IJHOSCR

Original Article

International Journal of Hematology-Oncology and Stem Cell Research

The Effects of Released Exosomes from NK-92 Cells with IL15 on the Apoptosis of HL-60 Cell Line

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Received: 14, May, 2024
Accepted: 11, Mar, 2025

ABSTRACT

Background: Most cancers are treated through chemotherapy and radiotherapy. However, these methods have limitations due to cancer cells evading immune detection, prompting researchers to explore alternatives such as immunotherapy. Nonetheless, cancer cells can weaken the immune response, necessitating improvements in immunotherapy methods. Exosomes, tiny cell-derived nanoparticles, reflect the traits of their originating cells. Natural Killer NK cells produce exosomes comprising perforin, granzyme, Fas-L, etc. The small size, proximity to tumors, and stability of these exosomes enable easy absorption by cancer cells. This study demonstrates that IL-15 impacts NK-derived exosomes, enhancing their ability to kill cancer cells.

Materials and Methods: With the addition of 100 nanograms per milliliter of IL-15 to NK-92 cell culture, the cells are incubated for 48 hours. Exosomes are then isolated from treated and non-treated NK-92 cell lines through the ultracentrifuge method. After isolation, different concentrations of exosomes from both groups are added to HL-60 cells for treatment. After 24 hours, the apoptosis rate is assessed through the Annexin-V method.

Results: Increased light absorption in the BCA test, along with thicker bands of CD63 and CD81 in the Western blotting test, indicates a higher yield of exosomes after adding IL-15 to the source cells. The low p-value from the t-test demonstrates that exosomes derived from stimulated NK cells are more cytotoxic than those from the control group. Further, two-way ANOVA confirms differences between the control and treatment groups at each concentration, and Welch's t-test proves that all differences in the ANOVA test are significant.

Conclusion: This article presents evidence that exosomes obtained from IL-15-induced NK cells not only increase in quantity but also demonstrate significant cytotoxicity against leukemic cells compared to exosomes obtained from non-stimulated NK cells.

Keywords: Exosome; Natural killer (NK) cells; Immunotherapy; Leukemia

INTRODUCTION

Leukemia, commonly referred to as blood cancer, is caused by the abnormal proliferation of hematological cell lines. Acute promyelocytic leukemia (APL), classified as M3 in the categorization of acute myeloid leukemia (AML), is characterized by the proliferation of neoplastic hypergranular promyelocytes and blast cells, caused primarily by t(15;17)[1]. APL constitutes approximately 5–10% of all AML cases[2,3]. About 10-20% of APL patients suffer from disseminated intravascular coagulopathy (DIC), thrombocytopenia, and bleeding, which are life-threatening. Currently, the most potent drug for APL treatment is arsenic trioxide, followed in effectiveness by ATRA, Gemtuzumab Ozogamicin (GO), and Anthracyclines[4]. However,

ATRA is not an effective treatment for patients with translocations other than t(15;17) [5].

Immunotherapy seeks to enhance the precision of the patient's immune system response to cancer while minimizing its negative impacts. It can boost or modify how the immune system works to identify and attack cancer cells. NK cells from the innate immune system are among the first lines of defense in recognizing cancerous and viral cells, regardless of previous contact[6,7]. NK cells initiate apoptosis through perforin and granzyme molecules[8,9], induce programmed cell death by engaging with TNF family molecules (such as FasL, TRAIL, and mTNF) present on tumor cell membranes, mediate membranes[9,10], antibody-dependent cell-mediated cytotoxicity (ADCC) through interactions with the Fab region of antibodies via CD16[11] and generate IFN-γ[12]. However, NK cells may struggle against cancer cells in low-pH environments due to the tumor microenvironment (TME) which contains TGFβ[13], release MICA/B into the TME, and engage with NKG2D, leading to NK cell exhaustionNKG2D[14–20] and impaired function, ultimately contributing to cancer development[21].

Exosomes are the nanoparticles with biostability and low immunogenicity that released by both healthy and unhealthy cells and contain proteins, nucleic acids, similar to the cells from which they originated[22–24]. Exosomes can impact nearby cells by being involved in intercellular interactions, signal transduction, tumor immune evasion and transmitting genetic information to target cells[25–27]. Articles have documented that exosomes derived from NK cells exhibit features of both exosomes and NK cells. They display exosome markers like CD9, CD63, and CD81, while also exhibiting NK cell characteristics such as CD56, NCRs, NKG2D, perforin, granzyme A-B, granulysin, Fas-L, and DNAM1. The studies reported that NK exosomes contain miR-186[28],which impairs neuroblastoma cells and miR-lit-7b-5b, which has anti28-proliferation effect on cancer cells[29]. Exosomes could be alternative to cell therapy due to their nanoscale size, more tissue penetration, low immunogenicity and resistance to immune clearance[30]. Moreover, articles addressed that NK-derived exosomes have a greater toxic effect than their originating cells (NK cells)[31,32]. Some researches demonstrated that being in an immunosuppressive environment mimicking TGF-b and IL-10 did not diminish NK-Exo's original affinity and anti-tumor abilities[33,34] and exosomes prefer low-pH environments and internalize better into cancer cells due to their acidic environments[35]. Another interesting future for exosome is inducing selective cell death of Leukemia cell-lines[36] . It is suggested that the exosome or the source cell can be engineered to express specific ligands targeting tumor tissues[37]. IL-15 is a more effective cytokine in activating NK cells compared to others. NK cells pre-treated with IL-15 are more effective at targeting tumors and more efficient in attacking cancer cells[38] but the effect of exosomes isolated from this parental cell is unclear.

To summarize, NK-derived exosomes are noteworthy for their ability to transport perforin and granzymes, along with crucial ligands such as Fas-L and NKG2D, their tendency to thrive in acidic environments, and their small size, which facilitates tumor cell penetration with selection. In this article, we try to compare the effect of exosomes on the apoptosis rate when their original cells are stimulated by IL-15 versus non-stimulated NK-92.

MATERIALS AND METHODS

NK-92 cell line

In this study, we use the NK-92 cell line to represent NK cells, which was obtained from Dr. Masoud Soleimani. NK-92, a natural killer cell line reliant on interleukin-2 (IL-2), is derived from the peripheral blood of patients with advanced non-Hodgkin's lymphoma. The cell culture medium contains αMEM, β-Mercaptoethanol, P/S penicillin-streptomycin, Inositol, Folic Acid, Horse Serum (HS), and Fetal Bovine Serum (FBS) at a final concentration of 12.5%, along with 100 IU/mL of IL-2 and 10 IU/mL of IL-15. When the cell density is low, the culture flask should be kept upright to increase cell density and promote cell aggregation. Once the cell mass grows normally, the culture flask can be kept flat to enhance air exchange. For subculturing, we begin by centrifuging the liquid at 950 RPM for 4 minutes. After resuspending the cell pellet with medium, the cells are transferred to a flask. Care should be taken when resuspending NK-92 cells to avoid vigorous agitation.

Cells should be maintained at 37°C with 5% CO2 and monitored daily (NK-92 Cell Culture Protocol from protocols.io).

HL-60 cell line

In this study, the HL-60 cell line used was obtained from the Pasteur Institute (ECACC Number: 85011431). HL-60 is a human cell line derived from peripheral blood lymphocytes of acute promyelocytic leukemia patients (APL). It exhibits a suspension cell line with lymphoblastic morphology. HL-60 cells were cultured in RPMI 1640 medium supplemented with 15% heat-inactivated Fetal Bovine Serum (FBS) and 1% penicillin. Incubation is done at 37°C with 5% CO2, with daily monitoring. Typically, 5-7 mL of culture medium is added to a T25 culture flask, and approximately 7-9 mL to a T75 culture flask. Subculturing can be performed by centrifuging at 1200 RPM for 5 minutes.

NK-92 stimulation

This study has two groups of NK-92 cells: a treatment group and a control group. The treatment group receives 100 nanograms per milliliter of IL-15 (Miltenyi Biotec, Catalogue Number: 130-095-760) to stimulate NK-92 cells while the control group does not receive any supplementary treatment. After 48 hours of adding IL-15 to the medium, the control and treatment groups are centrifuged at 1500 RPM for 5 minutes, and the supernatant is collected in separate falcon tubes. The collected samples are stored at -80°C until exosome isolation.

Exosome isolation, Quantification, and Confirmation

Exosome Isolation

Ultracentrifugation is one of the most popular methods for exosome isolation and applies to large-scale samples. The principle of ultracentrifugation relies on differences in density and size between exosomes and impurities in the sample. Initially, the sample is centrifuged for 10 minutes at 300 g, resulting in a pellet containing cells. The supernatant is then centrifuged for 10 minutes at 2000 g, yielding a pellet containing dead cells. These two steps are referred to as low-speed centrifugation in this process. The next step involves removing the supernatant and subjecting it to high-speed centrifugation at 10,000 g for 30 minutes to separate cell debris, which forms a pellet. The final high-speed centrifugation is performed at 100,000 g for 70 minutes, yielding a pellet containing exosomes and potentially contaminated proteins. To further purify the exosomes, the pellet should be washed with PBS and centrifuged at 100,000 g for 70 minutes. After these steps, a pellet containing exosomes is obtained. Importantly, all these procedures should be conducted at 4°C, and the isolated exosomes stored at -80°C to maintain their quality. Some protocols, particularly for large-scale preparations, replace low-speed centrifugation with a 0.22-micron filter [22,39,40.]

Exosome concentration assessment

As the exosome concentration following isolation is initially unknown, the concentration in each vial of exosomes should be determined. The Thermo Scientific Pierce BCA Protein Assay Kit is used to quantify the concentration of exosomes obtained from both the control group of NK-92 and the IL-15-treated NK-92 groups. After completing the kit's procedure, absorption is measured at 562 nm using spectrophotometry.

Dynamic light scattering (DLS)

Dynamic Light Scattering (DLS) is used to determine the size distribution of particles or molecules in a liquid or suspension by evaluating fluctuations in light resulting from Brownian motion. For this test, at least 2 µL of extracted exosomes suspended in PBS are filtered twice through a 0.22-micron filter. Measurements are obtained through the Horiba Scientific device.

Western blotting

Western blotting is an essential method for confirming the presence of exosomes. The presence of tetraspanins such as CD81, CD63, and CD9 is demonstrated in exosomes. Identifying exosome markers is crucial for verifying the effectiveness of isolation and purification. Notably, the same volume of each group is loaded in the gel. The first antibody for detecting CD81 is anti-CD81 antibody (Catalog Number: sc-166029), and the second is m-IgGκ BP-

HRP (Catalog Number: sc-516102). For CD63 recognition, the first antibody is anti-CD63 antibody (Catalog Number: sc-5275), and the second is mouse anti-rabbit IgG-HRP (Catalog Number: sc-2357).

Apoptotic efficacy assessment of cancer cells

As mentioned, HL-60 cells are cultured in RPMI1640 medium supplemented with 15% FBS. When the confluency in the flask reaches 80%, the target cells are seeded in a 48-well plate, with a recommended range of 100×10^3 to 120×10^3 cells per well. After seeding, the plate should be incubated for 24 hours to enable the cells to stabilize. Subsequently, different exosome concentrations (1 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL, and 50 µg/mL)[42]obtained from both NK-92 treated with IL-15 and the non-treated group are added to the respective wells. After 24 hours of incubation, apoptosis is measured through the Annexin-V method.

RESULT

Particle size analysis through Dynamic Light Scattering (DLS) technique

The DLS test was selected to determine the size of exosomes by measuring the scattering of light therefrom. As the sample of exosomes is covered by a water layer, the DLS results typically show sizes slightly larger than the actual size of exosomes, in an effect known as the hydrodynamic diameter effect. On the x-axis, the diameter of the particles is presented in nanometers (nm) while the y-axis on the left represents the frequency of the particle size, with the right y-axis correlating with the percentage of undersized particles. In the context of the graph, the intensity of the peak, positioned at approximately 100 nm, suggests that most of the particles in the sample have diameters close to this measurement. The DLS results indicate that the sample contains a predominant population of exosome-sized vesicles, besides some polydisperse particles, confirming the presence of exosomes, although further testing is necessary for validation.

Quantitative analysis of exosome concentration across comparative groups

Exosome extraction can be reported in different units, such as micrograms (µg), nanograms (ng), or particle counts. In this study, it was quantified at 1×10^9 particles per milliliter (mL). However, for subsequent research steps, it is essential to express exosome concentration in µg/mL. To establish the sample's concentration level, Bovine Serum Albumin (BSA) was used as a reference standard. Through a standard protein curve, we determined the unknown protein concentration. Serial dilutions of Albumin were used to construct a standard curve. Due to the concentration of albumin and its light absorbance chart, we can determine the slope of the line for further analysis. The Y-axis of the graph represents the sample's Optical Density (OD) and the X-axis the unknown protein concentration. The results of absorption and concentration are shown in Equation 1. Due to the finer correlation between protein concentration and light adsorption, results indicate that adding IL-15 leads to more exosomes being obtained.

Verification of exosome isolation purity

As mentioned previously, the hydrodynamic effect and microvesicle aggregation can modify the DLS test results. Other methods recommended to assess appropriate exosome isolation include immune blotting. Western blotting can be used to confirm acceptable exosome isolation based on exosome-specific CD markers such as CD63 and CD81. The results of the Western blotting analysis are presented in Figure 2. Both groups show exosome CD markers, confirming the success of the isolation.

Enhanced cytotoxicity of exosomes derived from pre-treated NK cells

The Annexin-V test is chosen for the apoptosis assay. The statistical tests include the t-test and kernel density plot to provide an overview of the effect of NK-exosomes derived from the pre-treated group with IL-15 on the HL-60 apoptosis rate, and the ANOVA test to determine the differences in apoptosis rates among concentrations from both groups. Additionally, we use Welch’s t-test, a post-analysis test, to validate the results of the ANOVA

test. The results of the t-test (Figure -A) indicate that HL-60 cells treated with exosomes isolated from the pre-treated group show an increased apoptosis rate, compared to the control group, and the low p-value confirms significance. The plot (Figure -B) shifts to the right, confirming the differences in death rates between groups. The ANOVA test shows that the pre-treated group enhances the apoptosis rate at almost all concentrations, although this relation is not linear (Figure - C). The results of Welch's t-test (Figure - D) prove that in all concentrations, except 5 µg/mL, the apoptosis rate is significant. The confidence interval line does not include 0, indicating that the p-value is low across all concentrations. However, at a concentration of 5 µg/mL, the effect of the exosome from the pre-treated group is negative.

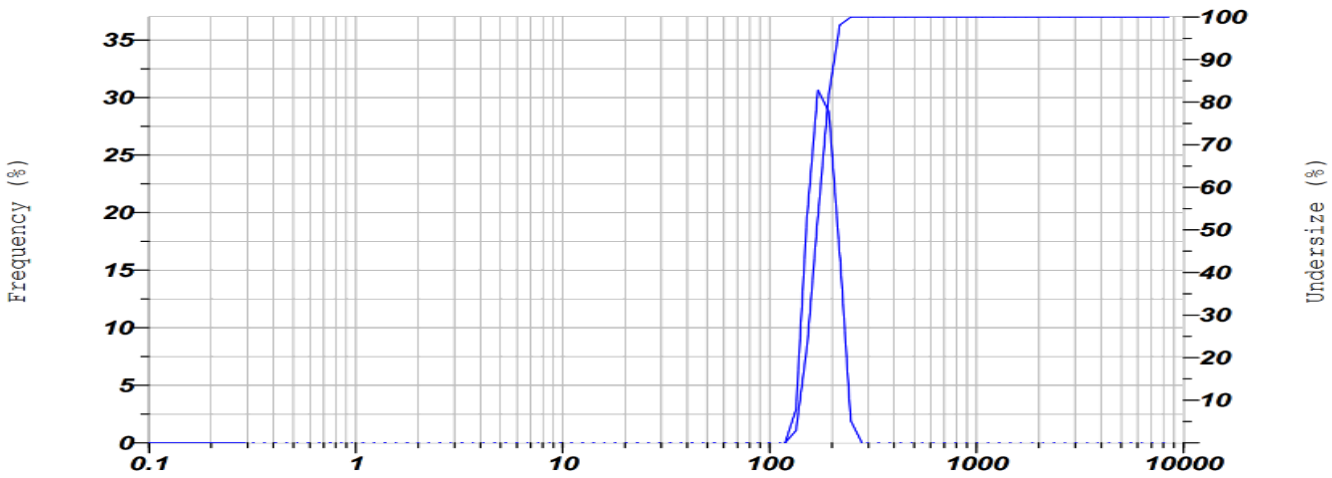


Figure 1. DLS Results indicate that the single prominent peak, with an S.P.Area Ratio of 1.00, suggests a uniform population of vesicles within the sample, with polydispersity under the standard distribution form. The mean is 171 nm and by considering hydrodynamic effect, the size is near to large range of exosome size. PI index and slight rise and fall of the peak also imply aggregation which is present alongside the exosome.

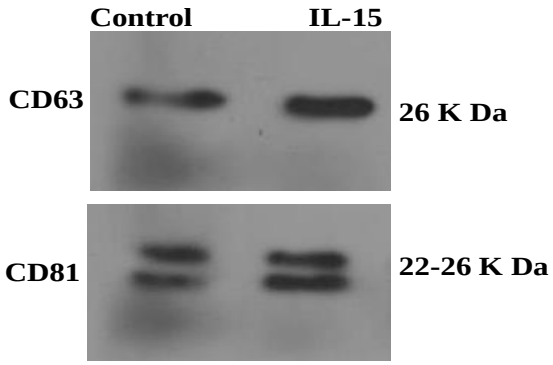


Figure 2. In this case, western blotting was used to confirm the exosome presence in sample. The band in IL-15 group is thicker than control group. It is obvious that the extra amount of IL-15 positively effect on final exosome concentration.

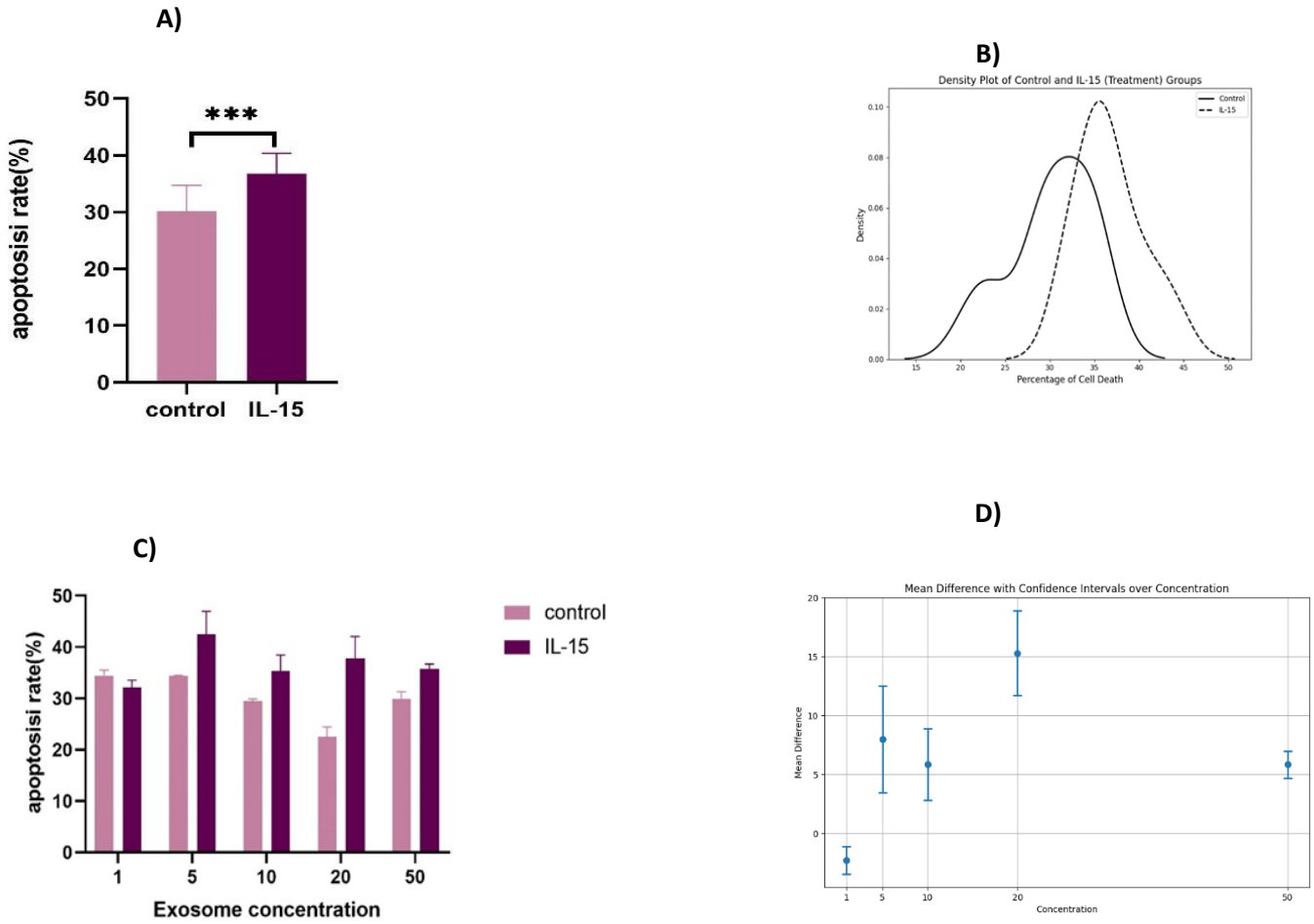
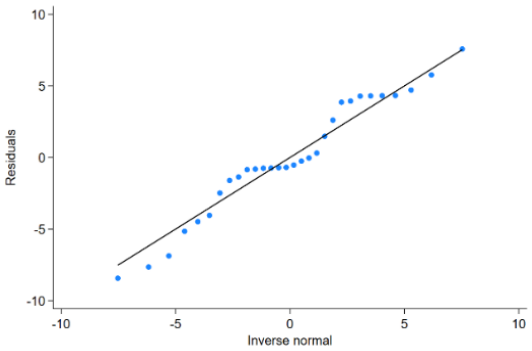
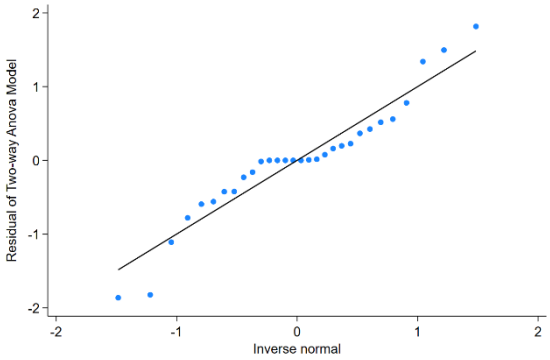


Figure 3. Result analysis: A) The results of the t-test derived from the analysis of the data demonstrate that the results are significant. B) Kernel density plot, the IL-15 group shifted to right, which indicates the apoptosis rate increment in IL-15 group in comparison to control group. C) ANOVA test shows the difference between the control and treatment group in each concentration. D) The P-values associated with IL-15 concentrations are notably low. The R-squared values for all concentrations are very high, ranging from about 0.89 to 0.98. This suggests that a significant proportion of the variability in apoptosis rate can be explained by the model, which likely accounts for the effect of IL-15 at different concentrations. (*: p-value<0.05, **: p-value<0.01, ***: p-value<0.001)



Shapiro-Wilk W test for normal data for t-test

Variable	Obs	W	V	z	Prob>z
Residuals	30	0.95583	1.404	0.702	0.24146



Shapiro-Wilk W test for normal data for ANOVA test

Variable	Obs	W	V	z	Prob>z
Residuals	30	0.93911	1.935	1.365	0.08607

Figure 3. Q-Q plot and Shapiro-Wilk W test for A) t-test and B) for ANOVA test. Q-Q plot of t-test and ANOVA test indicates that the residuals are close to the normality line, suggesting a reasonable approximation to a normal distribution.

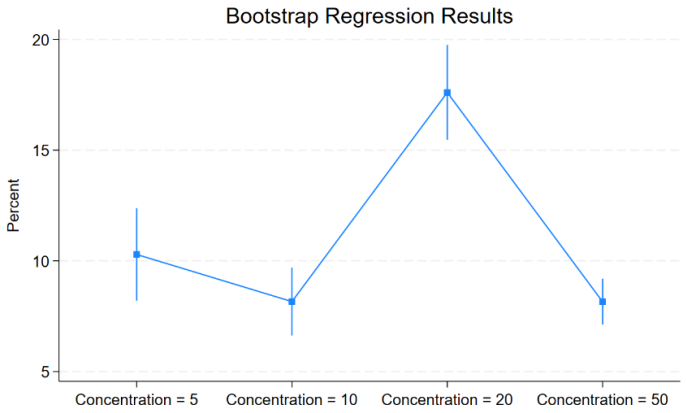
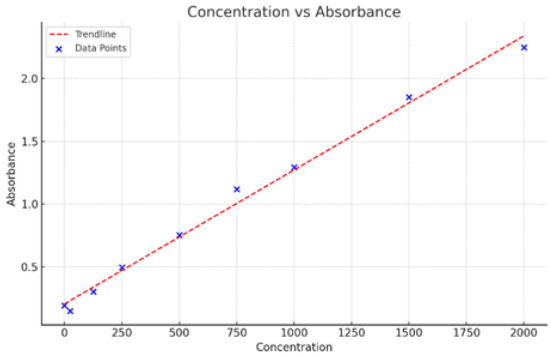


Figure 4. Bootstrap regression analysis for ANOVA results. This graph displays the differences in apoptosis rates between the IL-15 and control groups at each concentration, compared to the differences observed at a concentration of 1 µg/mL.

Concentration of albumin (µgr/mL)	OD of albumin(nm)
0	0.192
25	0.148
125	0.303
250	0.495
500	0.753
750	1.118
1000	1.293
1500	1.852



	first read	second read	average of OD	sample concentration
OD of exosome treats by IL-15	0.433	0.458	0.4455	**219.5455**
OD of exosome of control	0.276	0.263	0.2695	**59.54545**

Equation 1 Finding exosome concentration

DISCUSSION

Exosome immunotherapy: A novel approach to addressing cancer

Given the limitations associated with NK therapy, NK exosome therapy has been proposed as a potential alternative. However, the effectiveness of exosomes remains a concern in this field. [41] This study aims to determine whether adding activating interleukins, such as IL-15 to the NK-92 cell line can affect the apoptosis rate of AML-M3 cells through isolated exosomes. One of the points of this research concerns exosome quantity, which suggests that IL-15 positively influences the final exosome yield. As we know, low exosome yield in exosome therapy is a challenge,[41] and based on our research, adding activated interleukins into the cell culture may enhance exosome final yield. The higher optical density and concentration observed in the BCA test, along with the thicker bands corresponding to CD63 and CD81 in the Western blotting test, as between the exosomes derived from the NK cells pre-treated with IL-15 and those isolated from non-treated NK cells, can confirm this idea, which could improve exosome extraction and address challenges to exosome therapy[42,43].

The study's second focus is on exosome quality, indicating that NK exosomes have anti-tumor activity, especially after treatment with stimulating interleukin such as IL-15. Earlier research has demonstrated that Perforin, TNF-α, and Fas-L increase in exosomes compared to NK cells alone [31] and even the micro-RNA content differs between NK exosomes and NK cells. Based on these researches, exosomes derived from NK cells have a greater impact on the apoptosis rate than NK cells themselves[31] and their enrichment with let-7b-5p could reduce proliferation in pancreatic cancer cells[29]. Although other articles have shown that NK exosomes possess sufficient potential for use as an immunotherapy method and adding IL-15 and IL-21 has a synergistic effect on the anti-tumor activity of NK-exosomes stimulated with IL-15 and IL-21 in the Hep3B cell line[44], the effect of exosome derived from stimulated NK with IL-15 on AML is unclear. In our research, the results of the t-test derived from Annexin-V show that exosomes from the pre-treated group with IL-15 significantly enhance the apoptosis rate, compared to the control group. Besides the t-test, the kernel density plot indicates the IL-15 group shifting to the right, suggesting that the overall death rate in the IL-15 group is higher than in the control group and significant. According to the ANOVA test, the treated group has a higher apoptosis rate at all concentrations, and based on Welch's t-test, these differences are significant across all concentrations.

Our results align with previous claims that NK-exosome has cytotoxic potential against leukemia[36,45] and demonstrate that by adding an activating interleukin, such as IL-15, this effect increases, which could be used to expand NK exosome therapy by enhancing exosome yield and efficacy. Consider that our results did not reveal a linear relationship between exosome concentration and cell death rate, and at high concentrations, the death rate does not change significantly. We are unable to statistically find the underlying reason of this relation, but the most probable biological explanation is that in high concentration of exosome, the cells the cytolytic activity may trigger adaptive mechanisms in target cells that reduce their susceptibility to apoptosis, possibly through induced resistance or exhaustion of apoptotic pathways. As a result, even low concentrations of NK cell exosomes can have an apoptotic effect on the HL-60 cell line.[36]

This research has some limitations that may provide avenues for future investigation. For instance, we can explore whether the concentration of cytotoxic proteins differs or which pathways are distinct between these two groups of exosomes. The combination of exosomes with anti-cancer treatments, due to their capability to function as drug carriers, could be more effective than solo treatments, a topic that has not been sufficiently studied. NK cell therapy has a few limitations, such as TME characteristics that lead to NK cell dysfunction. However, NK cell-derived exosomes present a promising alternative due to their stability and ease of storage, compared to NK cells themselves. Nevertheless, further research is necessary to enhance their safety and efficacy and to address these challenges.

Robustness Analysis

Due to the small size of our dataset, the normality assumption for our analysis is crucial. We use both Q-Q (quantile-quantile) plots to visualize and the Shapiro-Wilk W test to quantify and check normality for the mean difference and two-way ANOVA tests. As observed from both sets of graphs, the Q-Q plot of the t-test and ANOVA test indicates that the residuals are close to the normality line, suggesting a reasonable approximation to a normal distribution.**Error! Reference source not found.**) However, the Shapiro-Wilk W test shows a significant violation of the normality assumption for the two-way ANOVA test at the ten percent level. This statistically significant deviation from normality cannot be ignored, as it could compromise the reliability of the F-tests and p-values derived from the ANOVA model. To address this issue, we turn to regression analysis but face challenges due to our limited sample size. With just 30 observations, traditional regression methods might yield unreliable estimates. Therefore, we implement a bootstrapping regression (Figure 4) approach with replacement and 10,000 repetitions. Bootstrapping is particularly well-suited for this situation because it does not rely on strict parametric assumptions and can generate a more accurate estimation of the sampling distribution of our regression coefficients. By repeatedly resampling from our small dataset, we can better assess the variability of our estimates and construct more reliable confidence intervals, making our analysis more robust despite the limited data.

CONCLUSION

The study focuses on exosome therapy, an aspect of immunotherapy. We have suggested that the activated NK-92 cell line produces more cytotoxic exosomes in higher quantities than exosomes derived from non-treated NK cells and NK cells alone. This method could be used as a potential therapy for hematological malignancies.

Ethical approval: The project was approved by the ethical committee at Iran by IR.TBZMED.REC.1401.669 ID approval.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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