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ORIGINAL ARTICLE

The Effect of Erythropoietin Treatment on Gene Expression Profile of Mesenchymal Stem Cells

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ABSTRACT

Introduction: Mesenchymal Stem Cells are one of the most important elements of bone marrow microenvironment, which has a role in stromal support and stem cell differentiation. Exosomes are small vesicles that responsible from various cellular roles such as cell-cell communication and cell signaling, which may affect nearby and distant cells/tissues. Mesenchymal Stem Cells have therapeutic importance because of their multipotency and immune modulation potentials also with their exosomes. Erythropoietin, produced by liver hepatocytes, is responsible for erythroid differentiation (erythropoiesis) in bone marrow. In addition, Erythropoietin treatment of several cell types including Mesenchymal Stem Cells, showed therapeutic effects in various diseases.

Objective: The aim of this study is to examine the effect of erythropoietin on bone marrow Mesenchymal Stem Cells transcriptome and exosome derived miRNA profile.

Materials and Methods: Effect of 3 different doses of Erythropoietin (1 IU/ml, 10 IU/ml and 100 IU/ml) for 48 hours on Mesenchymal Stem Cells transcriptome profile was analyzed. The results illustrated that 10 IU/ml Erythropoietin treatment has the most effective concentration in terms of gene expression profile. Therefore, small RNA libraries targeting miRNA was analyzed with 10 IU/ml Erythropoietin treated versus non treated groups with next generation sequencing.

Results: We found that Erythropoietin treatment slightly changed global gene expression profile. On the other hand, it was observed that Erythropoietin treated Mesenchymal Stem Cells have different exosomal miRNA profile.

Conclusion: Differentially expressed exosomal miRNAs may have therapeutic effects in different conditions. It will be important to perform further studies with in vitro models, mimicking different physiological conditions and diseases for Mesenchymal Stem Cells and exosome biology.

Key words: Mesenchymal stem cells (MSCs), erythropoietin (Epo), transcriptomics, exosome, miRNA.

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INTRODUCTION

Mesenchymal Stem Cells (MSCs), which have multipotent character, have the capacity to differentiate into mesoderm-derived bone, fat, cartilage and muscle cells [1]. MSCs were positive for surface markers CD90, CD73, and CD105 and negative for CD34, CD45, and CD14 [1]. MSCs are one of the most main members of the bone marrow microenvironment. Not only does it support stromal support, but it also affects stem cell fate, such as differentiation, proliferation and staying quiescent with secreted cytokines [2]. Not only the MSCs themselves but also the molecules they secrete provide a therapeutic effect [3]. One of the most important biological tools that have this effect are vesicles which called

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extracellular vesicles [3]. These vesicles include exosomes and microvesicles. Exosomes are small vesicles of 50-100 nm size [4]. They are involved in cell-cell communication and cell signaling. This mechanism affects the metabolism of nearby and distant cells or tissues [3]. The exosomes are also rich and varied in terms of content and can carry many types of molecules such as growth factors, signal lipids, mRNAs and miRNAs [5]. In previous studies, MSC-derived exosomes have been analyzed in many different diseases and tissues, and it has been observed that it supports the proliferation and migration of tissue-specific stem cells that accelerates the healing of scar tissues, protects various tissues and decreases the inflammatory effect, protects against apoptosis and crosses the blood brain barrier [6-8].

Transplantation of exosomes, which are the most important elements of MSCs to produce therapeutic effects, has the potential to be a safe option. It is thought that there is a direct relationship between the effect and content of exosomes. They provide a therapeutic effect with mRNA and miRNAs involved in gene expression control. [9]. In this way, it is thought that the therapeutic effect of MSCs can be mimicked by exosomes, avoiding the risks of transplanting MSCs [10].

Human erythropoietin (Epo) hormone is a glycosylated protein weighing 30.4 kDa [11]. Epo is produced by liver hepatocytes during embryonic development. It is produced by peritubular capillary endothelial cells in the liver and kidney in the adult period after birth [12]. However, peritubular fibroblast-like cells in the cortex of the kidney are responsible for the production of mostly large parts of the kidney [12]. Epo gene encoding erythropoietin is found in chromosome 7q22 region [13], and has an oxygen-dependent expression [12]. Epo, a glycoprotein hormone, allows the differentiation of hematopoietic stem cells in the bone marrow into erythroid precursor cells (erythropoiesis) [14]. This function is stimulated through the receptor called EpoR.

In previous studies, it was observed that different cell types such as neuron, endothelium, and cardiomyocytes were started to express EpoR as a result of treatment with Epo [12]. This suggests that Epo is also effective in addition to erythropoiesis. Although the role of Epo in erythropoiesis is the most well-known, it has been observed in the studies conducted that it also has an effect on hematopoietic cells, macrophages and endothelial cells [15]. It has also been shown in studies that Epo has an effect on neural, muscle and cardiovascular tissues. For example, by the effects of increasing the expression of antioxidant enzymes and reducing the release of free radicals, the brain also shows neutrophic and neuron protective properties [16].

Various Epo doses have been tried in different disease models and various effects have been observed in many studies [17-19]. The study by Wang et al. in 2015, it was thought that the Epo treatment on MSCs that help protect kidney function from chronic kidney diseases will increase their effect [17]. This positive result was thought to be related to the load of microvesicles released from MSCs. The effect of Epo on these microvesicles and their protective function in chronic kidney diseases were investigated [17]. Microvesicles obtained from MSCs that were treated with Epo at different concentrations (1, 10, 100, and 500 IU/ml), and microvesicles obtained from MSCs that are not treated with Epo were used in the treatment of kidney damage, both in vivo and in vitro [17]. In the dose range of 1-100 IU/ml Epo, a dose-dependent increase in the microvesicles was observed. Microvesicles obtained from MSCs treated with Epo have been shown to have a superior protective effect, particularly in fibrosis-associated kidney injury [17]. In a microarray study to examine the miRNA profile, it was observed that the expression of 212 miRNA was increased. It has been shown that the changes have a protective effect on renal function.

Treatment of mesenchymal stem cells with erythropoietin has been studied by many researchers and has been shown to increase the therapeutic effect. However, these studies were limited because they examined the effect on specific genes. In this study, the alteration of the gene expression profile of mesenchymal stem cells caused by erythropoietin will be examined with a more detailed and broad perspective by revealing the transcriptome profile, and exosomal miRNAs.

MATERIALS and METHODS

Cell Culture and Erythropoietin (Epo) Treatment

Human bone marrow derived mesenchymal stem cells (BM-MSCs) were purchased from American Type Culture Collection (ATCC, Cat. No: PCS-500-012TM, Lot No: 63208778), which were obtained from 24 years old Caucasian male bone marrow donor. BM-MSCs were cultured in DMEM-LG (supplemented with 10% FBS, 1% penicillin-streptomycin and 1% l-glutamine) and 37^{0}C, 5% CO_2. Culture medium was changed twice a week; passage 3 cells

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were used for further experiments.

MSCs were seeded on a 6-well cell culture plates with density of 40.000 cells per cm^2. After reaching 70-80% confluency 1, 10 and 100 IU/ml Erythropoietin (Epo) (Prospec Cyt-325) was added into culture medium. All samples were seeded in triplicates and incubated for 48 hours at 37^0C, 5% CO_2. Culture medium was collected for exosome isolation. For exosome studies, culture medium was prepared with exosome-depleted FBS (Thermo Fisher Scientific).

Total RNA, Exosome and Exosomal miRNA Isolation

Culture medium was removed (collected for exosome isolation), cells were washed with PBS, 1 ml TRIzol (Sigma) was added and RNA was isolated according to the manufacturer's instructions. RNA quality and quantity were measured with spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific).

Culture medium with 10 IU/ml Epo was collected and exosome isolation was performed with Total Exosome Isolation Solution (Cat. No: 4478359, Thermo Fisher Scientific) according to the manufacturer's instructions. Exosome pellet was resuspended in PBS and kept at -20^0C for further experiments. miRNA was isolated with Total Exosome RNA and Protein Kit (Cat. No: 4478545, Thermo Fisher Scientific) according to the manufacturer's instructions with small RNA enrichment protocol. miRNA pellet was resuspended in 15 µl TE buffer. These experiments were carried out in three biological replicates and the samples were continued by combining after the exosome isolation.

Quantitative Transcriptomics and Exosomal miRNA Profiling

cDNA was synthesized from 10 µl of each RNA samples with VILO Superscript cDNA synthesis Kit (Thermo Fisher Scientific). The libraries were prepared by using the Ampliseq Transcriptome Human Gene Expression kit (Thermo Fisher Scientific) and the Ion Chef instrument (Thermo Fisher Scientific). All samples were studied in duplicates. On the other hand, for exosomal miRNA profiling, Ion Total RNA-Seq Kit (version 2) was used for library preparation.

After library preparation, clonal amplification was performed with Ion PI Hi-Q OT2 200 kit (Thermo Fisher Scientific). The next generation sequencing reaction was performed on the Ion Proton device (Thermo Fisher Scientific) by using the Ion PI Hi-Q Sequencing 200 Kit (Thermo Fisher Scientific). For data analysis, all subsequent reads were normalized by the "read per millions" method and experimental groups were analyzed comparatively. Reads were mapped to the human genome assembly hg19. Differentially expressed genes (DEG) were determined with iDEP, fold changes $\log_2 2$ (4-fold) and above were considered as significant and FDR cut-off was 0.1.

RESULTS

Epo Treatment Affect the Gene Expression Profile of MSCs

MSCs were treated with 3 different concentrations (1 IU/ml, 10 IU/ml and 100 IU/ml) of Epo. Epo-treated and untreated MSCs were comparatively analyzed, 10 IU/ml Epo treated has the most DEG numbers (Figure 1). Gene expression changes (at least 4-fold and above) which are dependent on 10 IU/ml Epo treatment are listed in Tables 1 and 2. In this analysis, it was shown that the expression of 82 genes increased and the expression of 86 genes decreased as a result of 10 IU/ml Epo treatment. Therefore, this concentration (10 IU/ml) was selected for further experiments.

Figure 1: Analysis results of Epo-treated and untreated MSCs.Differentially expressed gene numbers (DEGs) of Epo treated groups with respect to control MSCs.

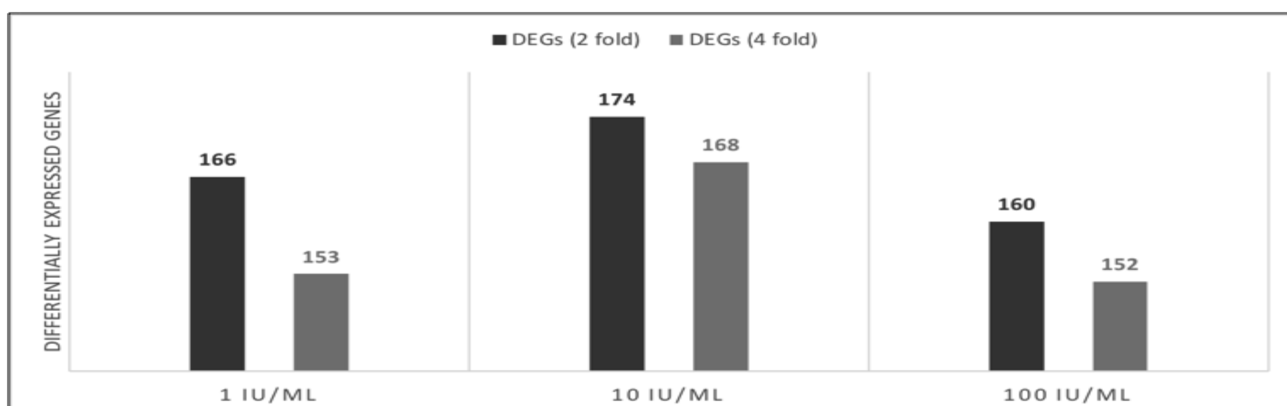

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Table 1: Log$_2$2 (4-fold) increased gene list after 10 IU/ml Epo treatment. Genes were listed alphabetically.

AA06	FAM159B	IL3	NFATC4	SH2D3C
ACTL9	FAM180B	IL31RA	NOXRED1	SLC15A2
ADRA2B	FAM222A	IQSEC3	NPC1L1	SLC18A3
BHLHA15	FAM71A	KIAA1804	NR2E3	SLC6A4
BPI	FBP1	KIF12	OR10V1	SNX10
C12ORF70	FGF11	KRT37	PANX2	STAR
C12ORF77	FGF12	KYNU	PDZD2	TMED6
C3ORF45	FGFBP3	LCN2	PDZD7	TMLHE-AS1
C8G	FLJ34503	LEKR1	PKLR	TRIML2
CPA6	FRAS1	LINC00111	PLA2G2A	UTS2R
DCSTAMP	GFI1	LINC00176	PRLR	VANGL2
DLEC1	GOLGA7B	LINC00547	PSG1	WDR86
DLG3	HAPLN4	LRRC61	RAB40A	WEE2
DLL3	HMP19	LUZP2	RASAL3	XIRP1
DLL4	HRASLS	NACA2	RFPL3	ZBP1
EEF1A2	IGF1	NEK5	RINL	ZMAT1
ERVMER34-1	IL27			

Table 2: Log$_2$2 (4-fold) decreased gene list after 10 IU/ml Epo treatment. Genes were listed alphabetically.

ALPK2	CDH3	KEL	NR0B1	SLAMF8
ANGPT2	CDK18	KRTAP5-11	NR6A1	SLC13A5
ANO2	CHST13	LILRA5	OR5AP2	SPRYD4
ANXA8	CKMT1A	LOC100133669	PARD6A	ST6GALNAC5
APOBEC3A	COLEC10	LOC100499183	PCDH9	SVOPL
ARHGAP15	CYP21A2	LOC100507034	PI3	SYT2
ARID3C	DCAF8L2	LOC255130	PIP5K1B	TBXAS1
ATAD3C	DOK6	LOC283050	PRDX1	TFF2
ATRNL1	DRD4	LOC387895	PRM3	TMEFF2
BEST1	DTX4	LOC402160	PXDNL	TMEM163
BIN2	FAM162B	LRG1	RAB24	TMEM244
C21ORF62	GIP	MATK	RAD51B	TMPRSS9
CA5A	GUCY1A2	MRGPRE	RHCE	TRIM7
CACNG7	HEATR4	MTMR8	SAA2	UGT8
CADPS	HLA-J	MYF6	SEMA4D	UPK1B
CARNS1	IL20RA	NOD2	SERPINB3	VNN2
CCL4	ILDR2	NOSTRIN	SFMBT2	ZNF219
CDC42BPG				

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Altered Exosomal miRNA Profile of MSCs After Epo Treatment

We hypothesized that the Erythropoietin might influence the exosomal contents of MSCs. We analyzed the exosomal miRNAs in MSCs before and after Epo treatment. We found critical changes in miRNA profile. As shown in Table 3, some of the miRNAs were only found in Epo (10 IU/ml) treated exosomes, but not in untreated group. It was noted that although 4 miRNAs were present in the control group, they were not in exosomes as a result of Epo treatment (Table 4). However, after Epo treatment, there was a decrease in 14 miRNA and an increase in 2 miRNAs compared to exosomes isolated from untreated group (Table 5).

Table 3: Exosome related miRNAs after 10 IU/ml Epo treatment.

miRNA	miRNA Sequence	Read Count (Control)	Read Count (10 IU/ml Epo)
hsa-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG	0	151
hsa-miR-140-5p	CAGUGGUUUUACCCUAUGGUAG	0	123
hsa-miR-99a-5p	AACCCGUAGAUCCGAUCUUGUG	0	107
hsa-miR-199a-3p	ACAGUAGUCUGCACAUUGGUUA	0	86
hsa-miR-199b-3p	ACAGUAGUCUGCACAUUGGUUA	0	86
hsa-miR-27b-3p	UUCACAGUGGCUAAGUUCUGC	0	64
hsa-miR-455-5p	UAUGUGCCUUUGGACUACAUCG	0	46
hsa-miR-376c-3p	AACAUAGAGGAAAUUCCACGU	0	45
hsa-miR-423-3p	AGCUCGGUCUGAGGCCCCUCAGU	0	44
hsa-miR-574-3p	CACGCUCAUGCACACACCCACA	0	43
hsa-miR-93-5p	CAAAGUGCUGUUCGUGCAGGUAG	0	37
hsa-miR-485-3p	GUCAUACACGGCUCUCCUCUCU	0	33
hsa-miR-574-5p	UGAGUGUGUGUGUGUGAGUGUGU	0	30
hsa-let-7a-5p	UGAGGUAGUAGGUUGUAUAGUU	0	28
hsa-let-7g-5p	UGAGGUAGUAGUUUGUACAGUU	0	24
hsa-let-7c-5p	UGAGGUAGUAGGUUGUAUGGUU	0	23
hsa-miR-199a-5p	CCCAGUGUUCAGACUACCUGUUC	0	22
hsa-let-7f-5p	UGAGGUAGUAGAUUGUAUAGUU	0	15
hsa-miR-126-3p	UCGUACCGUGAGUAAUAAUGCG	0	14

Table 4: Exosome related miRNAs after 10 IU/ml Epo treatment.

miRNA	miRNA Sequence	Read Count (Control)	Read Count (10 IU/ml Epo)
hsa-miR-19a-3p	UGUGCAAAUCUAUGCAAAACUGA	321	0
hsa-miR-103a-3p	AGCAGCAUUGUACAGGGCUAUGA	273	0
hsa-let-7i-5p	UGAGGUAGUAGUUUGUGCUGUU	241	0
hsa-miR-151a-5p	UCGAGGAGCUCACAGUCUAGU	114	0

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Table 5: Altered miRNAs depending on 10 IU/ml Epo treatment.

miRNA	miRNA Sequence	Read Count (Control)	Read Count (10 IU/ml Epo)
hsa-miR-145-5p	GUCCAGUUUUCCCAGGAAUCCCU	2232	51
hsa-miR-24-3p	UGGCUCAGUUCAGCAGGAACAG	2066	230
hsa-miR-23a-3p	AUCACAUUGCCAGGGAUUUCC	2008	119
hsa-miR-195-5p	UAGCAGCACAGAAAUAUUGGC	1335	101
hsa-miR-26a-5p	UUCAAGUAAUCCAGGAUAGGCU	1033	43
hsa-miR-10b-5p	UACCCUGUAGAACCGAAUUUGUG	930	7
hsa-miR-214-3p	ACAGCAGGCACAGACAGGCAGU	804	62
hsa-miR-140-3p	UACCACAGGGUAGAACCACGG	795	266
hsa-let-7b-5p	UGAGGUAGUAGGUUGUGUGGUU	473	118
hsa-miR-125b-5p	UCCCUGAGACCCUAACUUGUGA	322	17
hsa-miR-320a-3p	AAAAGCUGGGUUGAGAGGGCGA	321	77
hsa-miR-143-3p	UGAGAUGAAGCACUGUAGCUC	278	174
hsa-miR-29a-3p	UAGCACCAUCUGAAAUCGGUUA	262	1
hsa-miR-451a	AAACCGUUACCAUUACUGAGUU	256	3
hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA	17	59
hsa-miR-101-3p	UACAGUACUGUGAUAACUGAA	2	61

DISCUSSION

In this study, the effects of erythropoietin on MSCs from bone marrow were investigated at the level of gene expression. Transcriptome and exosome-derived miRNA profiles of Epo treated MSCs were investigated using the next generation sequencing method. It is important to clarify the content of these exosomes, as it is shown in previous studies that MSC-induced exosomes may have a therapeutic potential [3-5].

According to the previous studies, 3 different Epo doses were selected [17, 20]. In the first part of the study, the effect of 48 hours of Epo treatment on 3 different concentrations (1 IU/ml, 10 IU/ml and 100 IU/ml) of Epo transcriptome profile was investigated. Expression changes were generally found to be of low copy transcripts. In other words, selected Epo concentrations result in minimal transcriptomic differences in MSCs at 48 hours of incubation. According to comparative gene expression analysis, 10 IU/ml Epo treatment has found to be the highest number of DEGs. Therefore, in the second part of the study, this concentration (10 IU/ml) was used to investigate the effect of Epo treatment on miRNA expression originated from exosomes. In order to investigate the effect of Epo treatment on the miRNA profile in exosomes of MSCs, exosome isolation and subsequent RNA isolation from MSCs cultured with Epo at a concentration of 10 IU/ml for 48 hours were performed. By using next generation sequencing method, small RNA libraries targeting "miRNAs" were investigated under Epo treatment. The comparative analysis revealed a change in the number of several different miRNAs. Nineteen miRNAs were found to be present in the exosomes due to Epo treatment, but not in the control samples (Table 3), and it was noted that although 4 miRNAs were present in the control group, they were not in exosomes as a result of Epo treatment (Table 4). However, the reading number of 14 miRNAs decreased due to Epo, while 2 miRNAs increased (Table 5).

As a result of these analyzes, it is noteworthy that changes, especially in more than one element of the Let-7 miRNA family, were observed (Table 3). This miRNA family has different tasks and targets in various tissues and cells. Let-7e-5p and Let-7f-5p found in MSC originated from exosomes as a result of Epo treatment are known to support angiogenesis [12, 15]. Let-7g-5p that are detected in the MSC-exosomes due to the Epo effect has been shown to be negatively regulating events such as angiogenesis, inflammation, migration, and senescence [21]. Let-7i-5p, which is not found in exosomes originated

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in MSC as a result of Epo treatment positively affects its expression by bonding to TATA motive in the promotor region of IL-2, which triggers inflammation in CD4 positive T cells [22]. However, it may also act to trigger the immune system by a change in the microenvironment caused by disease and/or the cell type it is in. In another study, Let-7i-5p was reported to suppress apoptosis via FASL inhibition [23]. Let-7b-5p, which was found to decrease as a post-Epo cargo, has been shown to positively affect angiogenesis in the heart by targeting TGFBR1 in the endothelial cells [24]. When Let-7 miRNA family members with an increase/decrease in the exosomes content are examined, it can be said that a result of Epo treatment results in a miRNA profile in favor of angiogenesis.

Some of the exosomes of miRNAs are associated with tumor biology. It has been shown that miR-140-5p, which begins to be transported on exosomes due to Epo treatment, plays a role as a tumor suppressor [25]. Another miRNA with similar effects and gets triggered with Epo treatment is miR16-5p. It supports apoptosis by targeting Bcl2. Another miRNA cargo associated with cancer is miR-99a-5β detected in exosomes due to Epo treatment. This molecule has been shown to increase miR-99a-5p expression of leukemia stem cells to resist chemotherapy in acute myeloid leukemia (AML) and has the potential to become a biomarker for AML [26]. miR-485-3p, found in exosomes as a result of Epo treatment, controls the expression of DNA topoisomerase 2 molecule, which is targeted by many chemotherapy drugs, in the negative direction by targeting Nuclear transcription factor Y subunit beta (NF-YB). Decreased miR-485-3p expression in lymphoblastic leukemia is a mechanism used to provide drug resistance [27].

Anti-tumor effects of the molecules, in whose miRNA profiles a reduction of the exosomes was observed (miR-10b-5p, miR-145-5p, miR-140, miR195-5p, miR29a-3p, mir-26a-5p, and miR-451), has been reported. It has been shown that these molecules have negative effects on cell proliferation, invasion, and migration while having apoptosis-promoting effects. [28-34]. Contribution to tumor development may be shown as a common feature of miRNAs, which are not included in the exosomes as a result of Epo treatment (miR151a-5p, miR-103a-3p, miR-19a-3p). These miRNAs act in favor of tumor development by suppressing molecules that negatively affect tumor growth. For this reason, in many types of cancers, increased expressions are found [35-37].

Some of the miRNAs detected in the study are associated with the immune system. It is noteworthy that miR-19-3p, which is not involved in exosomes as a result of Epo treatment, is involved in communication between the natural and acquired immune system. It exhibits immunosuppressive properties by targeting the 5-Lipoxygenase (5-LO) enzyme produced by myeloid cells, responsible for the synthesis of leukotriene. But if miR-19a-3p and miR-125b-5p expressions are inhibited, it indicates the potential for initiation of inflammation if the myeloid cells are present in the target area [38]. mir-19-3p works to reduce tumor development and invasion in breast cancer. By reducing the expression of Fos-related antigen-1 (Fra-1), negatively affects the anti-inflammatory M2 macrophage phenotype and polarization. In this way, it activates the immune system to fight against breast cancer [37].

Among the detected miRNA molecules, those associated with stem cell biology and regeneration were also noted. miR-26a-5p has been shown to target iNOS and suppress expression in human cartilage cells [30]. In terms of this mechanism, the effect of Epo on pathological conditions such as osteoarthritis is worth examining. miR-199a-3p is a molecule that is detected in exosomes in the presence of Epo and stands out due to its function. This miRNA, whose expression is controlled by p53, allows the heart muscle cells that exited the cell cycle in the adult period, to re-enter the cell cycle [39]. It not only does help the heart muscle cells avoid apoptosis, but also supports their proliferation [40]. In this way, it has great potential to repair the damage in the heart, which cannot be regenerated, caused by heart attack or other reasons.

What makes this study original is the fact that miRNA content of exosomes collected by treatment of human bone marrow-derived MSCs with Epo is analyzed using the next generation sequencing technology. In addition, the exosome-specific miRNA analysis and the usage of next generation sequencing resulted in highly reliable and sensitive results. Although minimal amount of changes was detected at the transcriptomic level in the earlier studies, the fact that the expression change in miRNA in the exosome cargo was determined is critical in terms of the targets of the miRNA originated in exosomes. The fact that miRNAs with changes in exosomal cargo were found to play a role especially

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in critical biological diseases and pathways, such as cancer, angiogenesis, cell migration/invasion, are critical for the widespread influence of the data obtained in this study. The therapeutic potential of the detected miRNAs will shed light on the interdisciplinary studies that may occur in the future.

The potentials of the miRNAs identified in this study may be used as biomarkers in various experimental/pathological conditions should also be investigated and their specific functions should be elucidated in different cell/tissue models. This study demonstrates how the exosomal cargo of MSCs treated with Epo affects the miRNA content. However, it is another issue that needs to be elucidated about how this effect affects the content of exosomal cargo at the level of proteomics and metabolomes.

Considering the biological functions of MSCs such as immunomodulation and the function of supporting cells in the bone marrow niche, it is critical to reveal the contents of exosomes. Research in in vitro systems that mimic different physiological conditions and disease models will be important for MSC and exosome biology.

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CONFLICT of INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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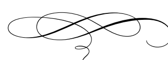

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