Original Article

L-carnitine in a certain concentration increases expression of cell surface marker CD34 and apoptosis in the rat bone marrow CD34$^+$ hematopoietic stem cells

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Abstract

Background: Stem cell based therapy has been encouraged as an attractive method in regenerative medicine. Poor survival and maintenance of the cells transferred into the damaged tissue are broadly accepted as serious barriers to enhancing the efficacy of regenerative medicine. For this reason, some antioxidants such as L-carnitine (LC) are used as a favorite strategy to improve cell survival and retention properties. **Aims:** This study aims to evaluate the effect of LC on the expression of CD34 marker and its effect on apoptosis and *SUZ12* gene expression. **Methods:** Rat bone marrow mono-nuclear cells (rBMNCs) were isolated. Then, CD34$^+$ hematopoietic stem cells (HSCs) were enriched using the magnetic activated cell sorting (MACS) method. The cells were treated with 0.2 and 0.4 mM LC. Gene and protein expression levels of the CD34 were then measured by real-time PCR and flow cytometry, respectively. The percentage of apoptosis and *SUZ12* gene expression were measured using the Annexin V/PI method and real-time PCR, respectively. **Results:** The results showed that in the experimental group, of the CD34$^+$ HSCs treated with 0.2 mM LC, gene and protein expressions of CD34 increased by 1.7 fold and 0.49%, respectively. At the concentration of 0.4 mM, the early cell apoptosis increased by 25.9% ($P<0.05$). Also, in the concentration of 0.2 and 0.4 mM LC, the *SUZ12* gene expression increased by 1.10 and 1.75 folds compared to the control group ($P<0.05$ and $P<0.01$), respectively. **Conclusion:** The results of this study could be used to improve chronic myeloid leukemia (CML) as a multidirectional therapeutic strategy.

Key words: Apoptosis, CD34, Hematopoietic stem cells, L-carnitine, SUZ12

Introduction

As a powerful antioxidant, L-carnitine (LC) has many biological effects such as increasing the activities of antioxidant enzymes, decreasing population doubling time and aging, improving lifespan of mesenchymal stem cells (MSCs), metabolism of fatty acids, and decreasing lipid peroxidation (Mobarak *et al*., 2017). Bone marrow contains a number of cell surface antigens for identification, isolation and enumeration of hematopoietic stem cells (HSCs) and progenitors called transmembrane CD34 glycoprotein with a molecular weight of 110 kDa (Attar, 2014). This marker is expressed in the vascular endothelium of 30% of patients with acute leukemia. CD34, as a specific selection marker, belongs to the sialomucin family and plays an important role in the adhesion of progenitors to bone marrow stroma via L-selectin, with the ability to produce and differentiate all blood cells including immune cells (de Fabritiis *et al*., 1993; Lin *et al*., 2020). Contrary to the abundance of CD34 markers in progenitor cells, this marker is rarely seen in peripheral blood (Nielsen *et al*., 2009). For this reason, in order to isolate the marker-containing cells from peripheral blood, progenitor stem cells must be stimulated to enter the peripheral blood (Walz *et al*., 2006).

Apoptosis is a type of precise, natural, and physiological cell death in the body that is genetically encoded and causes cell destruction during which structural changes and disintegration of cellular DNA occurs (Ghose *et al*., 2020). It is an energy dependent process and some anticancer therapies are based on activating this pathway. It occurs during differentiation, cell count regulation, embryonic and organ development, tissue homeostasis and defense against pathogens and immune regulation (Obeng, 2021). Caspase enzymes are

cysteine proteases that play a major role in initiating and performing apoptosis. By activating caspases, a number of key enzymes, including polymerase, are involved in repairing damaged DNA, and the structural proteins of the cell are broken down, leading to cell death (Kesavardhana *et al*., 2020).

Polycomb group (PcG) proteins contain PcG proteins such as enhancer of zeste homolog 2 (EZH2), suppressor of zeste 12 protein homolog (SUZ12), and embryonic ectoderm development (EED) (Lee *et al*., 2015; Penfornis *et al*., 2018). Deregulation of PcG proteins has been reported in several cancer types (Bracken *et al*., 2003). SUZ12 is essential for polycomb repressive complex 2 (PRC2), and its inactivation results in early lethality of mouse embryos. SUZ12 is often up-regulated in liver, breast, and colon tumors (Pizzatti *et al*., 2010). The role of signaling pathways such as E2F/Rb and Wnt/β-catenin in the regulation of SUZ12 in solid tumors has been previously indicated. For the first time, Pizzatti *et al*. (2010) found that SUZ12 is over-expressed in the bone marrow of patients with CML-blastic phase due to the activation of a non-canonical Wnt signaling pathway (Pizzatti *et al*., 2010). Pasini *et al*. (2007) showed that SUZ12 is essential for the proper differentiation of embryonic stem cells (ESCs), most likely by directly controlling specific gene expressions during cellular commitment (Pasini *et al*., 2007). In another study, it was pointed out that complete loss of SUZ12 resulted in failure of hematopoiesis and loss of HSCs maintenance (Lee *et al*., 2015). Also, it was indicated that because PRC2 is a potential target for cancer therapy, the significant consequences of modest changes in PRC2 activity, as well as the cell and developmental stage-specific effects, will need to be carefully considered in any therapeutic context (Lee *et al*., 2015).

This study aims to evaluate the effect of LC on the *CD34* gene and protein expression, apoptosis and *SUZ12* gene expression on $CD34^+$ HSCs as a therapeutic approach in CML.

Materials and Methods

Reagents

All culture plates and reagents related to cell culture, if not otherwise specified, were purchased from SPL Life Sciences Co., Ltd. (South Korea) and Gibco Co. (UK), respectively.

Bone marrow mononuclear cells isolation and $CD34^+$ HSCs enrichment

Ethical consent was applied by an ethics committee at Tabriz University of Medical Sciences, Tabriz, Iran (Ethic Code No.: IR.TBZMED.VCR.REC.1396.849) according to guidelines from Helsinki-Ethical Principles for medical research and experiments on animals. 3 male Rattus norvegicus were purchased and euthanized using ketamine/xylazin as previously reported by Fathi *et al*. (2020c). In brief, bone marrow contents from the tibia and femur were collected by flushing with a syringe needle containing washing buffer (PBS supplemented with 5% FBS). Bone marrow contents were washed with washing buffer and were layered over the same volume of Ficoll-Paque (Innotrain, Germany). Thereafter, the middle phase, containing the mononuclear cells (MNCs) layer was transferred to a new 15-ml falcon tube and o-incubated with 100 µL of $CD34^+$ micro beads (Miltenyi Biotech) for 30 min. Re-suspended cells were then passed through the magnetic-activated cell sorting (MACS) column (Miltenyi Biotec) and enriched $CD34^+$ cells were retrieved by flushing the column (Fathi *et al*., 2020b).

Purity assessment of $CD34^+$ HSCs

Purity assessment of the enriched cells was performed by flow cytometry as previously explained by Fathi *et al*. (2020b). In brief, approximately 20×10^4 enriched $CD34^+$ HSCs were incubated by 5 µL of FITC-conjugated antibody CD34 (Santacruz, Lifespan BioSciences, USA) (1 µg/10^6 cells) and were then subjected to the flow cytometry instrument. The output data were processed using FlowJo software version X.0.7.

Quantitative real-time PCR

In this study, the cells were divided into three groups: group I as the control group ($CD34^+$ HSCs without any LC treatment) and group II and III as experimental groups ($CD34^+$ HSCs with 0.2 and 0.4 mM LC treatment). The suitable concentration of LC was previously investigated by Farahzadi *et al*. (2016). They reported that LC increased cell proliferation at 0.2, and 0.4 concentrations at 48 h. Accordingly, 48 h was used in this study as well. In another study reported by Mobarak *et al*. (2017), it was shown that 0.2 mM LC increased cell proliferation. For this reason, two concentrations, 0.2 and 0.4 mM LC, were chosen for the present study (Farahzadi *et al*., 2016). For this purpose, LC was dissolved in PBS and prepared as 1 M concentration. In the following, LC was added to the wells at final concentrations of 0.2 and 0.4 mM for up to 48 h at 37°C in 5% CO_2. At the end of the treatment period, $CD34^+$ HSCs from the three control and experimental groups were collected. Total RNA was extracted and cDNA was synthesized. The mRNA expressions of target genes included CD34, SUZ12 and β-actin. Fluorescence data was calculated in relation to b-actin CT values by the $2^{-\Delta\Delta CT}$ method. Primers (Table 1) were designed using Oligo 7 v.7.52 software (Adibkia *et al*., 2021; Heidari *et al*., 2021).

Flow cytometry analysis of CD34 cell surface marker assessment

After treatment with LC, the $CD34^+$ HSCs from control and experimental groups were collected and subjected to flow cytometry to evaluate the CD34 cell surface marker. In brief, approximately 50×10^4 $CD34^+$ HSCs were incubated with an appropriate amount of FITC-conjugated antibody CD34 (Santacruz, Lifespan BioSciences, USA) (1 µg/10^6 cells) in washing buffer for 30 min on ice. After washing the cells, FACS instrument

Table 1: Primer sequences used for the real-time PCR assays

No.	Gene	Primer pair sequence	Product length (bp)
XM_039087645.1	*SUZ12*	GCTGTTCAGAGTAACTCGTCC CAAACACTGTCATTTGTGCAAC	158
Nm_001107202.2	*CD34*	CAGAACTTTCCAGCAAACTCC ACTCCCGAGGTAACCAATGC	145

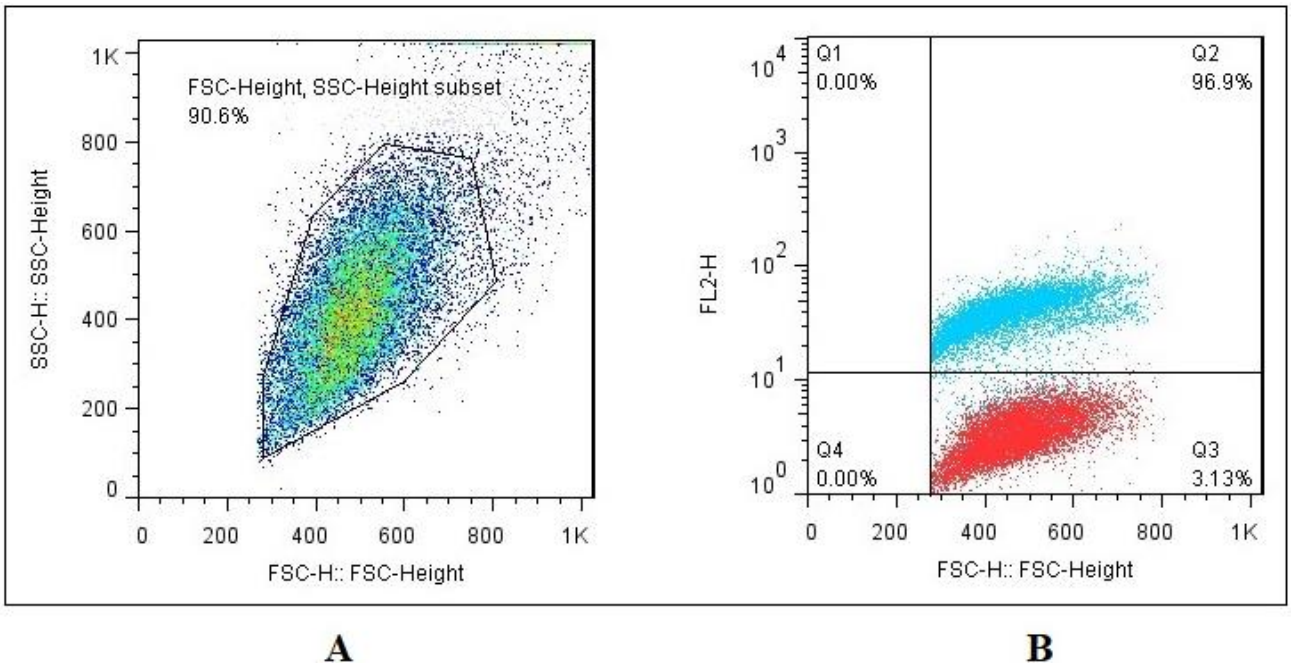

Fig. 1: Characterization of enriched bone marrow-derived CD34$^+$-expressing cells by flow cytometry. (**A**) A total population of cells for CD34 evaluation, and (**B**) Flow cytometry showed that 96.9% of cells were positive for CD34

was used to quantify the fluorescence intensity, and the data were analyzed with a FlowJo software (version 6.2) (Montazersaheb *et al*., 2020; Fathi *et al*., 2021).

Flow cytometry detection of apoptosis by Annexin V/PI assay

As mentioned above, the CD34$^+$ HSCs from control and experimental groups were trypsinized, washed twice with PBS, re-suspended in the binding buffer (Ref No.: 00-0055-56, ebioscience) and kept for 20 min. Then, CD34$^+$ HSCs were incubated with the binding buffer containing 5 µL of FITC-conjugated Annexin V (Ref. No.: 11-8005-74, ebioscience) for 15 min. Next, cells were washed with the binding buffer and exposed to PI solution in 100 µL binding buffer. Flow cytometry was performed by FACSCalibur (BD Bioscience), and the data were analyzed using FlowJo software ver. X.0.7 (Montazersaheb *et al*., 2019; Fathi *et al*., 2021).

Statistical analysis

The results were analyzed using the Graph Pad Prism version 6.01 software program. T-tests and one-way ANOVA were used to determine any significant differences among the groups and flow-cytometry was analyzed by FlowJo software. Statistical significance was determined to be $P<0.05$. All experimental procedures were repeated three times.

Results

Identification of CD34$^+$ HSCs

As shown in Figs. 1A and B, flow cytometry analysis indicated that enriched bone marrow-derived CD34$^+$ cells by MACS had high levels of expression of CD34 (96.9%). In other words, the enriched cells had a high level of CD34 expression.

LC caused to change the *CD34* and *SUZ12* gene expression

To evaluate the effect of LC on *CD34* and *SUZ12* gene expressions of CD34$^+$ HSCs, the mRNA was examined by real-time PCR. As shown in Fig. 2A, the mRNA expression level of CD34 in the presence of 0.2 and 0.4 mM LC significantly increased by about 1.7 and 1.5 folds, respectively ($P<0.01$ and $P<0.05$). In addition, a significant increase in the mRNA expression level of SUZ12 was shown by about 1.10 and 1.75 folds in the presence of 0.2 and 0.4 mM LC, respectively ($P<0.05$ and $P<0.01$) (Fig. 2B).

LC caused to change the CD34 protein expression of CD34$^+$ HSCs

At the end of culture time in the absence and presence of LC, the CD34$^+$ HSCs were analyzed for

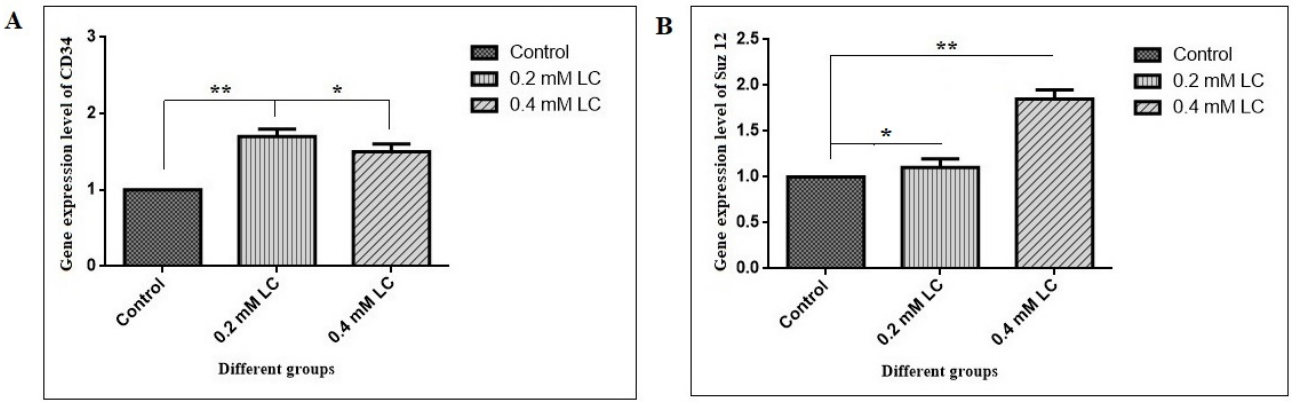

Fig. 2: Relative (**A**) CD34 and (**B**) SUZ12 mRNA expression levels of $CD34^+$ hematopoietic stem cells in the presence of 0.2 and 0.4 mM LC for 48 h of incubation. $CD34^+$ HSCs were cultured for 48 h in the presence of 0.2 and 0.4 mM LC. Total RNA was then extracted from cultured $CD34^+$ cells in group I as control group ($CD34^+$ HSCs without any LC treatment) and group II and III as experimental groups ($CD34^+$ HSCs with 0.2 and 0.4 mM LC treatment) as described in the methods section and subjected to real-time PCR assay, mean±SEM, n=3, $^*P<0.05$, and $^{**}P<0.01$. The Y-axis shows the fold of the mRNA expression level

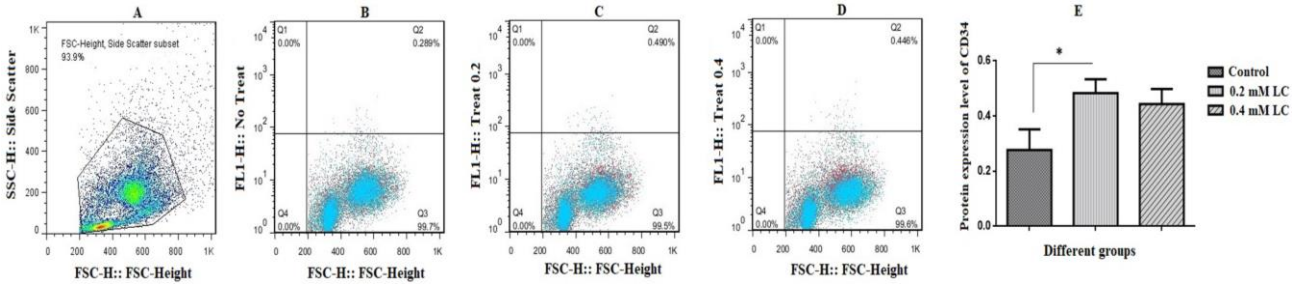

Fig. 3: CD34 cell surface marker protein expression levels of $CD34^+$ hematopoietic stem cells in the presence of 0.2 and 0.4 mM LC for 48 h of incubation. At the end of treatment period, $CD34^+$ HSCs were collected in both control ($CD34^+$ HSCs without any LC treatment) and experimental groups ($CD34^+$ HSCs with 0.2 and 0.4 mM LC treatment). (**A**) Cell population, (**B**) CD34 marker assessment in the control group, (**C**) CD34 marker assessment in the presence of 0.2 mM LC, (**D**) CD34 marker assessment in the presence of 0.4 mM LC. The quantification was shown in part **E**. As shown in part **C**, in the presence of 0.2 mM LC, the expression of CD34 significantly increased by about 1.75 folds in the experimental group compared to the control group, mean±SEM, n=3, and $^*P<0.05$

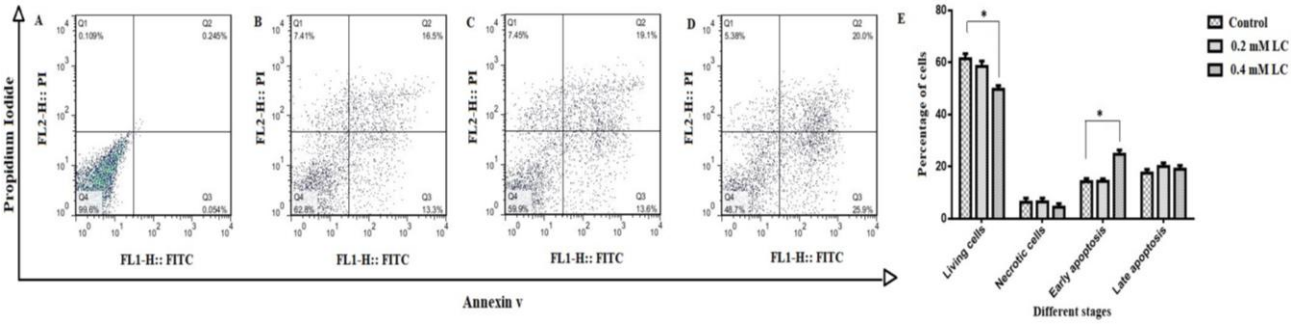

Fig. 4: Flow cytometry analysis of $CD34^+$ hematopoietic stem cells treated with LC with a combination of Annexin V-FITC, propidium iodide (PI). A shift from bottom-right quadrant panel (early apoptosis) to top-right quadrant panel (late apoptosis) and top-left quadrant panel (necrosis) was observed. (**A**) Unstained cells, (**B**) Control group ($CD34^+$ HSCs without any LC treatment), (**C**) Experimental group in the presence of 0.2 mM LC, (**D**) Experimental group in the presence of 0.4 mM LC, and (**E**) Statistical analysis

expressions of CD34 cell surface markers as shown in Figs. 3A-E. The results revealed that the expression of CD34 surface marker increased and decreased in the presence of 0.2 and 0.4 mM LC, respectively. This change in cell surface marker expression was only significant at 0.2 mM LC ($P<0.05$).

Investigation of apoptosis percentage by Annexin V/PI assay

As we know, early apoptotic cells are $Annexin^+$ and PI^- and late apoptotic or necrotic cells are positive to both $Annexin^+$ and PI^+. In order to assess the effect of LC on apoptosis, $CD34^+$ HSCs was treated with 0.2 and

0.4 mM for 48 h. Figs. 4A-E show the contour diagrams of Annexin V and PI stained $CD34^+$ HSCs using flow cytometry after 48 h of treatment with LC. As illustrated in Figs. 4A-E, in the presence of 0.4 mM LC, about 25.9% of the $CD34^+$ HSCs were in early apoptosis ($Annexin^+$, PI^-), which was 1.94 times higher than that of the control group ($P<0.05$).

Discussion

Reactive oxygen species (ROS) is one of the most important free radicals, which is generated through various pathways and plays an important role in the progression of leukemia (Thomas *et al*., 2013). One of the important challenges in the treatment of leukemia is the predominance of cells resistant to common chemical compounds that increase the ineffectiveness of anticancer drugs (Wang *et al*., 2011; Wu *et al*., 2011). In leukemia patients, the response to treatment is positive at first but the cells increase their effect resistance so that the chemotherapy is not effective (Druker *et al*., 2006). Therefore, the use of new therapies such as cell-based therapy and anti-cancer drugs is considered necessary and important (Fathi *et al*., 2019). In addition, poor survival, aging and maintenance of transplanted cells are broadly accepted as serious barriers against the efficacy of regenerative therapy (Fathi *et al*., 2020a). The use of external factors such as antioxidants to improve cell survival and retention properties is a favorite strategy for researchers, therefore finding effective and suitable agents such as vitamins and antioxidants to increase the viability of these cells could help promote their function in clinical applications (Farahzadi *et al*., 2016). Recently, many studies have been conducted to investigate the antioxidant effects of LC and its role in cell maintenance, differentiation and inhibition of aging, which proves the important function of this substance in the body (Mobarak *et al*., 2017). Several studies were conducted on the role of LC in promoting cell viability and maintenance (Fathi *et al*., 2020a). Hematopoiesis is a process that often takes place in the bone marrow, during whose various stages progenitor cells and eventually blood cells are produced. CD34 is one of the important cell markers that undergoes changes in the expression of cell surface in hematopoiesis and cell differentiation (Attar, 2014). Civin (1984) first found in 1981 that 1 to 4% of bone marrow cells with the potential of self-renewal capability express a type of cell surface marker that was later called CD34. Primary hematopoietic cells that lose self-renewal properties begin to express CD34 markers on their surface. This occurs in the transformation of LT-HSC (long term-HSC) into the common myeloid and lymphoid progenitor cells, which also have a higher ability to differentiate. Therefore, primary hematopoietic cells can be called $CD34^+$ and $CD38^-$ cells (Genovese *et al*., 2014). HSCs, as pluripotent and self-renewal cells, contain $CD34^-$ and $CD133^+$ that begin to express CD34 instead of CD133 by differentiating towards mature cells. Expression of this marker continues from long-term (LT)-HSC to blast cell lines and is not re-expressed again (Attar, 2014). Therefore, the differentiation of cells towards the lower and more distinguished cell lines increases the expression of CD34 cell surface markers in HSCs. Although the structure of the CD34 cell surface marker is not fully understood, its role in preventing or stimulating cell adhesion, proliferation and cell differentiation has been demonstrated (Nielsen *et al*., 2009; Scherberich *et al*., 2013). Production and differentiation of HSCs are strongly influenced by a group of hematopoietic cytokines (Montazersaheb *et al*., 2021). Each cytokine can have a variety of effects on the cells including cell preservation, proliferation, differentiation and growth (Metcalf, 2008). For example, cytokine interleukin (IL)-6, which aligns with granulocyte-macrophage colony-stimulating factor (GM-CSF), increases differentiation in myeloid cells (Tie *et al*., 2019). Few studies have been performed on the effect of LC on cytokine production. In one study reported by Kouttab *et al*. (1993), it was shown that LC increases production of IL-6, IL-1α, IL-1β, and tumor necrosis factor (TNF)-α. In the section of the present study which investigated the effect of LC on the expression of CD34 cell surface markers, an increase in the expression of this marker was observed. Based on previous studies and considering the role of LC in increasing the secretion of cytokines and consequently increasing cell differentiation, a significant relationship can be found between the effect of LC on rBMNCs and increased differentiation of pluripotent stem cells towards mature cells followed by increased expression of CD34 cell surface marker expressions. Induction of differentiation and prevention of cell self-renewals is one of the most important principles in the treatment of leukemia (Gambelli *et al*., 2012; Yang *et al*., 2020). Induction of apoptosis is one of the strategies of preventing the development of leukemia. Various factors are able to cause apoptosis in cells, each of which uses a specific pathway. For example Fas (CD95/Apo-1) antigen is a type 1 cell membrane protein and a part of the TNF/nerve growth factor (NGF) receptor superfamily which, by binding to its own ligand (Fas ligand (FasL), is from the TNF family) or reacting with the anti-Fas/IgM monoclonal antibody, sends apoptotic signals into the cell, creating a cascade that eventually causes cell death (Fouqué *et al*., 2015; Levoin *et al*., 2020). Fas and TNFR-1, in addition to the extra-cytoplasmic part, are very similar in the cytoplasmic part in that both are necessary for the ligands to bind and transmit death signals (Fouqué *et al*., 2015).

In the present study, which partially examined the effect of LC on the apoptosis of rBMNCs, these cells were treated with concentrations of 0.2 and 0.4 mM LC. One limitation of the study was that different concentrations of LC were not used. Flow cytometry analysis showed a significant increase in apoptosis in the treated groups compared to the control. In a study by Jiang *et al*. (2016), it was found that the rate of apoptosis in hepatocyte cancer cells increased in the LC treated group compared to the healthy group. These changes in the occurrence of apoptosis have been reported due to

increased levels of TNF-α, Fas, and caspase-8, which are involved in the external pathway of apoptosis, as well as decreased levels of the BCL-2 protein. Thus, LC increases apoptosis in hepatocyte cancer cells by decreasing BCL-2 as an anti-apoptotic protein and increasing the regulation of FasL (Fan *et al*., 2009). In another study on the effect of LC on apoptosis in myocardial and skeletal muscle cells, TNF-α decreases and apoptosis were observed (Vescovo *et al*., 2002). Therefore, it can be concluded that LC has different effects on the rate of apoptosis in different cells. This can be attributed to metabolic differences in cancerous and normal cells. Moreover, Vescovo *et al*. (2002) reported that free LC levels in cancer cells were lower compared to normal cells (Vescovo *et al*., 2002). In another study reported by Yazdanpanah *et al*. (1997), it was shown that the ratio of free carnitine to its esters in cancer patients differed from that of normal cells. The researchers attributed this to metabolic disorders caused by carnitine-related cancers.

According to one study, increases in the production of TNF-α are completely dependent on the dose of LC. Given that TNF-α is one of the important factors in causing apoptosis in cells, LC can be introduced as one of the inducers of apoptosis (Durazzo *et al*., 2020). In another study, Mutomba *et al*. (2000) investigated the effect of carnitine and palmitoyl carnitine on caspase activity and apoptosis rate, and obtained interesting results. In this study on Jurket cells (T-lymphocytes), apoptosis was induced by the Anti-Fas antibody. In the presence of LC, the rate of apoptosis in normal cells in a dose-dependent manner increased apoptosis, however, in apoptosis-induced cells, LC reduced apoptosis. In contrast, in the presence of Anti-Fas antibody, palmitoyl carnitine increased apoptosis. The study also found that LC and palmitoyl carnitine had an adverse effect on caspase activity (Vardiyan *et al*., 2020).

According to these studies as well as the results of the present study, it can be concluded that LC has a dual effect on the incidence of apoptosis. These effects vary depending on the cell type and whether the cell is cancerous or normal. In addition to activating apoptosis signaling pathways, LC increased apoptosis in all cancer cells, a finding that can be attributed to an increase in the energy available to cells through fatty acid oxidation (Fan *et al*., 2009).

In another part of the present study, the effect of LC on the expression of *SUZ12* gene as a polycomb protein was investigated. At the concentration of 0.4 mM LC, gene expression was almost doubled compared to the non-treated control. Polycomb group proteins are a group of proteins that regulate epigenetic changes in chromatin cells. They are essential for embryonic cell development and stem cell regeneration, and play a key role in establishing and maintaining the pattern of gene expression during development (Kanno *et al*., 2008). Leukemia progression is a process in which advanced genetic abnormalities occur, including the deletion or mutation of repressive genes, amplification of oncogenes and chromosomal abnormalities (Baylin *et al*., 2006). Epigenetic changes that do not affect the original DNA sequence can also alter gene expression and cause cancer (Lu *et al*., 2020).

In conclusion, this research indicates that 0.2 and 0.4 mM LC could promote the gene expression of SUZ12 as well as the protein expression of CD34 cell surface marker $CD34^+$ HSCs. LC could also, effect $CD34^+$ HSCs through the induction of apoptosis. Since the 0.4 mM LC caused early apoptosis and decreased the percentage of living cells, 0.2 mM LC is recommended for further investigations.

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Conflict of interest

The authors declare that they have no conflict of interest.

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