

The Involvement of Canonical NF κ B Pathway in Megakaryocyte Differentiation Induction by Nanocurcumin

Seyedeh Sahar Mortazavi Farsani¹, Dina Sadeghizadeh¹, Sadegh Babashah¹, Fariba Rad², Majid Sadeghizadeh^{1,3}

¹Department of Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran

²Cellular and Molecular Research Center, Yasuj University of Medical Sciences, Yasuj, Iran

³Alborz Nanomed Technology Company, Tehran, Iran

Corresponding Author: Majid Sadeghizadeh, Department of Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran

Tel: +9821 82884409

Fax: +9821 82884717

E-mail: sadeghma@modares.ac.ir

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ABSTRACT

Background: Megakaryopoiesis is characterized by progressive polyploidization and the expression of megakaryocytic markers. Numerous transcription factors and physiological signaling pathways regulate this phenomenon. Megakaryocyte differentiation induction in the K562 cell line and hematopoietic stem cells via nanocurcumin drug has been identified in our previous study. K562 cells are typical Chronic Myelogenous Leukemia (CML) cells that are resistant to apoptosis and express the bcr-abl fusion gene. These cells have the potential to differentiate into erythrocytes and megakaryocytes. Curcumin is well known as a component with strong potential to alter NF κ B activity in various cells. NF κ B pathway regulates various genes such as apoptotic and immune response genes. The aim of the current study is to evaluate the possible role of nanocurcumin in NF κ B pathway regulation during the megakaryopoiesis process in the K562 cell line.

Materials and Methods: Megakaryocyte markers expression and phenotype alteration of nanocurcumin-treated K562 cells have been detected by flow cytometry and microscopy imaging. The nuclear level of the RelA (p65) subunit of NF κ B was determined by western blot test in K562 cells during megakaryopoiesis induction via nanocurcumin treatment at different times. The expression of NF κ B target genes including *c-MYC*, *BAX*, and *NQO1* was also analyzed in nanocurcumin-treated K562 cells by quantitative RT-PCR assay at different times.

Results: It was demonstrated that nanocurcumin leads to an increase in NF κ B activity transiently during megakaryocyte differentiation, which is followed by a change in the expression of *c-MYC*, *BAX*, and *NQO1* target genes.

Conclusion: The NF κ B pathway can be considered a new pathway for inducing megakaryocyte differentiation by nanocurcumin for the purposes of in vitro and in vivo megakaryopoiesis experiments.

Keywords: Megakaryopoiesis; Nanocurcumin; NF κ B pathway; Chronic Myelogenous Leukemia (CML)

INTRODUCTION

Megakaryopoiesis is a complex and important process that produces megakaryocytes cells (MKs)

from hematopoietic stem cells in bone marrow¹. Megakaryocytes are responsible to generate platelet in our body. Decreased platelet levels lead to

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thrombocytopenia problem. Megakaryocytes have specific properties such as large cell size, polyploidy, membrane protuberance and surface MK protein markers².

Many of transcription factors, cytokines and chemokines regulate megakaryocyte production. Several pathways are also triggered in this phenomenon. Activity of NFκB (nuclear factor κB) pathway in this process is highly recognized³⁻⁶.

The limitations of current thrombocytopenia treatment strategies encourages scientists to fine a safe component to induce megakaryopoiesis⁷.

Curcumin is a safe plant natural product that derived from the root of the *Curcuma longa*. This agent can effect in numerous cellular physiological processes such as inflammation and oxidation in a dose dependent manner⁸. Moreover, curcumin has various molecular targets and can regulate many of transcription factors, cytokines, enzymes, etc^{9,10}. Nanocurcumin containing curcumin and nanocarrier has previously synthesized in our laboratory in order to improve solubility, bioavailability and cellular uptake of curcumin. This nano carrier prepared from oleic acid and PEG400¹¹. Significant effect of nanocurcumin on megakaryopoiesis induction in hematopoietic stem cells and K562 cell line indicated in our previous study¹². This function of nanocurcumin was associated with intracellular reactive oxygen species alteration and MAPKs pathways modulation¹².

Curcumin has been well known as NFκB pathway modulator¹³. Many of biological processes such as inflammation, cell proliferation, survival, differentiation and apoptosis can be affected by NFκB activity. Five subunits including RelA (p65), RelB (p68), c-Rel, p50 and p52 make active homodimers and heterodimers of NFκB in cells. NFκB activity is classified in two "canonical" and "noncanonical" pathways. In canonical pathway a dimer of NFκB contain Rel A (p65) transfer into cell nucleus¹⁴. The most abundant dimer of NFκB in mammalian cells is p50/p65 heterodimer.

It has shown that p50 homodimers and of p50-p65 heterodimers of NFκB have fundamental function in proliferation of megakaryocytes¹⁵. It has also indicated that NFκB pathway participates in megakaryocytic differentiation and involves in

thrombopoietin (TPO) receptor and MPL signaling^{16,17}. TPO is a cytokine that is essential for megakaryocyte differentiation regulation in the body. This factor signals through the receptor MPL (myeloproliferative leukemia protein), and is essential for megakaryocyte differentiation¹⁸.

There are some contradictory reports about effects of NFκB on megakaryocyte differentiation. For example, inhibition of NFκB activity under the influence of Diosgenin in megakaryocyte production process of HEL cells has demonstrated¹⁹.

In this study, NFκB activity during megakaryocyte differentiation of K562 Chronic Myelogenous Leukemia (CML) cell line under the influence of nanocurcumin has examined. CML is a chronic myeloproliferative disorder with an specific chromosomal abnormality called Philadelphia chromosome that have a translocation between chromosomes 9 and 22 (t(9;22)(q34;q11)). K562 cells have potential to differentiate to erythrocyte and megakaryocyte. These cells have been used in many studies as model cells to recognize the mechanisms of megakaryocyte differentiation mechanisms^{20, 21}. The expression of three NFκB downstream genes include BAX, c-MYC, NQO has also evaluated in this study.

The outcome of this study showed the increasing of nuclear localization of P65 (RelA) subunit of NFκB at the beginning of nanocurcumin treatment, and then followed by a decrease in the activity of this factor. Thus, it seems that nanocurcumin can alter canonical activity of NFκB during megakaryopoiesis transiently. Gene expression analysis has demonstrated upregulation of NFκB target genes by nanocurcumin treatment in a time-dependent manner.

MATERIALS AND METHODS

Cell culture and reagents

K562 CML cell line was obtained from Pasteur Institute of Tehran, Iran. These cells were cultured in RPMI1640 medium (Gibco BRL, Life technology) contain FBS (10% v/v) and penicillin/streptomycin (1% v/v) (Gibco BRL, Life technology). Incubation was performed at 37°C and 5% CO₂. Cells were counted by hemocytometer using trypan blue (Sigma-Aldrich, St. Louis, MO) staining. Nanocurcumin was synthesized in professor Sadeghizadeh's laboratory.

Its nanocarrier contain Oleoyl chloride and PEG400 [22]. RiboEx purchased from GeneAll Biotechnology company, Korea. May-Grunwald Giemsa was prepared from Sigma Aldrich Company, St. Louis, MO. Antibodies include anti CD41-FITC, anti CD61-FITC and PCNA (Proliferating Cell Nuclear Antigen) were purchased from Miltenyi Biotec Company, Germany. NFκB activation assay kit was purchased from FIVEphoton Biochemicals™, San Diego, CA 92117.

Megakaryocyte differentiation analysis

The best concentration of nanocurcumin (15μM) for megakaryopoiesis triggering in K562 cells was identified in our previous study [12]. In current study 15μM of nanocurcumin has used for K562 cells treatment in order to NFκB activity evaluation during megakaryocyte differentiation. For this purpose 2 × 10⁵ cells per well have seeded in a 6 well plate containing RPMI media, FBS (10%) and penicillin/streptomycin (1%). These cells treated with nanocurcumin for 24, 48 and 72 hours. According to our previous study, 72 hours is the best period time for detection of megakaryocyte differentiation of K562 [12], so phenotype alteration of K562 was evaluated up to 72 hours. In order to evaluate the induction of megakaryocyte differentiation, cell surface megakaryocyte markers including CD41 and CD61 expression was analyzed by flow cytometry (FACS Calibur, BD Biosciences, San Jose, CA 95131) using anti-human FITC-conjugated CD41 and CD61 monoclonal antibodies after 24, 48, and 72 hours. Flow cytometry results were analysis by FlowJo7.6.1 software. The morphology of treated and untreated cells was also determined by May-Grunwald Giemsa 1× staining and visualized via light microscopy.

Nuclear and cytoplasmic protein extraction

After treatment of K562 cells with nanocurcumin for different times (24h, 48h, 72h and 96h), the cells were harvested by centrifuge. Nuclear protein and cytoplasmic proteins were separated by nuclear fractionation Reagent (NER-1) and cytoplasmic fractionation reagent (CER-1) respectively. Dithiothreitol (DTT from Sigma-Aldrich, St. Louis, MO) and protease inhibitor cocktail (Sigma Aldrich,

St. Louis, MO) have been added to CER-1 buffer. All of these protocols performed according to the manufacture of NFκB activation assay kit (FIVEphoton Biochemicals™, San Diego, CA 92117). Protein concentration of each sample was determined using Bradford assay²³.

Western blot assay

Equal amount of nuclear proteins samples (30 μg) were heated to 60 °C for 5 min in the presence of loading buffer and vortex was done for 1 min, then proteins loaded in SDS-PAGE (SDS–polyacrylamide gel electrophoresis), and transferred to PVDF membrane. The membranes were blocked by 5% BSA (Merk Company, Germany) for 1 hour at room temperature and washed by TBST buffer. The blocked membranes were incubated with P65 primary antibody (1:1000) overnight at 4 °C according to antibody manufacture (FIVEphoton Biochemicals™, San Diego, CA 92117). After washing with TBST buffer, incubation with anti-rabbit horseradish peroxidase conjugated secondary antibody from Santa Cruz Biotechnology, Inc. Texas, U.S.A (1:1000) was performed to detection of primary antibody by enhance chemiluminescence (ECL) detection system (Cyto Matin Gene Immune Company, Isfahan, Iran) . PCNA protein used as internal control for nuclear protein and detected by anti PCNA antibody (Santa Cruz, Texas, U.S.A, 1:1000) and anti-mouse HRC conjugated antibody (1:1000) like previous explained protocol. Western blot results were qualified by Image j software (SCR_003070).

Quantitative real time RT-PCR

The expression of c-MYC, BAX and NQO1 genes was determined by quantitative real time RT-PCR after 24h, 48h, 72h and 96h of nanocurcumin treatment. GAPDH gene expression utilized as internal control. Total RNA was extracted from samples using RioEx solution (GeneAll Biotechnology company, Korea) and reverse transcribed to cDNA by reverse transcriptase (RT) according to the manufacturer's instructions (from Sina clone Tehran, Iran). Quantitative PCR was done by Step One real time PCR equipment. The amplification condition was one cycle 94° C for 10min, followed by 40 cycles at 94° C

for 30 s, 63° C for 30 s and 72° C for 30 s. Primers have designed via OLIGO Primer Analysis Software Version 7 and ordered from Macrogen Company of Tehran. Each primer was used at 10pM concentration and their sequences are listed in table1. Threshold cycle

numbers were determined using Step one manager software (RRID: SCR_014281) and data analyzed using delta-delta CT method. Statistical analysis was done by GraphPad prism7 software.

Table 1: Primer sequences for real time RT-PCR

Gene	Forward primer	Reverse primer	Product length
c-MYC	CGTCCTCGGATTCTCTGCTCTC	GTTCCCTCCTCAGAGTCGCTGC	114
BAX	GCAAACCTGGTGCTCAAGG	CAGCCACAAAGATGGTCA	183
GAPDH	CCGAGCCACATCGCACAG	GGCAACAATATCCACTTTACCAG	119
NQO1	CCAATTCAGAGTGGCATTCTGC	GGAAGTTTAGGTCAAAGAGGCTG	190

Statistical analyses

One-way repeated measure analysis of variance (one-way RM anova) has used for analysis of difference between groups by GraphPad Prism7 software. Probability values of $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) were considered statistically significant

RESULTS

Nanocurcumin can increase megakaryocyte markers expression

In order to analyze the rate of megakaryocyte differentiation in K562 cells, the expression level of CD41 and CD61 megakaryocyte markers were detected by flow cytometry after nanocurcumin treatment at 24, 48 and 72 hours. Expression of these markers has increased significantly in the presence of nanocurcumin after 72h (67.4% and 49.3% of cells were CD41 and CD61 positive respectively) (Figure 1). Expression alteration of these markers after 24h and 48h of treatment were not significant (results have not shown). In addition to surface markers, treated cells were stained with May-Grunwald Giemsa and visualized via light microscopy after 48 and 72 hours. Cells imaging have demonstrated increasing of cell size and protuberances of cells membranes after nanocurcumin treatment. These alterations indicated specific characteristics of megakaryocyte cells (Figure 2).

NFκB activity analysis

The involvement of NFκB pathway in megakaryocyte differentiation process under the treatment of nanocurcumin has evaluated via western blot analysis of nuclear p65 subunit. As shown in Figure 3, the nuclear localization of p65 subunit of NFκB increase at 48h after treatment with 15 μM nanocurcumin and followed by significantly decrease after 72 and 96 hours. Amount of nuclear PCNA protein as internal control was similar in all of samples. Results have qualified by image j software.

Gene expression alteration under the influence of nanocurcumin

mRNA expression level of BAX, c-MYC, NQO1 and GAPDH genes were measured by real time RT-PCR. Data have illustrated that c-MYC gene expression was enhanced after 48 hours and NQO1 and BAX upregulated significantly after 96 hours in nanocurcumin treated K562 cells (Figure 4).

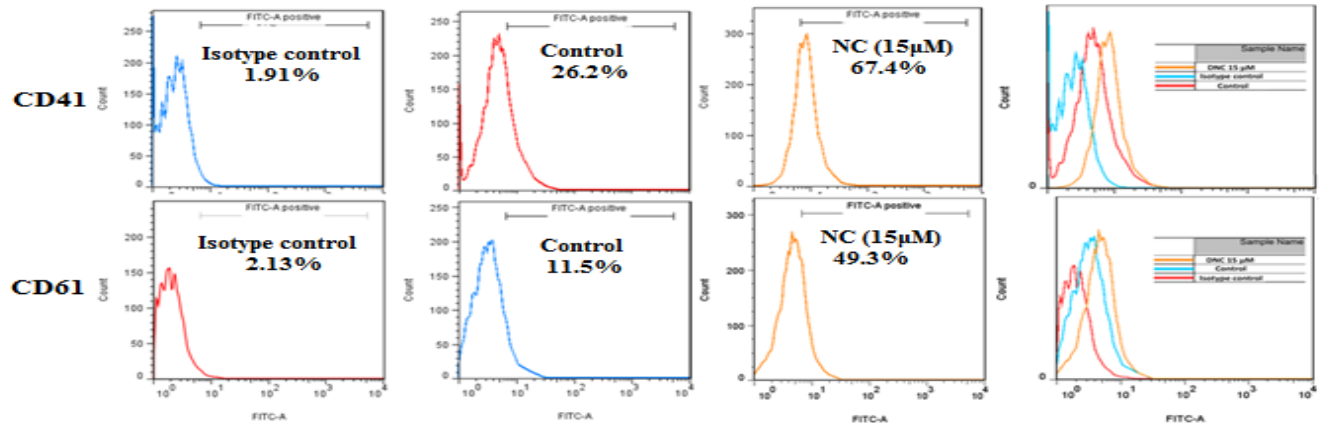
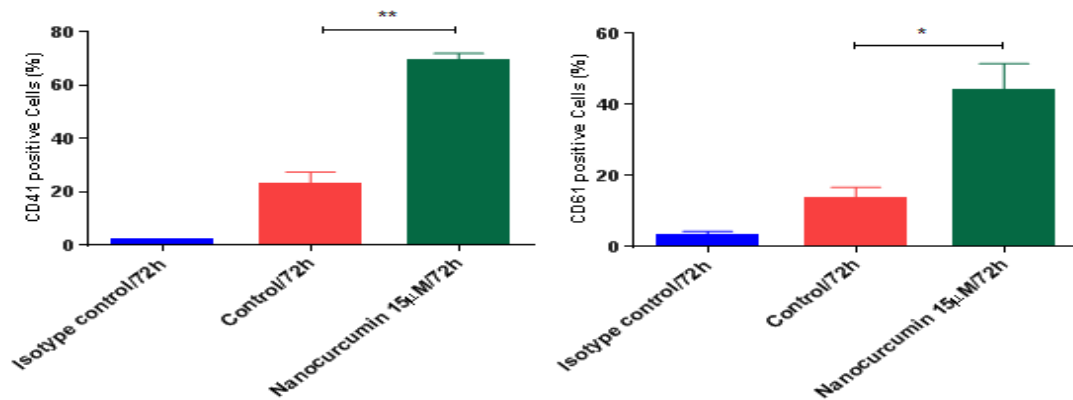
(A)**(B)**

Figure 1. Flowcytometry results of K562 cells stained with FITC-CD41 and FITC-CD61 antibodies. (A) The percentage of CD41 and CD61 positive of control (untreated) and treated cells after 72 hours are shown. (B) The diagrams demonstrated that after 72 hours of nanocurcumin treatment, protein expression levels of CD41 and CD61 have increased significantly. Data was not significant for 24 and 48 hours (not shown). (Sample size (n) = 4, *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001, NC: nanocurcumin, CD: cluster of differentiation, FITC: Fluorescein isothiocyanate).

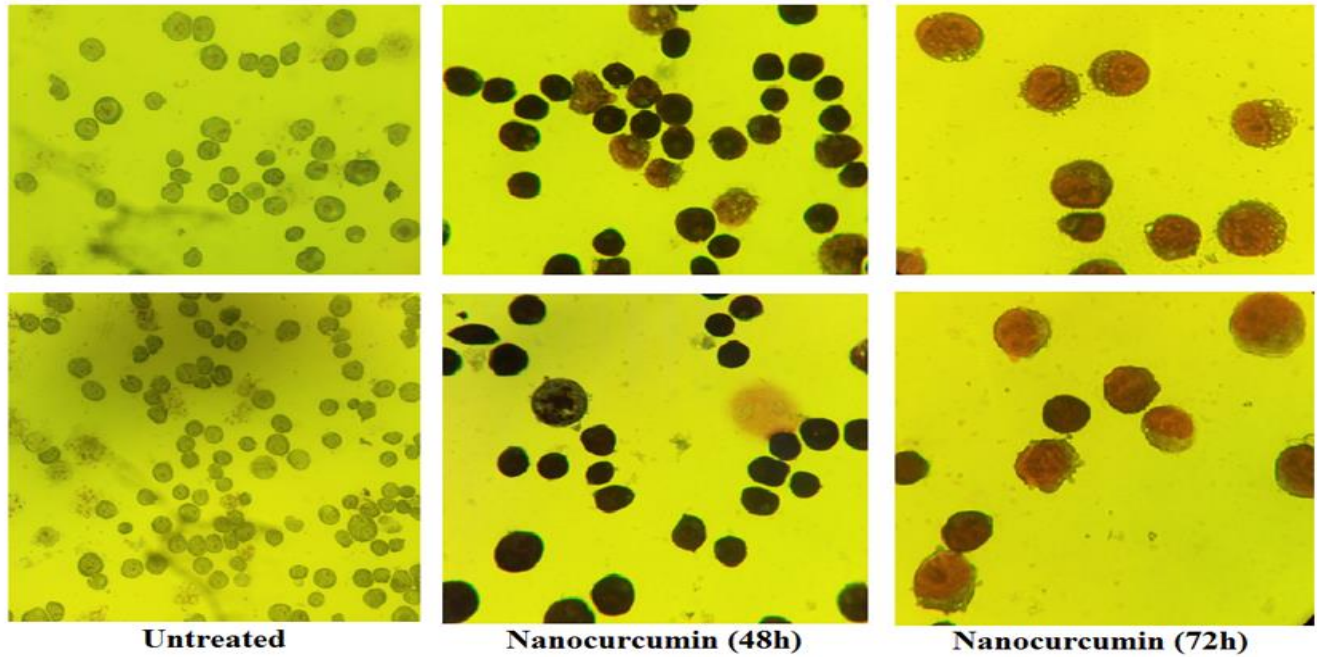


Figure 2. Alteration morphology of k562 cells that treated with nanocurcumin (15μM). Pictures have shown control (untreated) and treated cell with nanocurcumin (15μM) for 48 and 72 hours. Images have been taken by light microscopy after Giemsa staining. As it is evident the cell size increased significantly after 72 hours by nanocurcumin treatment.

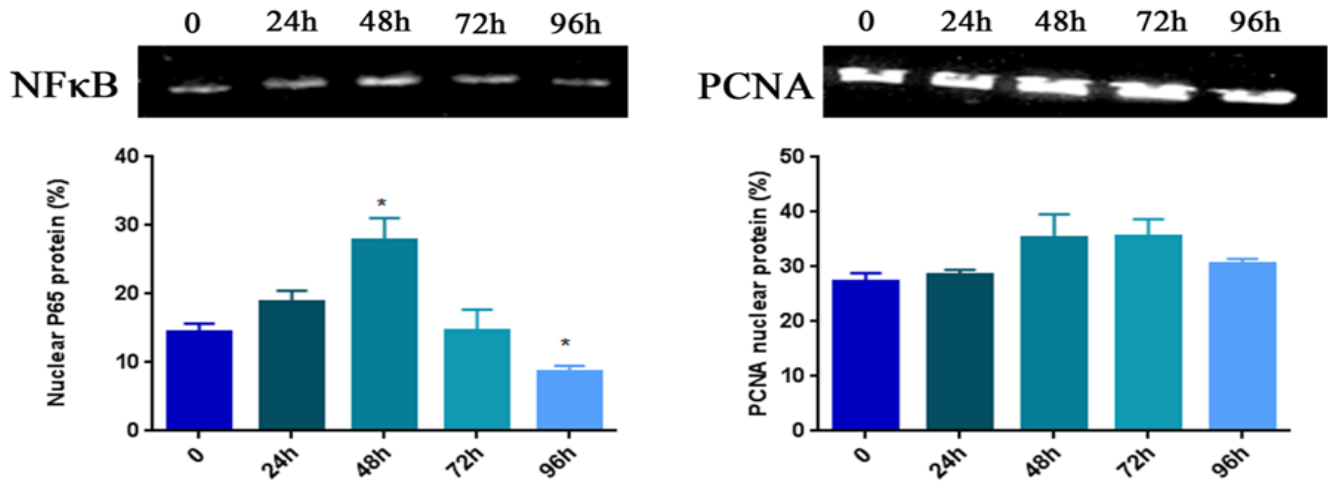


Figure3. Western blot analysis of nuclear NFκB (P65 subunit) in K562 cells treated with nanocurcumin for different time intervals (0, 24, 48, 72 and 96 hours). PCNA protein used as nuclear internal control. Data are expressed as means ± SEM, *P < 0.05 was considered statistically significant.

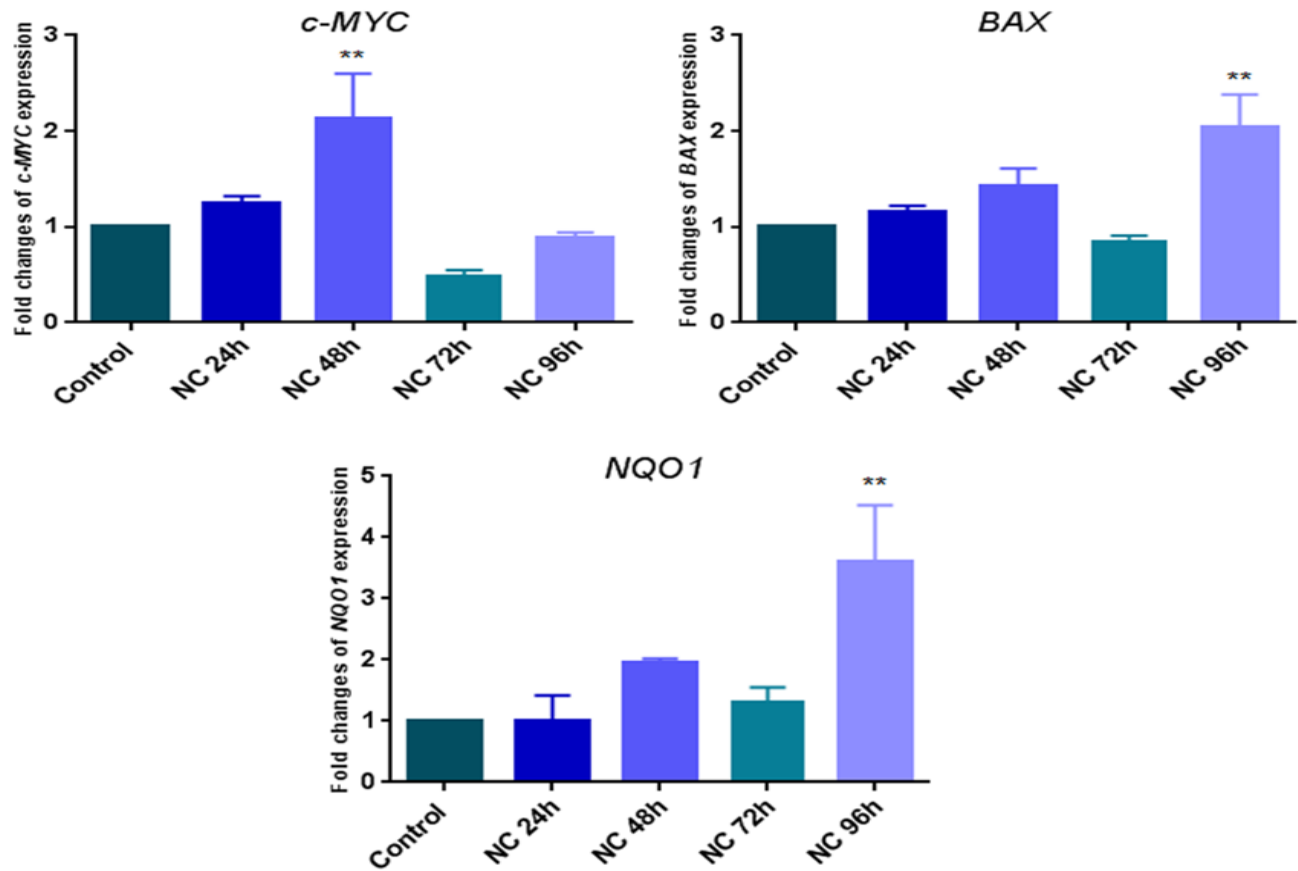


Figure 4. Gene expression analysis of cMYC, BAX and NQO1 factors. Fold change expression of genes in control and treated K562 cells with nanocurcumin (15µM) at different times were measured by real-time RT-PCR. The data was analyzed using the $2^{-\Delta\Delta CT}$ method. (Sample size (n) = 4, *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001, NC: nanocurcumin).

DISCUSSION

Megakaryopoiesis is a phenomenon that is responsible for producing megakaryocytes and platelets for our body. This process is triggered via thrombopoietin²⁴. Various factors include cytokines and growth factors regulate megakaryopoiesis in physiological conditions. Scientists demonstrated that many chemical components such as Phorbol-12 myristate-13 (PMA) and nanocurcumin can induce megakaryocyte differentiation from megakaryocyte progenitors or K562 leukemia cell lines. PMA is a small molecule that induces differentiation of hematopoietic stem cells and K562 cells towards megakaryocytes²⁵. Nanocurcumin contain curcumin and nanocarrier. Void curcumin has many limitations

for therapeutic application because of the low bioavailability, water insolubility, and low cellular uptake. In previous experiments, nanocurcumin was synthesized in our laboratory to overcome these limitations²².

NFκB is a transcription factor that regulates the expression of thousands of genes involved in main cellular processes. NFκB signaling has been known as a central mediator of inflammation and involved in the thrombotic processes, platelet production and platelet activity [26]. NFκB activity induces megakaryocyte progenitor cells to convert systemic or local inflammatory conditions to a transcriptional response, which may has consequences on the phenotype of megakaryocyte and released

platelets^{27,28}. Available studies showed that activation of thrombopoietin receptor (MPL) by interaction with its ligand leads to immediate induction of IκB kinase (IKK) activity during megakaryocyte differentiation in physiological condition. However, over time leading to the significant reduction in IKK and NFκB activities^{27,29}. IKK factor is responsible for the phosphorylation of IκBα inhibitory protein, dissociation of IκBα from NFκB and formation of active NFκB. It has also showed that NFκB pathway regulates the TPO production through the stimulation of transferrin³⁰. Results of current study demonstrated the induction of MK differentiation by nanocurcumin. In this experiment nanocurcumin increased the expression of CD41 and CD61 markers more than other megakaryopoiesis inducers such as phytosphingosine, 3-Hydrogenkwadaphnin³¹, Lapatinib³² and many of small molecules³³.

The outcome of NFκB activity experiments supports that nanocurcumin can increase transiently nuclear localization of NFκB (P65 subunit) during megakaryopoiesis induction. This finding is consistent with the process that takes place in normal physiological condition¹⁷, so activation of NFκB after 48 hours of nanocurcumin treatment possibly participates in megakaryocyte differentiation triggering.

Curcumin is widely used in diet and nutritional supplements and can regulate various cellular pathways³⁴. The inhibitory effect of curcumin on canonical NFκB signaling pathway is well-described previously^{35,36}. Scientists indicated that among 84 target genes of NFκB, 29 mRNAs was significantly down-regulated in Curcumin treated K562 cells³⁷.

In accordance with current study, the ability of a number of anti-inflammatory agents such as 15-deoxy-Δ12,14-Prostaglandin J2 and parthenolide (a feverfew plant-derived compound) to induce megakaryocyte differentiation and platelet production through NFκB modulation have been indicated [38, 39]. Differentiation of K562 cells also induced by 8-Hydroxydaidzein, an Isoflavone from Fermented Soybean by activation of NFκB signaling pathway⁴⁰.

The expression of NFκB target genes including BAX, NQO1 and c-MYC have altered in different times

during nanocurcumin treatment in current investigation. C-MYC gene expression enhanced earlier than NQO1 and BAX genes. Previous studies have demonstrated that NFκB target genes have distinct kinetic patterns. Early and late-activated P65 target genes have different chromatin configuration. Early target genes express directly by P65 activity and late target genes respond indirectly to other P65-activated transcription factors⁴¹. Hence, it seems that c-MYC gene is a member of early-activated target genes of NFκB whereas NQO1 and BAX are late-activated genes.

CONCLUSION

According to the results of present experiment NFκB pathway can be a new mechanism in the megakaryopoiesis induction by nanocurcumin. Transiently nuclear localization of NFκB (P65) maybe related to upregulation of NFκB target genes during this process. Therefore NFκB pathway could be considered for future studies about small molecules that induce megakaryopoiesis. Further experiments are also needed to determine whether inhibition of NFκB causes megakaryopoiesis inhibition in the cells treated with nanocurcumin or other MK differentiation inducers.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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