Original Article:

Evaluation of CXCR4, VLA4, and VLA5 expression in peripheral mobilized hematopoietic stem cells in presence of Sotalol

Fatemeh Taghavirad [1, 2] , Mohsen Hamidpour [1,*], Masoud Soleimani [3], Abass Hjifathali [4]

[1]Department of labratory Hematology and Blood Bank, School of Allied Medical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2]Royan Cord Blood Bank, Tehran, Iran
[3]Department of labratory Hematology, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[4]Taleghani Bone Marrow Transplantation Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

[*]Corresponding author: e-mail: mohsenhp@sbmu.ac.ir (M. Hamidpour)

ABSTRACT

Hematopoietic stem cells (HSC), are considered as an important source for HSCT transplantation. There are several regulators for stem cells migration and homing, among which the nervous system is an important one. This study is going to investigate the role of nervous system in homing of hematopoietic stem cells.
In an experimental study, mononuclear cells were isolated from peripheral blood and treated by a beta adrenergic receptors blocker (Sotalol). After treating the cells with different concentrations 0.5- 500 (mM/ml) for 1- 24 hours, RNA was extracted and the expression of VLA4, VLA5 and CXCR4 genes were determinated through RT-PCR method.The results of this study demonstrated high expression in genes associated with homing of the cells after being treated with drug for 1 hour: CXCR4 gene expression increases in 10 and 50 mM/ml, VLA4 gene expression in 50mM/ml and VLA5 gene expression in 10 mM/ml concentration of Sotalol.Using beta adrenergic receptors blockers in appropriate time and dose of drug can affect the expression of genes which involved in HSCs homing and will lead to high success rate of transplantation of these cells.

Key words: Hematopoietic Stem Cells; CXCR4; VLA4; VLA5; Sotalol

INTRODUCTION

Recently, HSCs have been widely used to treat many diseases including bone marrow failure. One of the bone marrow transplantation applications is recovery (remodeling) after chemotherapy and radiotherapy [1-4] . Bone marrow repopulation through HSCs can be mentioned as one of the important properties of these cells. In order for this repopulation to happen, HSCs respond to signals from their environment or even far organs to regulate their movement, location and position in cell cycle. These regulations include changes in level of cytokines, cell receptors, adhesive molecules and cytoskeleton remodeling, and finally, in migration, cell growth and development and their transforming from silence to active form[5]. One of the HSCs regulators is nervous system. This system acts directly in developement of HSCs and mobility through neurotransmitter extraction and indirectly in bone remodeling process through myeloid cytokines [6-8]. Neurotransmitters will be induced via second messenger activity through interaction with G-proteins receptors [9-10]. B2 Adrenergic receptors are expressed on osteoblasts and hematopoietic stem cells surface, while B3 Adrenergic receptors are expressed on bone marrow stromal cells surface[9]. These cells activate the signaling pathway and consequently increase B-Catenin in cells. This signaling pathway beside Notch and Hedgehog play an important role in bone marrow function and act as an intermediate for neurotransmitter effect on HSCs [10-13]. Neurotransmitters play three important roles in bone marrow functions as: proliferation, mobility and repopulation. It has been identified that among

current and available transplantations in bone marrow niche, SDF1 interaction with its receptor (CXCR4) produce signals which play important roles in motility bone marrow progenitor cells. Recent studies demonstrated that using neurotransmitters such as epinephrine, increase expression of CXCR4 in a logarithmic pattern and homing of hematopoietic Stem Cells in bone marrow niches is a fast process which happens in only few hours, in which HSCs of peripheral bloods are transferred across the endothelium. This transmission requires interactions between HSCs and different adhesive molecules. P/E selections should be available, but for more attachment, integrins are required, such as: CD11a/CD18,Very Late Antigen4 (VLA-4) and Very Late Antigen 5(VLA-5). There are many studies showing the VLA4 and VLA5 importance in HSCs migrations [14-16]. Homing of hematopoietic Stem Cells in bone marrow niches is a fast process which happens in only few hours, through which HSCs of peripheral bloods are transferred across the endothelium. This transmission requires interactions between HSCs and different adhesive molecules. P/E selections should be available, but for more attachment, more integrins are required too, such as: CD11a/CD18 and VLA 4 and VLD 5 that play importance roles in HSCs migrations [17-19]. The aim of the current study is to investigate nervous system regulatory effect on bone marrow functions and effect of HSCs Beta adrenergic receptors blockers on genes related to these cells homing in bone marrow niches. This study used Sotalol as a Beta adrenergic receptors blocker.

MATERIALS AND METHODS

HSCs Isolation

The sample (HSCs) was collected from peripheral blood of a normal volunteer, who had taken G-CSF single dose. Before sampling, the participant gave informed consent in accordance with the Deceleration of Helsinki. The mononuclear cells (including HSCs) were isolated by gradient centrifuging using Ficol-Hypaquegradient (Inno-train).

Colony forming assay

In order to find out cells colony forming property, 10^4 cells per milliliter were counted and the suspense in 200 micL PBS. 1.8 mL of methyl cellulose medium (Stemtech,USA) was added to cells. Then, this combination was added to 6 wells' plates and well vortexed. The plates were incubated in 37°c and 5% CO2 for 14 days. Then the number and type of colonies were analyzed.

Flowcytometry analysis

Cells were stained with CD34PE/CD45-FITC (BD, USA) and HSCs with (CD34+/CD45 dim) were analyzed (Partec).

Treating cells with Sotalol

Samples were stained by Trypan blue and counted using Hemocytometer chamber. The cells treated by Sotalol (FARABI) in 0.5, 1, 10, 50, 100, 300 and 500 µmol concentrations in plates. A plate with 12 wells containing IMDEM (sigma) medium with 10% FBS were used. Then different concentrations of drug including 0.5,1, 10, 50, 100, 300 and 500 µmol/ml were added to cells and gene expression pattern was analyzed in 1, 3, 5 and 24 hours.

mRNA extraction and cDNA synthesis

Total RNA was extracted by TRIZOL (QIAZOL Lysis Reagent.USA). At the first stage, cells were digested by Trizol and then 200 µl Chloroform and Isoamyl alcohol (1:49) were added and centrifuged at 1200 g for 20 minutes in 4°C. Upper layer liquid (contains RNA) was separated; Isopropanol was added for the purpose of getting pellet and washed by 75% alcohol and RNA pellet was allowed to get dried at room temperature. The pelt resolved in 20 µl DEPC Buffer. RNA was qualified by agarose gel electrophoresis and Nanodrop (Nanodrop2000 spectrophotometer). cDNA synthesis was done through Thermo Scientific kit method.

Real time PCR

Real-time PCR primers were designed for CXCr4, VLA4, VLA5 and Abelson murine leukemia viral oncogene homolog 1 (ABL, as an internal control or housekeeping gene), using Gene Runner (version 3) software. Primer sequences for amplifying cDNA of CXCr4, VLA4, VLA5 and ABL are listed in table 1. Real Q Plus 2X Master Mix Green without ROX Dye (Ampliqon, Denmark) was used for all real-time PCR reactions. Real-time PCR reactions were carried out by Roche real-time PCR instrument (Light Cycler 96 Roche) using the following cycling conditions: initiation at 95˚C for 15 minutes, amplification for 40 cycles with denaturation at 95˚C for 15 seconds, annealing at 60˚C for 25 seconds and extension at 72˚C for 30 seconds. All tests were performed in triplicate on treated cells with 0.5- 500 mM concentrations of Sotalol and

compare with untreated cells. The expressions of the genes were defined based on the threshold cycle (Ct) and relative expression levels were determined by ddCT method.

Table 1. Sequence of primers used in the quantitative analysis through real-time PCR

primer	sequence	Size Primer	Annealing Time °c
ABL Forward:	TTCAGCGGCCAGTAGCATCTGACTT	276	60
ABL Reverse	TGTTGACTGGCGTGATGTAGTTGCTTGG	276	60
CXCR4 Forward	TTA CCA TGG AGG GGA TCA G	121	60
CXCR4 Reveres	TAG ATG GTG GGC AGG AAG	121	60
VLA4 Forward	CAA GCA TTT ATG CGG AAA GA	117	60
VLA4 Reveres	AAG TGG TGG GAA TTC CTC TG	117	60
VLA5 Forward	CCC TGA ACC TCA CTT TCC AT	123	60
VLA5 Reveres	AGA AGT TCC CTG GGT GTC TG	123	60

Statistical analysis

All experiments were repeated three times and statistical analyses were performed with SPSS software (version 21). A $P<0.05$ was considered statistically significant.

RESULTS

To find out the number of HSCs that mobilized to peripheral blood of the donor, isolated mononuclear of blood were stained by CD34-PE and CD45-FITC. The results show that 1.94% of peripheral cells are CD 34+. Figure 1 demonstrates the flow cytometry analysis for HSCs counted. To determinate whether the gene expression is time dependent, cDNA synthesize from cells which treated for 1, 3, 5 and 24 hours. The mean data show that maximum increase in gene expression after 1 hour. The gene expression in cells treated with Sotalol in other times (3,5, 24 hours) significantly decreased ($p<0.01$); Figure 2 demonstrates the gene expression in different times.

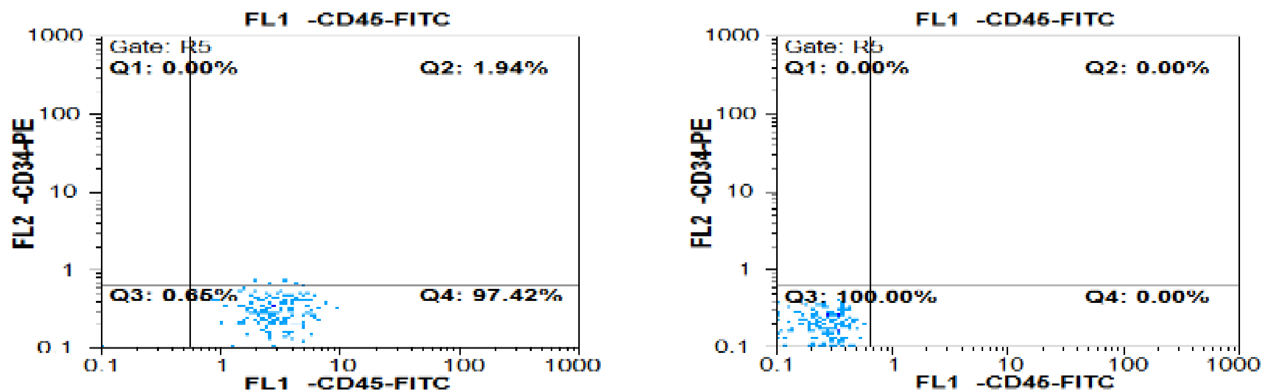

Figure 1. Right dot plat shows negative control in HSCs mobilized with peripheral blood and left dot plat shows two linage cells: 97% of cells with CD45+ and 1.94% of cells CD34, CD45+.

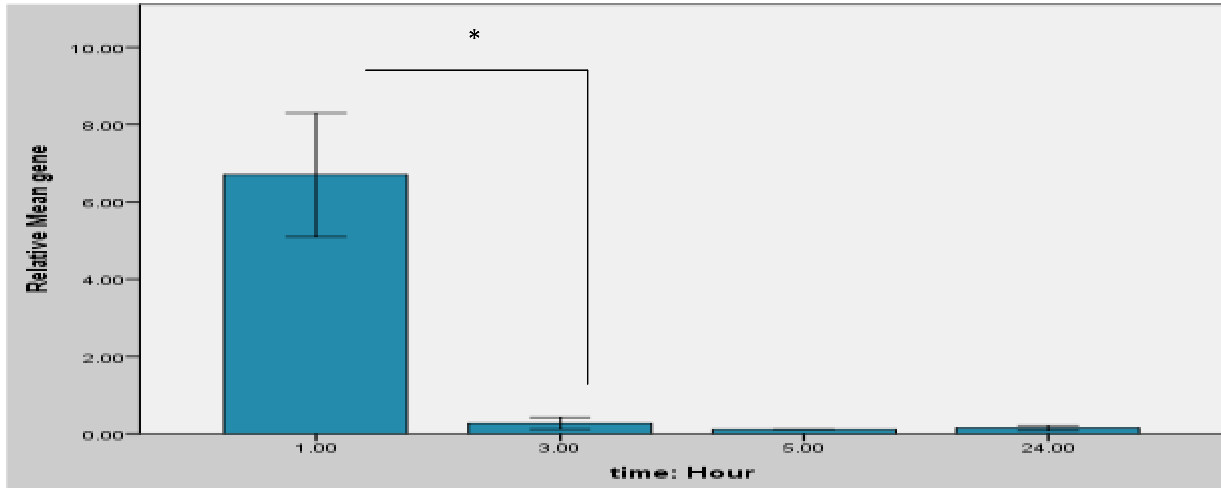

Figure 2. The gene expression trend in different time points: Time 1 revealed a significant increase in gene expression, * P<0.01.

To determinate if the gene expression is dose dependent, RNA extraction and cDNA were synthesized from cells which were treated by 0.5-500 µmol of Sotalol. The mean data shows maximum increase in gene expression of CXCR4 in concentration of 10 and 50 µmol in comparison with other concentrations (p <0.01), Figure 2 demonstrates the gene expression in different concentrations of the drug.

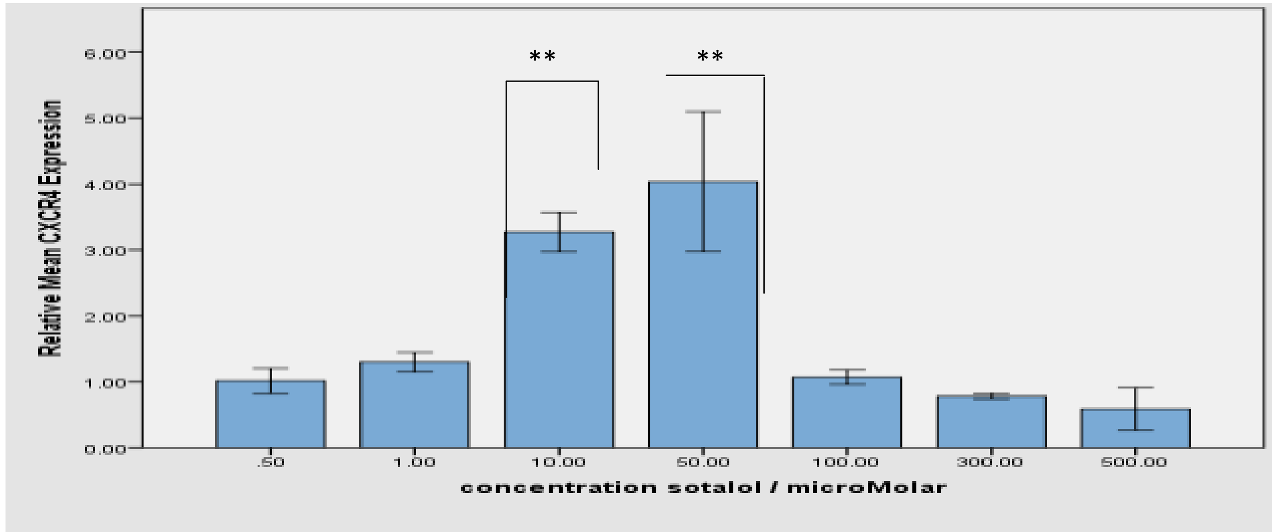

Figure 3. Demonstration of significant increase in gene expression of CXCR4 in the presence of 10 and 50 µmol concentrations of Sotalol, ** P<0.01.

About the gene expression of VLA4 and VLA5 in different concentrations of Sotalol, the results showed maximum increase for VLA4 in 50 µmol and for VLA5 in 10 µmol, compared with other concentrations (p <0.01); Figure 4 demonstrates the gene expression in VLA4 and figure 5 shows the gene expression in VLA 5 with different concentrations of drug.

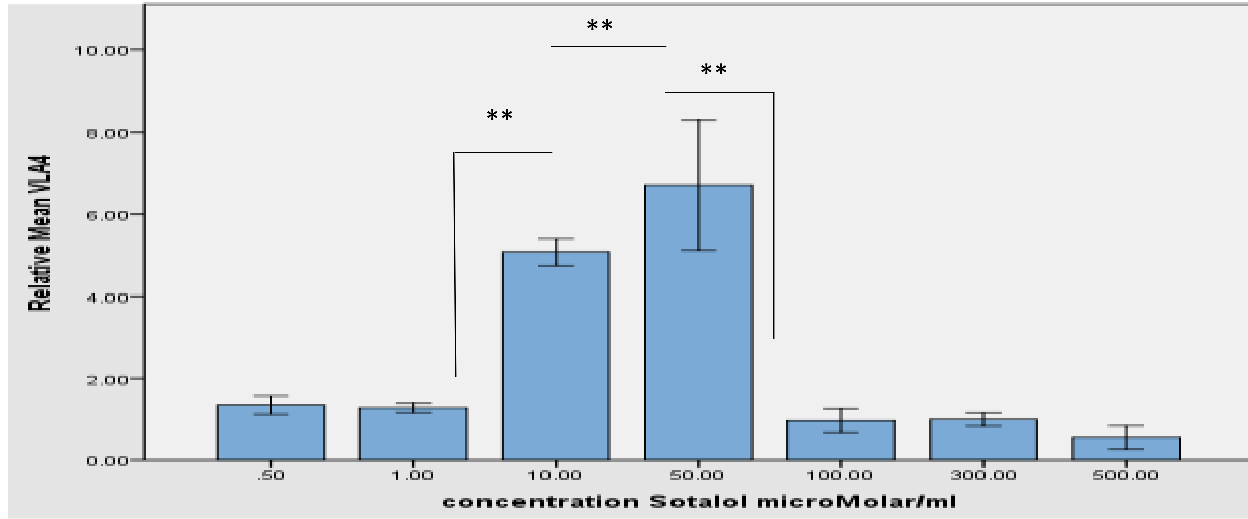

Figure 4. Demonstrates a significant increase in gene expression of VLA 4 in 50 µmol Sotalol. ** P < 0.01.

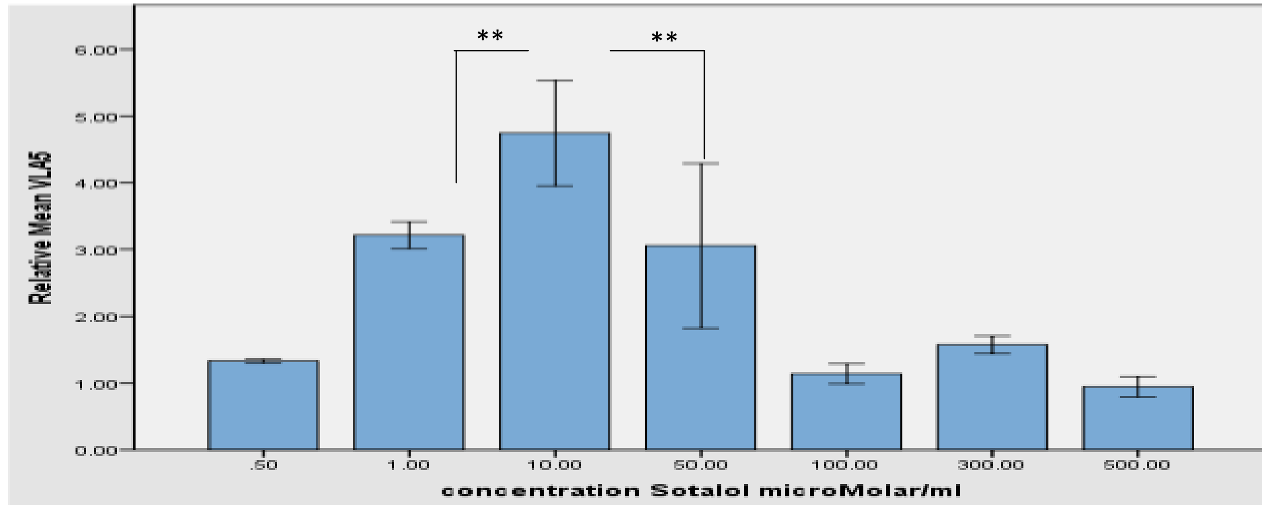

Figure 5. The demonstration of the significant increase in gene expression of VLA 5 in 10 µmol Sotalol . ** P <0.01.

DISCUSSION

Sotalol (Betapore) is an adrenergic receptors antagonist (B-blockers) similar to propranolol. Satalol inhibits interaction between neurotransmitters and their receptors; therefore, receptor G-protein related will not be activated and will be unable to produce c-AMP and also decrease intracellular calcium). In this study, the effect of B-blockers on cell surface adrenergic receptors was investigated on HSCs via Sotalol. Results demonstrated that this mechanism increases expression of genes related to cells homing, and therefore is helpful in homing of HSCs after transplantation. The role of nervous system in regulating bone marrow functions have been shown previously [17- 19]. Jonas Larson reported in a similar study that HSCs functions such as mobilization and homing are affected by adrenergic receptors agonists and antagonists[7]. In 1998, George SJ.M. Maestorni reported that rhythmic function of bone marrow is because of rhythmic function of nerve system located in bone marrow. Increase in epinephrine and norepinephrine in darkness causes cell proliferation and mobilization [20]. Kuzhali Muthu and colleagues demonstrated the presence

of nervous system receptors such as α1 and β2 on the surface of HSCs and their progenitors whose activation, for example on the surface of monocytes, differentiates them with macrophage. Thus, bone marrow progenitor cells not only express adrenergic receptors, but also respond to their stimulation and even change their phenotype [21-24]. Tsvee and his colleagues reported that the effects of catecholamines such as epinephrine, norepinephrine and dopamine change some phenotypes in hematopoietic progenitor cells, including mobility, proliferation and repopulation[10]. Recently, it was determined that Norepinephrine increases the amount of hematopoietic progenitor cells and their mobilization in peripheral blood after exercising through acting on adrenergic receptors, specially α1 and epinephrine through affecting both α and β adrenergic receptors [25-28]. In this regard, Paul S. Frentte and his colleagues showed that both β2 and β3 play important roles in mobilization induced by G-CSF. Consequently, in this study, B2 adrenergic receptors were blocked to increase expression of genes related to homing. In order to find out the role of this drug on HSCs homing, effective molecular factors should be analyzed. However, to evaluate the impact of blocking these receptors by Sotalol on homing of the HSCs, molecular factors should also be evaluated, among which chemokine and adhesive molecules are noteworthy. Tsvee Lapidot showed that repopulation potential in human and mouse is dependent on CXCR4 signaling and HSCs homing needs adhesive molecules and their receptors in transmitting across the vessels wall,similar to their mobilization [5, 29, 30]. In recent years, CXCR4 signaling is called stage-specific, meaning that this signaling does not play important role in speeding up and homing of HSCs but is very important in repopulation that can be important in transplantation [31]. In another study, CXCR4 was analyzed after blocking β-adrenergic receptor, showing that blocking β-adrenergic receptors on HSCs surface increased the CXCR4 amount. Fakhredin Saba and colleagues claimed that stimulating adrenergic receptors with agonists on immature hematopoietic cells increases CXCR4 expression and their mobilization [32]. It was reported that increased mobilization is because of reduced induction of SDF1 in bone marrow. Thus, catecholamine affected molecular particles of bone marrow such as CXCR4 and SDF-1 and helped HSCs to exit from vessels. Since homing is a multiple-step process, different mechanisms and molecules are influential in the process [32]. SDF1 helps adhesion of HSCs to endothelial cells through integrins such as VLA4, VLA5 and CD49f/CD29 and help cells transmission through endothelial cells and basal lamina (Fibronectin, Collagen and Laminin) and subsequently, HSCs migrate toward osteoblast cells due to SDF1 concentration gradient [33]. The role of VLA5 in homing and mobilization was proved by treating HSCs with VLA5 antagonist antibody [14, 34]. In this study, the peripheral HSCs ($CD34^+$) collected through aphrase procedure was employed. During the initial phase of the study, after aphrasing the peripheral blood samples, the mononuclear was isolated and in the later procedure, the cells confronted with concentration of 0.5,1,10,50,100,300 and 500 microMolar/ml of Sotalol as β-adrenergic receptor blockers and expression genes CXCR4,VLA4 and VLA5, playing a role in homing, were evaluated in the first, third, fifth and twenty-forth time. The result reveals that the mentioned genes represented an increasing expression in the first time whereas it slows down through the third, fifth and twenty-forth time. This result shows the dependency of this drug to time as one of β-adrenergic receptor blockers. It should be noted that the expression of these genes show an increase in particular concentration of the drug, and CXCR4 shows a significant increase in concentration of 10 and 50 microMolar/ml,VLA4 in concentration of 50 and VLA5 in concentration of 10. Low concentration of drug didn't leave a considerable impact on genes expression and also higher concentration didn't show any positive effect which shows a dependency on drug dose. Hence, the results reveal that blocking the β-adrenergic receptors blocking can play an effective role in increasing gene expressions involved with hematopoietic stem cells homing.

CONCLUSION

Since the role of nervous system on bone marrow function is proved, their use in therapeutic purposes is suggested. Suitable blocking of this system receptors plays an important role in HSCs homing. Using these blockers (β-blockers), can increase expression of genes related to homing and

therefore can increase success during transplantation.

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"The authors declare no conflict of interest"

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