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In Vitro Assessment of MDS-derived Microvesicles on Umbilical Cord Hematopoietic Stem Cell Phenotype

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ABSTRACT

Introduction:

Myelodysplastic syndrome (MDS) is a heterogeneous group of hematologic clonal malignancies. As MDS cells transmit microvesicles in the bone marrow microenvironment along with other cells, the aim of this study was to investigate the effects of MDS bone marrow-derived microvesicles that have the ability to change the phenotype of a healthy hematopoietic stem cell to a leukemic-like phenotype invitro.

Method:

The bone marrow sample of MDS patients and healthy individuals was collected to prepare microvesicles and hematopoietic stem cells were identified as target cells.After culturing hematopoietic stem cells in the presence or absence of prepared microvesicles for 7 days, stem cell markers including CD34, CD90, CD38 and CD117 were detected and colony formation ability was observed. In addition, Cell survival, changes in microRNA-21, 10a, 181a / b expression and specific markers of leukemic stem cells including CD32, 25, 45RA were performed detect the leukemic-like phenotype.

Results:

In this study, healthy umbilical cord hematopoietic stem cells exposed to MDS bone marrow microvesicles for 7 days showed changes not seen in the other study groups. These changes, which include increased cell survival, increased expression of microRNA-21 and microRNA-10a, were observed while the stem ness of hematopoietic stem cells was preserved.

Discussion:

In this study, MDS-like changes in target cells were investigated and observed. It is also recommended that the effect of MDS microvesicles on hematopoietic stem cells be investigated for more than 7 days to show changes that require more time.

KEYWORDS

MDS, Cord Hematopoietic Stem Cells, Microvesicles, MicroRNA-21, MicroRNA-10a.

Introduction

Myelodysplastic syndrome (MDS) is a heterogeneous group of hematologic clonal malignancies associated with ineffective hematopoiesis, single- or multigrade dysplasia, and cytopenia[1]. The prevalence of MDS is estimated at approximately 3.4 per 100,000 and the three-year survival rate is about 35%. About 10,000 new cases of MDS are diagnosed each year [2, 3] . The exact mechanism of onset and progression of MDS is unknown, but its origin is early hematopoietic stem cell (HSC) with somatic mutations[4].In the cells of MDS patients, overactive apoptotic mechanisms are seen, but as the disease progresses to AML, the pathways move toward reducing apoptosis and increasing survival[5].In the progression of MDS to AML, increased expression of miR-155 and miR-125b is seen[6, 7].In the stage of MDS progression to acute myeloid leukemia (AML), the goal of treatment is to delay the conversion of MDS to AML, and it is one of the treatment strategies for hematopoietic stem cell transplantation (HSCT)[8].Hematopoietic stem cells (HSCs) have the ability to self-renew and differentiate into different types of cells, including blood cells, as well as to differentiate into other non-hematopoietic cells under certain conditions[9].Hematopoietic stem cells, due to their ability in regeneration and repair of various tissues of body during tissue damage, are very important in therapeutic purposes.During tissue damage, they are transplanted into damaged areas where most of the cells have been destroyed and are replaced the damaged cells for reforming the

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new functional tissue[10].The ability to self-renew and differentiate hematopoietic stem cells often takes place in the bone marrow niche, and maintaining the communication and activity between hematopoietic stem cells and cells in the bone marrow niche is really essential[11-14]. In bone marrow microenvironment, MDS cells and other cells transmit microvesicles towards each other. microvesicles are a type of extracellular vesicles that germinate from cell membranes during growth, proliferation, cellular stress, and apoptosis[15].microvesicles have a variety of functions that are completely dependent on their contents, and these contents also depend on the type of cell from which the microvesicles are derived[16].microvesicles can bind specifically to the surface molecules of the target cell and activate associated signaling pathways[17]. On the other hand, they have the ability to change the expression profile of bone marrow cell genes so that the target cells find a different phenotype using advanced regulatory mechanisms[18]. Numerous studies have investigated the types of functions of microvesicles and especially the effect they have on the target cell[19]. Studies have shown the ability of microvesicles to change the phenotype of the target cell, but the effect of whole leukemic bone marrow cells on hematopoietic stem cells by microvesicles has not been investigated. Given that in the bone marrow microenvironment, all healthy cells and MDS are together, and microvesicles can be transported by all cell populations, including blood and non-blood cells, as well as healthy MDS cells, examining the relationships and effects that exist in this field is really essential. The aim of this study was to investigate the effects of bone marrow-derived microvesicles on MDS cells that have the ability to change the phenotype of a healthy hematopoietic stem cell to a leukemic-like phenotype in vitro.

Materials and Methods

Preparation of Microvesicles from Bone Marrow Samples

Bone marrow aspirate samples from patients with AML and bone marrow aspiration samples from healthy individuals who volunteered for bone marrow transplantation were collected in tubes containing heparin anticoagulants. Red blood cells aspiration samples of bone marrow of patients and healthy individuals were removed lysed using ammonium chloride.Samples with more than 95% viability were used for culture (RPMI-1640 containing 0.5% BSA was used for culture of bone marrow nucleated cells). Cell culture flasks containing more than 95% of living cells were used to isolate microvesicles. The microvesicles were isolated using a centrifuge (at ambient temperature and 2000 rpm for 10 minutes) and stored for use at 4 ° C.

By using Zestasizer Nano(Malvern Company), the particle diameter of 1 ml of solvent is determined using radiation and light scattering. The solvent used in this study was phosphate buffer and its refractive index was 1.334 with a viscosity of 1.08. The refractive index of microcycles was selected as 1.33 due to its similarity to the structure of the liposome, which is the refractive index of the liposome and its absorption was measured at a wavelength of 630 nm, 0.05. In order to determine the amount of isolated microcycles, the protein content of the microcycles was measured. Also, qualitative quantitative evaluation of isolated microcycles was performed using DLS (Vasco) technique and qualitative evaluation was performed using TEM (Philips TEM) technique.

Preparation of Hematopoietic Stem Cells from Cord Blood Samples

Umbilical cord blood samples were obtained from the Umbilical Cord Blood Bank in bags containing CPDA1 anticoagulants. These cells were isolated from umbilical cord blood samples using MACS (milteny biotech) technique.First, mononuclear cells were isolated based on density gradient using a Ficol (innotriane) centrifuge at 1500 RPM.Hematopoietic stem cells were then isolated using CD34 magnetic antibody (eBioscience company) and a special column based on MACS technique. Cord blood mononuclear cells were isolated first by centrifugation and then by MACS technique.For every 200-100 million cells, 70 µl of magnetic CD34 antibody and 70 µl of blocking antibody were considered. Cells were stored in 4 ° C for 30 min. After this time, the cells were mixed with 10 ml of MACS buffer to remove unbound and excess antibodies and centrifuged for 10 minutes at 1500 rpm at 4 ° C. The cell pellet was suspended in an appropriate amount (500 µl per 108 cells) of MACS buffer and transferred to the large column (LS) of MACS previously washed with 3 mL of MACS buffer and placed inside the magnetic field. After complete transfer of cells to the column and attachment of CD34 positive cells to it and washing of non-adherent cells with MACS buffer, the column was removed from the field and placed on a sterile Falcon tube and all cells adhered to the column were physically pressurized by MACS buffer under special piston Columns. The mixture was centrifuged at 1500 rpm at 4 ° C for 10 minutes. Cell precipitate was suspended in 200 µl of serum-free Stemline medium. This isolated cell population, in addition to being used for treatment with microvesicles, was also examined as a sample of zero day cells in this study.

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Counting, Purification and Viability of Isolated Hematopoietic Stem Cell Population

The number of cells was calculated using a light microscope slide and a cell count formula. To evaluate the purity of isolated hematopoietic stem cells, the isolated cells were stained and evaluated by flow cytometry (FACS Aria) to evaluate the level of CD34 surface indicator, which represents their purity. And the results were analyzed by Flowjo software (version). If the percentage of $CD34^+$ cells was more than 90%, the method would be continued. To more accurately determine the percentage of living cells, flow cytometry technique was selected using propidium iodide staining (PI (Cmg. Company)).

Treatment of Hematopoietic Stem Cells with Microvisicles

Stemline (Sigma Aldrich) base culture medium was used for treatment with minimal damage. In this study, isolated hematopoietic stem cells were examined at two time points: The first time point on day zero is when these cells were immediately isolated from the umbilical cord blood sample, and the second time point on day 7, is when the cells were cultured (in vitro) for seven days. The study groups included: The control group consisted of hematopoietic stem cells that were cultured with basic culture medium for 7 days and no microvesicles were added to their culture medium. The normal group included hematopoietic stem cells that were cultured for 7 days with basal culture medium and bone marrow derived microvesicles of healthyindividuals were added to their culture medium. Also, leukemia group consisted of hematopoietic stem cells that were cultured for 7 days with basal culture medium and bone marrow derivedmicrovesiclesof AML patients were added to their culture medium.

Hematopoietic Stem Cell Culture and Microvesicles Treatment

A total of 55,000 hematopoietic stem cells were placed in each 24-well plate well in the presence of 500 µl of basal culture medium.For the control group, only the same conditions were defined, but for the normal and leukemia groups, 15 micrograms of normal and leukemic bone marrow derived microvesicles were added to the wells. Which by considering 0.5 ml of culture medium, A dose of 30 µg/ml of microvesicles be obtained. The cells were incubated for 7 days at 37° C, 5% CO2 and complete humidity. In order to compensate the evaporated culture medium and provide fresh medium, 50 µl of culture medium was added to all wells daily.

Cell Harvest on day 7 of Culture

After 7 days of culture, all the contents of the well were transferred to sterile microtubes, cell sediment was prepared by centrifugation (at 1500 rpm at 25 ° C) and then the cell sediment was suspended in phosphate buffer solution (PBS). This cell suspension was used for all subsequent steps.

Evaluation of Hematopoietic Stem Cell Survival on Day 7 of Culture

Using neubauer chamber, the number of cells was calculated. Also, by using flow cytometry (Facs Aria/ Partec) and PI dye(Flow cytometry technique was selected using propidium iodide (PI) staining).More, the percentage of living cells was determined. The results were analyzed with Flowjo software (version).

Evaluation of Specific Surface Markers of Hematopoietic and Leukemic Stem Cells on Days 0 and 7 of Culture

For staining, at least 3,000 stem cells from day 0 and 7 of culture were considered for study. Hematopoietic and leukemic stem cell surface markers were determined using flow cytometry technique. In this study, 6 different colors were used for cell staining. Hematopoietic stem cell antibodies including CD34-PE (eBioscience), CD38-PE-CY5 (eBioscience), CD117-PE (eBioscience), CD90-APC (eBioscience) and specific leukemic stem cell antibodies including CD25-PE-CY7 (eBiosc), CD45RA-eFluor 450 (eBioscience), CD32-FITC (eBioscience) were used in this study.

Assessing of the Differentiation and Colony Formation Ability in Stem Cells on Day 0 and 7 of Culture

Using CFU assay (STEMCELL Technologies), 3000 hematopoietic stem cells on day 0 and 7 were mixed with

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RPMI culture medium and then homogenized with Methocult medium (STEMCELL Technologies) in a ratio of 10:1 (according to the manufacturer's protocol) and 12 wells were poured and the plate was incubated for 14 days at 4 ° C, 5% CO2 and complete humidity. Then, the colonies containing at least 50 cells (erythroid colonies) and at least 40 cells (monocyte and granulocyte colonies) and at least 20 cells (mixed erythroid, megakaryocyte, granulocyte and monocyte colonies) were counted according to the instructions for using this culture medium.

Evaluation of the Expression of the Target Micrornasin Stem Cells on Days 0 and 7 of Culture

Real Time PCR technique was used to evaluate the expression of microRNAs. To perform this technique, whole cell RNA (Favorgen) was extracted and synthesized into cDNA (Fermentase company).

Extraction of RNA and cDNA Fabrication from Stem Cells on Day 0 and 7 of Culture

At least 10,000 cells were used to extract RNA from hematopoietic stem cells on days 0 and 7 of culture. To evaluate the quality and quantity of the extracted RNA, RNA samples were analyzed using an spectrophotometer (Eppendor) with a ratio of 260/280. The constructed cDNAs were used to study the expression of microRNAs. The cDNAs made at this stage are used to study the expression of microRNAs.

Measurement of Desired MicroRNAs Expression by Real Time PCR Technique

The PCR Real Time PCR (ABI Plus one) technique was used to determine the expression of genes.Ingredients of Master mix included: H2O (5.4 µl), Takara Master mixi (6.25 µl), Rox color (0.25 µl), F-primer (0.3 µl) and R-primer (0.3 µl). 12.5 µl of Master Mix was poured into microplate wells for ABI Plus one and 0.5 µl of cDNA was added to each well. For each sample, 3 repetitions were considered in each procedure. Time and temperature schedule in each cycle of Real Time PCR includes: Holding stage (95 ° C, for 30s), Denaturation stage (95 ° C, for 15s), Annealing and Extension (61 ° C, for 60s). Real Time PCR reaction was repeated 40 times.

Also, drawing the melting curve of the amplified fragments was considered to ensure the specific binding of the primer and the specific amplification of the desired microRNA. The sequence of primers used for each gene is listed in Table 1. In this study, stem loop primer was used to make cDNA. To confirm the size of the amplified fragments, the contents of a well of any amplified microRNA were electrophoresed after the Real Time PCR steps were completed.From the cDNA of each microRNA, serial dilutions with a ratio of 1.2 were prepared and the reaction mixture was prepared and the Real Time PCR technique (Kit by Takara) was performed.Using the "Standard Curve" panel in the ABI Plus One software, the standard curve of each sample was drawn and its efficiency was calculated. If the efficiency of the standard charts was more than 90% and the difference between this amount for microRNAs primer and housekeeping genes was not more than 10%, the formula -ΔΔCt2 was used to determine the amount of changes in gene expression.

Table 1. Sequence of primers used for each gene

Sequence ('3 ---' 5)	**Direction**	**Gene name**
CGC CGT AGC TTA TCA GAC T	forward	microRNA-21
GAG CAG GGT CCG AGG T	reverse	
GTC GTA TGC AGA GCA GGG TCC GAG GTA TTC GCA CTG CAT ACG ACT CAA CA	Stem loop primer	
ACC TAG CAC CAT CTG AAA TC	forward	microRNA-29a
GAG CAG GGT CCG AGG T	reverse	
GTC GTA TGC AGA GCA GGG TCC GAG GTA TTC GCA CTG CAT ACG ACT AAC C	Stem loop primer	
GGG CAA CAT TCA ACG CTG TC	forward	microRNA-181a/b
GAG CAG GGT CCG AGG T	reverse	
GTC GTA TGC AGA GCA GGG TCC GAG GTA TTC GCA CTG CAT ACG ACA CTC AC	Stem loop primer	
ATC ACT GTA AAA CCG TTC CA	forward	Snord47
GAG CAG GGT CCG AGG T	reverse	

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GTC GTA TGC AGA GCA GGG TCC GAG GTA TTC GCA CTG CAT ACG ACA ACC TC	Stem loop primer	

Statistical Analysis

This study was performed based on 3 samples of patient bone marrow, 3 samples of healthy bone marrow and 5 samples of cord blood.All data were analyzed as mean ± SD. First, the type of data distribution was determined using Kolmogorov Smirnov statistical test. Then, parametric statistical tests were used to analyze the data with normal distribution and non-parametric statistical tests were used for the data with abnormal distribution. In general, in this study, One Way ANOVA, Kruskal Wallis and Mann Whitney statistical tests were used and P value less than 0.05 was the significant basis of the results.In cases where the comparison of the mean of one variable in all studied groups and in general, was less than 0.05, the comparison of the means of the groups was also performed to determine the significant differences in means is related to which group.

Results

Bone Marrow Samples from Sick and Healthy People

1.5 ml bone marrow sample were collected from 3 MDS patient individually (1.5 ml sample per person) at the time of diagnosis and before taking any medication.Definitive diagnosis of the type and subgroup of the disease was made by referring to the patients' medical records and it was confirmed by additional tests such as immunophenotyping and cytogenetic examination. In this study, 2 male and 1 female patients with a mean age of 63±7.5 and also 2 healthy males and 1 female with a mean age of 38±11 participated. About 5 ml of bone marrow samples were collected from 3 healthy individuals (5 ml sample from each person) who volunteered to donate bone marrow for transplantation and their healthiness was confirmed by tests in the file.

Preparation of Bone Marrow Cell Supernatant

To count and determine the percentage of living bone marrow cells, after lysis of red blood cells, the number of nucleated cells in patients' bone marrow samples was 8/12±6/116 million, and the viability of them was 196 ±1%.
Also, the number of nucleated cells in healthy bone marrow samples was 71±10.7 million, and the viability of them was 97 ± 0.5%.In order to prepare the cell culture supernatant, the living cells (more than 95% viability) were used. To examine the culture of bone marrow nucleated cells, the cells were cultured for 20 hours and then examined using inverted microscope. As shown in Figure 1, both adherent and suspended cell populations, representing a heterogeneous population of bone marrow, were present in the culture flask.The percentage of viable cells after culture was 96±1%. Since more than 95% of the cells were alive, their supernatant was used to isolate microvesicles.

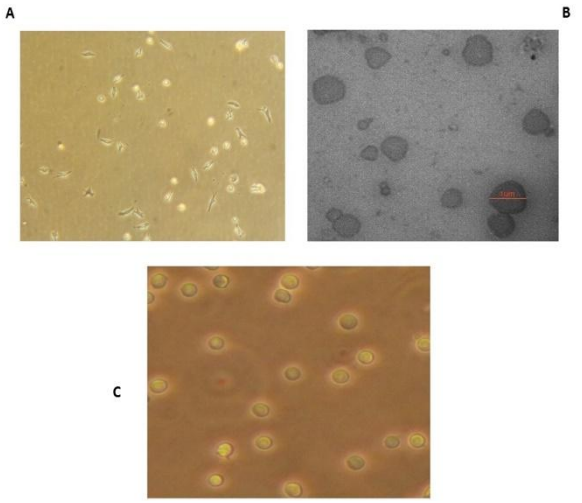

Figure 1.
A-Flask bottom image after culturing bone marrow nucleated cells (magnification 400).

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B- Microscopic image of microcyclic suspension with TEM technique (uranyl acetate staining).
C- Microscopic image of hematopoietic stem cells isolated from umbilical cord specimens by MACS technique (magnification 400).

Investigation of Isolated Microvesicles

Using the DLS technique, the particle diameter of the microcyclic suspension isolated from the cells was determined. As shown in figure 2, 85.8% of the particles have an average diameter of 304.4 nm and14.2% of the particles have an average diameter of 95.77 nm. In total, the average total diameter of the particles in the microvesicles suspension was 274.4 nm. These results quantitatively confirmed the method of separating the microvesicles and the diameter of the isolated microvesicles. Using TEM technique, the structure of isolated microvesicles was examined. As shown in Figure 1, the membrane integrity of the microvesicles is preserved during the separation process and the spherical and vesicular structure of the microcycles is not damaged. Also in this image, the diameter of the microvesicles is 1000 nm and less, which qualitatively confirms the size of their diameter.Using Bradford's solution and preparing successive dilutions of BSA protein whose concentration was known, a standard protein concentration curve was obtained (Figure 2). Also, the concentration values of proteins isolated from microvesicles are calculated according to their light absorption (Table 2).

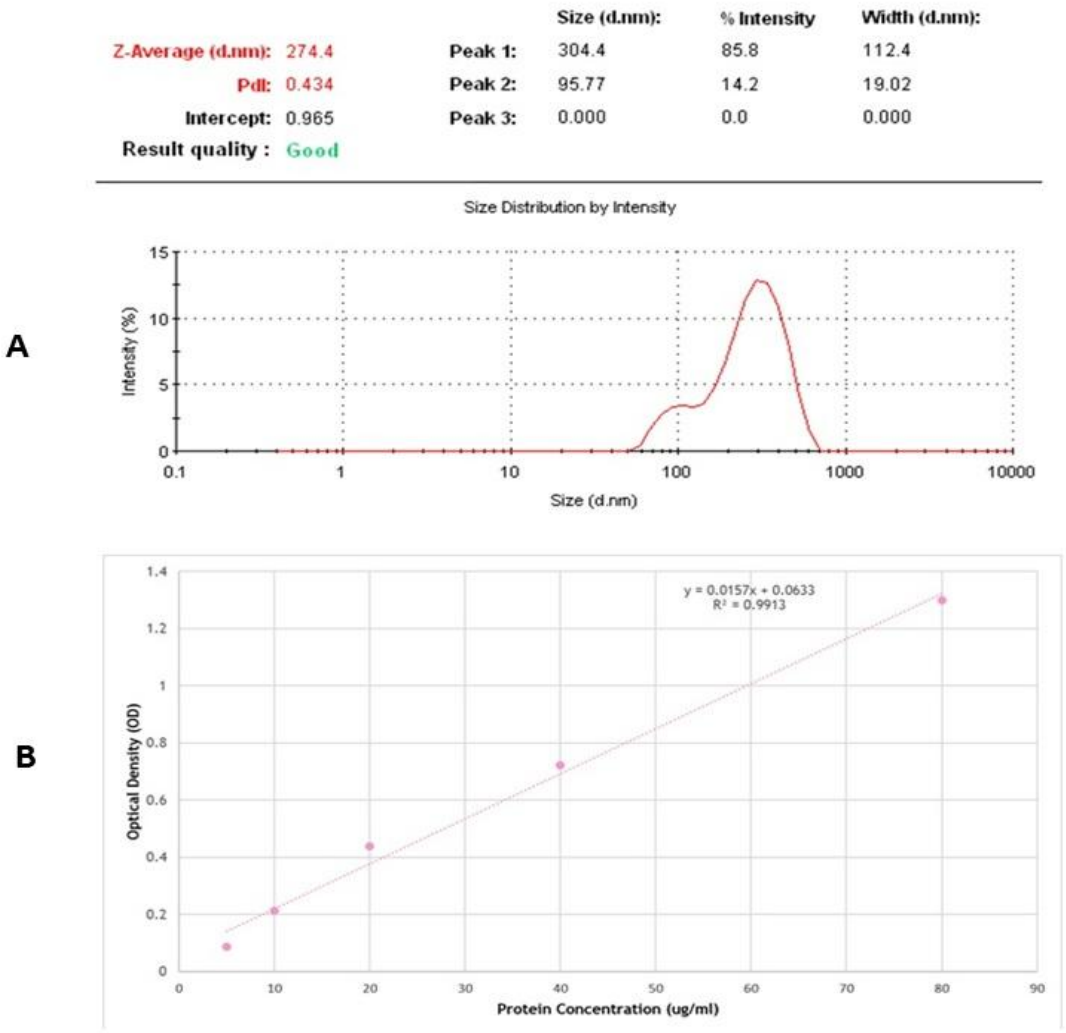

Figure 2.
A- Results obtained from the examination of microcyclic suspension by DLS technique.
B- Standard chart for determining protein concentration. The correlation coefficient of this diagram (R2) is 0.99.

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Table 2. Protein values calculated from microcyclic suspension samples. The values of A1-A3 are related to the microcycle of the patient samples and the values of N1-N3 are related to the microcycle of the normal normal samples

Samples	Protein (ug)
A1	22.071
A2	21.821
A3	18.845
N1	14.918
N2	16.643
N3	15.172

Hematopoietic Stem Cells Isolated from Umbilical Cord Samples

A total of 5 umbilical cord blood bags were used that had been sampled for a maximum of 48 hours, and totally, the HSC isolation procedures were performed three times from these samples and their cell count was 700,000, 800,000, and 1,800,000, respectively.Figure 1 shows isolated hematopoietic stem cells, which are considered for this study for zero day.The purity of isolated cells was measured by detecting the CD34 marker on the cell surface by flowcytometry (Figure 4). The purity of isolated hematopoietic stem cells was 97±1%.The amount of living cells was measured based on the lack of dye entering the living cell and its results was assessed using PI dye and flow cytometry device (Figure 4). After three times of the isolation process, the amount of cells was 96% ± 0.5 have been.

Evaluation of Hematopoietic Stem Cells after 7 Days of Culture in the Presence or Absence of Microvesicles

Hematopoietic stem cells were counted on day 7 in the study groups including control, normal and MDS (Figure 3). The results of this study showed that the mean cell count of leukemia and normal group (P = 0.02) and also leukemia and control group (P = 0.03) were significantly different, while there was no significant difference between leukemia group and zero day cells (4.4). 0P =). On the other hand, the mean cell count of day 0 showed a significant difference with each of the control (P = 0.01) and normal (P = 0.009) groups. According to studies, the survival of MDS group cells after 7 days of culture is higher than normal and control group cells (Figure 3). To evaluate the percentage of viable cells on day 7 of culture, PI dye and flow cytometry were used and the results showed that the percentage of viable cells in the study groups on day 7 of culture was about 80%. Kruskal Wallis non-parametric statistical test was used to compare the mean percentage of viable cells in the study groups which showed that there was not a significant difference between the viability of cells in control, MDS and normal groups after 7 days of culture and also compared to zero day cells. None Figure 3(P=0.12).

Evaluation of Cell Surface Markers

The level of cell surface markers and their changes in the study groups was evaluated using flow cytometry and monoclonal antibodies against specific surface markers of hematopoietic stem cells (CD34, 38, 117, 90).

The results of CD34 changes showed that the rate of CD34 changes in the study groups during 7 days of culture in the presence or absence of **microvesicles** is very small and still show high expression of it. Statistical analysis of the results with One Way ANOVA test did not show a significant difference in CD34 levels between the studied groups on days 0 and 7 of culture (P value = 0.5)). Figure 4shows the flow cytometric result of one of the samples.

$CD34^{+}$ $CD38^{-}$ phenotype is the main representative of hematopoietic stem cells, therefore, the changes in this phenotype was investigated in hematopoietic stem cells before and after culture. Statistical analysis of the results with One Way ANOVA test showed that there were no significant changes of this phenotype in the studied groups on days 0 and 7 of culture (P value = 0.35). Figure 4 shows the flow cytometry result of one of the samples.

Another surface indicator of hematopoietic stem cells studied in this study is CD117, the results of which showed no significant changes among the study groups and also compared to zero day cells using One Way ANOVA. Figure 4 shows the flow cytometry result of one of the samples.
Comparison of the results of CD90 test with One Way ANOVA showed that there were no significant changes in the

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studied groups on day 7 and also on day 0 cells (P = 0.15).

CD45RA surface marker was also evaluated.The distribution of the results of the CD45RA study in different groups was normal. One Way ANOVA parametric test was used to compare the mean CD45RA between the studied groups, but no significant difference was observed (P = 0.18) Figure 4shows the flow cytometric result of one of the samples.

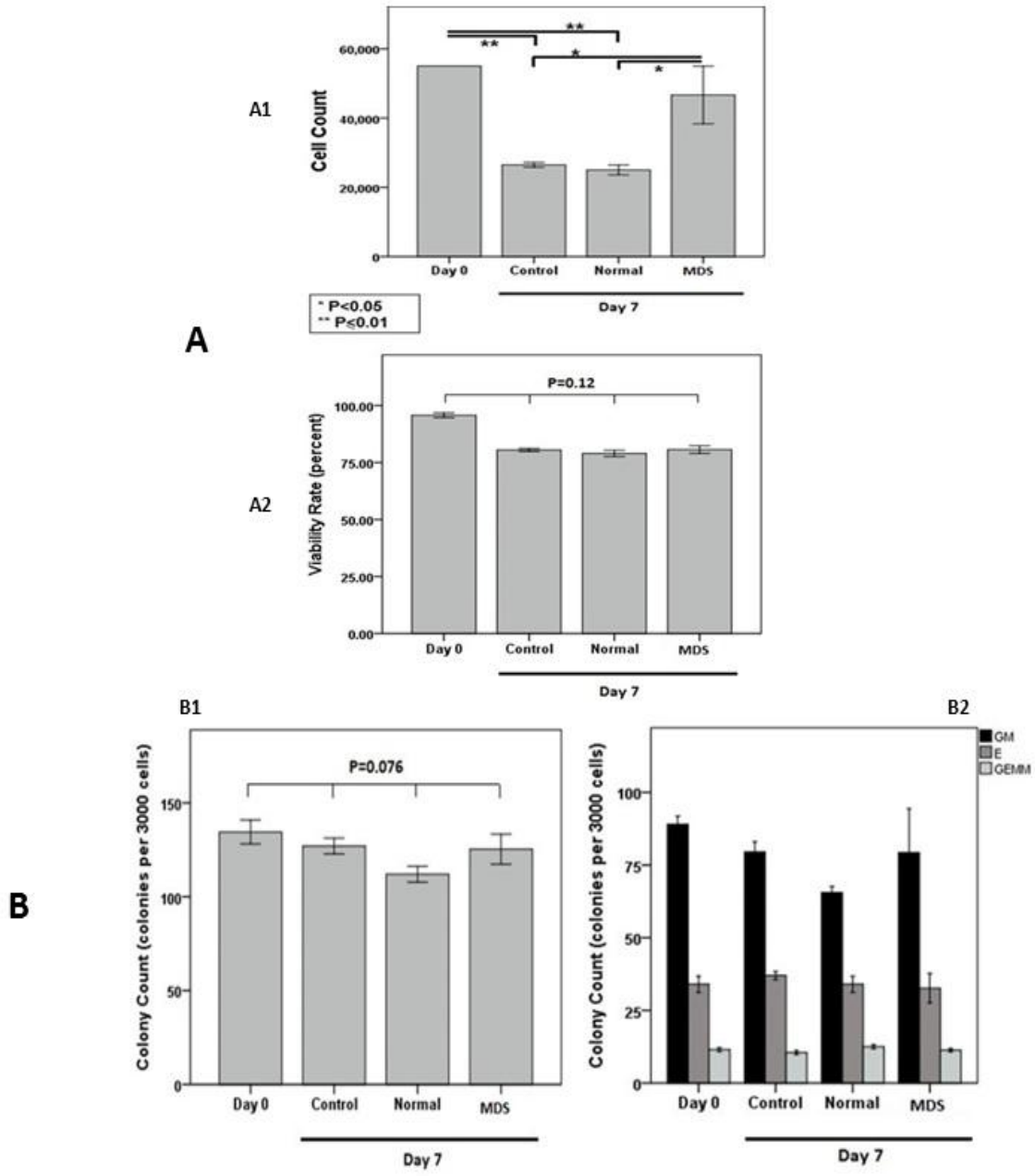

Figure 3.
A1- Results of hematopoietic stem cell count in the studied groups on days 0 and 7 of culture.
A2- Hematopoietic stem cell viability in the studied groups on days 0 and 7 of culture.
B1- Colony count results from hematopoietic stem cell differentiation in the studied groups on days 0 and 7 of culture.
B2- Results of separate counting of colonies in the studied groups on days 0 and 7 of culture.

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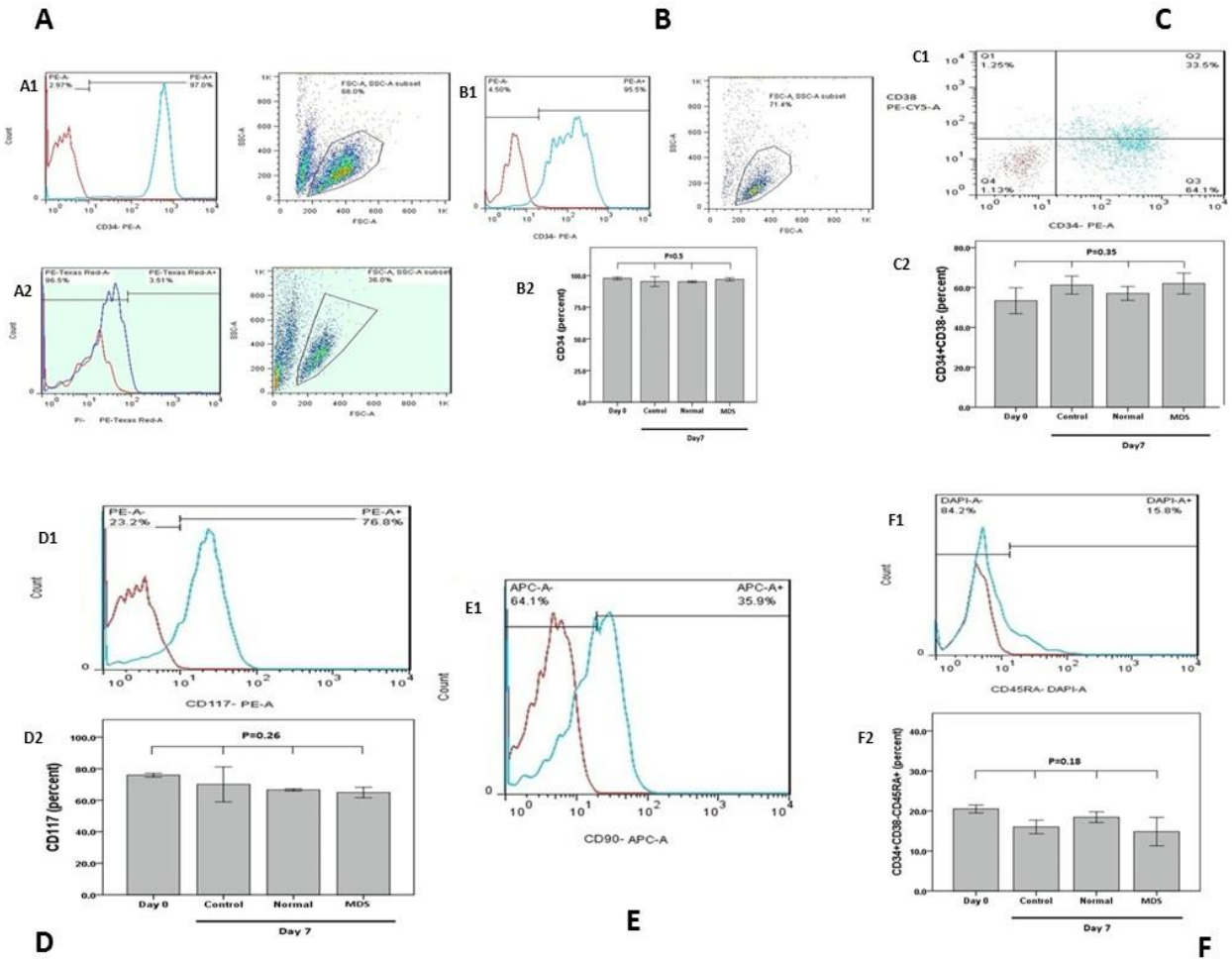

Figure 4.

A1- The purity of isolated hematopoietic stem cells. Image on the right: shows the selected cell population. Left image: shows the percentage of cells with CD34 surface index from the selected population. (In all forms of cell flow cytometry, the red histogram represents the negative control sample (isotype) and the blue histogram represents the sample to be examined, which appear in a frame.)

A2- The viability of isolated hematopoietic stem cells. Image on the right: shows the selected cell population. Left image: Shows the percentage of living cells identified by the blue histogram and includes cells that have not entered the PI dye.

B1- Comparison of CD34 surface index of hematopoietic stem cells in the studied groups on days 0 and 7 of culture.

B2- The result of cell flow cytometry for CD34 surface index in one of the samples. The image on the right shows the selected cell population (the same population was used to examine other surface indicators in subsequent images). Left image: shows the percentage of cells with a surface index of CD34.

C1- The result of cell flow cytometry for CD34 + CD38- phenotype in one of the samples. Q1 includes CD34-CD38 + phenotype, Q2 includes CD34 + CD38 + phenotype, Q3 includes CD34 + CD38- phenotype, Q4 includes CD34-CD38- phenotype.

C2- Comparison of CD34 + CD38-hematopoietic stem cell phenotype in the studied groups on days 0 and 7 of culture.

D1- The result of cell flow cytometry for CD117 surface index in one of the samples.

D2- Comparison of CD117 surface index of hematopoietic stem cells in the studied groups on days 0 and 7 of culture.

E1- The result of flow cytometry of cells for D90 surface index in one of the samples.

F1- The result of cell flow cytometry for CD34 + CD38-CD45RA + phenotype in one of the samples.

F2- Comparison of CD34 + CD38-CD45RA + hematopoietic stem cell phenotype in the studied groups on days 0 and 7 of culture.

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Study of Differentiation and Colony Forming Assay

Cells are divided into erythroid colony (total CFU-E and BFU-E), granulocyte and monocyte (CFU-GM) and a mixture of granulocyte, erythroid, myeloid and monocyte (CFU-GEMM) based on the type of distribution and the presence of cells containing hemoglobin. (Figure 5).

One Way ANOVA parametric statistical test was used to evaluate the results due to the normal distribution of colony count results in the studied groups. Based on this statistical test, no significant difference was observed between the mean colony counts of the different groups. (P =0.076) (Figure 6). Also, the average count of each type of colony was compared among the groups separately. Comparison of mean CFU-E colony count (P = 0.66), mean CFU-GM colony count (P = 0.23) and mean CFU-GEMM colony count (P = 0.12) showed no significant difference between all groups. (figure5).

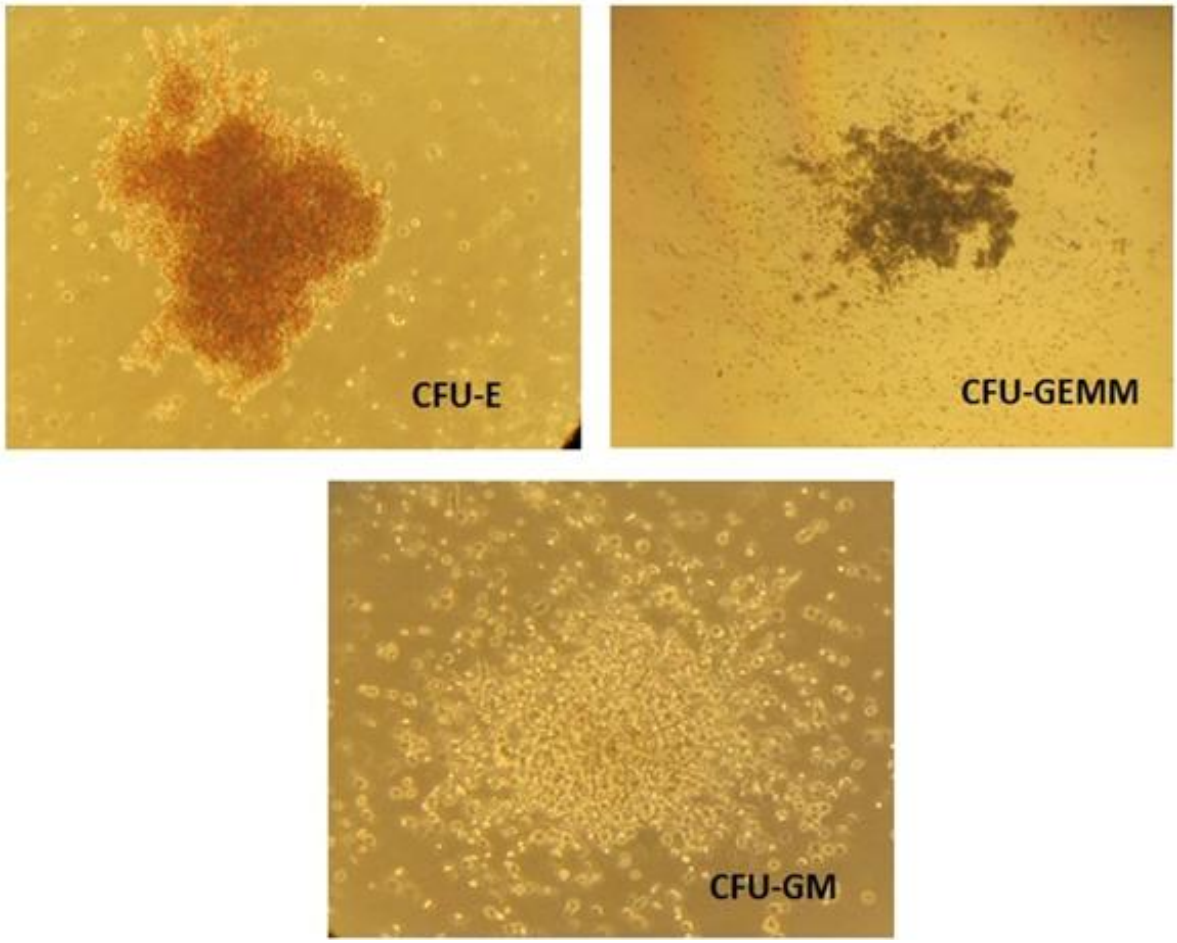

Figure 5. Colonies resulting from hematopoietic stem cell differentiation (magnification 400).

Investigation of MicroRNA-10a, 21, 181a / b Expression Changes

Using spectrophotometer, the extracted RNA concentration was determined. Its value for day zero samples was 1.61± 0.48 Micrograms per microliter of the RNA sample, and for day 7, due to low number of cells, the amount was 35.2±6.5 ng/µl. The ratio of 260.280 was measured with a spectrophotometer. The samples were in the range of 1.7-2.

Using the formula $2^{-\Delta\Delta Ct}$, the efficiency of Real Time PCR reaction for the studied genes was between 90% to 110%, which is in the acceptable range, and also the difference between the reaction efficiency of each gene and the housekeeping gene is less than 10%. According to the formula $2^{-\Delta\Delta Ct}$, it is permissible to study the changes in gene

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expression (figure 6). Quantitative analysis of SNORD47 housekeeping gene expression was performed to normalize the RNA levels of each sample. The amplification curve for this gene in each sample, which was logarithmically generated over a specified number of periods, showed that the amplification path was well developed. Using the melting curve of PCR products, it was ensured that only one product of a certain size was amplified and that there was no non-specific binding between the primer and the undesired amplified fragment. Also, the negative control sample (NTC), which did not have cDNA, did not have an amplification curve that confirmed that there was no contamination in the materials and equipment (Figure 7).

MicroRNA-21 Gene Expression

The proliferation curve, melting curve and negative control diagram for microRNA-21 gene expression were controlled and confirmed (Figure 4). The rate of microRNA-21 expression changes in the study groups compared to the control group was calculated using the formula $2^{-\Delta\Delta Ct.}$ Due to the fact that the distribution of results obtained from the study groups was normal, One Way ANOVA parametric test was used to compare the mean expression of microRNA-21 between the groups, which showed a significant difference (P = 0.009). To find the groups that showed a significant difference in the mean expression of microRNA-21, Tukey test, which is a subgroup of the One Way ANOVA test, was used and the results showed that this difference in expression between leukemia group and zero day cells (P = 0.009) between leukemia group and control (P = 0.03) and between MDS and normal groups (P = 0.03) is significant (Figure 8).In summary, the results showed that the expression of microRNA-21 in the cells of the leukemia group after 7 days of culture was higher than the other groups.

MicroRNA-10a Gene Expression

After ensuring the quality of the Real Time PCR process, the amount of microRNA-10a expression changes in the study groups compared to the control group was calculated using the formula $2^{-\Delta\Delta Ct.}$ The amplification curve, melting curve and negative control diagram for this gene were also controlled and confirmed (Figure 4). Considering that the distribution of results obtained from the studied groups was not normal, the Kruskal Wallis non-parametric test was used to compare the mean expression of microRNA-10a between the groups which showed a significant difference (P = 0.041).Mann Whitney non-parametric test was used to find the groups that showed a significant difference in the mean expression of microRNA-10a and the results were significant between the MDS group and zero day cells (P = 0.046), MDS group and the control group P = 0.037) and leukemia and normal groups (P = 0.046) (Figure 5).

These results showed that the expression of microRNA-10a in the MDS group is higher than other groups.

Expression of MicroRNA-181a / b Gene

In this study, a specific primer fot microRNA-181a/b gene, identified both isoforms a and b simultaneously, and changes in the expression of both isoforms were generally investigated.First, the amplification curve, melting curve and negative control chart for microRNA-181a/b gene were controlled and confirmed (Figure 4). The rate of changes in microRNA-181a/b expression in the study groups compared to the control group was calculated using the formula $2^{-\Delta\Delta Ct.}$ Since the distribution of results obtained from the study groups was normal, One Way ANOVA parametric test was used to compare the mean expression of microRNA-181a/b between the groups, which did not show a significant difference (P = 0.49) (Figure 5).

In Real Time PCR, the length of amplified fragments of each gene should contain 75-70 pairs of nucleotides. The products were electrophoresed (figure 9) to evaluate the length of gene amplification. The size of fragments was between 50 to 100 base pair on DNA ladder, which shows that the approximate size of Real Time PCR products for each gene can be 70-75 pairs of nucleotides.

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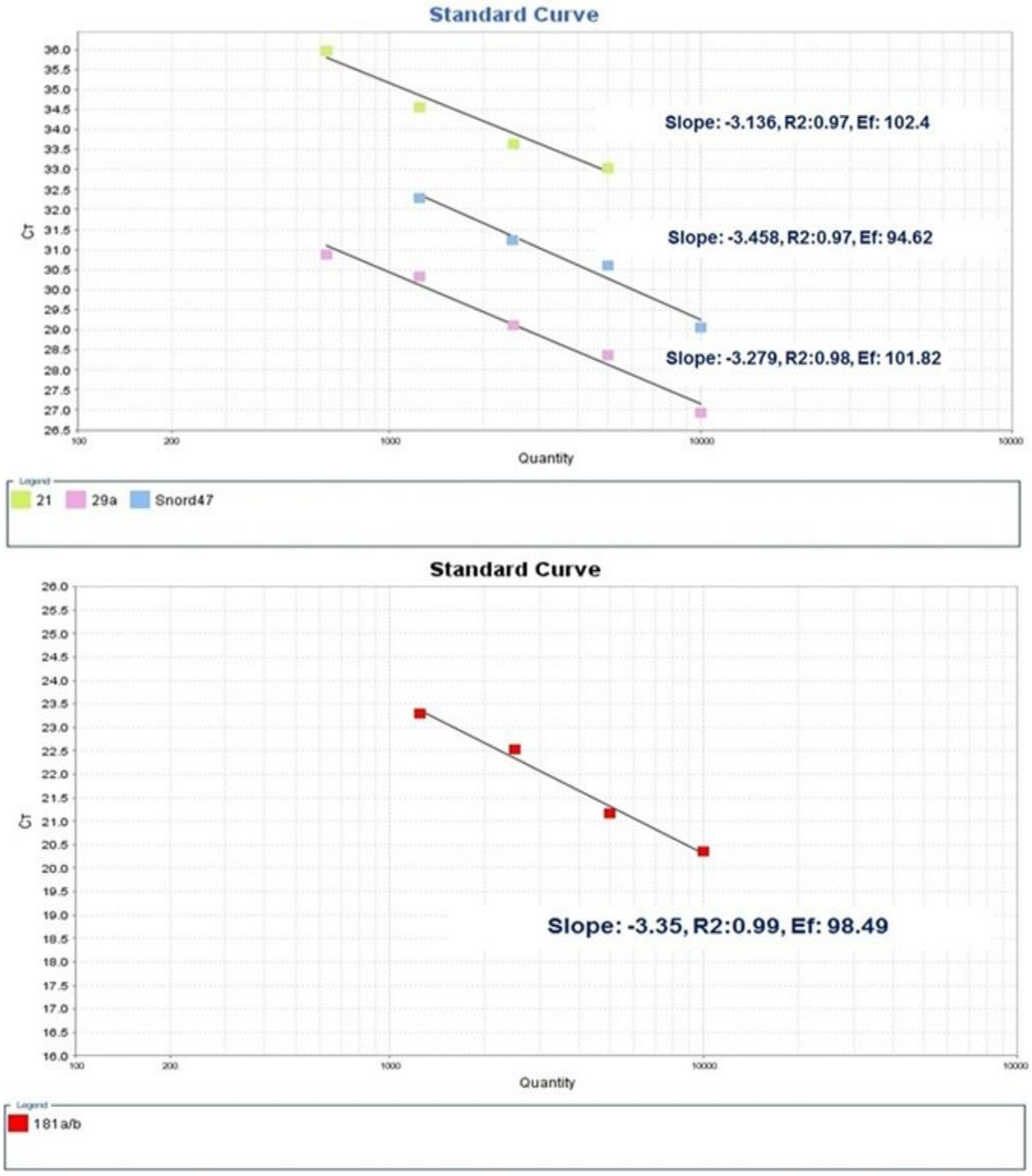

Figure 6.
A- Standard diagram prepared from consecutive cDNA dilutions for microRNA-21, 10a and Snord47 genes (above: microRNA-21, Snord47 and microRNA-29a, respectively). Ef: reaction efficiency, R2: graph correlation coefficient, Slope: graph slope.
B- Standard diagram prepared from consecutive dilutions of cDNA for microRNA-181a / b gene. Ef: reaction efficiency, R2: graph correlation coefficient, Slope: graph slope.

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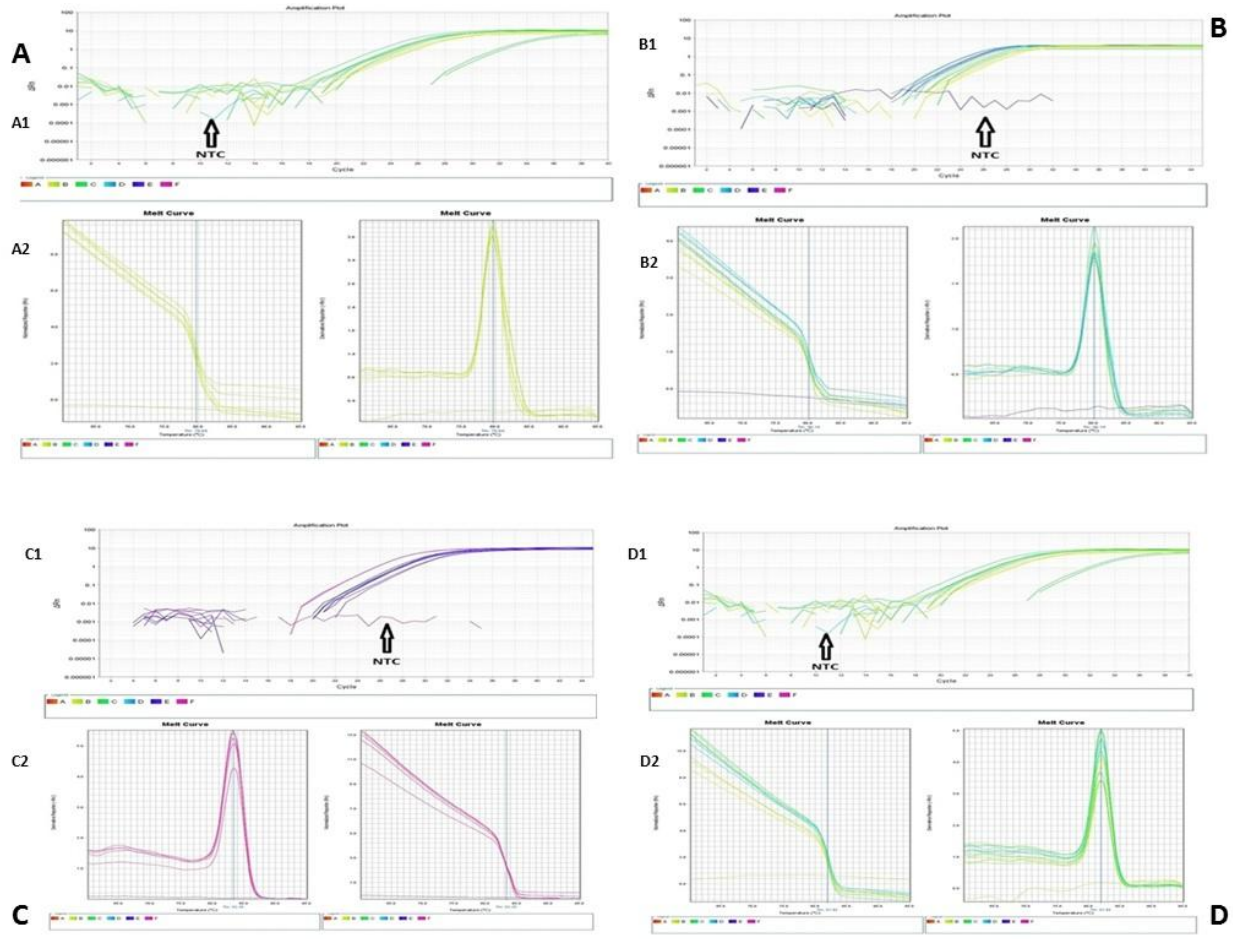

Figure 7.

A1- Snord47 gene amplification diagram. The Negative Control Chart (NTC) shows no amplification. Horizontal axis: Number of total PCR process cycles, Vertical axis: The amount of fluorescence light received by the device.

A2- Melting curve of PCR products of SNORD47 gene. Left: Melting curve showing fluorescence light changes of products (vertical axis) with increasing temperature (horizontal axis). Right: Melts the curve after calculating the derivative of fluorescence light changes per unit time (vertical axis) during temperature increase (horizontal axis).

B1- MicroRNA-21 gene amplification diagram. The Negative Control Chart (NTC) shows no amplification. Horizontal axis: Number of total PCR process cycles, Vertical axis: The amount of fluorescence light received by the device.

B2- Melting curve of PCR products of microRNA-21 gene. Left: Melting curve showing fluorescence light changes of products (vertical axis) with increasing temperature (horizontal axis). Right: Melts the curve after calculating the derivative of fluorescence light changes per unit time (vertical axis) during temperature increase (horizontal axis).

C1- MicroRNA-10a gene amplification diagram. The Negative Control Chart (NTC) shows no amplification. Horizontal axis: Number of total PCR process cycles, Vertical axis: The amount of fluorescence light received by the device.

C2- Melting curve of PCR products of microRNA-10a gene. Left: Melting curve showing fluorescence light changes of products (vertical axis) with increasing temperature (horizontal axis). Right: Melts the curve after calculating the derivative of fluorescence light changes per unit time (vertical axis) during temperature increase (horizontal axis).

D1- MicroRNA-181a / b gene amplification diagram. The Negative Control Chart (NTC) shows no amplification. Horizontal axis: Number of total PCR process cycles, Vertical axis: The amount of fluorescence light received by the device.

D2- Melting curve of PCR products of microRNA-181a / b gene. Left: Melting curve showing fluorescence light changes of products (vertical axis) with increasing temperature (horizontal axis). Right: Melts the curve after calculating the derivative of fluorescence light changes per unit time (vertical axis) during temperature increase (horizontal axis).

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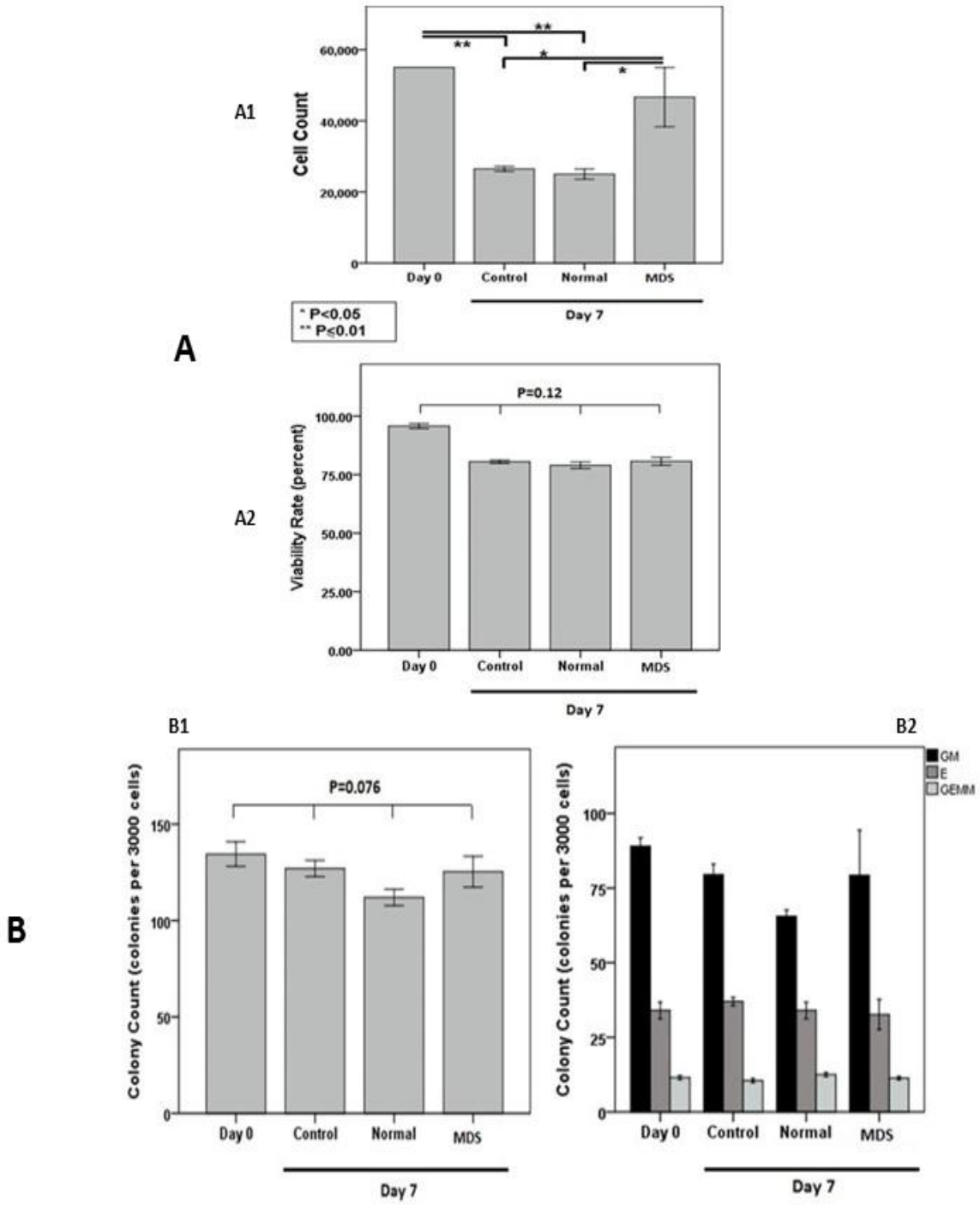

Figure 8.
A- The rate of microRNA-21 expression changes in the studied groups on days 0 and 7 of culture.
B- The rate of microRNA-10a expression changes in the studied groups on days 0 and 7 of culture.
C- Comparison of microRNA-181a / b expression expression changes in study groups on days 0 and 7 of culture.

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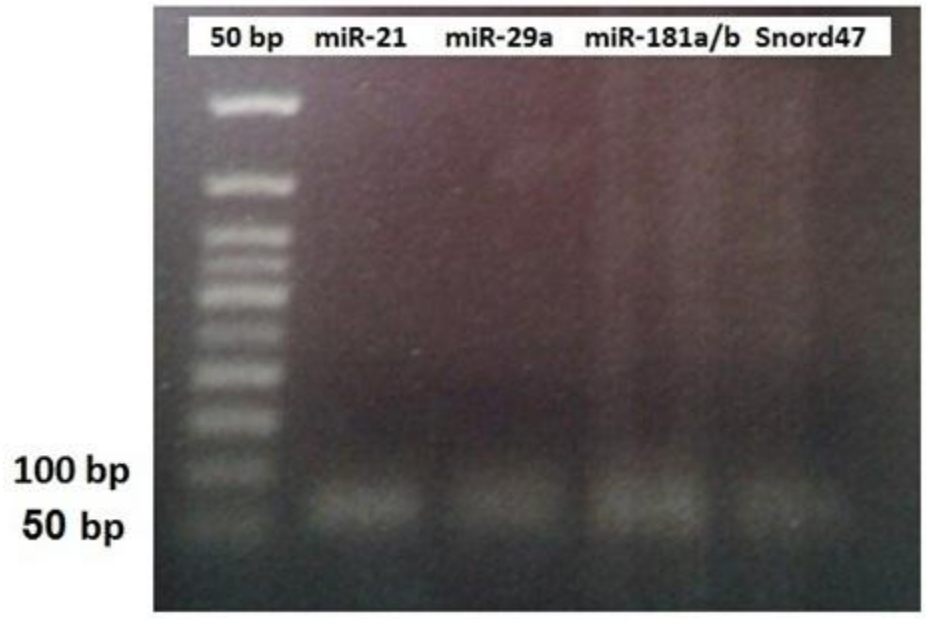

Figure 9.
Electrophoresis of Real Time PCR products. The product of each gene is in the range of 50-100 nucleotides, which confirms the expected size of 70-75 nucleotides.

Discussion

In general, umbilical cord hematopoietic stem cells that were exposed to bone marrow MDS microvesicles for 7 days, retained their originality. Also, the viability rate and their expression of microRNA-21 and microRNA-10a was increased. Although this evidence is not sufficient to name this cell a leukemic stem cell, it can certainly be said that it was influenced by MDS microvesicles. In this study, the ability of MDS bone marrow microcycles to make changes in healthy hematopoietic stem cells was investigated. Many in vivo studies of MDS and AML have shown that improper regulation of miRNAs may be a key factor in differentiation, increased apoptosis, and increased risk of disease progression.

Although many studies have identified a number of pathogenic miRNAs, their results are not adequate enough due to small sample size, inadequate control sample and low number of selected miRNAs. MRNAs with altered expression in MDS may be involved in autoimmunity, differentiation disorders, altered apoptosis, and increased proliferation.The effects of these changes on disease characteristics, such as HSC MDS role in bone marrow niche, impaired hematopoietic efficiency, and leukemic transformation, all require the expression of miRNAs at different stages of cell differentiation and disease development. Studying of the specific population of cells, such as HSCs and progenitors, seems to be the ideal method for studying the biology of MDS. Isolation of CD34+ cells alone is insufficient to understand specific changes in miRNA expression at different stages of MDS. In addition, miRNA dysregulation is also seen in the aging of the hematopoietic system, so the control sample used must be age-matched with patient.In a study published by Zhu et al[20], leukemic cell line K562 derived microvesicles were used and shown to be affect the umbilical cord, bone marrow, and peripheral blood mononuclear cells after 14 days and cause genetic instability. These cells were then transplanted into immunocompromised mice, and clinical manifestations similar to leukemia were observed, including splenomegaly, changes in blood index values, and cell morphology. According to these findings, healthy cells, under the influence of leukemic microvesicles, turn into cells that develop leukemia after transplantation in immunocompromised mice, which are called "leukemic-like cells." This group showed that patient’s cell’s microvesicles are able to affect healthy cells and change them. In comparison with their method, in this study, instead of K562 cell line, MDS bone marrow cells were used to prepare leukemic microvesicles.Also, healthy cells targeted by these microvesicles were selected directly from umbilical cord hematopoietic stem cells instead of peripheral blood, bone marrow and umbilical cord mononuclear cells.

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A study by Rademakers et al (2020). On bone marrow endothelium function and vascular permeability in BM[21]states that the consolidation of cross-vascular endothelial cadmium in vivo reduces bone marrow permeability, but does not prevent hematopoietic stem and stem cell migration to the bone marrow. This suggests that hematopoietic stem and stem cells use the intracellular migration pathway to enter the bone marrow.This study states that vascular endothelial-cadherin plays a vital role in BM vascular homeostasis but is very important for the home and stem cell formation of hematopoietic stem cells.

The findings of this study are important for improving the home-building strategies of hematopoietic stem and stem cells.

Zhu et al. observed the first results of genetic instability on day 14, while in this study, changes were examined on day 7, and results such as better survival of healthy cells and increased expression of microRNA-21 and microRNA-29a in these cells which were observed in culturing with MDS microvesicles.

However, during these changes, the colony forming ability of blood cells and the level of hematopoietic stem cell surface markers in healthy cells were preserved and not affected.

Increased microRNA-21 expression has been reported in almost all solid tumors such as colon, prostate, pancreatic, breast, lung, head and neck cancers[22]. This microRNA has also been shown to increase expression in blood malignancies including leukemia, lymphoma, and multiple myeloma[23, 24].

Another study on 540 human samples for six different types of tumors, including lung, breast, intestinal, prostate, stomach and pancreatic cancers, showed that among all selected microRNAs, microRNA-21 was increased in all cancers[25]. microRNA-21 targets genes involved in processes such as induction of angiogenesis, mutation, genetic instability, cell death resistance, invasion and metastasis. The most targeted microRNA-21 genes are the genes that control cell death and survival, and by reducing their expression, the cell acquires one of the most important characteristics of cancer, namely resistance to death. Some genes that play an important controlling role for the cell and are referred to as "tumor inhibitory genes" are also targeted by this microRNA, including PTEN and PDCD4 genes[26]. Decreased expression of these genes by increasing the expression of microRNA-21 leads the cell to become cancerous and therefore this microRNA can be included in the Oncomir group. In this study, the expression of microRNA-21 in hematopoietic stem cells that were exposed to MDS microvesicles for 7 days showed a 2.7-fold increase compared to the control group with no microvesicles, while its expression in the normal group did not show a significant change.In fact, MDS microvesicles were able to alter the expression of this key cancer microRNA in hematopoietic stem cells after 7 days, and since this was not seen in the other study groups, it can be concluded that this effect is due to MDS microvesicles. The role of microRNA-21 in cell survival has been investigated in many studies. In a study by Zhou et al., They found that inhibition of microRNA-21 expression in glioma cells inhibited their proliferation and, in turn, induced cell apoptosis by activating caspases 9 and 3 [27]. Also, a study by Smigielska-Czepiel et al. Showed that inhibition of microRNA-21 expression in memory T lymphocytes directly affects cell proliferation and survival and induces apoptosis in the cell[28]. One of the limitations of using mesenchymal stem cells in transplantation is the low survival of these cells in hypoxic conditions. Nie et al. Showed that induction of microRNA-21 expression in rat mesenchymal stem cells leads to better cell survival[29]. In 2011, Zhang et al. Showed that inhibition of microRNA-21 expression in renal cell carcinoma cells activates the enzyme caspase and induces apoptosis[30]. By comparing these results, it can be conducted that the rate of microRNA-21 expression and cell survival are directly related together. Cell survival improves as microRNA-21 expression increases, and cell survival decreases as microRNA-21 expression decreases. In this study, the survival rate of hematopoietic stem cells after 7 days of culture in different conditions was investigated. The number of surviving cells in the MDS group was higher than the other groups, which was also statistically significant. Thus, it can be concluded that MDS microvesicles also increased cell survival by increasing the expression of microRNA-21.

In 2020, Hrustincova et al. Conducted a study on 42 patients with myelodysplastic syndrome (MDS) and 17 healthy individuals to examine the expression profile of sncRNAs in healthy and healthy MDS patients[31].Hrustincova et al. Observed that many hematopoietic miRNAs, such as miR-34a, miR-125a, and miR-150, were significantly more pronounced in patients with MDS than in healthy individuals, and also showed that sncRNAsIn circulation follow certain patterns in MDS, and their expression changes in advanced stages. In 2006, Valenti et al. Showed that tumor cell microvesicles were able to make peripheral blood $CD14^{+}$ cells resistant to apoptosis[32]. It seems that tumor cells transfer one of their most important features, which is greater survival, to the target cell through microvesicles.

Thus, the increase in cell survival in the presence of leukemic microvesicles in this study, both due to the cancerous nature of the microvesicles and due to the increased expression of microRNA-21, is a result that is supported by both directions. In general, umbilical cord hematopoietic stem cells that were exposed to MDS bone marrow microvesicles for 7 days, were changed to the cells that still retained their renewal, but the rate of cell survival and expression of microRNA-21 and microRNA-10a was increased in them. Although this evidence is not enough to name this cell a leukemic stem cell, it can certainly be said that it was influenced by MDS microvesicles.

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Conflict of Interests

None declared.

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References

[1] Benkhadra, R., Nayfeh, T., Bakalov, V., Hilton, C., Khan, C., Prokop, L., Shah, M.V., Alkhateeb, H., Litzow, M.R., &Murad, M.H. (2020). Intensive chemotherapy in patients with high grade myelodysplastic syndromes (MDS): A systematic review and meta-analysis. *American Society of Clinical Oncology*.
[2] Cogle, C.R. (2015). Incidence and burden of the myelodysplastic syndromes. *Current hematologic malignancy reports*, *10*(3), 272-281.
[3] Buckstein, R. (2020). Epidemiology, Etiology, and Clinical Presentation of Myelodysplastic Syndromes. *In Diagnosis and Management of Myelodysplastic Syndromes*, *Springer, Cham*, 3-17.
[4] Ogawa, S. (2020). Genetic basis of myelodysplastic syndromes. *Proceedings of the Japan Academy, Series B*, *96*(3), 107-121.
[5] Badar, T., Szabo, A., Sallman, D., Komrojki, R., Lancet, J., Padron, E., & Hussaini, M.O. (2020). Interrogation of molecular profiles can help in differentiating between MDS and AML with MDS-related changes. *Leukemia & lymphoma*, *61*(6), 1418-1427.
[6] Wan, C., Wen, J., Huang, Y., Li, H., Wu, W., Xie, Q., & Liu, Z. (2020). Microarray analysis of differentially expressed microRNAs in myelodysplastic syndromes. *Medicine*, *99*(27).
[7] Saberinia, A., Alinezhad, A., Jafari, F., Soltany, S., & Sigari, R.A. (2020). Oncogenic miRNAs and target therapies in colorectal cancer. *Clinica Chimica Acta*.
[8] Kubasch, A.S., & Platzbecker, U. (2020). Hematopoietic Cell Transplantation for MDS Patients. In *Diagnosis and Management of Myelodysplastic Syndromes*, *Springer, Cham*, 165-173.
[9] Hofer, T., Rodewald, H.R., Busch, K., Fanti, A.K., Greco, A., Wang, X., & Morrison, S.J. (2020). Hematopoietic stem cells self-renew symmetrically or gradually proceed to differentiation. *bioRxiv*.
[10] Fenaux, P., Platzbecker, U., & Ades, L. (2020). How we manage adults with myelodysplastic syndrome. *British journal of haematology*, *189*(6), 1016-1027.
[11] Aly, R.M. (2020). Current state of stem cell-based therapies: An overview. *Stem cell investigation*, *7*.
[12] Méndez-Ferrer, S., Bonnet, D., Steensma, D.P., Hasserjian, R.P., Ghobrial, I.M., Gribben, J.G., & Krause, D.S. (2020). Bone marrow niches in haematological malignancies. *Nature Reviews Cancer*, *20*(5), 285-298.
[13] Montazer, F., &Alizadeh-Navaei, R. (2019). Expression of GLUT1 in Neoplastic Cells of Papillary Thyroid Cancer. *Turkish Journal of Oncology*, *34*(4), 243–247.
[14] Soltany, S., Abdolahpour, A., & Gholamalian, E. (2019). Investigating the effect of internal sphincterotomy on wound healing and postoperative pain after open hemorrhoidectomy. *International Journal of Pharmaceutical Research*, *11*, 848-854.
[15] Kokkaliaris, K.D., & Scadden, D.T. (2020). Cell interactions in the bone marrow microenvironment

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 3, 2021, Pages. 8208 - 8225
Received 16 February 2021; Accepted 08 March 2021.

affecting myeloid malignancies. *Blood advances*, *4*(15), 3795-3803.
[16] Meunier, M., Guttin, A., Ancelet, S., Laurin, D., Zannoni, J., Lefebvre, C., & Park, S. (2020). Extracellular vesicles from myelodysplastic mesenchymal stromal cells induce DNA damage and mutagenesis of hematopoietic stem cells through miRNA transfer. *Leukemia*, *34*(8), 2249-2253.
[17] Samii, A., & Razmkhah, F. (2020). Transformation of hematopoietic stem and progenitor cells by leukemia extracellular vesicles: a step toward leukemogenesis. *Stem Cell Reviews and Reports*, *16*, 1081-1091.
[18] Preciado, S., Muntión, S., & Sánchez-Guijo, F. (2021). Improving hematopoietic engraftment: Potential role of mesenchymal stromal cell-derived extracellular vesicles. *Stem Cells*, *39*(1), 26-32.
[19] Teodorescu, P., Pasca, S., Dima, D., Tomuleasa, C., & Ghiaur, G. (2020). Targeting the Microenvironment in MDS: The Final Frontier. *Frontiers in Pharmacology*, *11*, 1044.
[20] Zhu, X., You, Y., Li, Q., Zeng, C., Fu, F., Guo, A., & Chen, Z. (2014). BCR-ABL1–positive microvesicles transform normal hematopoietic transplants through genomic instability: Implications for donor cell leukemia. *Leukemia*, *28*(8), 1666-1675.
[21] Rademakers, T., Goedhart, M., Hoogenboezem, M., Ponce, A.G., Van Rijssel, J., Samus, M., Schnoor, M., Butz, S., Huveneers, S., Vestweber, D. (2018). Hematopoietic Stem and Progenitor Cells Use Podosomes to Transcellularly Home to the Bone Marrow. *bioRxiv*, 269704.
[22] Bautista-Sánchez, D., Arriaga-Canon, C., Pedroza-Torres, A., De La Rosa-Velázquez, I.A., González-Barrios, R., Contreras-Espinosa, L., & Herrera, L.A. (2020). The promising role of miR-21 as a cancer biomarker and its importance in RNA-based therapeutics. *Molecular Therapy-Nucleic Acids*, *20*, 409-420.
[23] Han, Z., Rosen, S.T., & Querfeld, C. (2020). Targeting microRNA in hematologic malignancies. *Current Opinion in Oncology*, *32*(5), 535-544.
[24] Sun, X.Q., Bi, F.Q., & Cui, X. (2020). Reaserch Progress on Non-Coding RNA in Multiple Myeloma--Review. *Zhongguo shi yan xue ye xue za zhi*, *28*(2), 713-716.
[25] Volinia, S., Calin, G.A., Liu, C.G., Ambs, S., Cimmino, A., Petrocca, F., & Croce, C.M. (2006). A microRNA expression signature of human solid tumors defines cancer gene targets. *Proceedings of the National Academy of Sciences*, *103*(7), 2257-2261.
[26] Wu, Y., Song, Y., Xiong, Y., Wang, X., Xu, K., Han, B., & Zhou, L. (2017). MicroRNA-21 (Mir-21) promotes cell growth and invasion by repressing tumor suppressor PTEN in colorectal cancer. *Cellular Physiology and Biochemistry*, *43*(3), 945-958.
[27] Zhou, X., Zhang, J., Jia, Q., Ren, Y., Wang, Y., Shi, L., & Kang, C. (2010). Reduction of miR-21 induces glioma cell apoptosis via activating caspase 9 and 3. *Oncology reports*, *24*(1), 195-201.
[28] Smigielska-Czepiel, K., Van Den Berg, A., Jellema, P., Slezak-Prochazka, I., Maat, H., Van Den Bos, H., & Kroesen, B.J. (2013). Dual role of miR-21 in CD4+ T-cells: activation-induced miR-21 supports survival of memory T-cells and regulates CCR7 expression in naive T-cells. *PloS one*, *8*(10), e76217.
[29] Nie, Y., Han, B.M., Liu, X.B., Yang, J.J., Wang, F., Cong, X.F., & Chen, X. (2011). Identification of MicroRNAs involved in hypoxia-and serum deprivation-induced apoptosis in mesenchymal stem cells. *International journal of biological sciences*, *7*(6), 762-768.
[30] Zhang, A., Liu, Y., Shen, Y., Xu, Y., & Li, X. (2011). miR-21 modulates cell apoptosis by targeting multiple genes in renal cell carcinoma. *Urology*, *78*(2), 474-e13.
[31] Hrustincova, A., Krejcik, Z., Kundrat, D., Szikszai, K., Belickova, M., Pecherkova, P., & Merkerova, M. D. (2020). Circulating small noncoding RNAs have specific expression patterns in plasma and extracellular vesicles in myelodysplastic syndromes and are predictive of patient outcome. *Cells*, *9*(4), 794.
[32] Valenti, R., Huber, V., Filipazzi, P., Pilla, L., Sovena, G., Villa, A., & Rivoltini, L. (2006). Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-β–mediated suppressive activity on T lymphocytes. *Cancer research*, *66*(18), 9290-9298.