Cardiac differentiation of bone-marrow-resident c-kit\(^+\) stem cells by L-carnitine increases through secretion of VEGF, IL6, IGF-1, and TGF-\(\beta\) as clinical agents in cardiac regeneration

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The idea of regenerating lost myocardium via cell-based therapies remains as highly considerable. C-kit\(^+\) stem/progenitor cells are represented to be suitable candidates for cardiac regeneration compared to other stem cells. A multitude of cytokines from these cells are known to give such multifunctional properties; however, the associated mechanisms of these factors are yet to be totally understood. The aim of the present study was to investigate the \textit{in vitro} effect of L-carnitine (LC) on cardiac differentiation of c-kit\(^+\) cells using a cytokines secretion assay. For this purpose, bone-marrow-resident-c-kit\(^+\) cells were enriched by MACS method, and were differentiated to cardiac cells using cardiomyocyte differentiation medium in the absence (control group) and presence of LC (experimental group). Also, characterization of enriched c-kit\(^+\) cells was performed using flow cytometry and immunocytochemistry. In the following, the cells were subjected to real-time PCR and Western blotting assay for gene and protein assessment, respectively. Afterward, culture medium was collected from both control (\(-\)LC) and experimental groups (\(+\) LC) for cytokine measurement. It was found that 0.2 mM LC significantly increased the mRNA and protein expression of cardiac markers of Ang-1, Ang-2, C-TnI, VEGF, vWF, and SMA in c-kit\(^+\)-cardiomyogenic-differentiated cells. Also, the significant presence of IL-6, IGF-1, TGF-\(\beta\), and VEGF were obvious in the cultured media from the experimental group compared with the control group. It can be concluded that the mentioned \textit{in vitro} effects of LC on cardiac differentiation of c-kit\(^+\) cells could have resulted from the secreted cytokines IL-6, IGF-1, TGF-\(\beta\), and VEGF.

Keywords. Bone-marrow-resident c-kit\(^+\) stem cells; cardiac differentiation; cell therapy; cytokine secretion; L-carnitine

1. Introduction

Heart failure is known to be the main cause of mortality worldwide. Dysfunctions of the heart as well as significant reduction in cardiac myocytes are the eminent signs in acute myocardial infarction (AMI). Also, impotence of cardiomyocytes and insufficiency of healthy cardiomyocytes to compensate for heart function have resulted in considerable attention being paid to new therapeutic approaches (Wencker \textit{et al.} 2003). In other words, neovascularization and cardiac regeneration are important processes to rescue organ from acute ischemia (Isner and Asahara 1999). Hence, finding suitable methods to recover cardiac cells after AMI is the final purpose at the heart research centers (Miyamoto \textit{et al.} 2010). Recently, stem cells usage has been known as an attractive method, which can regenerate and repair the lost heart organ (Traister 2018). Nowadays, broadly used cells in animal modeling are somatic stem cells from various adult tissues, embryonic stem (ES) cells,
and induced pluripotent stem (iPS) cells (Ishigami et al. 2018; Jackson et al. 2017). As mentioned earlier, somatic stem cells derived from various sources have been used to regenerate dead myocardium; however, its efficiency is low due to the progressive loss of functional role and the reduced output (Mohsin et al. 2011). In addition, ES cells can effectively produce functional cardiomyocytes; however, due to retroviral gene transfer, the safety of ES cells is still controversial (Nussbaum et al. 2007). Among the cells used currently for cell therapy, endothelial progenitor cells (EPCs) and bone-marrow (BM)-resident progenitor cells (BMPCs) have been significantly contributed to myocardium regeneration, and were most widely examined in clinical trials (Jeevanantham et al. 2013). Among the cells used currently for cell therapy, endothelial progenitor cells (EPCs) and bone-marrow (BM)-resident progenitor cells (BMPCs) have been significantly contributed to myocardium regeneration, and were most widely examined in clinical trials (Jeevanantham et al. 2013). Concerning the studies related to the impacts of BMPCs on the heart, some contradictory effects on the left ventricle (LV) remodeling have been reported (Honold et al. 2013). Apparently, it seems that, the role of BMPCs in cardiac regeneration and improvement are still controversial (Matuszczak et al. 2014). In other words, despite comprehensive research performed in the past 10 years, it is still unknown whether BMPCs are the best cell sources. So, subsequent studies are required to investigate different types of these progenitor cells for the cell-based therapy in the heart failure. Among the BMPCs, c-kit+ hematopoietic stem cells (HSCs) are reported to have the most noteworthy recovery of cardiac after AMI (Van Berlo et al. 2014). Poor survival and retention of transferred cells into the pathologically damaged heart are widely accepted as significant barriers against enhancing the efficacy of regenerative therapy. However, the use of external factors such as antioxidants to improve the cell survival properties is a favorite strategy for researchers. L-carnitine (3-hydroxy-4-N-trimethylammoniumbutyrate) (LC) as an essential substance plays an effective role in the mitochondria. Given the biological effect of LC, it is considered to be important for supplying energy in the heart tissue. As reported earlier, heart failure along with reduction in ATP levels mostly cause insufficiency in mechanical work (Abozguia et al. 2009). Therefore, LC has been considered to preserve cardiac function and cell viability (Mingorance et al. 2011). Another identified effect of LC is on increasing the flow of acyl/acyl groups into the plasma, which plays an important role in the reduction of free radicals. Accumulation of these intermediates can lead to heart failure and ischemia (Zhang et al. 2010). Accordingly, we can hypothesize this point that LC as an endogenous antioxidant agent, could improve the potential of c-kit+ BM-derived stem cells in regenerative medicine via the greater cardiomyogenic differentiation of these cells. Also, in this research, we reported that increasing cardiomyogenesis of c-kit+ cells in the presence of LC, is correlated with the cytokine secretion.

2. Materials and methods

2.1 Isolation of rat BM-resident c-kit+ cells

C-kit+ (CD117+) cells were isolated from BM of About 8 (5- to 8-week-old) rats by positive selection with anti-CD117 MicroBeads. Rats were euthanized and mononuclear cells (MNCs) were isolated. The isolated MNCS was incubated with 100 μl of CD117+ micro beads (Miltenyi Biotec, Germany; Cat no: 130–091–224) for 30 min. Thereafter, re-suspended cells were passed through one LS MACS column (Miltenyi Biotec, Germany) and enriched CD117+ cells were retrieved by flushing the column (Czarna et al. 2017).

2.2 Purity assessment of BM-resident c-kit+ cells

Purity assessment of the enriched cells was performed by flow cytometry. Briefly, approximately 20 × 10⁴ enriched BM-resident c-kit+ cells were incubated by 5 μl of fluorescein isothiocyanate (FITC)-conjugated antibody CD117 (c-kit) (Biocompare, Lifespan Biosciences, USA) (1μg/10⁶cells) for 30 min on ice and were then subjected to FACS instrument. The output data were processed using FlowJo software version X.0.7. In addition to flow cytometry, immunocytochemistry (ICC) was also done. The protocols used for ICC were described below in the immunofluorescence staining. In addition, the antibody used for ICC was PE-conjugated c-Kit/CD117 antibody (LS-C78828, Lifespan Biosciences, Inc., USA).

2.3 Cardiomyogenic differentiation of BM-resident c-kit+ cells

Cardiomyogenic differentiation of BM-resident c-kit+ cells were induced by the cardiomyocyte differentiation medium (Millipore-Sigma, USA; Cat no: SCM102). For this purpose, 10 × 10⁵ BM-resident c-kit+ cells/6-well plates were cultured in cardiomyocyte differentiation medium for a duration of 14 days. The medium was then refreshed with fresh cardiomyocyte differentiation medium three times per week. Afterward, Cardiomyocyte induction was confirmed by immunocytochemistry (ICC).
2.4 Immunofluorescence staining

ICC was done as previously reported by Fathi and Farahzadi (2018) (Fathi and Farahzadi 2018). In brief, a total of $4 \times 10^3$ BM-resident c-kit$^+$ cells were plated in chamber slide containing cardiomyocyte differentiation medium for 14 days. The control group was assessed in the absence of cardiomyocyte differentiation medium. At the end of 14th days incubation, the cells were fixed in 4% paraformaldehyde for 30–60 min at 25°C. In the following, the cells were incubated 16 hours at 4°C with mAbs against $\alpha$-Actinin and Desmin (all from cardiomyocyte characterization kit, Chemicon International, USA; Cat no: SCR059) in PBS. The cells were then incubated with a goat anti-mouse IgG-PE as secondary antibody (sc-3738, Santa Cruz Biootechnology, USA) for 2 h. Then, cells were washed with PBS and nuclei were stained with DAPI at 30 s. After washing, the fluorescent cells were visualized under a fluorescence microscope.

2.5 Cell proliferation assay

The MTT test was done to define proper concentration of LC. BM-resident c-kit$^+$ cells were seeded at $2 \times 10^3$ cells/wells. In the following, LC was used at final concentrations of 0.1, 0.2 and 0.4 mM and incubated under the same culture conditions for 7, 14 and 21 days. At the end of incubation time (7, 14 and 21 days), the culture media was removed and MTT dye solution was added for 4 hours. At the end of 4 hours, the MTT solution was removed and formazan crystals were dissolved in DMSO. The OD of each well was measured at a wavelength of 570 nm by an ELISA reader (Labsystems, Helsinki, Finland).

In this study, the cells were divided two groups: group I as control group (cardiomyogenic differentiated BM-resident c-kit$^+$ cells without any LC treatment) and group II as experimental group (cardiomyogenic differentiated BM-resident c-kit$^+$ cells with LC treatment).

2.6 Western blot analysis for protein expression assessment

BM-resident c-kit$^+$ cells from both control and experimental groups were collected and protein was extracted as previously explained by Valipour et al. (2018). Cell protein sample was electrophoresed on 12% polyacrylamide slab gels and transferred to PVDF membrane. Next, the membranes were incubated 16 hours at 4°C with primary antibodies (1:1000) against $\beta$-actin (sc-69879), Angiopoietin-1 (Ang-1) (sc-517593), Angiopoietin-2 (Ang-2) (sc-74403), Cardiac Troponin-I (C-TnI) (sc-133117), VEGF (sc-7269), von Willebrand Factor (vWF) (sc-365712) and SMA (sc-53015), and were incubated with secon-dary antibody for 60 min at 25°C. Next, the membranes were washed and protein bands were detected using ECL with X-ray film.

2.7 Quantitative real time-PCR

The BM-resident c-kit$^+$ cells from both control and experimental groups were collected. Next, total RNA was extracted and cDNA was synthesized using molecular kits (Yekta Tajhiz Azma, IRAN). The mRNA expressions of target genes included Ang-1, Ang-2, C-TnI, VEGF, vWF, SMA and $\beta$-actin. Fluorescence data was calculated in relation to $\beta$-actin CT values by the $2^{-\Delta\Delta CT}$ method. Primers (table 1) were designed using Oligo 7 v.7.52 software.

2.8 Cytokine measuring by ELISA

Culture media was collected from each group including, control group without any LC treatment, and experimental group with LC treatment. ELISA was performed according to the manufactures guidelines (R&D Systems, China). In brief, a 96-well plate was coated with detection Reagent A for 16 h at 4°C. Then, cell culture media was added into the 96-well plate, which had been coated with mouse transforming growth factor-$\beta$ (TGF-$\beta$), interleukin (IL)-6, vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF-1) antibodies, and detected via the ELISA sandwich technique.

2.9 Statistical analysis

The results from figures 3 and 6 were analyzed using two-way ANOVA followed by Sidak’s multiple comparisons test. The results from figures 4 and 5 were analyzed using unpaired t-test. The statistical significance was determined at $p < 0.05$ by Graph Pad Prism version 6.01.

3. Results

3.1 Identification of BM-resident c-kit$^+$ cells

BM contents were collected, c-kit$^+$ cells were enriched by MACS method, and the purity of the cell
enrichment was assessed using flow cytometry analysis. The BM-resident c-kit\(^+\) cells enriched by MACS showed a purity of 89.8\%. In other word, the enriched cells had the high levels of expression of CD117 (c-kit) (figure 1A), these results are in agreement with the previous reports (Ellison et al. 2007; Miyamoto et al. 2010). In addition to flow cytometry, immunocytochemistry (ICC) was performed to identify the enriched c-kit\(^+\) cells. In this regard, monitoring the protein level of c-kit-related marker in the enriched c-kit\(^+\) cells by ICC revealed the PE-conjugated CD117 (c-kit) cells (figure 1B).

3.2 Cardiomyogenic differentiation confirmation of BM-resident c-kit\(^+\) cells by ICC

The cardiomyogenic differentiation was confirmed by ICC. In this regard, when the c-kit\(^+\) cells were cultured in cardiomyocyte differentiation medium for a period of 14 days, they exhibited the cardiac markers of Desmin and \(\alpha\)-Actinin. Briefly, as shown in figure 2A–F, the cardiomyogenic differentiated BM-resident c-kit\(^+\) cells have expressed the Desmin and \(\alpha\)-Actinin as the cardiomyogenic markers.

3.3 Effect of LC on the BM-resident c-kit\(^+\) cells proliferation

To recognize the LC effective concentration and to investigate the viability of BM-resident c-kit\(^+\) cells, the cells were treated by 0.1, 0.2, and 0.4 mM LC for 7, 14, and 21 days and cell proliferation was examined by MTT assay as described earlier by Farahzadi et al. (2016). It became clear that, under the 0.1 and 0.4 mM LC concentrations, no significant increase was observed on the viability of BM-resident c-kit\(^+\) cells. In figure 3, the significant differences in BM-resident c-kit\(^+\) cells proliferation between the treated group with 0.2 mM of LC and the control group (\(**p < 0.01\)) after 14 days, is shown. In other words, BM-resident c-kit\(^+\) cells after 14 days’ treatment in the presence of 0.2 mM LC significantly showed more rapid growth compared to the 7 and 21 days treatment (\(**P < 0.01\)). Also, no significant effect was seen on the proliferation of treated cells compared to the control in the presence of 0.1 and 0.4 mM LC.

3.4 Induction of gene and protein expression of cardiac markers in BM-resident c-kit\(^+\) cells in the presence of LC

To evaluate the effect of LC on the cardiomyogenic differentiation of BM-resident c-kit\(^+\) cells, the mRNA and protein expression were examined by real time-PCR and western blot, respectively (Al-Maqtari et al. 2017). Because VEGF is considered as a main factor involved in angiogenesis as well as vasculogenesis, we investigated the mRNA and protein expression of VEGF. Also, consistent with the mRNA expression, the protein expression of VEGF significantly increased in the experimental group as compared to the control group by about 3.5 and 3.6, respectively. Other cardiac differentiation markers, like C-TnI (as cardiomyocyte cell marker), vWF (as endothelial cell marker), SMA (as smooth muscle cell marker), and Ang-1 and Ang-2 (as potent angiogenic markers), were investigated in this panel. As shown in figure 4, the mRNA

| No.     | Gene | Primer pair sequence (5’–3’) | Product length (bp) |
|---------|------|------------------------------|---------------------|
| NM_001146.5 | Ang-I | CTCGCTGCCATTCTGACTCAC GACAGTTGCCCATGCTTGTTCTG | 136 |
| XM_017013318.1 | Ang-2 | TCTTGGCCGCAGCCTTATAAC TGCTGGACCTGATATGCTTCT | 73 |
| XM_006716677.4 | C-TnI | GCAGGTGAAGAAGGAGGACA CGATGATGGCAGGACATGC | 139 |
| NM_001204384.1 | VEGF | ATCACGAACTGGTGAAGTTC TGCTGTAGGAAGCTCATTC | 117 |
| NM_000552.4 | vWF | ATCGTTGGAGAAGCTGGTG GCTGACTGGCTGGAACAAAC | 134 |
| NM_001613.4 | SMA | ATCACGAACTGGTGAAGTTC TGCTGTAGGAAGCTCATTC | 175 |
| NM_001101.5 | \(\beta\)-actin | TCTCTTCCCAAGTGCTTCCACACAGG GGCACGAAGGCTCATCCATT | 131 |
expressions of Ang-1, Ang-2, C-TnI, vWF, and SMA have significantly increased by about 1.2-, 2-, 1.8-, 1.6-, and 2.1-folds, respectively. In addition, the protein expression levels of Ang-1, Ang-2, C-TnI, VEGF, vWF, and SMA have significantly increased by about 1.2, 1.4, 1.9, 1.5, and 1.8-folds, respectively (figure 5).

3.5 Measuring cytokine secretion of BM-resident c-kit+ cells by ELISA

The data of the present study demonstrate that, in the presence of LC, BM-resident c-kit+ cells express and then secrete a variety of growth factors, which stimulate the cardiomyogenesis. Cytokines derived the cultured media (IL-6, IGF-1, TGF-β, and VEGF) from two groups of cells as follows: group I (the control group with no LC treatment) and group II (the experimental group with LC treatment) were assayed using ELISA sandwich technique. For this purpose, culture media was collected from each one of the control and experimental groups, and then, ELISA method was performed in terms of the manufactures guidelines. The results obtained from ELISA revealed that, the secretion of IL-6, IGF-1, TGF-β, and VEGF significantly increased in group II (the experimental group with LC treatment) compared to group I (the control group with no LC treatment) (figure 6) (*p < 0.05 and **p < 0.001, respectively). In other words, increases of 3.39, 1.63, 1.79, and 2 times of IL-6, IGF-1, TGF-β, and VEGF were observed in the culture media of the experimental group compared to the control group, respectively.

4. Discussion

Following the first published paper reporting BM-derived c-kit+ cells capability in cardiomyocytes regeneration in the mouse model infarcted heart, various publications with elaborated affairs showed the effect of these cells on myocardial healing (Orlic et al. 2001). Also, the ability of BM-derived c-kit+ cells in generating the specialized types of heart cells during cardiogenesis, have been shown (Matsuda 2011). In the present study, it was shown that, BM-resident c-kit+ cells have the ability of being differentiated into cardiac cells when cultured in cardiomyocyte differentiation medium. The results of this study are in line with the results of a pilot study performed by Kubo et al. (2009), who provided evidence that BM-resident c-kit+ cells have the ability of being trans-differentiated into functional cardiac myocytes when co-cultured with neonatal rat ventricular myocytes (NRVM) (Kubo et al. 2009). They also showed that, in the presence of NRVM, BM-resident c-kit+ cells could express the Actinin and α-tropomyosin (Kubo et al. 2009). In addition, some studies have focused and examined other cell populations including BM mononuclear cells (BM-MNCs), mesenchymal stem cells (MSCs), EPCs, and BM-derived CD133+ cells (Afzal et al. 2015). The use of separate pools of BM cells has made the

Figure 1. Characterization of isolated BM-resident c-kit+ cells by flow cytometry and immunofluorescence analyses. (A) Flow cytometry showed that more than 89% of cells were positive for CD117 (c-kit). Data are expressed as mean ± SD. (B) Data confirmed the existence of CD117 (c-kit) indicated by positive color cells by immunofluorescence imaging; blue = DAPI; red = PE-conjugated CD117 (bar = 20 μm).
comparison among studies rather intricate (van der Spoel et al. 2011). In spite of this, an agreement has been reached on the mechanisms of actions of various BM cell classes. It is well known that the majority of BM cells are the sources of many growth factors and cytokines that affect paracrine cardiac stem cells and vascular cells (Afzal et al. 2015).

By considering all of these interpretations, the potential role of the subset of BM-derived c-kit\(^+\) cells in myocardial regeneration remains still controversial. One study suggested that, BM-derived c-kit\(^+\) cells cannot be adopted to a cardiac phenotype, and hematopoietic identity of c-kit\(^+\) cells is preserved (Balsam et al. 2004). Therefore, comprehension of the base of these controversial results is important to recognize the clinical function of BM-derived c-kit\(^+\) cells.

Also, cell senescence in patients with coronary artery disease may partially underlie the inefficient cardiac repair. So, finding some effective and suitable agents such as vitamins and antioxidants to increase cardiomyogenic differentiation potential and cell viability of these progenitor cells can promote cardiomyocyte function in clinical.

As mentioned earlier, in this study, LC was used as a natural antioxidant that is useful for cardiac function. The potential therapeutic effects of LC and its derivatives on the cardiovascular diseases treatment have been considered since the mid-1980s (Paulson et al. 1986). For the first time, Spagnoli et al. (1982) reported a low LC concentration in the heart of the patients who died of MI, so it was shown that, LC and its analogues may be beneficial on the prevention of damage to the heart after an ischemic (Spagnoli et al. 1982). In following, Iliceto et al. (1995) indicated that, LC treatment reduced the necrosis and apoptosis of myocardiacocytes within the left ventricle (Iliceto et al.}
A study by Sack et al. (1996) showed the recovery of heart arrhythmia following the treatment by LC (Sack et al. 1996). In another study, Pauly and Pepine (2003) demonstrated that supplementation with LC has a specified protective effect through the attenuated oxygen deficiency and the generated reactive oxygen species (ROS) that result in ameliorative impact on ventricular functional impairment and cardiac oxygen deficiency (Pauly and Pepine 2003). Moreover, in another study by Ogawa et al. (2008), it
was shown that, LC treatment induced some protective anti-oxidative and anti-inflammatory effects and decreased the myocardial dysfunction in choline-deficient rats (Ogawa et al. 2008). In the present study, 0.2 mM was used as the final concentration of LC, as mentioned in the results section. To explore the LC effect mechanism on the cardiomyogenic differentiation of BM-resident c-kit\(^+\) cells at the cellular and molecular levels, in this study, we explored the gene and protein expressions of Ang-1, Ang-2, C-TnI, VEGF, vWF, and SMA as cardiac differentiation markers, as well as the secreted cytokines such as IL-6, IGF-1, TGF-\(\beta\), and VEGF in response to LC treatment. The results of this study showed that, in group II (the experimental group with LC treatment), LC promoted the mRNA expression as well as protein expression of Ang-1, Ang-2, C-TnI, VEGF, vWF, and SMA as compared with group I (the control group with no LC treatment). In other words, in the present study, we showed that 0.2 Mm LC can enhance the cardiac differentiation of BM-resident c-kit\(^+\) cells by the expression of VEGF and vWF as endothelial cell markers, SMA as smooth muscle cell marker, and Troponin T as cardiomyocyte marker. As previously demonstrated by Pauly and Pepine (2003), the protective mechanism of LC on cardiomyogenesis may be through the reduction of ROS generation (Pauly and Pepine 2003). Of course, to prove this assumption related to the LC effect on BM-resident c-kit\(^+\) cells, it is recommended to measure ROS in future studies. Because of angiogenesis is an important aspect of tissue repairing, we also investigated the angiogenic factors (Fazel et al. 2006). In another part of the result section, we explained that, LC can increase the mRNA and protein expressions of Ang-1 and Ang-2. Accordingly, this section of our study is consistent with the previous data obtained by Fazel et al. (2006) showing that, BM-resident c-kit\(^+\) cells act as key regulators of the angiogenic switch in MI by increasing VEGF and by reversing the cardiac ratio of Ang-1 to Ang-2 in favor of Ang-2 (Fazel et al. 2006). In this regard, Ang-2 is critical in angiogenesis because it blocks the tonic quiescent signal delivered by Ang-1. The increased Ang-2/Ang-1 ratio potentiates the endothelial cells responsiveness to VEGF and also promotes blood vessel (Hanahan 1997). Altogether, our data suggest that, in the presence of 0.2 mM LC, along with increasing the mRNA and protein expression of VEGF, Ang-2/Ang-1 ratio has increased. This finding could emphasize on the effect of LC to mediate the important transition of quiescent to active endothelium. We also observed that the increase in the expression of cardiac markers is correlated with the increasing presence of IL-6, IGF-1, TGF-\(\beta\), and VEGF. Importantly, it was indicated that, the treatment of BM-resident c-kit\(^+\) cells with LC can induce cardiac differentiation and angiogenesis, suggesting that this result may be due to the cytokine secretion induction. In addition to differentiation capacity of BM stem and progenitor cells toward cardiac cell lineages, release of some cytokines and paracrine factors, which have anti-remodeling, anti-inflammatory, and anti-apoptotic properties, have become them as promising candidates for regenerative medicine (Toth et al. 2011). In other words, it was reported that, under heart tissue ischemic condition, stem and progenitor cells can release cytokines. Accordingly, out of these factors, the most important ones are as follows: TGF-\(\beta\), IL-6, VEGF, angiopoietin (Ang)-1, IGF, fibroblast growth factor (FGF)-2, and TNF-\(\alpha\). These secreted cytokines exert important anti-remodeling, anti-apoptosis, and anti-inflammatory effects on paracrine manner cytokines, which play pivotal roles in stem cell-related cardiac repair mechanisms (Duran et al. 2013). Among the above-mentioned cytokines, TGF-\(\beta\), IL-6, IGF-1, and VEGF are the most widely regulators in cardiomyocyte regeneration (Li et al. 2014; Park et al. 2011). In a study, Bujak and Frangogiannis (2007) reported that, after MI the TGF-\(\beta\) was upregulated, especially in the infarct border zone (Bujak and Frangogiannis 2007). This upregulation was associated with the expressions of Smad 2, 3, and 4 pathways (Bujak and Frangogiannis 2007). In another study by Abarbanell et al. (2009), it was indicated that, in ischemic damage after MI, TGF-\(\beta\) plays a considerable role in cardiomyocyte repair by promoting the myofibroblast phenotype and

**Figure 6.** The secretion levels of cytokines IL-6, IGF-1, TGF-\(\beta\) and VEGF from two groups; control (without any LC treatment) and experimental (with 0.2 mM LC treatment). (*) \(p < 0.05\) and *** \(p < 0.001\); compared with control group.
suppressing inflammation. Moreover, its role lead to restoration of cardiac function after MI (Abarbanell et al. 2009). Recently, Rouhi et al. (2013) indicated that, autologous serum promoted cardiomyogenesis of rat BM-resident stem cells in the presence of TGF-β. (Rouhi et al. 2013). Besides, various studies were conducted on the fundamental effects of IL-6 family as a key regulatory role in cardiac regeneration (Li et al. 2014). In a preliminary study by Freed et al. (2005), it was shown that, the upregulation of cardiotrophin-1 (CT-1), as a member of the IL-6 cytokine family inhibited the fibroblast proliferation in the infarcted myocardium zone (Freed et al. 2005). In a previous study by Tang et al. (2018), it was indicated that, IL-6 as a pro-inflammatory cytokine has several good effects on improving the cardiac function in mice model with cardiac injury (Tang et al. 2018). Among the mechanisms resulting in some beneficial effects from stem cell transplantation on heart regeneration, it is obvious that neovascularization may be considered that was regulated by a variety of cytokines, such as the FGF and VEGF (Boomsma and Geenen 2012). In a study by Mathison et al. (2012), it was indicated that, pretreatment of cardiac scars with gene transfer of VEGF boosted the trans-differentiation of fibroblasts into cardiomyocytes in rat animal model (Mathison et al. 2012). Also, in another study, Ye et al. (2013) reported that, VEGF as a neovascularization key factor is required for the effective cardiomyocyte differentiation of the human iPS cells (Ye et al. 2013). Moreover, it was proved that, FGF is upregulated early after MI, which suggests that, it may be involved in the regulation of the early inflammatory reaction after cardiac ischemic injury. In addition, it has been indicated that, FGF’s angiogenic effects are VEGF dependent and these actions are highly correlated with each other (Holmes and Zachary 2008). According to the aforementioned results, interestingly, we are the first reporting that 0.2 mM LC as endogenous antioxidant agent could induce the cardiac differentiation of BM-resident c-kit+ cells via increasing the cardiac markers expressions as Ang-1, Ang-2, C-TnI, VEGF, vWF, and SMA. In this regard, its effects may be due to the secreted cytokines of IL-6, IGF-1, TGF-β, and VEGF.

5. Conclusion

In conclusion, this research indicates that, 0.2 mM LC could promote the gene and protein expressions of cardiac-related markers in c-kit+ stem/progenitor cells, which are correlated with the presence of cytokines as IL-6, IGF-1, TGF-β, and VEGF. As tyrosine kinase activity to be critical for the in vitro functional cardiomyogenic differentiation of c-kit+ stem/progenitor cells, examining the c-kit receptor expression using tyrosine kinase activity assay is suggested for future studies.

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Ethics statement

Ethical consent was approved by an ethics committee at Tabriz University of Medical Sciences, Tabriz, Iran (Ethic Code No: IR.TBZMED.REC.1396.607) in accordance with the guidelines of Helsinki-Ethical Principles.

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