IJHOSCR

Original Article

International Journal of Hematology-Oncology and Stem Cell Research

Antileukemia Activity of Human Natural Killer Cell-Derived Nanomagic Bullets against Acute Myeloid Leukemia (AML)

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Received: 24, Feb, 2022
Accepted: 26, Apr, 2022

ABSTRACT
Background: Cancer is among the serious health problems of the medical world, for treatment of which severe treatments are used. However, the prognosis of cancer patients is still poor. The application of NK cell-derived exosomes (NK-Exo) is a new method for cancer immunotherapy. These nanoparticles with a size range of 30-120 nm are a small model of mother cells. In this study, the anti-tumor activity of NK-Exo and LAK-Exo (activated NK cell-derived exosome) against acute myeloid leukemia (AML) is investigated in vitro.
Materials and Methods: The MACS method was performed for the separation of NK cells from the buffy coats of healthy donors, and an EXOCIBE kit was used for the isolation of NK-Exo. After treating the KG-1 cell line with different doses of NK-Exo, MTT assay, and annexin V-PE were done to evaluate cell proliferation and apoptosis, respectively, and for confirmation of involved proteins, Real-Time PCR and western blotting were performed.
Results: Anti-tumor activity of NK-Exo and LAK-Exo was dose- and time-dependent. Their highest activities were observed following 48 hours of incubation with 50 µg/ml exosome ($p<0.0001$). However, this cytotoxic activity was also seen over a short period of time with low concentrations of NK-Exo ($p<0.05$) and LAK-Exo ($p<0.001$).The cytotoxic effect of LAK-Exo on target cells was significantly higher than NK-EXO. The induction of apoptosis by different pathways was time-point dependent. Total apoptosis was 34.56% and 51.6% after 48 hours of tumor cell coculture with 50µg/ml NK-Exo and LAK-Exo, respectively. Significant expression of *CASPASE3*, *P38,* and *CYTOCHROME C* genes was observed in the cells treated with 50 µg/ml NK-Exo and LAK-Exo.
Conclusion: Our study confirmed the antileukemia activity of NK-Exo against AML tumor cells in vitro. Therefore, NK-Exo can be considered as a promising and effective treatment for leukemia therapy.

Keywords: Natural Killer Cell; Exosomes; Leukemia; Immunotherapy

INTRODUCTION

Cancer is a malignant disorder that leads to the death of many people all over the world each year[1]. Lung, breast, colorectal, and prostate cancers are the most frequent cancers among both sexes with the highest mortality rates among various types of cancer. Leukemia is one of the most common causes of mortality due to cancer in the world[1-3]. Many

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inherited and acquired factors are involved in the development of this disease. Chemical, physical and biological factors are known as the etiological agents of tumorigenesis. However, the main cause of cancer pathogenesis is genetic cell damages such as inactivation of tumor suppressor genes, mutations, impaired gene expression, which result in uncontrolled proliferation of tumor cells[4-7].

Several efforts have been dedicated to control this global problem over the past 50 years using different methods such as cytotoxic drugs, small molecule inhibitors and targeted antibodies. However, there are still several gaps in the treatment of cancers, and there are limitations in the application of common methods for treating cancers. Patients' quality of life along with complete treatment of the disease is among the important factors in selection of relevant therapy. Chemotherapy agents lead to various side effects in the patients, including cardiovascular complications (like ischemia, thromboembolism, etc.) or damage to central nervous system (CNS) . Damage of normal tissues by radiotherapy and failure of surgery in metastatic stages of cancer are other problems associated with the application of conventional treatments[7-10]. In addition, prevailing conditions of tumor microenvironment (TME) such as hypoxia, acidic pH, nutrient deficiency and necrosis that are created in most solid tumors contribute to further development of tumor cells as well as their resistance to treatment[11]. Immunotherapy is a new and promising method for tumor therapy that reduces many side effects of conventional treatments using patient's immune system. Identification of tumor-specific or tumor-associated antigens by the immune system is the main factor in successful management of the tumor. The first reported case of immunotherapy was performed with high-dose interleukin 2 (IL-2) for the treatment of advanced melanoma and renal cancer, which led to long-term and complete response (CR) in the patients (10). Nowadays, NK cells are used for tumor immunotherapy in different forms like autologous, allogeneic or genetic engineered cells[12].

NK cells are BM-derived large granular lymphocytes and a subset of innate lymphoid cells (ILCs).These cells can combat infectious and tumor cells through a variety pathways such as assessing the amount of major histocompatibility complex (MHC) class I proteins on the surface of tumor cells[13-15]. Unlike T-cells , activation of NK cells is not dependent upon antigen presentation by dendritic cells (DCs) and MHC I molecules[14]. Activation of NK cells requires a number of pro-inflammatory cytokines such as IL-15, IL-12, IL-21, IL-18, INF-α and β as well as a balance between activating and inhibitory receptors[16]. NK cell activating receptors include DNAX accessory molecule-1 (DNAM-1), C-type lectin receptors (NKG2D,NKG2C, NKG2F and NKG2E/H), cytotoxic receptors (NCRs- NKp30, NKp44 and (NKp46), killer cell immunoglobulin-like receptors (KIRs- KIR-2DS and KIR-3DS), and some of their inhibitory receptors are KIR-2DL, KIR-3DL and NKG2A/B[14,17]. Most peripheral blood NK cells, which are CD56dim/CD16$^+$/NKP46$^+$, show high cytotoxicity against tumor cells [12, 18, 19]. However, there are many problems in the application of NK cells, including low pH that is one of the most noticeable factors in the tumor microenvironment impairing the function of immune cells such as NK cells. The disruption of NK cell responses can be observed in the form of perforin, granzyme and Nkp46 reduction along with prevented upregulation of nuclear factor of activated T-cell (NFAT), reduction of metabolic activity and apoptosis of NK cells by different mechanisms such as stimulation of poly ADP ribose polymerase-1 (PARP) dependent cell apoptosis through reactive oxygen species (ROS) production by malignant monocytes. A characteristic feature of peripheral blood NK cells is the reduction of NKp46, NKp30 and DNAM-1, which are activating receptors for these cells [20-23].

There are different types of extracellular vesicles such as apoptotic bodies, microvesicles and exosomes. Exosome secretion is a new way for communication between different cells. The size range of exosomes is 30-120 nm with a wide variety of contents. In fact, the contents of exosomes are similar to their donor cells. All cells secrete exosome under physiological and pathological conditions [24-26]. Due to the unique structure and function of exosomes, these nanovesicles are highly attractive for the treatment of various diseases, including cancer [26, 27].

Acute myeloid leukemia is a disorder of leukemogenesis, which has been described with increasing number of myeloid blasts in bone marrow (BM) and peripheral blood (PB). One of the main treatments for this disease is chemotherapy; however, resistance to these drugs in most patients leads to the failure in treatment and decreases life expectancy[22]. Various therapies have been developed in recent years for AML, including stem-cell transplantation (SCT), mutation-specific targeted treatments such as fMS-like tyrosine kinase 3 (*FLT3*) (midostaurin), inhibitors of isocitrate dehydrogenase 1 (*IDH1*) (ivosidenib) and *IDH2* (enasidenib), and immunotherapy. Nevertheless, no effective treatment has been found to be be used as the first line of therapy[22, 28-30]. As mentioned, immunotherapy has injected new hopes into the field of cancer therapy. Researchers have sought different methods of immunotherapy such as bi-specific T- cell engagers (BiTEs), chimeric antigen receptor (CAR) T-cells and immune checkpoint blockers (ICBs) to treat this disease with different success rates[31]. Several studies have investigated the effectiveness of NK cells with different methods for AML therapy [32-34]. On the other hand, the effectiveness of NK-Exo against various cancers has been demonstrated [35, 36]. However, few studies have been performed on cytotoxic effect of exosomes against leukemic blasts. Our main goal in the present study was to evaluate the cytotoxicity of these exosomes against AML tumor cells in vitro.

MATERIALS AND METHODS

Cell line and cell culture

Fifty milliliters of blood were taken from two healthy donors by Blood Transfusion Organization of Iran. This study was approved by ethics committee of Blood Transfusion Organization of Iran (IR.TMI.REC.1398.018), and the donors signed the informed consent form. Peripheral blood mononuclear cells (PBMCs) were extracted from separated buffy coats by Ficoll-Histopaque 1077 gradient. Then, NK cells were isolated from PBMCs by negative immunomagnetic selection and a panel of anti-CD4, anti-CD3, anti-CD15, anti-CD19, anti-CD36, anti-CD123, anti-CD14 and anti-CD235 mAbs (Mojosort, Biolegend, USA). To examine the purity of isolated cell population, NK cells were incubated with magnetic beads and were then subject to flow cytometry (BD FACSCalibur II, BD Biosciences, USA).

Cell viability was evaluated by trypan blue (Sigma Aldrich, Germany) assay. Isolated NK cells were cultured for one day in RPMI 1640 medium (Gibco, USA) supplemented with exosome-depleted fetal bovine serum (FBS). To obtain the activated NK cells (LAK cells), we cultured them with 500 IU/ml IL-2 (Promokine, Germany) in RPMI 1640 medium that was supplemented with 2%FBS for two days.

In this study, KG-1 (acute myeloid leukemia) cell line purchased from Pasteur Institute of Iran was used.KG-1 cell line was cultured in RPMI 1640 medium supplemented with 10% exosome-free FBS and 1% penicillin-streptomycin. The conditions of cell culture were same for all the cells (37° C and 5% CO_2).

Isolation of exosomes

Supernatants from culture of NK and LAK cells were centrifuged with different speeds to remove the cells and debris in accordance with the standard protocol of exosome separation. Briefly, cell culture medium was centrifuged for 5 minutes at 300 g, 20 min at 1200 g and finally 30 min at 10000 g at 4°C(37). The next step was exosome isolation using EXOCIBE kit (CibBiotech Co, Iran, LOT: 3603) as instructed by kit protocol. The isolated exosomes were pooled, aliquoted and stored at -80°C.

Scanning Electron Microscope (SEM), Transmission Electron Microscope (TEM) and Dynamic Light Scattering (DLS)

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were performed to determine the morphology of isolated exosomes based on what has been described in previous studies[37] by scanning (Zeiss, DSM-960A, Germany) and transmission (Zeiss, EM900, Germany) electron microscopes. We first re-suspended the isolated NK-Exo in 100 µl of 2% paraformaldehyde (PFA) to prepare the sample for examination by an electron microscope (EM). After fixing, 5 µl exosome suspension was placed on formvar-carbon coated EM grids. This step of membranes adsorption lasts 20 min at room temperature. Then, 100 µl phosphate buffered saline (PBS) was used to wash the grids.

Afterward, the grids were placed in 50 µl 1% glutaraldehyde and washed seven times for two minutes using 100 µl deionized water.

Dynamic light scattering (DLS) was used to investigate the size and distribution of purified exosomes by zeta potential and size analyzer (HORIBA SZ-100, Japan).1:1000 dilution was employed for DLS experiment.

Bradford assay using Coomassie Brilliant Blue G-250 stain was done to determine the protein content of exosomes and western blotting was used to confirm exosome markers such as CD63. All experiments were done in triplicate.

In vitro cytotoxicity assay

Firstly, we co-cultured ~8×10^3 target cells (KG-1 cell line) within the wells of 96-well plates in triplicate with different doses of isolated exosomes for 24 and 48 hours at 37°C. Then, cell proliferation was measured by MTT assay (Sigma, USA). Briefly, we added 100 µl exosome-free medium and 0.5 mg/ml MTT solution to each of the wells containing exosomes and cells, which were incubated for 4 hours. After removing the medium, we added 100 µl dimethyl sulfoxide to the wells and the changes in absorption were measured at 550 nm by a multi plate reader (Biotek, USA). The results were reported as percentage compared to control.

Apoptosis detection by flow cytometry

We used Annexin V-FITC (Sigma Aldrich, Germany) to confirm the cytotoxic effect of NK-Exo on tumor cells. In brief, the target cell line was incubated with 50 µg/ml NK-Exo for 48 h. Next, the cells were washed by PBS and were then centrifuged. A cell concentration of ~1×10^6 cell/ml was prepared using the binding buffer. Annexin V and 7ADD stains were added, mixed and incubated with the cells at the defined conditions (room temperature and darkness). Finally, the results were analyzed by flow cytometry device (BD FACSCalibur II, BD Biosciences, USA).

Western Blot Analysis

Firstly, PBS and phenylmethylsulfonyl fluoride (PMSF) anti-protease were used to suspend the cells after their co-culture with 50 µg/ml NK-Exo for 48h at the mentioned conditions. The following steps were solving the cell lysates in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transfection to polyvinylidene fluoride (PVDF) membranes (Amarsham, England). After blotting, the membranes were washed by Tris Buffered Saline with Tween® 20 (TBST) (Sigma Aldrich, Germany) and stained using Ponceau S. The next step was PVDF membranes blocking using TBST with 1% BSA (6-7 ml solution) at room temperature for 1h.To probe the blots, we used anti-CD63 (exosome marker), anti-caspase3, p38,phosphorylated extracellular signal-regulated kinase (p-ERK), pAKT, cleaved-caspase3, cleaved poly ADP ribose polymerase(PARP), HMG2, cytochrome c, PTEN and anti-GAPDH as primary antibodies for 24h at 4°C. After washing the membranes with TBST, incubation of them with antimouse-HRP (secondary antibody conjugate) was performed for 1h at room temperature. Finally, gel documentation system (Vilber, E-Box, France) was employed for the detection of proteins.

RNA Isolation, cDNA Synthesis

We extracted total RNA from the cell line that was treated with different doses of exosomes using RNeasy Plus Mini kit (QIAGEN, Hilden, Germany). We then determined the quantity of total RNA by NanoDrop device (Thermo Scientific, Wilmington, DE, USA) and its quality using gel electrophoresis. Finally, we used a kit (TaKaRa, Japan) to synthesize cDNAs.

Quantitative Real-Time PCR

To investigate the expression of genes involved in cell proliferation and apoptosis, we first designed their specific primers by Oligo v7.60 software. The sequences of designed primers have been shown in Table 1.

ABI StepOnePlus detection system (Applied Biosystems, Foster City, CA, USA) and Ampliqon SYBR Green PCR kit were used to perform quantitative Real-Time-PCR. The reaction mixture contained 2x Real-Time master mix, the equal mixture of forward and reverse primers, nuclease-free water, cDNA, and the reaction conditions are listed in Table 1. Fold changes were calculated as the relative expression of

genes and normalization of gene expression was performed using GAPDH threshold cycles.

Statistical analysis

All quantitative data were expressed as mean ± standard error using SPSS version 22 (SPSS, Inc., Chicago, IL, USA). Two-tailed student́s t-test, one-way ANOVA or Kruskal–Wallis tests were used to analyze the differences between groups. Statistical significance was considered at $p<0.05$.

A

Table 1 (A): The properties of primers used in this study

Gene	Product	Primer	TM
MAPK1(ERK)	221	F: CTGTGGTGCAGATGAGAAGC	58.91
		R: AGGCACCAACAGTACAAAGC	58.68
PTEN	162	F: CCAGTCAGAGGCGCTATGTG	60.53
		R:TCACCTTTAGCTGGCAGACC	59.68
MAPK14(p38)	224	F:AACAGGATGCCAAGCCATGAG	60.96
		R: GGATCGGCCACTGGTTCATC	60.82
CASPASE3	172	F: AGAACTGGACTGTGGCATTGAG	60.55
		R: CAGCATGGCACAAAGCGAC	60.44
AKT1(AKT)	137	F: TACGAGAAGAAGCTCAGCCC	59.18
		R: TCCACACACTCCATGCTGTC	59.96
HMG2(HMGB2)	81	F: ATGTCCTCGTACGCCTTCTTC	59.87
		R: CGCGAAATTGACGGAAGAGTC	59.94
CYTOCHROME C	110	F: TATTCAGATGCCACCTACATGC	58.26
		R: AGTGGCTACCACACTGGAC	58.94
GAPDH	187	F: GTTGCAACCGGGAAGGAAATG	60.34
		R: GCCCAATACGACCAAATCAGAG	59.39

B

Table 1 (B): Reaction conditions of Real-Time PCR (B)

The number of cycles	1 cycle	40 cycles	
Steps	Primary denaturation	Denaturation	Annealing/Extension
Time	10 minutes	15 seconds	60 seconds
Temperature	95°C	95°C	60°C

RESULTS

Characterization of NK-Exo

The purity of NK cells isolated by MACS was more than 94% CD56-positive and CD3-negative cells, and cell viability was more than 99% by trypan blue assay. DLS, SEM, TEM and western blotting were used to evaluate the successful isolation of released exosomes into supernatant medium. The spherical shape of exosomes and their complete membrane structure were evident in the obtained images from SEM and TEM (Figures 1A and B). TEM and DLS results showed an exosome size distribution <100 nm with the highest frequency at 37.3 nm (Figures 1B and C). The presence of exosome markers such as CD63 was confirmed by western blotting (Figure 1D). The obtained results from all of these methods showed that the separation of exosome from NK cells was completely correct and successful.

Cytotoxicity of NK-Exo against tumor cells

After isolation of NK-Exo, we co-cultured the target cell line with different concentrations of exosome at various time intervals and MTT assay was then performed. Proliferation in the cell line treated with NK-Exo was lower than control group ($p<0.05$) (Figures 2A and B).The cytotoxic effect of NK-Exo was dose- and time-dependent. Cell viability decreased with increasing concentration and incubation time of co-culture. The greatest reduction (20.9%±2.26) of cell viability occurred in the cells treated with 50 µg/ml NK-Exo at 48h incubation ($p<0.0001$) (Figure 2B).

Our results showed that the low concentration of NK-Exo in short time incubation was effective on hematological tumors ($p<0.05$) (Figure 2A).

Comparison of cytotoxic effects of NK-Exo and LAK-Exo

After activation of NK cells by IL-2 and isolation of their exosomes, co-culture of them at defined concentrations with KG-1 cell line was performed at 24 and 48 hour intervals. The MTT results showed a significant reduction in cell proliferation due to the low concentration of LAK-Exo (10µg/ml) at short time ($p<0.001$) (Figure 2C).

The greatest reduction (35.61%±1.43) occurred in the cells treated with 50 µg/ml LAK-Exo at 48h incubation ($p<0.0001$) (Figure 2D).

The difference in cell proliferation was significant between the two groups (resting and activated NK cells), which was dose- and time-dependent. Most important difference in proliferation was observed at 50 µg/ml exosomes after 48 hours of incubation ($p<0.01$) (Figure 2F).

The effect of NK-Exo and LAK-EXO on cell apoptosis

Annexin V-FITC was used to investigate the created cytotoxic effects of NK-Exo and LAK-Exo in stimulation of tumor cell lysis. Real-Time PCR and western blotting were performed to confirm the proteins involved in cell apoptosis. After cell staining and analysis by flow cytometry, apoptotic cells (FITC+/7ADD-), necrotic cells (FITC-/7ADD+) and live cells (FITC-/7ADD-) were determined. Our results showed the induction of target cells apoptosis by NK-Exo and LAK-Exo in vitro. However, the induced apoptosis was time-point dependent. The highest cell death was observed after 48h incubation (Figures 3B and C).

The percentage of $FITC^{+}/7ADD^{-}$ apoptotic cells in control (untreated KG-1 cell line) and KG-1 cells after treatment with 50µg/ml NK-Exo and LAK-Exo was 0.64%, 27.72% ($p<0.01$) and 37.86% ($p<0.001$), respectively (Figure 3). Total early and late apoptosis in the cells treated with 50µg/ml NK-Exo and LAK-Exo were 34.56% and 51.6%, respectively (Figures 3B and C).

Real-Time PCR and western blotting were performed to assess the levels of proteins involved in cell proliferation and apoptosis pathways (Figure 5). A slight decrease in the expression of *ERK*, *AKT* and *HMG2* genes was visible but this decrease was not significant ($p>0.05$) (Figure 4).

PTEN gene expression slightly increased at 50µg/ml NK-Exo, which was not significant. However, increased expression of *P38* gene was observed with two concentrations of NK-Exo (30 and 50 µg/ml); however, this increase was significant only at 50 µg/ml NK-Exo. Increased expression of *CASPASE3* gene was significant in all three concentrations of NK-Exo. The highest level (7.46 fold) of its expression was at 50 µg/ml NK-Exo ($p<0.001$). Finally, increased

expression of *CYTOCHROME C* gene was visible and significant in all three concentrations of NK-Exo (p<0.05) (Figure 4A).

Considerable increase in the expression of genes involved in apoptosis like *CASPASE3* (16 fold, p<0.0001), *P38* (12.12 fold, p<0.0001), *CYTOCHROME C* (12.99 fold, p<0.001) was observed in the KG-1 cell line treated with 50 µg/ml LAK-Exo after 48h incubation. *PTEN* level increased and was significant with all doses of LAK-Exo but 4.92-fold increase at 50 µg/ml LAK-Exo dose was considerable (p<0.001) (Figure 4B). Therefore, NK cell activation is a good option for enhancing the cytotoxicity of NK-Exo against tumor cells.

We used anti-caspase3, cleaved-caspase3, p38 (MAPK signaling protein), cleaved PARP, HMG2, cytochrome c, p-AKT, p-ERK and PTEN for western blot to confirm the proteins involved in cell apoptosis mechanisms. In line with Real-Time PCR results, western blot analysis showed the increase in apoptotic proteins such as cleaved caspase3, cleaved PARP, and cytochrome C in tumor cells treated with 50 µg/ml NK-Exo. The level of all these proteins along with PTEN was significantly increased in tumor cells treated with 50 µg/ml LAK-Exo. Moreover, western blot analysis showed a noticeable increase of p38 protein expression in KG-1 cells that were treated with LAK-Exo (Figure 4).

The decrease in the level of p-ERK and p-AKT proteins was visible in western blot results with both NK-Exo and LAK-Exo. However, the level of HMG2 was not remarkably decreased in target cells co-cultured with NK-Exo and LAK-Exo (Figure 4C).

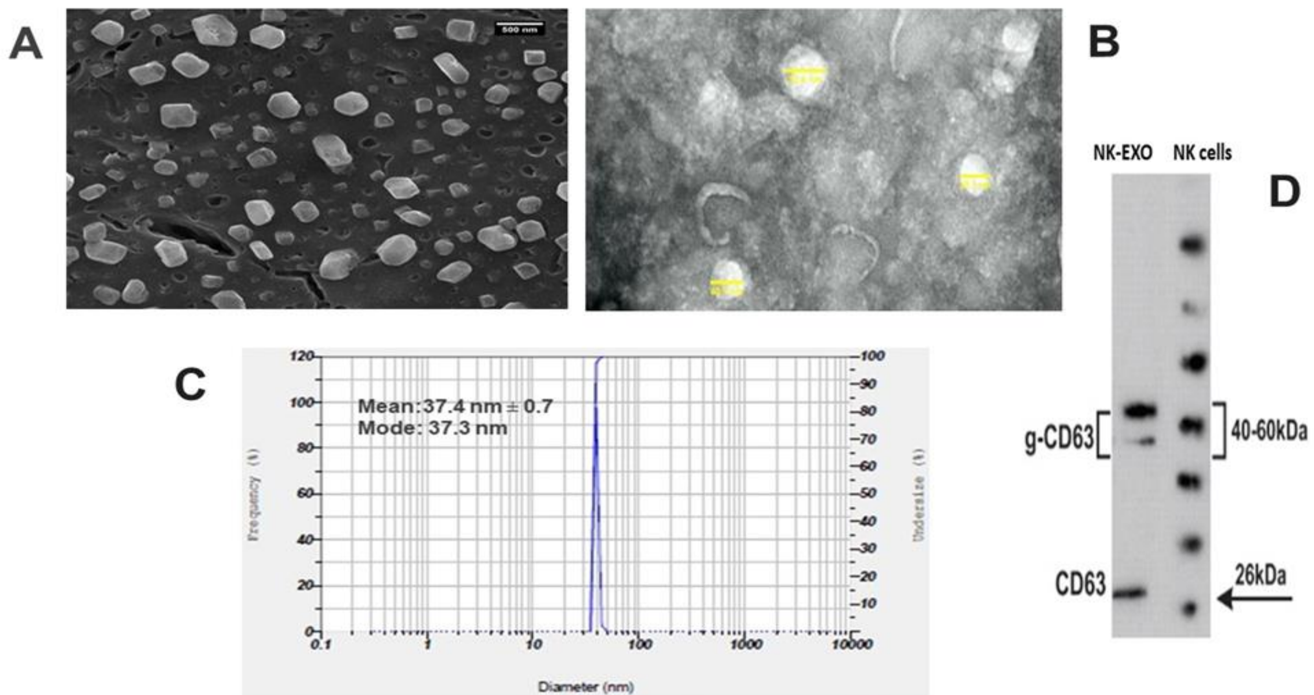

Figure 1: Characterization of Natural killer (NK)-Exo.
The investigation of NK-Exo morphology using Scanning Electron Microscope (SEM) (scale bar, 500 nm) (A) and Transmission electron microscope (TEM) (scale bar, 200 nm) (B).The size of NK-Exo was determined by Dynamic Light Scattering (DLS) (C).The confirmation of main exosome marker (CD63) using western blotting (D).

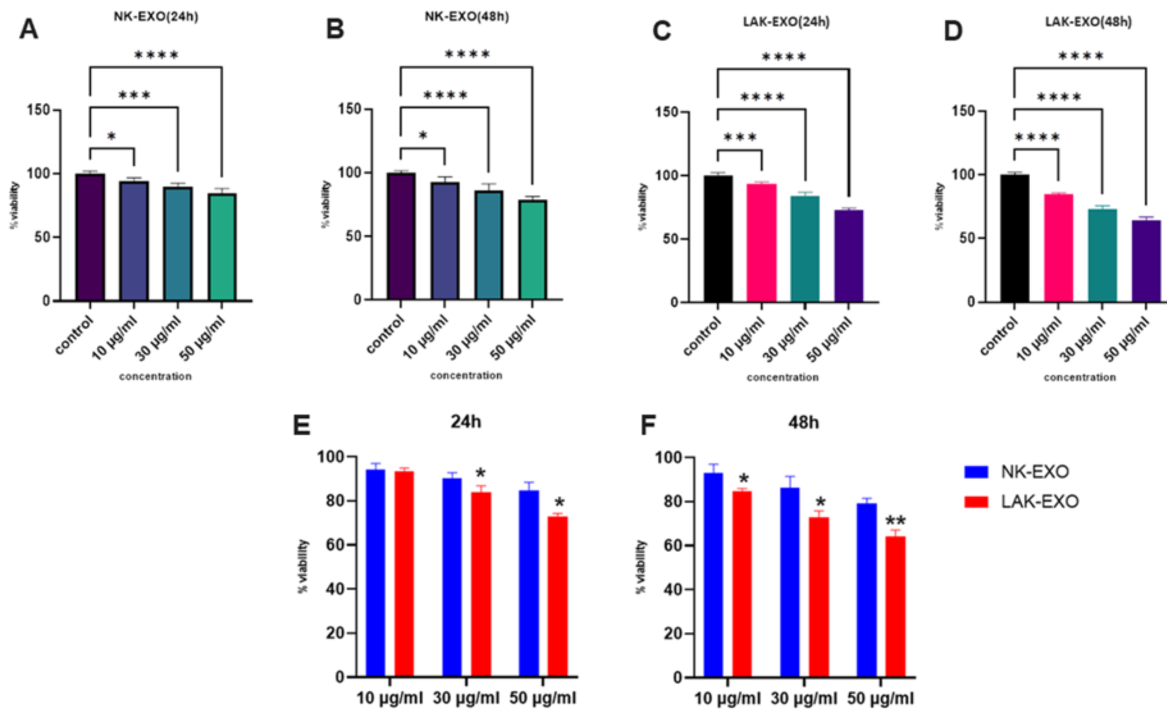

Figure 2: The cytotoxic effect of NK-Exo and LAK-Exo against KG-1 cell line. MTT assay was performed after co-culture of KG-1 cell line with three doses of NK-Exo and LAK-Exo at two time points. Treatment with NK-Exo for 24 and 48h incubation (A,B) and treatment with LAK-Exo for 24 and 48h incubation(C,D). The comparison of cytotoxic effects of NK-Exo and LAK-Exo at two time points 24h(E) and 48h (F).Graphs were plotted with collected data (mean±SE) from experiments in triplicate *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

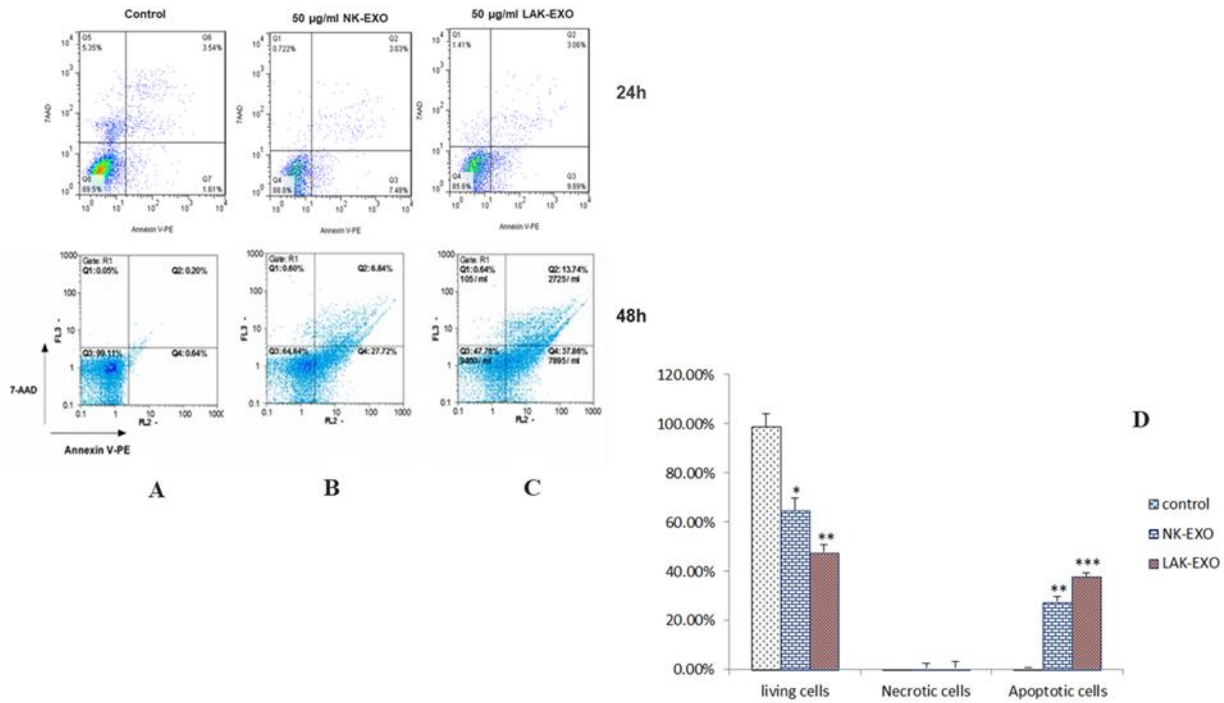

Figure 3: The effect of NK-Exo and LAK-Exo on programmed death of KG-1 cell line after 24 and 48 hours of treatment. Treated KG-1 cell line was stained using Annexin V /7-AAD and then flow cytometry was performed. (A) Untreated KG-1 cell line as control group (B) Treated KG-1 cell line with 50 µg/ml NK-Exo (C) Treated KG-1 cell line with 50 µg/ml LAK-Exo (D) Cell separation based on early and late apoptosis (48h).The graph was plotted with collected data (Mean ± SE) from experiments in triplicate *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

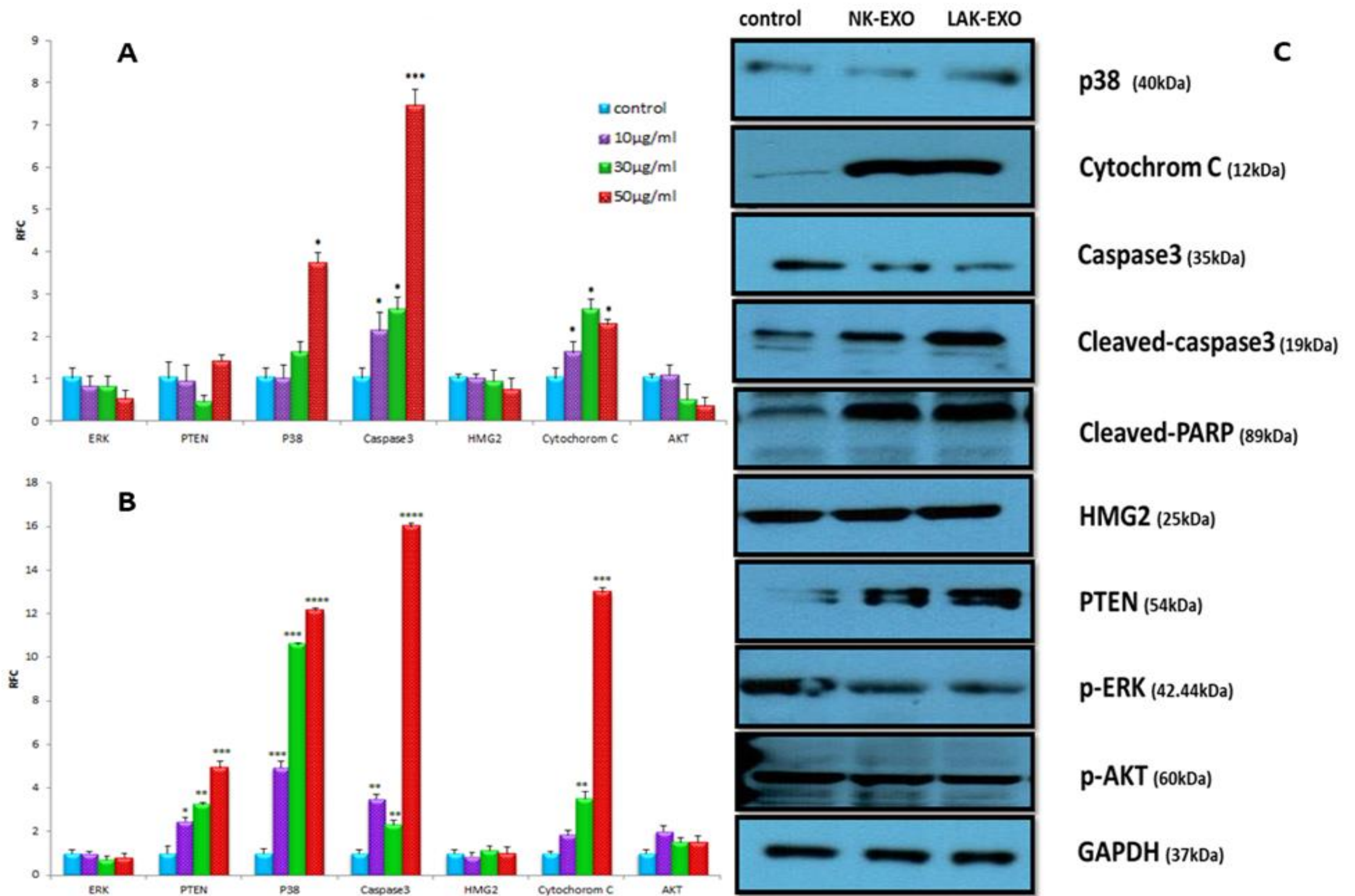

Figure 4: The expression of genes involved in cell proliferation and apoptosis was measured by Real-Time PCR after 48h incubation of KG-1 cell line with NK-Exo (A) and LAK-Exo (B). The investigation of proteins involved in apoptosis and proliferation of target cells treated with 50 µg/ml NK-Exo and LAK-Exo at 48h incubation by western blotting (C).Graphs were plotted with collected data (mean±SE) from experiments conducted in triplicate *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

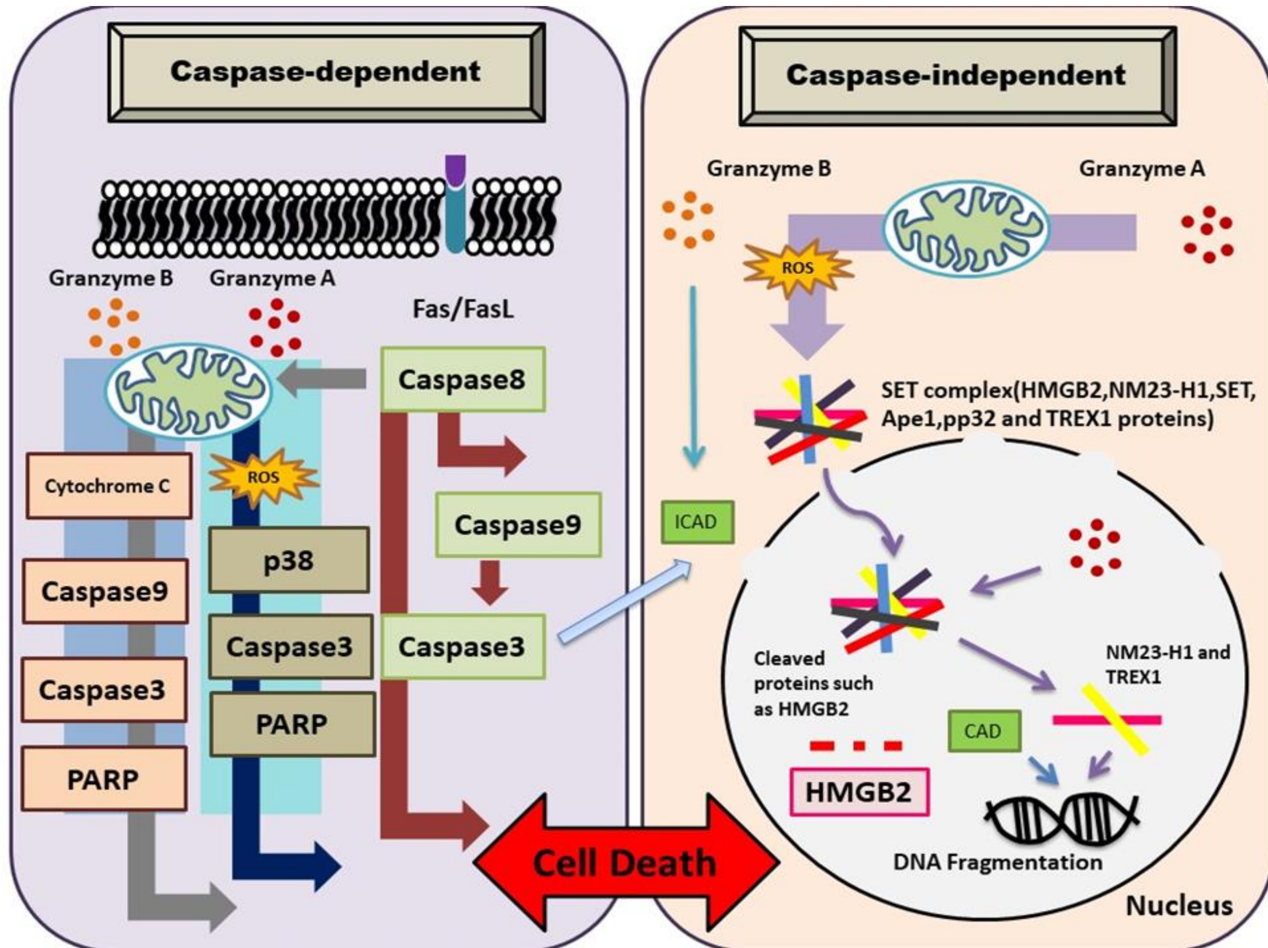

Figure 5: Mechanisms and signaling pathways involved in apoptosis of target cells[52, 53, 63, 68, 76-82].
Fas/FasL, granzyme A and B that exist in NK-Exo can cause death of tumor cells in caspase-dependent and independent pathways; HMGB2: high mobility globulin box2, PARP: Poly ADP ribose polymerase, ROS: Reactive oxygen species

DISCUSSION

NK cell therapy is used for the treatment of multiple malignancies such as neuroblastoma, lymphocytic leukemia, multiple myeloma, acute myeloid leukemia (AML), as well as colon, ovarian, renal, and gastric cancers. In a clinical study, haploidentical NK cells were used in combination with different immune suppressors (like cyclophosphamide and fludarabine) along with subcutaneous interleukin 2 (IL-2) in patients with acute myeloid leukemia (AML). Twenty-six percent of patients with poor prognosis showed complete improvement [38-42]. There are considerable problems in cancer treatment using NK cells such as difficult transfer of NK cells to the site of solid tumors, impaired cytotoxic activity of NK cells because of acidic conditions of tumor microenvironment[20-23,43]. Acidic pH causes accumulation and further infusion of exosomes with the membrane of tumor cells. Application of exosomes for immunotherapy is a novel method in tumor immunotherapy[44]. Exosomes are extracellular nanoparticles produced by a variety of cell types under different conditions, which are involved in many cellular processes such as immune responses as well as signal transduction, and their contents depend on the type of cell from which they are derived. For example, the exosomes derived from NK cells express CD63, CD81 and Rab5B as typical markers of exosomes as well as NKG2D, CD56, NKp46, NKp30 and NKp44 as NK cells markers. The higher expression of FasL and perforin as cytotoxic molecules in NK-Exo than NK cells has been reported in previous studies [35, 36, 45, 46]. Therefore, exosomes are appropriate selective agents for tumor immunotherapy.

The effectiveness of exosomes isolated from NK cell lines and peripheral blood NK cells against different tumor cells has been reported in various studies [36, 47]. Therefore, for further investigation of their cytotoxic effects, we used the exosomes derived from NK cells that were isolated from blood of healthy donors as well as from a hematological cell line, namely KG-1 acute myeloid leukemia cell line. Afterward, we studied the tumor cells sensitivity to NK-Exo with different methods.

Our data showed that the size of NK-Exo was <100 nm and that their membrane structure was intact along with positive CD63 as exosome marker (Figure 1), which has been introduced as a typical marker of exosomes [35, 46].

In line with previous studies, the sensitivity of hematological tumor cells to NK-Exo was one of the important results of our research[35,46,48]. The dependence of this effect on time and dose was obvious in our study as well as other investigations. Through the evaluation of NK-Exo concentration at 24h incubation, Anna Laura Di Pace et al. reported that the cytotoxicity of NK-Exo against K562 (erythroleukemia cell line) and Nalm-18 was increased and that the highest rate of cell lysis was reported at 50γ NK-Exo [46].

A significant point in the present research and previous studies is the sensitivity of hematological tumor cells to exosomes derived from NK cells [35, 46]. Despite good sensitivity of leukemic cells, solid tumor cells are more resistant to NK-Exo[35]. Therefore, the use of NK-Exo is a proper treatment choice for hematological malignancies, especially AML.

NK cells use different pathways to destroy the tumor cells such as granules that contain perforin and granzymes, factors such as FasL and TNF-related apoptosis inducing ligand (TRAIL), production of cytokines such as INFγ or antibody-dependent cellular cytotoxicity (ADCC) mechanism[17,18,21,49]. Many of these compounds have been reported in the exosomes derived from NK cells[50,51] and each of them kills the tumor cells in a specific manner.

Granzyme A is one of the most important cytotoxic molecules in NK cell granules, and high levels of it has been reported in extracellular vesicles isolated from NK cells (EV-NK)[51]. Granzyme A is a tryptase and serine protease known to be involved in cellular apoptosis [52]. Three members of SET complex, namely high mobility group protein 2 (HMG2), nucleosome assembly protein, SET and base excision repair enzyme apurinic/apyrimidinic endonuclease 1 (Ape 1) are direct substrates of this enzyme[53]. Chun-Hua Wu et al. reported a rapid decrease in HMG2 (or HMGB2) level in leukemic cells treated with EV-NK[51]. However, no significant reduction was observed in our data (Figure 4). According to Figure 5, p38 is another substrate of this enzyme that does not show a significant increase after treatment (Figure 4C).

Granzyme A activity is likely to decline for various reasons.

Alpha-2-macroglobulin (α2M) is an endoproteinase inhibitor that is mostly found in human plasma[54]. The production of α2M by tumor cells has been reported by a number of previous studies[55, 56]. This protein can inactivate granzyme A but granzyme A binds proteoglycans to solve this problem[52]. Expression regulation of proteoglycans can change in tumor cells, which contributes to progression of these cells57 [57]. HSP27, 40, 60, 70 and HSP90 belong to the group called Heat shock proteins (HSPs)[58]. The high expression of HSP27 has been reported in some tumors such as breast and prostate cancers and contributes to further development of them [59-61]. On the other hand, granzyme A can bind HSP27 [62].

Another content of NK cell granules called granzyme B causes DNA fragmentation and cell death through a caspase dependent pathway. Indeed, granzyme B induces the release of mitochondrial cytochrome C by converting Bid to tBid and its interaction with Bak and Bax. Cytochrome C actives Apaf-1/caspase9/caspase3/iCAD pathway that results in DNA fragmentation [51, 63, 64]. Moreover, Fas/FasL causes cell apoptosis in a caspase-dependent pathway (Figure 5) [63].

Increased level of cytochrome *C* in each of three doses of NK-Exo was significant, indicating that this signaling pathway is an important and active pathway in myeloid leukemia cells treated with NK-Exo (Figure 4A and C). Higher levels of *CASPASE3* were also observed in each of three doses of NK-Exo, and the highest level of it (7.46 fold) was at 50 mg/ml NK-Exo (Figure 4A). Caspase 3 is activated in several killing mechanisms (extrinsic and intrinsic pathways) (Figure 5)[63]. Therefore, further increase of Caspase 3 mRNA along with the decrease in its protein as well as augmentation of cleaved caspase 3 indicate the high activity of caspase3 in target cells (Figures 4A and C). In a study conducted by Liya Zhu et al., an increase in cleaved caspase3 (3.78-fold) and cytochrome C (1.66-fold) was reported in B16F10 cells (melanoma cell line) treated with NK-92 Exo[36].

Mitogen-activated protein kinase (MAPK) cascade is one of the important pathways involved in cellular proliferation, apoptosis, differentiation, development and inflammatory responses. ERK (classical MAPK), p38 kinase and C-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) are three members of MAPK family. Following the stimulation by cytokines and growth factors, Raf-MEK-ERK pathway is activated and leads to cellular proliferation. Heat shock, lipopolysaccharide, UV irradiation, high osmotic stress, pro inflammatory cytokines, specific mitogens and protein synthesis inhibitors can activate p38 MAPK family[65]. TGF-β activating kinase 1 (TAK1)-MKK6-p38 kinase pathway has been introduced as a negative regulator of cyclinD1 expression [66].

Reactive oxygen species (ROS) production is another important function of granzyme A in tumor cells[53]. ROS (e.g. hydrogen peroxide) can induce p38, ERKs and JNKs MAPK [67]. The activation of p38 is a caspase 3 dependent pathway and PARP is a substrate of caspase 3 [68]. In our results, 3.73 fold increase of *P38* was observed in target cells treated with 50 mg/ml NK-Exo (Figure 4A). However, no significant increase was observed in western blot results of this protein (Fig.4C), which we discussed earlier. In Liya Zhu's study, 4.71-fold increase of p38 protein was reported in tumor cells (B16F10 cell line) treated with NK-92 Exo [36].

Phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway has a crucial role in cellular proliferation and cell cycle via activation of different downstream molecules. AKT is an important substrate of PI3K and one of the main keys in the survival of tumor cells [69].

When there is no cell stimulation by growth factors, PTEN (TEP1) is activated and subsequently leads to the inhibition of PI3K–AKT pathway. In fact, PTEN is a negative regulator of this pathway with its phosphatase activity that can inactivate PI3K and AKT[69-72].

In our study, the slight increase of *PTEN* level and negligible decrease of *AKT* and *ERK* levels were seen in the treated cells and optimal results were observed in the cells treated with 50 mg/ml NK-Exo; however, none of them was significant (Figure 4A). The increase of PTEN protein and the reduction of p-ERK protein were also visible in western blot analysis. Nevertheless, the decrease in p-AKT protein was not observed (Figure 4C). In a previous study, decreased levels of p-AKT and p-ERK proteins were reported in

human glioblastoma (D54) cells treated with exosome mimetics that were derived from NK cells. Also, in this study, the levels of AKT and ERK proteins were not significantly different from control group[50]. Promoter methylation, protein destruction and gene mutations can disrupt PTEN activity. Mutation of PTEN gene is commonly seen in cancers. Disrupted activity of this protein has been reported in many cancers such as prostate, breast, lung, hematopoietic, and lymphoid tumors [69-72].

PIK3CA (phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit alpha), PIK3R2 and PIK3R1 mutations that further increase the activity of PI3K cause progression of cancers [69].

NK cells in peripheral blood are mostly in resting phase and can enter into tissues after activation by cytokines [21]. There are several ways for activation of NK cells and their in vitro study; for example, use of cytokines such as IL-2, IL-12, IL-15 and IL-21 or co-culture of PBMC with an irradiated cell line. There is no difference between cytokines in activation of NK cells with regard to the amount of released exosomes and their contents[35, 46,73]. Moreover, NK-Exo can induce resting NK cells and lead to the expression of NKG2D and KIR2DL2 on them, and the combination of cytokines and NK-Exo is highly effective [73].

We investigated the cytotoxic effect of NK-Exo in both activated and inactivated states on KG-1 cell line. This study demonstrated that the cytotoxic activity of LAK-Exo was significantly higher than resting NK-Exo (Figures 2F and E). Interleukin -2 activates JAK/STAT signaling pathway in NK cell and is effective on their cytotoxicity[74]. Activation of NK cells by IL-2 increases the gene expression of *FasL*, which results in further cytotoxicity. Previous studies demonstrated the high cytotoxicity of LAK-Exo against hematologic tumor cell lines[75]. Luana Lugini et al. showed that the activation of NK cells increased the amount of released exosomes but that it was not significant. According to their results, cytotoxicity of LAK-Exo on lymphoblasts (Jurkat cell line) is fast and strong and the best result was observed at 60 µg LAK-Exo [35]. In other study, neuroblastoma derived exosomes were used to enhance the activity of NK cell against neuroblastoma cells. Their reports showed that the education of NK cells with this method was effective [73].

CONCLUSION

Our study proves the effectiveness of NK-Exo against acute myeloid leukemia (KG-1 cell line) in vitro. Therefore, it seems that NK-Exo therapy is a suitable method for tumor immunotherapy. The patient's immune system is the best weapon to fight the abnormal cells. Hence, immunotherapy can be the main key for treating many diseases, especially cancer.

Due to the desirable properties of NK-Exo, further studies about its cytotoxic effect on primary myeloblasts as well as other tumor cells are required both in vitro and in vivo. Examination of other aspects of exosomes derived from NK cell lines and peripheral blood NK cells with the help of animal models will be helpful in understanding the effectiveness of this new and different treatment.

CONFLICT OF INTEREST

The authors have no conflicts of interest for publication of this study.

Funding

This article is the result of a thesis project approved and supported by the research committee at Blood Transfusion Organization of Iran (IR.TMI.REC.1398.018).

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