Reports of Biochemistry & Molecular Biology
Vol.11, No.3, Oct 2022

Original article

www.RBMB.net

Synergistic Anti-Cancer Activity of the Combination of 1,25-Dihydroxyvitamin D3 and Retinoic Acid in U937 Cell Line

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Abstract

Background: MicroRNA is a form of non-coding RNAs that able to regulate gene expression. miR-424 is one of the members of the regulatory family, which plays an important role in the proliferation and differentiation of myeloid cells. Epigenetic changes can change the level of miR-424 under environmental factors. Therefore, the level of expression of miR-424 in U937 cells of the myeloid line was evaluated in this research under the influence of vitamin D3 (VitD3) and retinoic acid (RA).
Methods: In this study, U937 cells were cultured in the presence of VitD3, and RA to evaluate cell proliferation, viability via the trypan blue exclusion test, and expression level of miR-424 by real-time PCR at specific times.
Results: Cell proliferation has shown a significant decrease in the RA group versus other groups during incubation times ($P < 0.05$). In VitD3 group, there was a significant increase in cell proliferation after 24- and 48-hours incubation periods versus other groups. In the VitD3 and RA groups, the increase of cell proliferation caused the downregulation of miR-424. In addition, the upregulation of VitD3 group and downregulation of the RA group were significant versus the control group ($P < 0.05$).
Conclusions: We concluded that the expression level of miR-424 was critically affected in the dose- and time-dependent of RA and VitD3 treatment in the U937 cell line. Treatment with VitD3 decreased the expression of miR-424 and RA treatment increase miR-424 expression level in physiological doses.

Keywords: Cell Proliferation, Differentiation, miR-424, U937 Cells.

Introduction

MicroRNAs (miRNAs) are a highly conserved subgroup of non-coding RNA, which are 18-25 nucleotides in size. These molecules regulate the expression of genes after transcription by inhibiting mRNA translation or inducing their degradation. Approximately 30% of human genes control by miRNAs (1, 2). Some studies have shown miRNAs regulate many physiological and biological processes such as proliferation, differentiation, neurogenesis, apoptosis, hematopoiesis, immune response, and development (3, 4). Several of these miRNAs can act as oncogenes or tumor suppressors by inhibiting the expression of target genes related to cancers. Oncogenic miRNAs (oncomiRs) are upregulated in tumors whereas tumor-suppressive miRNAs are downregulated (5, 6).

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Received: 25 Apr, 2022; Accepted: 8 May, 2022

[DOI: 10.52547/rbmb.11.3.440]

The structure of miRNAs and their function have shown that many miRNAs are expressed differently in various cancers. In addition, functional differences between tumors and different stages of cancers are associated with miRNA expression, which can be due to variations in the origin of cancer cells and adjacent tissues (7). Recent studies have been reported that miR-424 is associated with proliferation and osteogenic differentiation of MSCs using miRNA microarrays, but the roles and mechanisms of this miRNA in these processes have not been examined. Moreover, Zhao et al. (2013) and Liu (2015) et al. have displayed that miR-424 is downregulated by oxidative stress in mice and patients after focal cerebral ischemia and reperfusion injury (8-10).

The development of myeloid cell lines depends on the activity of certain genetic details that are effective for decreasing cell proliferation, inducing apoptosis, and expressing myeloid-specific genes. Among the important regulators of cell differentiation, there are transcription factors in which miRNAs have specific regulatory roles in this process. The studies have shown that miRNAs play an important role in the development of blood cells by specific differentiation and cellular response to extracellular stimuli. Acute myeloid leukemia (AML) is a hematopoietic malignancy, in which immature myeloid progenitor cells accumulate in the bone marrow and even in tissues, which are involved in the formation of blood cells (11-13). Studies have shown that acute promyelocytic leukemia (APL) was due to the chromosome translocation in the retinoic acid receptor-alpha (RAR α) gene. RAR α led to the formation of colonies of hematopoietic precursors that have been stopped in the promyelocytic differentiation stage (14). Treatment of APL cells with all-trans retinoic acid (ATRA) showed that this problem could be overcome and eliminated by inducing granulocyte differentiation (15).

Recently, vitamin D has been shown to affect the immune system. The receptors of the active form of 1,25- dihydroxy vitamin D3 (VitD3), are in the cells including monocytes, T cells, and myeloid cells such as U937. Recent research has shown that the VitD3 hormone inhibits the proliferation of activated T cells. This activity occurs due to decreased IL-1 and IL-2 production by the macrophage and U937 cells. VitD3 induces the differentiation of myeloid cells, such as murine M1 and HL60, into a monocytic cell line (16-18). In addition, retinoids have an influential effect on cell proliferation and differentiation. Retinoic acid (RA) can be a factor in differentiating HL-60 cells into granulocytes, which is likely done by conflicting with specific cytoplasmic RA binding protein. Previously, the effect of VitD3 and RA on the differentiation of U937 cells has been investigated. It has been pointed that VitD3 and not other forms of vitamin D can inhibit the proliferation of U937 cells. These requirements led to the expression of CD14 as a monocytic marker. However, RA inhibits cell proliferation, but it does not lead to the expression of CD14 (19-22).

The purpose of this study was to determine the level of expression of miR-424 in U937 cells in the presence of VitD3 and RA, which was investigated by real-time PCR techniques.

Materials and Methods

Cell culture

U937 cells, a pro-monocytic, human myeloid leukaemia cell line, were obtained from Pasteur Institute (Tehran, Iran) and cultured in suspension culture in RPMI-1640 medium (GIBCO, Germany) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Germany), penicillin (100 units/ml), and streptomycin (100 µg/ml) (GIBCO, Germany) with the humidified atmosphere of 5% CO_2 at 37 °C. The media replaced every 48 hours with fresh media.

Differentiation of U937 cells and treatment preparation

To differentiate U937 cells, a proper concentration of VitD3 (10^{-5} M) was used. In addition, in order to prevent the differentiation of U937 cells, an appropriate concentration of

[DOI: 10.52547/rbmb.11.3.440]

RA (10^{-8} M) was used (23). Then, cell viability and proliferation were evaluated after 24, 48, and 72 -hours incubation periods.
In this study, the following groups have investigated.
1- Control group of U937 cells were seeded for 24, 48, and 72 hours in RPMI-1640 medium containing 10% FBS.
2- Placebo (DMSO) group of U937 cells were treated with DMSO (Sigma, Germany) as solvents for the same period of times.
3- RA group of U937 cells with appropriate RA concentration.
4- VitD3 group of U937 cells were treated with VitD3.

Trypan blue exclusion test of cell viability
The rate of cell viability and proliferation were investigated using trypan blue staining by counting cells with a hemocytometer to determine the number of viable cells present in a cell suspension. Based on Strober research, "A viable cell will have a clear cytoplasm whereas a non-viable cell with have a blue cytoplasm" (24).

Total RNA extraction and cDNA synthesis
Total RNAs were extracted from desired treated groups using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. Its purity was determined at 260 nm. For cDNA synthesis, 1 µg of total RNA sample for each isolate was reverse transcribed in a final volume of 20 µl using the miRNA 1st-Strand cDNA Synthesis Kit (Agilent Technologies, Santa Clara, California) according to the manufacturer's instruction. Briefly, the cDNA synthesis was accomplished in two steps. In the first step which called the polyadenylation phase, the materials included 500 to 1000 ng RNA, 4 µl Poly A Polymerase buffer, 1 µl *E. coli* poly A polymerase, and 1 µl of 10 mM rATP to obtain 20µl total reaction sample and incubated at 37 °C for 30 minutes and then for 5 minutes at 95 °C to stop polyadenylation. In the second step of cDNA synthesis, 4 µl of the first step solution with 2 µl of RT buffer, 0.8 µl of 100 mM dNTP, 1 µl of the 10 µM adaptor primer and finally 1 µl of RT / RNase Block enzyme mixture and DEPC water was used to obtain total volume to 20 µl. Then, the solution was incubated at 55 °C for 5 minutes, then 25 °C for 15 minutes, 42 °C for 30 minutes, and finally 95 °C for 5 minutes.

Real-Time Quantitative PCR
Real-Time qPCR was performed using the ABI StepOne™ (Applied Biosystems, Sequence Detection System Foster City, CA, USA) according to the manufacturer's instruction of High-Specificity miRNA QPCR Core Reagent Kit (Agilent Technologies, Santa Clara, California). The 25 µl reaction contained 1 µl of cDNA, 1 µl forward primer, 1 µl universal reverse primer, 0.375 µl reference dye, 0.5 µl enzyme, 2.75 µl $MgCl_2$, 1 µl dNTP, and 1.25 µl MasterMix+Eva green mixed. This technique was used to confirm quantitatively the expression of miR-424 in treatment groups. The primers for Real-time qPCR were: miR-424-F: 5'-GCAGCAGCAATTCATGTTTTGAA-3'; U48-F: 5'-CAGGTAACTCTGAGTGTGTCG-3'.
Conditions for amplification were followed by 40 cycles of 95 °C for 10 s, 60 °C for 15 s and 72 °C for 20 s. In addition, Amplification and melting curve was obtained to evaluate the presence of contamination in each reaction. The results were analyzed with Pfaffl method and the expression of target miRNA was normalized via reference (25).

Statistical analysis
Statistical analysis of the results was performed by SPSS software (version 19.0, Chicago, IL, USA). $P < 0.05$ was considered statistically significant. The results of RT-qPCR were analyzed using one-way ANOVA with Tukey's comparison of all groups. Data were displayed as the means ± standard deviation (mean ± SD) with five repeats for each group.

[DOI: 10.52547/rbmb.11.3.440]

Results

Cell proliferation of U937 in experimental groups during the incubation period

The proliferation of U937 cells was investigated after 24-, 48-, and 72-hours incubation times in different experimental groups. In that way, the proliferation rate of these cells on the first day has shown that the control group was proliferated 1.5 times. The proliferation rate for DMSO and RA was similar to the control group, but the VitD3 group has proliferated not the same as other experimental groups which were statistically significant compared to other experimental groups ($P < 0.05$). After 48 hours of incubation, all experimental groups were proliferated almost two times toward the first place. After 72 hours of incubation time, all groups toward the seeding time were proliferated 4 times, but the RA group was similar to 48 hours and has not shown any proliferation and the difference was significant toward other experimental groups ($P < 0.05$) (Fig. 1).

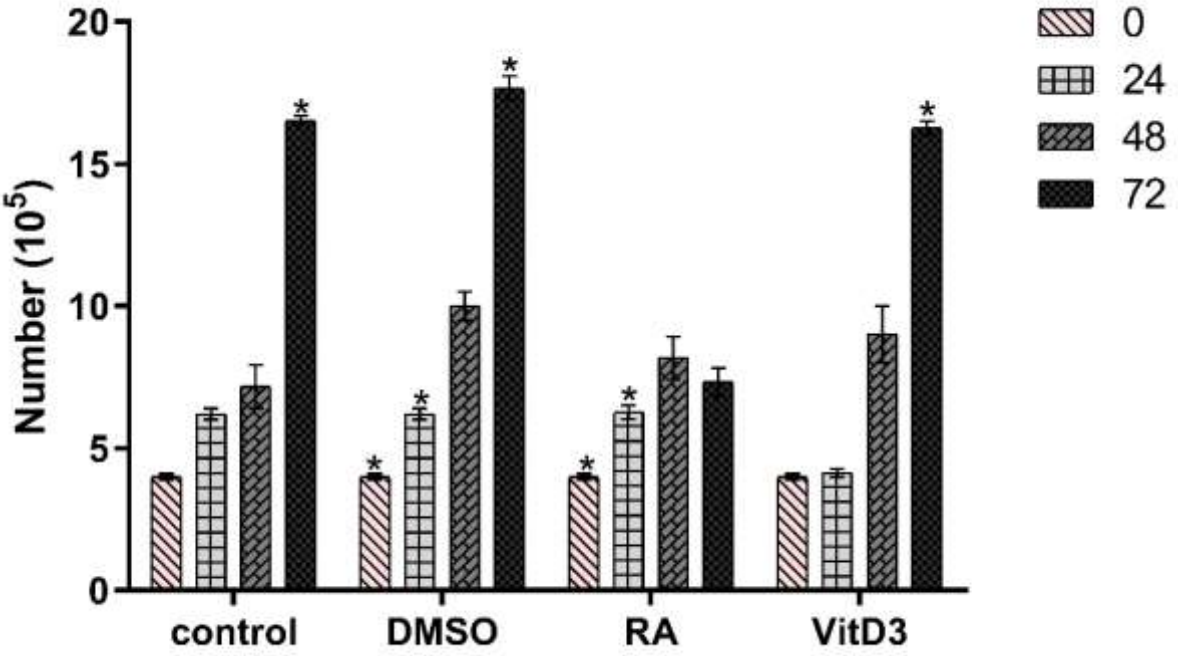

Fig. 1. The cell proliferation of U937 cells, treated with DMSO, RA, Vit D3 was measured by counting cells using a hemocytometer every 24 hours for 3 days. Data shown as means ± SD of triplicate cultures of three independent experiments. * $P < 0.05$ compared with control cells.

U937 cell viability in experimental groups during the incubation period

U937 cell viability was examined after 24-, 48-, and 72-hours incubation periods in different experimental groups. In that way, the rate of cell viability has shown that the control group was decreased after 24 hours of incubation. That was similar to the control group for DMSO, VitD3, and RA. After 48 hours of incubation, the reduction was observed in comparison to the first time but showed a slight increase compared to the 24 hours incubation period. After 72 hours of incubation time, all groups toward the seeding time were decreased, but compared to 24 and 48 hours, they were increased. This increase was statistically significant in the VitD3 group in comparison with VitD3 groups after 24- and 48-hours incubation ($P < 0.05$) (Fig. 2).

Expression of miR-424 in U937 cells in experimental groups during incubation the period

The results have shown that all experimental groups, including the control group were able to express miR-424 during 24-, 48-, and 72-

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hours incubation periods. Therefore, the expression level of miR-424 was evaluated toward U48 as a reference and analyzed in different experimental groups. According to the results, the expression of miR-424 in the control group was similar to DMSO, VitD3, and RA after 24 hours of incubation. This expression increased significantly in the control group after 48 hours of incubation compared to other experimental groups ($P < 0.05$). The results in the VitD3 group have shown significantly decreased expression of miR-424 compared to other experimental groups after 24 hours. However, the expression level of miR-424 was significantly decreased in the RA group compared to the control, DMSO groups, but it was relatively constant compared to the first time ($P < 0.05$). After 72 hours incubation period, it was found that both VitD3 and RA groups have shown a significant increase compared to the previous ones in the same groups ($P < 0.05$). However, the two groups have revealed a significant difference compared to each other at this time ($P < 0.05$). In addition, the control and DMSO groups have suggested a significant decrease compared to the 48 hours incubation period. Comparison of experimental groups at 24-, 48- and 72-hours incubation have shown that the control group had the highest expression level of miR-424 at 48 hours and the lowest was 24 hours. These increases and decreases were statistically significant ($P < 0.05$). In the DMSO group, the same pattern was observed with a decrease in the expression level of miR-424 in groups toward the control group. However, this expression pattern was completely different in the two VitD3 and RA groups. Hence, there was a significant increase in the RA group at 72 hours compared to other incubation times ($P < 0.05$). Nevertheless, in the VitD3 group, the expression level of miR-424 was significantly reduced at 48 hours ($P < 0.05$) (Fig. 3).

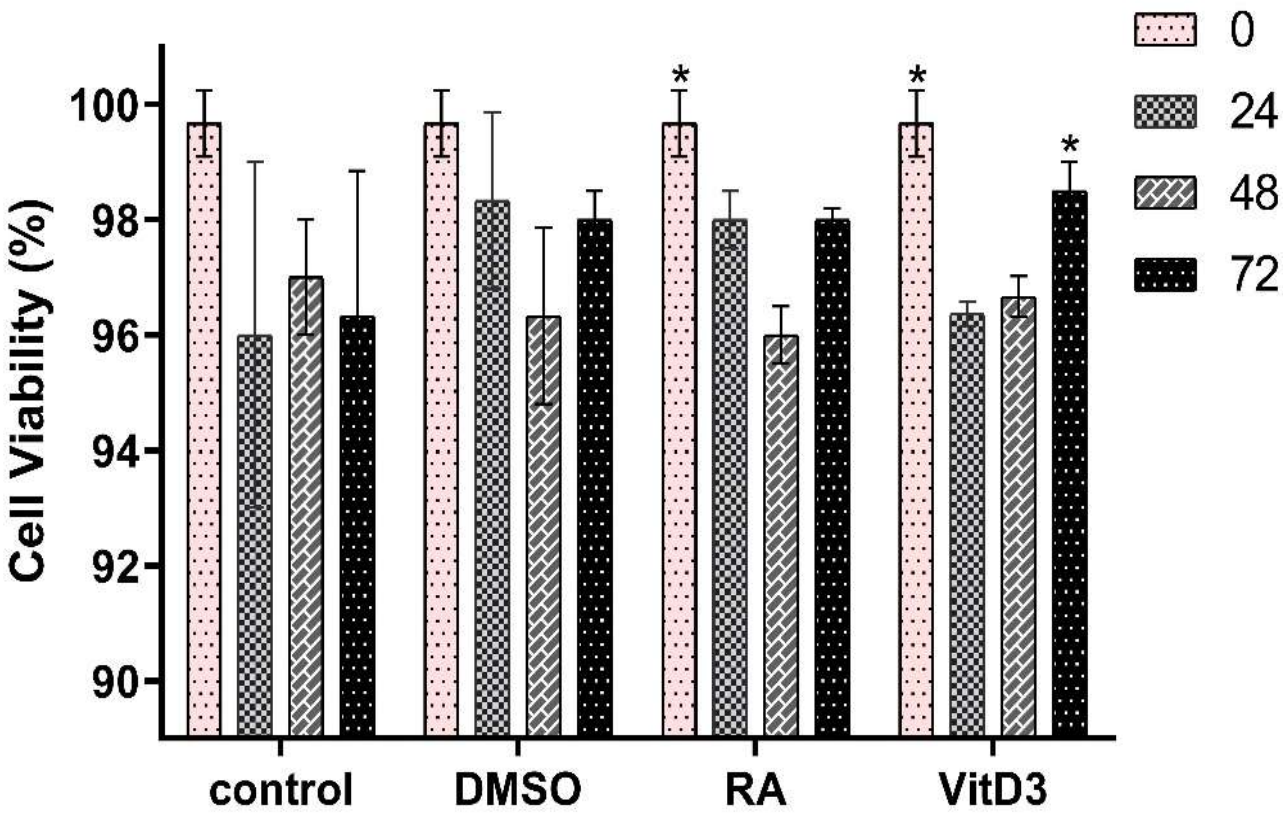

Fig. 2. The cell viability of U937 cells, treated with DMSO, RA, Vit D3 was measured by trypan blue exclusion test every 24 hours for 3 days. Data shown as means ± SD of triplicate cultures of three independent experiments. * $P < 0.05$ compared with control cells.

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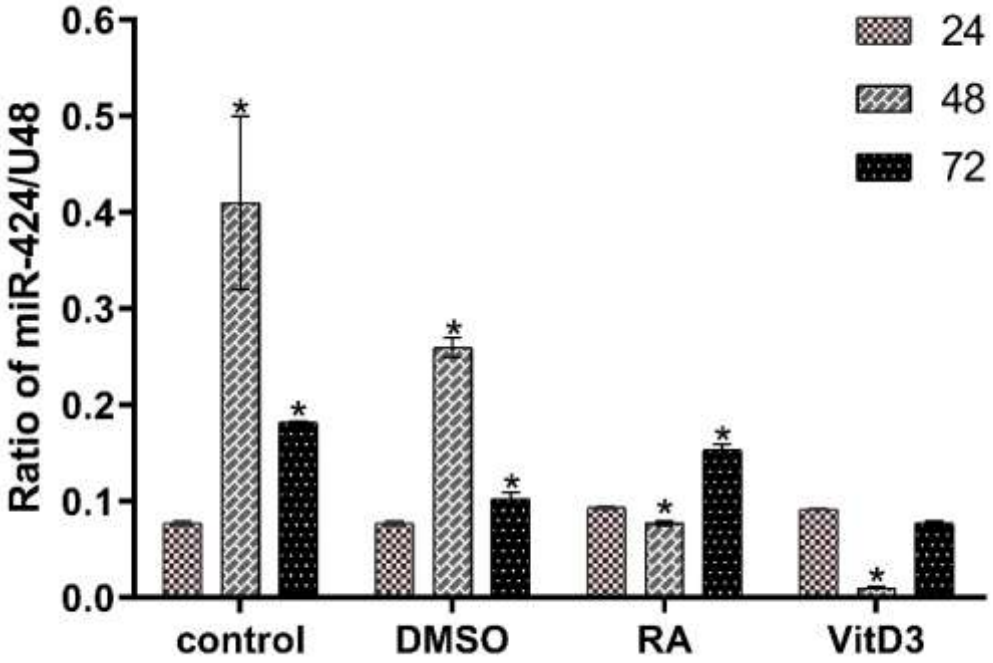

Fig. 3. The expression of miR-424 was measured in U937 cells, treated with DMSO, RA, Vit D3 every 24 hours for 3 days. Data shown as means ± SD of triplicate cultures of three independent experiments. * $P < 0.05$ compared with control cells.

Discussion

The role of microRNAs in regulating cell behavior, especially in life-threatening diseases such as cancer, has been strongly considered by researchers. Under some conditions, such as acute myeloid leukemia (AML), there is an increase in the number of myeloid cells and a stop in the maturation of these cells in the bone marrow. These conditions lead to hematopoiesis and hematological disorders (26). In AML, the accumulation of leukemia cells (Blasts) occurs from a defect in the myeloid progenitor cells to produce mature blood cells (27). Acute promyelocytic leukemia (APL) as a subtype of AML is an analogue model for investigation of granulopoiesis. U937 cells are a proper cell line to study inhibition of cellular differentiation in the development of myelogenous leukemia. This cell line is identified to be propagated or inhibited by epigenetic modifications. The way that expression of regulating pathways under the influence of induction factors can evaluate the proliferative and differentiation pathways of the cells. Alterations of proliferation or differentiation pathways are due to the regulatory activity of molecules such as miRNA. These regulatory factors, which influence cell viability and proliferation are able to affect the biogenesis of cancer. One of these microRNAs is the miR-424 (15, 27, 28). In this study, the U937 cell line, as a pro-monocytic, human myeloid leukemia cell line was exposed to factors such as RA and VitD3 for 24, 48, and 72 hours to investigate the expression of miR-424.

The results of treatment cells with VitD3 during 24, 48, and 72 hours have shown that there was no difference between the VitD3 treatment group with the control and DMSO group after 24 hours treatment. In addition, after 24 hours, all three-group proliferation were increased to 1.5 times in cell number. This proliferation doubled at 48 hours and more than 4 times during 72 hours. The proliferation rate was similar to control and DMSO groups, but it has shown a significant difference between the VitD3 and RA groups. Hence, it seems that VitD3 has not been able to show its inhibitory effect on proliferation

[DOI: 10.52547/rbmb.11.3.440]

during times. Moreover, this growth factor did not affect cell survival, and this was similar to the control group during treatment hours. Bollag et al. (1995) have studied the role of VitD3 on cell proliferation. They have evaluated the proliferation of epidermal keratinocyte cells in the presence of VitD3 and have shown that low concentrations of VitD3 were ineffective in differentiation and inhibition of cells. In addition, these researchers have found that a protein kinase C inhibitor, Ro 31-7549 which plays an important role in cell proliferation, is stimulated by a high concentration of VitD3 such as 1 μM. They reported that a high concentration of VitD3 affected on reduction in cell number and activation of rapid intracellular signaling for 4 hours. Therefore, this study has displayed that the physiological dose of VitD3 can stimulate cell proliferation in keratinocytes, but a non-physiological dose of this component leads to inhibition of proliferation and initiation of differentiation, which have been remarked in most of the cancer studies (29). This high concentration of VitD3 can conduct to harmful effects on normal cells. Therefore, maximum proliferation was observed at 72 hours in the VitD3 treatment group which can be due to physiological dose and proper time treatment. Then, intracellular signaling has changed during treatment hours and the cells rebound to their proliferation condition before exposing with VitD3, which may have occurred in the present study.

In part of the present study, it was investigated the role of VitD3 in the expression of miR-424 during different treatment times. It was observed that there is no significant difference in the expression of miRNA after 24 hours of treatment. However, there was a significant decrease in treatment after 48- and 72-hours incubation compared to other groups. These outcomes have shown that treatment of U937 with VitD3 led to decreased expression of miR-424 during treatment hours. According to the results of cell proliferation and cell survival in this group, it can be concluded that the reduction of expression of miR-424 occurs to prevent inhibition of cell proliferation and increase the number of cells.

The miR-424 leads to stop the monocytic differentiation pathway through suppression of the NFI-A translation target (30). A decrease in the NFI-level is essential for the purpose of the activity of differentiation-specific genes, such as the M-CSF receptor (M-CSFr) (11, 12). Previous studies have shown that the PU.1 factor communicated with the miR-424 promoter and is responsible for at least 50% of the activity when drugs such as tetradecanoylphorbol acetate (TPA) are used. miR-424 causes differentiation and the commitment of cells while performing an important stimulant effect. On the other hand, it has been shown that the improper expression of miR-424 in the myeloid precursors leads to the progression of monocytic differentiation, and the reason for this unusual activity is the lack of PU.1 inducer factor (30).

Growth factors such as RA and VitD3 can affect molecular pathways. As it has shown miR-32 can be induced by VitD3 and regulate Bim proapoptotic factors. Improper expression of miR-32 caused an increase in differentiation response of AML cells to VitD3 (31). In contrast, miR-181 expression is reduced by VitD3 treatment. This miRNA regulates the expression of p27kip1 of the cell cycle. P27kip1 is generally induced during monocytic differentiation (11). In this study, treatment of cells with VitD3 caused expression of miR-424 downregulated which enhanced cell proliferation and survival in myeloid cell lines. It looks that the intracellular pathway is related to what was discussed in the previous paragraph. The results of this part of the study have shown that treatment of U937 cells with RA for 24, 48, and 72 hours led to decrease cell proliferation and viability.

All Trans Retinoic Acid (ATRA) has been used in the differentiation of myeloid malignancies. In addition, some studies have considered the role of phorbol myristate acetate (PMA) and 12-O-13-

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acetatetradecanoylphorbol (TPA) in this concern (15). ATRA was made in Shanghai for the first time and used in the treatment of APL patients in 1987 (32). First, ATRA treatment was used on French patients, which treated with chemotherapy at 45 mg/m^2 dose **.** In this method, no hair loss, the aplastic phase, and drug resistance were observed. Progressive changes were detected in the shape of malignant cells in the bone marrow, which these changes included the differentiation of malignant cells, and the confirmation was performed by the FISH method. However, the use of this method alone has two harmful consequences: One during the treatment and one after the treatment. The activation of leukocyte differentiation led to an increase in their number in the blood. This status happened in one-third of the patients, which referred to ATRA syndrome (33). In other ways, after treatment, patients with resistance to ATRA did not respond to this method. According to the earlier study, it can be concluded that RA was able to differentiate in malignant cells and to help in the healing process of patients.

Grenier et al. (2007) explored the role of RA in the proliferation and differentiation of intestinal epithelial cells (IECs). They were found that these cells progressed with the process of differentiation. In this study, researchers have shown the treatment of Coca cell line with different concentrations of RA, such as 10 μM in 24 hours caused a decrease in cell proliferation. In addition, in this study, the effect of this factor on cell survival was examined and it was found that there was not found any negative effect on the cells. Then, it was determined that this factor led to inhibit proliferation and decreased expression of cyclin D1 and increased cyclin D3 (34). Najafzadeh et al. (2013) evaluated the concentration of 10 μM of RA in the differentiation of AGS cells. Therefore, it was found that the concurrent use of this factor with cisplatin caused an increase in differentiation and inhibit cell proliferation (35). Rosa et al. (2007) investigated the role of miR-424 on myeloid cells. They reported that the PU.1-dependent pathway was used to differentiate promyeloblast into monocyte and macrophage cells that interfered with miR-424 promoter and it is responsible for 50% of its function on TPA therapy (36).

In this study, the role of RA on the expression of miR-424 was examined. The results have displayed that the expression of miR-424 significantly increased with time-lapse. 24 hours treatment of cells with RA showed the lowest expression of miR-424 and the 72 hours treatment displayed the highest expression. On the other hand, it was revealed that the highest proliferation and survival was displayed at the lowest expression level of miR-424, while the highest expression level of miR-424 was observed with the lowest number of cell proliferation. In addition, in the present study, this molecular process is likely happened due to an increase in the expression of miR-424 in U937 cells, which ultimately led to a reduction in cell proliferation within 72 hours of treatment. However, more studies are needed to examine the intracellular molecular process.

The U937 myeloid cells were proliferated and differentiated after treatment with inducer factors such as RA and VitD3. This increased or inhibition of proliferation was probably associated with an increase or decrease of miR-424 expression. The initiation factors had no adverse effects on survival cells after 24, 48, and 72 hours of treatment. However, the results may be due to the dose of both compounds and may change with the dose or time-dependent of treatment. On the other hand, the RA and VitD3 factors will be affected on miR-424 expression by dose or time-dependent of treatment.

Acknowledgements

This study was approved by the Tarbiat Modares University Ethics Committee and supported financially by Tarbiat Modares University.

We would like to thank researchers in stem cell technology research center for their scientific assistance. All authors declare that they have no conflict of interest.

[DOI: 10.52547/rbmb.11.3.440]

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[DOI: 10.52547/rbmb.11.3.440]