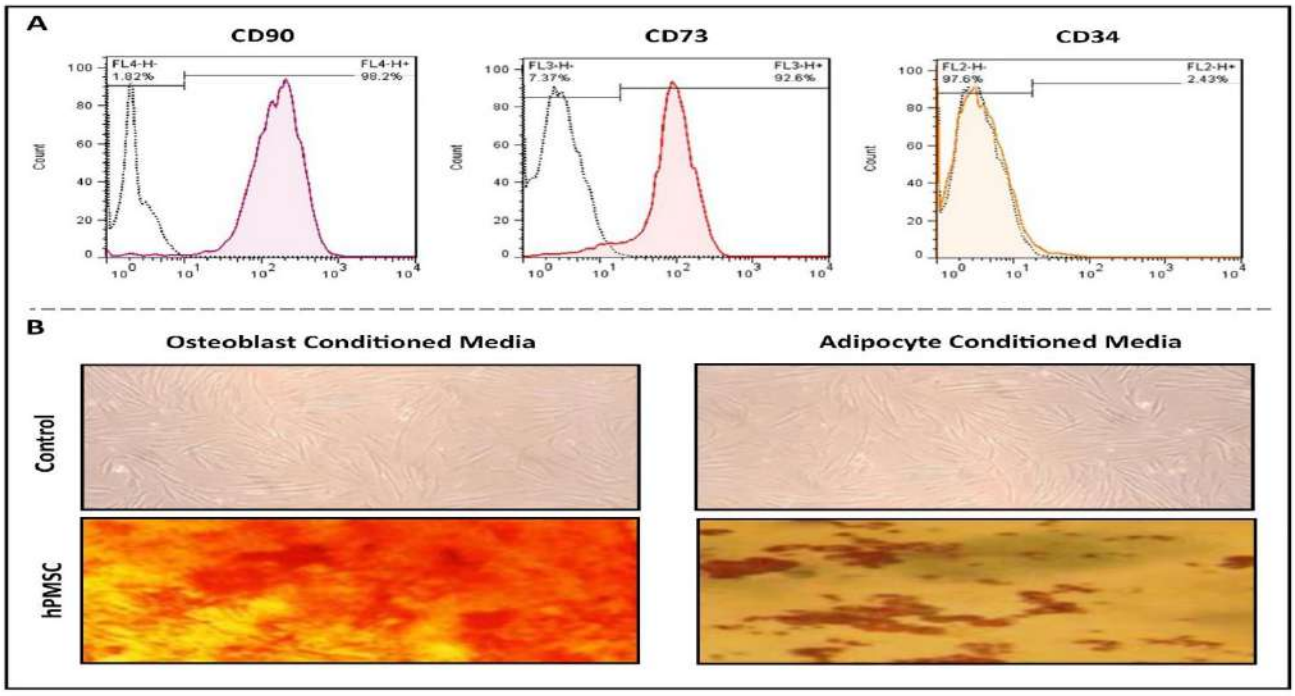

Figure 1. Immunophenotypic and differentiating characterization of hPMSCs. A, B) The expression of MSC-positive markers CD90 and CD73, respectively. C) The expression of MSC negative marker (CD34). Differentiation of hPMSCs to osteoblasts and adipocytes. D) The morphology of differentiated hPMSCs in osteoblast conditioned medium; Alizarin red depicting positive staining of differentiated cells and E) the negative control group for osteoblasts. F) Morphology of the differentiated hPMSCs in adipocyte conditioned medium; Oil red O depicting positive staining of adipogenic differentiated cells and G) the negative control group for adipocytes.

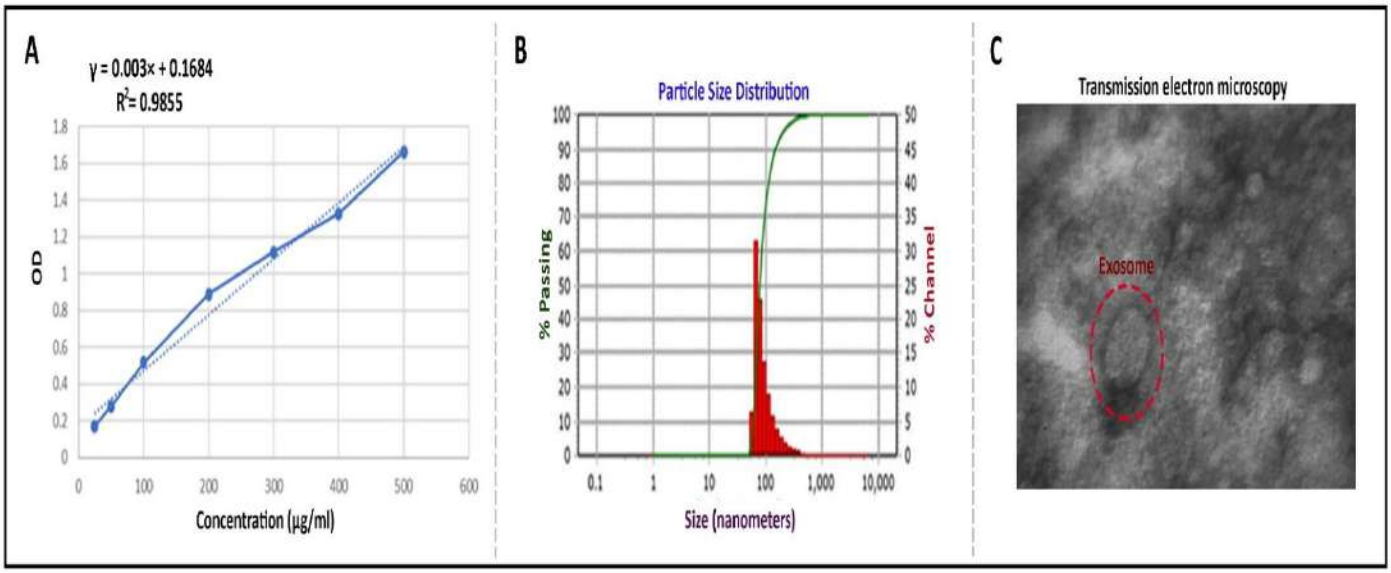

Figure 2. Characterization of hPMSCs- derived exosomes. **A)** BCA protein assay evaluates the protein concentration in the sample. **B)** DLS result depicts the size distribution of exosomes. **C)** Transmission electron microscopy images showing cup-shaped lipid-bilayer structure, marked with the red circle.

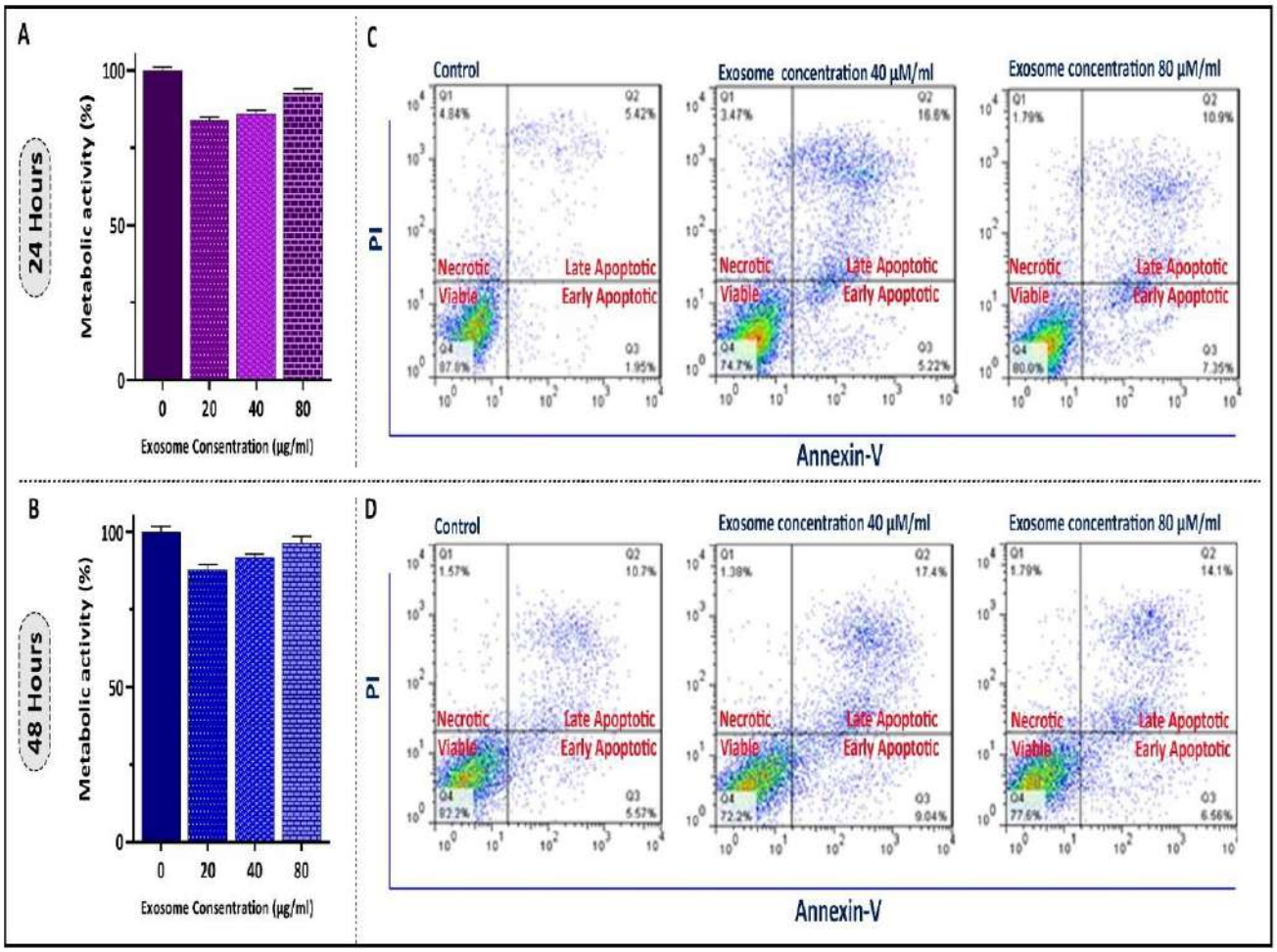

Figure 3. **A, B)** The results of the microculture tetrazolium assay revealed that hPMSCs- derived exosomes did not possess any significant effects on the metabolic activity of the U-266 cell line following 24h and 48h of treatment respectively. Values are given as mean ± standard deviation of three independent experiments. **C, D)** The results of the annexin-V/PI staining by flow cytometry acknowledged the results of the MTT assay and depicted that hPMSCs- derived exosomes did not impose significant apoptosis in the U-266 cell line following 24h and 48h treatment.

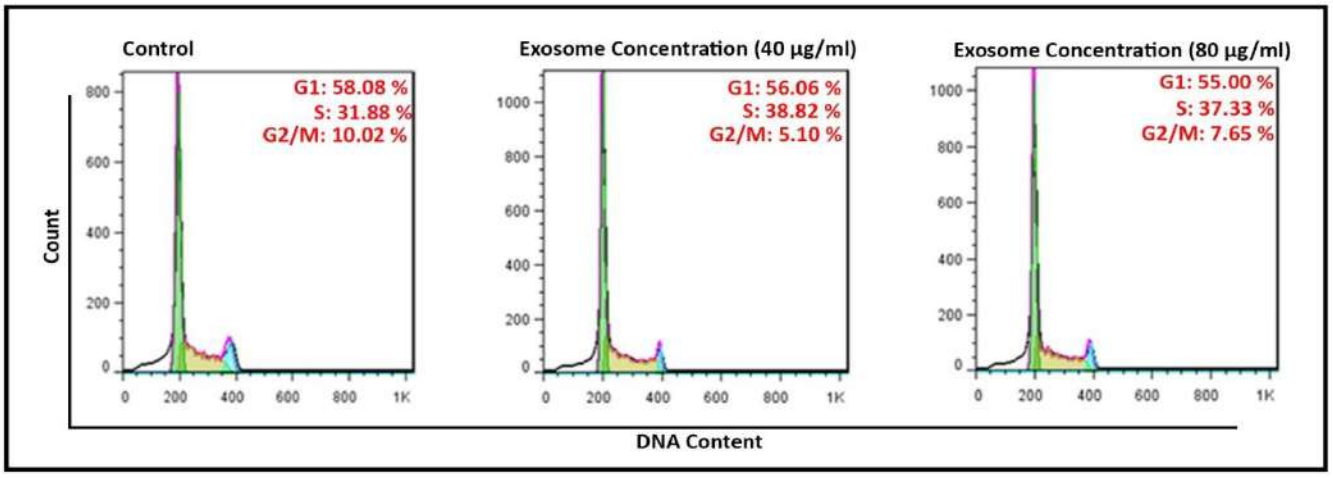

Figure 4. Percentage of cell populations in various phases of the cell cycle U-266 cells is plotted at different concentrations of exosomes following 48h treatment. We did not detect significant changes in cell cycle profiles in the cells following the treatment with 80 µg/ml **(B)** and 40 µg/ml **(C)** as compared to the control group **(A)**.

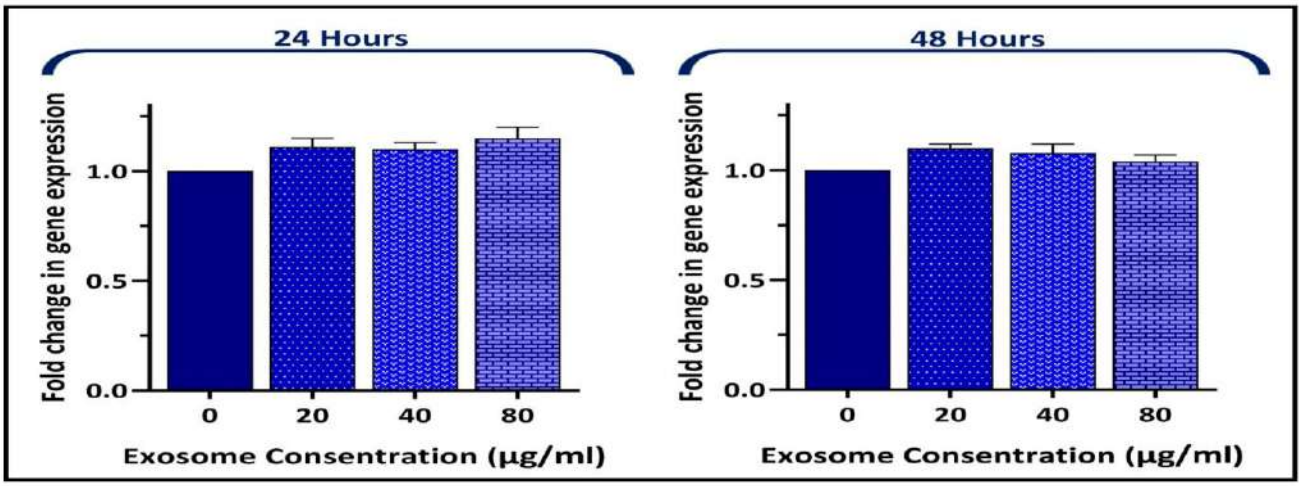

Figure 5. The expression levels of the IL-6 gene were examined using qRT-PCR after normalizing the cycle thresholds of each triplicate against their corresponding HPRT, and it is obvious that neither escalating levels of exosomes nor augmenting the time of treatment were not capable of regulating the gene expression in U-266 cells. Values are given as mean ± standard deviation of three independent experiments. *P ≤ 0.05 represents significant changes from untreated control.

DISCUSSION

MM is the second most prevalent hematologic malignancy diagnosed with the clonal proliferation of plasma cells in the BM as well as the presence of monoclonal proteins in blood and urine, anemia, bone lesions, and elevated calcium levels[23,35]. In the context of therapeutic strategies for MM, proteasome inhibitors and immunomodulatory drugs have been shown to be almost successful in improve the survival of the patients. However, MM is still an incurable disease[10].

Presently, ongoing therapeutic strategies based on the utilization of MSCs have drawn the attention owing to multipotentiality and low immunogenicity[12,20]. However, the uncontrollable growth of MSCs and unexpected differentiation may hamper their beneficial efficacy[24]. Fortunately, MSCs can exert their therapeutic functions via paracrine effects rather than cellular properties[3]. In the last decade, the secretory function of MSCs has been widely studied exhibiting that MSC-derived exosomes are superior to their parent cells because of several valuable characteristics such as their less complexity, smaller size, effortless production procedure, and immunogenic properties[16], which strengthen their application for therapeutic strategies in various diseases, especially cancer.

One of the rewarding strategies to tackle cancer is apoptosis induction in cancerous cells. Apoptosis induction has been under tight scrutiny from the early days of identification of the apoptosis process, and numerous attempts have been conducted to evaluate the efficacy of this pathway in different cells[44]. Although various anti-cancer drugs based on apoptosis induction were reported to be functional in different cells, cancer cells possess specific intelligence to surmount the impacts of such agents and counteract their fatality effects[29,42]. Furthermore, recognized anti-cancer drugs are not entirely safe and may threaten the patient's quality of life attributable to the various side effects[33]. In agreement with these statements, it is vital to discover agents with promising anti-cancer and immunomodulatory capabilities to surmount the present issue. Among all current therapeutic strategies, stem cell-based and exosome therapies are powerful tools to find novel anti-cancer treatments.

As stated, one of the most examined cells in the arena of cancer treatment are MSCs and their exosomes.

Among various subtypes of MSCs, several studies evaluated the different roles of hPMSCs in various aspects of therapeutic strategies, especially on apoptosis.

Accordingly, it was reported that hPMSCs were capable of expressing apoptosis-inducing molecules like Fas-L and TRAIL, which are considered apoptosis inducers in T cells. Still, surprisingly they inhibited apoptosis owing to the presence of other soluble factors or molecules[14]. Besides, in another study, it was revealed that the conditioned medium derived from hPMSCs could rescue the neutrophils from apoptosis and proposed the presence of other molecules in the conditioned medium of hPMSC which overshadowed the effects of the inhibitory molecules. Since the impact of such exosomes on myeloma cells and their apoptosis were not studied, we investigated the impacts of hPMSCs-derived exosomes on the apoptosis and cell cycle of U-266 cells in the present study. Surprisingly, our results revealed that different concentrations of the exosomes derived from hPMSCs were not capable of inducing apoptosis in U-266 cells. The MTT results revealed that the exosomes did not influence the viability of the cells. In addition to the MTT test, the Annexin/PI test did not depict any significant apoptosis rate in the treated cells with exosomes.

Another aspect of our study was to investigate the impact of such exosomes on the IL-6 gene expression level. With this regard, several studies were conducted to evaluate the effects of hPMSCs or their conditioned medium on the expression or production of IL-6. To begin with, according to the results of the phase 1 clinical trial, IV injection of high-dose hMSCs, from both the placenta and umbilical cord satisfactorily reduced the serum levels of IL-6 in patients suffering from COVID-19[17]. In another survey, treatment with CM of hPMSCs considerably reduced lung fibrosis and the lung content of MCP-1, MIP-1a, TNF-a, TGF-b1, and IL-6 in bleomycin-injured mice[4]. In another study, IV infusion of PMSCs and BMMSCs reduced the number

of infiltrated inflammatory cells and the molecules they secrete like IL-1, TNF-a, and IL-6[31]. According to the studies mentioned earlier, we hypothesized a variation, specifically a decrease, in the gene expression level of IL-6 in U-266 cells following the treatment with exosomes at different concentrations and time points. In contrast to our hypothesis and previous reports, our qRT-PCR results showed that no expression change was evident in the expression of IL-6 in U-266 cells following exosome treatment. We propose further investigation to find the reasons such as other soluble factors or signaling molecules or even novel characteristics of such exosomes in the supernatant of hPMSCs or even U-266 cells that may cause such results.

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CONFLICT OF INTERESTS

The authors declared that there is no conflict of interests.

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