

## Synergistic Effect of Sodium Butyrate and Thalidomide in the Induction of Fetal Hemoglobin Expression in Erythroid Progenitors Derived from Cord Blood CD133+ Cells

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Article information	Abstract
<p>Article history: Received: 26 Jun 2011 Accepted: 29 Aug 2011 Available online: 19 Dec 2011</p> <p>Keywords: CD133+ cells -globin gene Sodium butyrate Thalidomide</p> <p>*Corresponding author at: Department of Hematology, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran. E-mail: kavianis@modares.ac.ir</p>	<p><b>Background:</b> The use of drugs with the ability to induce production of fetal hemoglobin as a novel therapeutic approach in treating -Hemoglobinopathies is considered. -globin gene expression inducer drugs including sodium butyrate and thalidomide can reduce additional -globin chains accumulation in erythroid precursors.</p> <p><b>Materials and Methods:</b> In this experimental study, MACS kit was used to isolate CD133+ cells of umbilical cord blood. Further, the effect of two drugs of thalidomide and sodium butyrate were separately and combined studied on the induction of quantitative expression of -globin and -globin genes in erythroid precursor cells derived from CD133+ stem cells in-vitro. For this purpose, the technique SYBR green Real-time PCR was used.</p> <p><b>Results:</b> Flow cytometry results showed that approximately 95% of purified cells were CD133+. Real-time PCR results also showed the increased levels of -globin mRNA in the cell groups treated with thalidomide, sodium butyrate and combination of drugs as 2.6 and 1.2 and 3.5 times respectively, and for -globin gene, it is respectively 1.4 and 1.3 and 1.6 times compared with the control group (<math>p&lt;0.05</math>).</p> <p><b>Conclusion:</b> The study results showed that the mentioned drug combination can act as a pharmaceutical composition affecting the induction of fetal hemoglobin expression in erythroid precursor cells derived from CD133+ cells.</p> <p>Copyright © 2012 Zahedan University of Medical Sciences. All rights reserved.</p>

### Introduction

-hemoglobinopathies are genetic disorders of -globin chain that can cause significant mortality in different parts of the world. Beta-thalassemia and sickle cell disease (SCD) are two common cases of this group of disorders that occur following specific mutations in -globin gene. SCD is created following point mutations in the sixth codon of -globin gene that causes substitution of glutamic acid for valine [1]. -thalassemia also resulted from various types of mutations in -globin gene and result in reduction or lack of production of -globin chain [1, 2]. Novel therapeutic solutions based on changes in the epigenetic pattern of genes, which increases gene expression of -globin and thus, increase of fetal hemoglobin levels (HbF) are highly regarded. It has been seen in patients with SCD and phenotype HPFH (hereditary persistence of fetal hemoglobin), that high levels of HbF can reduce the disease complications [1-5]. -globin gene expression inducer drugs, including

hydroxyurea, [6] histone deacetylase (HDAC) inhibitors drugs, such as sodium butyrate [7] and azacitidine [8] and decitabine [9] as well as immunomodulator drugs such as pomalidomide and lenalidomide [10] and thalidomide [11] can reduce the accumulation of additional -globin chains in erythroid precursors and recovery of non-equilibrium status of alpha chains to non- alpha chains ratio [12].

Various studies have been conducted on the use of growth factors and drugs inducing HbF expression in erythroid precursors derived from hematopoietic stem cells [13-16]. It is seen that CD133+ stem cells are more immature than CD34+ stem cells and has a higher colongenicity potential [17]. While increase of low amounts of HbF in SCD patients can be very effective, increase of significant amounts of HbF in -thalassemia is required to improve disease symptoms [18]. Therefore, the uses of drugs with high potential in the HbF induction

and with fewer side effects are highly regarded [1, 19]. In this study, two drugs of sodium butyrate and thalidomide are used for induction of expression of  $\alpha$ -globin and  $\beta$ -globin genes in erythroid precursors derived from CD133+ cord blood stem cells and synergistic effects of these two drugs have also been studied.

## Materials and Methods

In this experimental study we used recombinant erythropoietin (EPO; R & D systems, Minneapolis, MN; USA), IL-3 (IL-3; stem cell Technology, Vancouver, BC; Canada), thalidomide (Tocris Bioscience, Missouri, USA) and sodium butyrate (Sigma, Saint Louis, MO, USA) for erythroid lineage differentiation and  $\alpha$ -globin gene induction.

Isolation of mononuclear cells from cord blood: after the completion of consent form by the pregnant women (Sarem Hospital, Tehran, Iran), human cord blood was collected in blood bags containing citrate phosphate dextrose (CPD) to prevent clotting. Next, cord blood was diluted by Hydroxyethyl Starch (HES) solution with one to six ratios. Then, it was slowly added to ficoll (Amersham Pharmacia, Piscataway, NJ) with one/two ratio (ficoll to cord blood ratio) with density 1.077. Then, centrifuge at round 400G was performed for 40 minutes. Difference in cell density led to the separation of red blood cell layer from the layer containing the mononuclear cells. Having been collected, mononuclear cell layer was mixed with three volumes of phosphate buffered saline (PBS) with pH=7.2 and centrifuge was performed in round 300G for 10 minutes. Finally, pelleted mononuclear cells were washed with PBS twice.

CD133+ cells isolation: The process of isolation of CD133+ cells from other mononuclear cells was performed by MACS Kit (Magnetic Activated Cell Sorting) (Miltenyi Biotech, Germany) and according to kit instructions. Briefly, about  $10^7$  mononuclear cells with 400 $\mu$ l PBS containing 3% of volume of human serum was incubated. After washing, 60 $\mu$ l of human monoclonal antibody against CD133+ antigen which is conjugated with the iron particles was added to the cell mixture and incubated for 30 min at 4°C temperature. Then, cells were washed with PBS containing 0.5% bovine serum albumin volume (BSA; Sigma Aldrich) and 2 mM EDTA. Cells treated with antibodies were passed through MiniMACS column.

CD133+ cells attached to antibodies are trapped in magnetic field and remain in the column walls. Then, the column was washed three times with PBS containing EDTA and BSA, in order to remove cells not bonded with antibodies. In the next stage, the column was separated from magnets and using the piston mounted on top of the column, a sudden pressure was applied to remove the cells attached to the column wall. Purified CD133+ cells were stored in stem span medium containing growth factors and some cells went through flow cytometry in order to determine the purity of CD133+ cells. Finally, about  $3 \times 10^4$  CD133+ cells with a purity of approximately 95% were isolated from mononuclear cells.

Cell culture and fetal hemoglobin induction: CD133+ cells obtained from the previous stage were cultured in IMDM medium containing 30% fetal bovine serum (FBS) (Cambrex, Belgium), 70 $\mu$ g/ml of transferrin, 2mM L-glutamine, beta-mercaptoethanol ( $10^{-5}$  M) and 100U/ml penicillin/streptomycin. Then, cells were treated with 3U/ml human recombinant erythropoietin (EPO) and 5ng/ml IL-3 for differentiation to the erythroid lineage. In order to obtain optimal expression of globin gene, cells were divided into four different groups, including groups of 10 $\mu$ M thalidomide, 10 $\mu$ M sodium butyrate, combination of thalidomide and sodium butyrate in the same concentrations of 10 $\mu$ M and 0.1% DMSO (Sigma, St Louis, MO) (as control) on the day 6 of the differentiation stage and were treated with the mentioned concentrations of drugs. Medium was changed once every three days.

Finally, cells were collected on day 14 of differentiation. In this study, CD133+ cells were isolated from umbilical cord blood of three donors and were separately cultured and treated.

Flow cytometry: after isolation of CD133+ cells with Mini MACS column, flow cytometry technique was used to study homogeneity of CD133+ cells population. For preparation, 100 $\mu$ l PBS was added to about  $10^4$  cells. Afterwards, 7 $\mu$ l CD133-PE antibody (Clone, AC141; Miltenyi Biotech, Germany) was added to it. Incubation was performed in 60 minutes at 4°C temperature. Then, 100 $\mu$ l Paraformaldehyde 1% solution was added to it. Rat IgG1-FITC Isotype (IQ-Products, the Netherlands; IQP-191F) was used as an isotype negative control. According to Figure 1, flow cytometry results show that about 95% of cells purified by Mini MACS column are CD133+.

Quantitative analysis of gene expression with Real-time PCR technique: Real-time PCR technique was used to examine  $\alpha$ -globin and  $\beta$ -globin genes expression. First, on the day 14 of differentiation, cells were collected and washed with PBS and finally RNA extraction processes were performed with RNA extraction kit (RNeasy Mini Kit, Qiagen, Valencia; CA). Approximately  $3 \times 10^6$  cells were used for RNA extraction. Then, cDNA molecules were synthesized using RT-PCR technique. Further, for quantitative evaluation of gene expression, SYBR green Real-time PCR technique (Qiagen Kit, Valencia, CA) was used.

In Table 1, sequences of primers used in Real-time PCR of  $\alpha$ -globin and  $\beta$ -globin genes are shown. To normalize the results obtained from  $\alpha$ -globin and  $\beta$ -globin genes expression,  $\beta$ -actin gene was used as a housekeeping gene. The relative values have been obtained based on CT method and were calculated using the formula  $2^{-Ct}$ . Statistical analysis was also performed using software SPSS-15 and *t* test. In addition, the results are obtained from repetition of three different samples (mean $\pm$ SD). The  $p < 0.05$  was considered statistically significant.

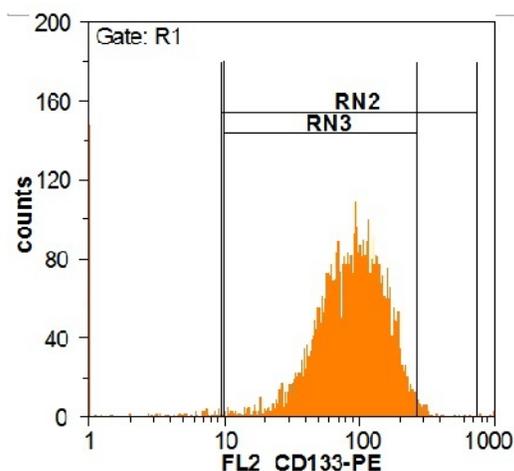
## Results

The effects of thalidomide, sodium butyrate and thalidomide/sodium butyrate combination in the induction

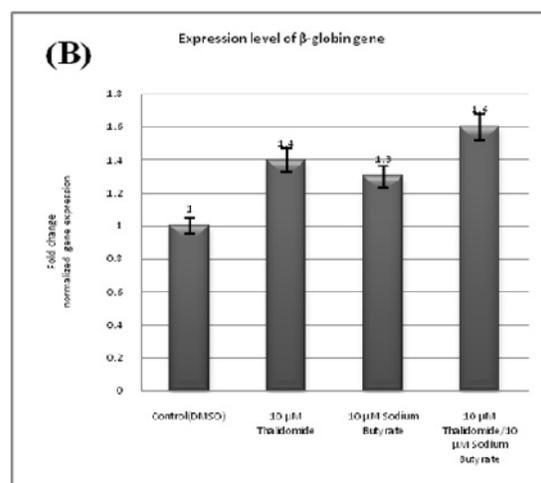
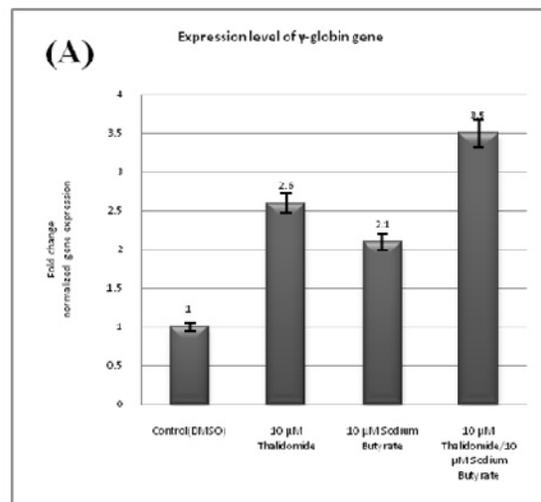
of  $\alpha$ -globin and  $\beta$ -globin genes expression in erythroid precursors: in order to study the effects of thalidomide and sodium butyrate on the increase of  $\alpha$ -globin and  $\beta$ -globin genes expression, CD133+ cells were cultured in medium containing 10 $\mu$ M thalidomide (10T), 10 $\mu$ M sodium butyrate (10S) and combination of 10 $\mu$ M thalidomide and 10 $\mu$ M sodium butyrate (10T/10S) for a period of 8 days. Then,  $\alpha$ -globin and  $\beta$ -globin genes expression was determined through the examination of mRNA of these two genes using quantitative Real-time RT-PCR technique. According to figure 2, thalidomide 10 $\mu$ M, sodium butyrate 10 $\mu$ M, and combination of thalidomide 10 $\mu$ M and sodium butyrate 10 $\mu$ M after treatment with erythroblast cells on the day 6 of differentiation, on the day 14 of differentiation increase  $\alpha$ -globin gene expression, respectively by 1.4, 1.3 and 1.6 times and increase  $\beta$ -globin gene expression 2.6, 2.1 and 3.5 respectively compared with control group. These results indicate the synergistic effect of thalidomide and sodium butyrate in  $\alpha$ -globin and  $\beta$ -globin genes expression. Also, comparison of the effects of thalidomide and sodium butyrate in the induction of  $\alpha$ -globin and  $\beta$ -globin genes expression shows thalidomide's higher ability in the increase of gene expression of these two genes. The results showed that thalidomide's ability in the induction of  $\alpha$ -globin and  $\beta$ -globin genes expression is respectively 1.23 and 1.07 times more than sodium butyrate ( $p < 0.05$ ).

**Table 1.** Sequence of primers  $\alpha$ -globin,  $\beta$ -globin and beta-actin

Gamma globin-F	GTCCTCTGCCTCTGCCATC
Gamma globin-R	CGGTCACCAGCACATTTCC
Beta Globin-F	CTCACCTGGACAACCTCAAG
Beta Globin-R	AGCCACCACTTTCTGATAGG
Beta actin-F	CCCTGGCGGCTAAGGACTC
Beta actin-R	CACATGCCGAGCCGTTGTC



**Figure 1.** Results of flow cytometry of cells isolated with column Mini MACS in terms of homogeneity of population of CD133+ cells



**Figure 2.** Results of quantitative study of  $\alpha$ -globin (A) and  $\beta$ -globin (B) genes expression using Real-time PCR technique in the studied groups (vertically oriented numbers indicate the relative increase in gene expression compared with the control group). \*  $p < 0.05$ , against control group cells (no drug treatment)

## Discussion

As fetal hemoglobin induction considers as novel therapeutic approach for  $\alpha$ -thalassemia and sickle cell disease treatment (1), the present study conducted on  $\alpha$ -globin and  $\beta$ -globin genes expression using single and combination of thalidomide and sodium butyrate. The results showed that the simultaneous use of two drugs of thalidomide and sodium butyrate has proper synergistic effects on the increase of  $\alpha$ -globin and  $\beta$ -globin genes expression in comparison with single-drug groups and control groups in erythroid precursor cells derived from cord blood CD133+ cells.

Creation of hematopoietic stress conditions is important in the effective induction of fetal hemoglobin [13]. It has been seen that thalidomide provides high levels of fetal hemoglobin by increasing cell proliferation in erythroid precursor cells derived from CD34+ cells [11] and

sodium butyrate through the increasing of histone acetylation in BFU-E cells. [12] However, choosing drug compounds that affect different molecular and epigenetic mechanisms can induce fetal hemoglobin production effectively [1, 20].

Various clinical studies indicate a high potential of hydroxyurea in treating patients with SCD. In addition to increasing fetal hemoglobin levels, especially in children, this drug causes a significant reduction in complications of this disease including painful crises and also reduces the need for blood transfusions in mild to severe states of the disease. However, about 30 percent of patients with SCD do not respond to this medication [1, 20-26]. Therefore, it is very important to consider new treatment procedures and the effect of various drugs and drug compounds in-vivo and in-vitro in order to assess the induction of fetal hemoglobin expression and improvement of disease-related complications in the treatment of  $\alpha$ -hemoglobinopathies.

Moreover, fetal hemoglobin induction has been studied in various studies by butyrate-derived drugs. Butyrate is a drug inhibiting histone deacetylase enzyme which increases fetal hemoglobin expression in sickle cell disease and  $\alpha$ -thalassemia through an unknown mechanism. Also, butyrate can increase  $\alpha$ -globin gene expression and reduce  $\beta$ -globin gene expression. It is seen that the final level of fetal hemoglobin production in continuous and intermittent treatment is the same. Therefore, the effect of butyrate on the increase of fetal hemoglobin expression is possibly inherited [7, 27].

A study has shown that thalidomide increases fetal hemoglobin levels by about 2 times higher than butyrate in erythroid precursor cells derived from CD34+ cells [10]. Our results also suggest a higher ability of thalidomide in the induction of fetal hemoglobin compared to sodium butyrate. Thus, considering high ability of immunomodulator drugs in the increase of  $\alpha$ -globin gene expression and cell proliferation [10, 11]. It seems that the use of an immunomodulator drug along or with other effective drugs for induction of fetal hemoglobin production can reduce hemolysis and improve anemia status in patients with  $\alpha$ -thalassemia [28].

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One of shortcomings of this research it has not investigated the effect of the drugs thalidomide and sodium butyrate on changes of expression of other genes such as  $\alpha$ -globin gene and transcription factor genes affecting hemoglobin production, such as GATA-1 and EKLF. In addition, the effect of the mentioned drugs on changes of gene expression of oncogenes and tumor suppressor genes (TSG) has been ignored. In this study, comparison of the effect of single and combination drug treatment in separate colony formation has been also ignored.

It is recommended to discuss the change of pattern of expression of  $\alpha$ -globin gene, transcription factor genes and genes involved in creation of tumor in further research. It is also recommended to use other pharmaceutical compounds affecting the induction of fetal hemoglobin production such as decitabine as single drug or along with immunomodulator drugs to verify the ability to produce high levels of fetal hemoglobin in erythroid precursors derived from various stem cells as well as erythroid precursors isolated from the blood of patients with sickle cell disease and  $\alpha$ -thalassemia.

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## Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

## Conflict of Interest

No conflict.

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