INTRODUCTION

Epigenetic changes are reversible alterations in function and expression of genes without any modification in their nucleotide sequence. Methylation and deacetylation of histones often cause the chromatin structure to become more compact, triggering gene silencing during the cell cycle. In contrast, histone acetylation and deacetylation are commonly associated with an open state of chromatin structure and active transcription of the gene, although there are some exceptions. DNA methylation is the most well-known epigenetic marker, comprising the addition of the methyl group to the cytosine base in the CpG-rich regions of the genome known as the CpG Islands.

In cancer cases, three major phenomena may affect the pattern of DNA methylation. Increased activity of methylating enzymes in malignant cells is mainly due to global demethylation of CpG in the body of the gene, leading to a general hypomethylation compared to a normal cell. In general, two types of enzymes are involved in the DNA methylation process, including DNA methyltransferases (DNMTs) and DNA demethylases. In mammals, CpG methylation is mediated by the methyltransferase family of genes which have 3 active members, including DNMT3B, DNMT3A, and DNMT1. Based on investigations DNMT1 and DNMT3B activity levels are abnormally increased in acute lymphoblastic leukemia (ALL) patients. Considering the critical role of diverse DNMT isoforms, particularly DNMT1 in adjusting of hematopoietic stem cell (HSC) proliferation, differentiation and survival, it is not unexpected that the aberrant methylation of the enzyme results in leukemogenesis.

Ara-C is one of the antineoplastic drugs, immune suppressants and a pyrimidine nucleoside. Though Ara-C is an important drug in the treatment of acute myeloid leukemia (AML), there are encouraged consequences about the therapeutic capability of Ara-C in acute lymphoblastic leukemia (ALL). Herein, we evaluated the effect of Ara-C on the expression of genes coding for the enzymes DNA methyltransferase (DNMT) 3A, DNMT 3B and histone deacetylase 3 (HDAC3) in the human B cell-ALL cell line Nalm6. Moreover, we investigated its effects on Nalm6 cells proliferation and apoptosis. Briefly, Nalm6 cells and also normal peripheral blood mononuclear cells (PBMCs) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, and treated with Ara-C at their exponential growth phase. Cell apoptosis rates were studied using Annexin-V/PI staining and fluorescence-activated cell sorting (FACS) analysis, and their proliferation levels were evaluated upon treatment with increasing concentration of Ara-C (5–80 nM) by MTT assay. Finally, the expressions of the above-mentioned 3 genes were quantified using real-time PCR. Based on analysis, Ara-C could powerfully trigger apoptosis and obstructs proliferation of Nalm6 cells upon treatment. After Ara-C treatment, expressions of the genes DNMT3A in Nalm6 cell line increased but DNMT3B and HDAC3 decreased significantly compared with the control group. Altered expressions of the above-mentioned genes in ALL cells under the effect of Ara-C suggests that epigenetic changes such as DNA hypermethylation and histone deacetylation may be appropriate goals in the development of new therapies.
Another epigenetic change associated with cancer is the aberrant pattern of change after translation of histones. Acetylation of histones is by enzymes with histone acetyltransferase (HAT) activity and their deacetylation by histone deacetylase (HDAC) enzymes. Increase or decrease in the expression of DNMTs and HDACs is likely to play a role in the development of leukemia or disease progression because they can turn on or off the suppressors or cell signaling pathway molecules that are effective in preventing cancer. According to a large number of studies, aberrant expression of HDACs is related to hematological disorders, in particular, leukemia. Specially, there exists tight association between overexpression of HDAC5 and HDAC7 and ALL. Increasing numbers of chemical compounds have now been identified that exhibit hypomethylation activity. These agents originally used for epigenetic research are now under investigation for their potential use in cancer treatment. In this regard, Ara-C, (possibly a histone deacetylase inhibitor) has attracted great worldwide attention. While Ara-C is an important drug in the treatment of acute myeloid leukemia (AML), there are hopeful results about the therapeutic potential of Ara-C in ALL. However, the underlying mechanism involved in anti-leukemic effects of Ara-C has not yet been entirely understood in ALL.

Accordingly, in this study we investigated the effect of Ara-C on the expression of HDAC3 and DNMT3A and 3B in the human B cell-ALL cell line Nalm6 using real-time PCR (RT-PCR). Moreover, the proliferation rates of the Nalm6 cells were evaluated upon exposure with various concentrations of Ara-C by MTT assay. Also, Ara-C cytotoxicity was examined in Nalm6 cell using Annexin-V/PI staining and fluorescence-activated cell sorting (FACS) analysis.

MATERIALS AND METHODS

Reagents and cell line

This lab trial experiment was conducted in Shahid Sadoughi University of Medical Sciences, Yazd, Iran, and approved by the ethics committee under the code IR.SSU.MEDICINE.REC.1396.133. The human B-cell precursor leukemia cell line Nalm6 was provided from Institute Pasteur, Tehran, Iran. As healthy control group, peripheral blood mononuclear cells (PBMCs) from a pool of 10 healthy asymptomatic children (<12 years old) referred to Yazd Central Medical Laboratory were taken by Ficoll gradient method. All cells were grown in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Sinaclon, Iran), penicillin (50 units/ml, Gibco, USA) and streptomycin (50 μg/mL, Gibco, USA) at 37°C in a 95% humidified atmosphere with 5% CO₂. The culture flasks were diluted at a ratio of 1:3 every 1 to 2 d. The Ara-C (Sigma-Aldrich, Germany) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany). The maximum final concentration of DMSO in medium was less than 0.02%. Also, MTT reagents were obtained from Sigma-Aldrich (Sigma-Aldrich, Germany).

MTT assay

In this step, the cytotoxicity of Ara-C treatment in the Nalm6 cells was investigated by the MTT assay based on the instructions from the manufacturer of the MTT kit. Concisely, 1.25 × 10⁴ Nalm6 cells/100 μL of RPMI-1640 medium were cultured into the wells of a 96-well plate and then the Ara-C was added at the various concentrations (5–80 nM) into the wells. After that, within the 24, 48 and 72 hours upon exposure with Ara-C, 20 μL of 5 mg MTT/ml medium were added into the wells. Then, optical density (OD) of wells was estimated at 570 nM wavelengths with ELISA reader upon cells storing at 37°C for 4 hours. The viability of exposed cells with Ara-C was measured according to the viability rate of control cells and showed as percentage. All viability sequences were descriptive of three independent experiments and values were represented in means ± SEM.

Flow cytometric analysis of apoptosis

An Annexin V-based kit (Apopotest™-FITC Kit, Dako, Glostrup, Denmark) was utilized to estimate the apoptosis levels. The Nalm6 cells were exposed with Ara-C at 20 nM for 24, 48 and 72 hours. Upon adding 5 μL of propidium iodide (PI) and 10 μL of fluorescein isothiocyanate (FITC)-conjugated Annexin-V to plates, the FITC-Annexin V, the apoptosis percentage was measured based on fluorescent signal emission from the FITC-Annexin bound phosphatidylserines. The fluorescent emission was identified by a FACSCalibur machine (BD Biosciences, Franklin Lakes, NJ, USA) and the consequences were plotted by FlowJo software version 10.4.1.

RNA Isolation from the cells treated with Ara-C

Total RNA from Ara-C -treated cells was extracted according to the RNeasy kit (Sinaclon, Iran). The quality and quantity of RNA content was evaluated using Nanodrop-2000 (Thermo Fisher Scientific, Waltham, MA, USA). Absorbance ratios and concentrations were determined as indicators of sample yield, quality and purity.

Reverse transcription and cDNA synthesis

To evaluate primers by RT-PCR and to measure the expression of genes by real-time PCR (quantitative PCR, QPCR), the isolated total RNA was transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific, USA). Briefly, 1 μg of RNA was mixed with 1 μL of the random hexamer prim-
er and nuclease-free water (Qiagen, Germany) added up to 12 μL, according to the kit instructions. The tubes were then incubated in a thermocycler at 65°C for 5 minutes after which the tubes were placed on ice (4°C) and the other reagents were added. First strand cDNA was then amplified with the following program: 5 minutes at 25°C, 60 minutes at 42°C, and 5 minutes at 70°C.

RT-PCR

The two most commonly used methods to analyze data from QPCR experiments are absolute quantification and relative quantification. The 2^ΔΔCT method is a convenient way to analyze the relative changes in gene expression from QPCR. To determine the amount of mRNA levels of target genes, QPCR was done using cDNA, forward and reverse primers, distilled water and EvaGreen qPCR Mastermix 5× (as a mixture of dNTPs, Hotstart Taq polymerase [HOT FIREPol®, Solis BioDyne, Tartu, Estonia], MgCl₂, fluorescent detection dye EvaGreen, reference dye, and proprietary buffer components), according to the kit instructions. Unlike SYBR® Green I, EvaGreen® dye is cell membrane impermeable, and therefore cannot bind DNA in living cells. It has much less PCR inhibition, is extremely stable dye, has been shown to be nonmutagenic and noncytotoxic, and imparts brilliant green fluorescence to dsDNA. The relative expression of target genes was performed by Step One Plus Real-time PCR (Applied Bio systems, USA) in duplicate to a final volume of 20 μL using pre-set cycling parameters (10 min at 95°C; 15 s at 95°C; 20 s at 60°C with the latter two steps repeated for 35 times), and was then quantified by the ΔΔCT method. The expression of Parp1 mRNAs was normalized to GAPDH (Applied Biosystems; assay ID: Mm99999915-g1), which was the endogenous reference in the corresponding samples, and relative to the untreated control cells. The primer sequences used in RT-PCR are listed in Table 1.

Statistical analysis

Statistical analyses were completed employing GraphPad Prism version 7.05. Data are expressed as means ± SEM of three independent tests (triplicate). One-way ANOVA was used to evaluate statistical differences among the experimental groups. P-values <0.05 were considered statistically significant.

RESULTS

Ara-C reduced Nalm6 cells proliferation

Based on MTT assay consequences, Ara-C 5, 10, 15, 20, 40 and 80 nM reduced Nalm6 cells growth, slightly or powerfully (P < 0.05) (Figs. 1A, 1B). Although Ara-C 5 nM lessened proliferation level of the Nalm6 cells during 24 hours of treatment, this attenuation was not significant (Figs. 1A, 1B). Moreover, the inhibitory effect of the Ara-C 5 nM on Nalm6 cell proliferation was obvious and significant during 48 and 72 hours of exposure (P < 0.05) (Figs. 1A, 1B). Also, results demonstrated a noticeable attenuation in proliferation of the Nalm6 cells upon treatment with 10, 15, 20, 40 and 80 nM concentration of Ara-C at 24, 48 and 72 hours of exposure (P < 0.05) (Figs. 1A, 1B). Accordingly, the inhibitory impacts of Ara-C on Nalm6 cell proliferation was more obvious within 72 hours and at 80 nM concentration than other experimental times and concentrations (P < 0.05) (Figs. 1A, 1B).

Ara-C induced Nalm6 cells apoptosis

Results revealed a significant shift in apoptosis percentages in Nalm6 cells treated with Ara-C 20 nM compared with the control group (untreated Nalm6 cells) at 24, 48 and 72 hours of treatment measured by Annexin-V/PI staining and FACS analysis (P < 0.05) (Figs. 2A, 2B). Accordingly, apoptosis percentages in Nalm6 cells (control) and cells treated with Ara-C 20 μM within 24, 48 and 72 hours of exposure were 3.11 ± 1.21, 12.98 ± 2.05, 32.44 ± 3.28, and 48.19 ± 2.91% of total cells, respectively (Figs. 2A, 2B).

Decreased expression of HDAC3 gene in lymphoid cell line Nalm6 treated with Ara-C

The expression of HDAC3 gene in the Nalm6 lymphoid group and normal human blood cells was measured before and after treatment with Ara-C 20 nM. RT-PCR results showed a significant decrease in Nalm6 cells 24, 48 and 72 h after treatment (P < 0.05) (Fig. 3A). However, there was no significant difference in HDAC3 expression before and after treatment (P > 0.05) (Fig. 3A).
between 3 groups treated with Ara-C 24, 48 and 72 hours after exposure.

**Decreased expression of DNMT3B gene in lymphoid cell line Nalm6 treated with Ara-C**

The expression of DNMT3B gene in Nalm6 lymphoid and normal human blood cells was measured before and after treatment with Ara-C 20 nM. Based on observations, we found a significant decrease in Nalm6 cells 24, 48 and 72 h after exposure with Ara-C 20 nM (P < 0.05) (Fig. 3B). Moreover, there was a significant difference in DNMT3B expression levels among groups treated with Ara-C 24, 48 and 72 hours after exposure (P < 0.05) (Fig. 3B). This reduction was more prominent within 72 hours than 24 and 48 hours of exposure (P < 0.05) (Fig. 3B).

**Increased expression of DNMT3A gene in lymphoid cell line Nalm6 treated with Ara-C**

The expression of DNMT3A gene in Nalm6 lymphoid and normal human blood cells was measured before and after treatment with Ara-C 20 nM. According to RT-PCR results, a significant augmentation was demonstrated in Nalm6 cells 24, 48 and 72 h after treatment with Ara-C 20 nM (P < 0.05) (Fig. 3C). Nonetheless, we found no significant difference in DNMT3A gene expression levels among groups treated with Ara-C within 24, 48 and 72 hours of treatment (Fig. 3C).

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**Fig. 1** Evaluation of the Ara-C effect on Nalm6 cell proliferation by MTT assay. Figures (A and B) show the proliferation level of the Nalm6 cells upon exposure with Ara-C 5, 10, 15, 20, 40 and 80 nM with 24, 48 and 72 hours of treatment. Data are representative of three independent tests and values are represented in mean ± SEM. One-Way ANOVA test was conducted to determine the experimental statistical differences. P values < 0.05 were considered statistically significant. (*; p < 0.05, **; p < 0.01, NS; non-significant)
DISCUSSION

Epigenetics is a biological system that pronounces phenotypes and befalls because of the disparity modification of genes (as contrasting to genetic mutations). Present epigenetic trainings comprise the regulation of basic biological activities, developmental biology, the cause of disorders, and cancer therapeutics. Examining the regulatory mechanisms driving these consequences contain genome-wide mapping of chromatin accessibility and conformation, transcription factor (TF) binding along with DNA methylation, and histone modifications.

Based on investigations, leukemia oncogenesis is an outcome of both genetic and epigenetic causes, whereby hematopoietic pathways are interrupted and leukemia cells can evade lineage commitment. In acute leukemia, somatic mutations are commonly categorized as nonsynonymous mutations in genes relevant for pathogenesis. Some of these alterations modify epigenetic changes, including mutations in DNA-methylation genes and chromatin-modifying genes. According to findings, aberrant epigenetic modifications, such as DNA methylation of promoter related CpG islands, show in ALL. Current data from various laboratories signify that wide spectrums of genes, containing pivotal molecular pathways, are epigenetically inhibited in ALL. It has been identified that mutations in DNMT3A occur in approximately 20% of cases of acute leukemia. Moreover, it was evidenced that DNMT3A is mutated in other hematopoietic disorders at a lower frequency. In addition to the myeloid lineage, DNMT3A mutations appear to be happened in T-cell lymphoma and T-ALL.

Also, in pediatric ALL, overexpression of HDAC1, HDAC2, HDAC3, HDAC6, HDAC7 and HDAC8 have been found compared with normal cells. Due to the importance of these modifications, targeting of epigenetic modification to achieve acceptable therapeutic outcome has attracted a growing attention in the world during last decade's. Ara-C is a deoxycytidine analogue that is a typical drug for treatment of patients suffering from AML. Moreover, there are hope-

Fig. 2 Assessment of the Ara-C cytotoxicity against Nalm6 cells. Figure 2A depicts a representable sample of study of apoptosis using Annexin-V/PI staining and FACS in Nalm6 cells upon exposure with optimum dosage of Ara-C (20 nM) within 24, 48 and 72 hours of exposure. The data expresses the three independent tests. The percentage of cells in each quadrant is shown (viable cells are in Q4). Figure 2B demonstrates apoptotic cells percentage triggered with Ara-C (20 nM) within 24, 48 and 72 hours of exposure. Data are expressed as mean ± SEM of three independent tests. One-Way ANOVA was used to determine the experimental statistical differences. P values <0.05 were considered statistically significant. (*; p < 0.05, **; p < 0.01)
ful results reflecting therapeutic potential of Ara-C in ALL\textsuperscript{29}. However, the underlying mechanisms involved in anti-leukemic effects of Ara-C have not yet been entirely understood in ALL. In this study, firstly, we evaluated Ara-C effects on the proliferation of human acute lymphoblastic leukemia Nalm6 cells line. Results revealed that the proliferation of Nalm6 cells was abrogated upon exposure with optimum dosages of Ara-C (20 nM) (Fig. 1). Also, Ara-C was able to induce apoptosis pathways in Nalm6 cells, according to FACS analysis (Fig. 2). On the

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Fig. 3 Real Time-PCR data for HDAC3 (A), DNMT3B (B) and DNMT3A (C) expression in healthy PBMCs sample (normal), and in Nalm6 cells before (pre-treat) and after treatment with Ara-C (20 nM) within 24, 48 and 72 hours of exposure. Data are representative of three independent experiments and values are expressed in mean ± SEM. GAPDH was used as the internal control. One-Way ANOVA was e to evaluate the observed statistical differences. P values <0.05 were considered statistically significant.
other hand, we found that it significantly reduced HDAC3 and DNMT3B and promoted DNMT3A expression at mRNA levels in treated cells. Regardless of the finding reporting that HDAC3 expression is strongly promoted in varied types of the human leukemia (e.g., ALL)\(^1\), there are studies delivering the proof of the concept that HDAC3 contributes to chemotherapy resistance by augmenting the activation of AKT, a well-recognized molecule in drug resistance progression. Accordingly, HDAC3 connects to AKT and deacetylates it at the site Lys20, thus stimulating the phosphorylation of AKT. Therefore, HDAC3 down regulation, as shown by our analysis, triggers apoptosis in leukemia cells by inhibition Akt/Pi3K pathways\(^2\). On the other hand, a large number of investigations confirmed that DNMT3B overexpression is a poor prognostic marker in acute leukemia. For example, Pole et al. reported that that in T-ALL and Burkitt’s lymphoma the MYC oncogene induces overexpression of DNMT3B, leading to the tumor development\(^3\). As well, it has been revealed that there is tight association between DNMT3B over expression and Akt activation in cancer cells\(^4\). In sum, regarding reports reflecting that inhibition of Akt pathways can lead to the desired therapeutic outcomes in leukemia, it seems that Ara-C could induce apoptosis and inhibit proliferation of Nalm6 cells most likely by down regulation of survival-involved pathways, in particular Akt/Pi3K signaling through down regulation of HDAC3 and DNMB3 in Nalm6 cells.

In sum, we showed that Ara-C could display notable cytotoxicity against leukemia Nalm6 cells. According to analysis, we suggest that Ara-C can suppress Nalm6 cells proliferation and attenuate their survival by up regulation of DNMT3B and HDAC3 expression and down regulation of DNMT3A expression at mRNA levels. It seems that inhibition of Akt activation, leading to the suppression of survival-involved Akt/Pi3K signaling pathways, possibly plays an important role in Ara-C exerted anti-leukemia effects in human Nalm6 cells. Due to the existence of some complexity in the identification of epigenetic modification in leukemia cells and their double sword role in leukemia incidence and progression, executing of more investigation is of paramount importance in this context.

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