

L-carnitine significantly decreased aging of rat adipose tissue-derived mesenchymal stem cells

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Abstract Mesenchymal stem cells are undifferentiated cells that have the ability to divide continuously and tissue regeneration potential during the transplantation. Aging and loss of cell survival, is one of the main problems in cell therapy. Since the production of free radicals in the aging process is effective, the use of antioxidant compounds can help in scavenging free radicals and prevent the aging of cells. The aim of this study is evaluate the effects of L-carnitine (LC) on proliferation and aging of rat adipose tissue-derived mesenchymal stem cells (rADSC). rADSCs were isolated from inguinal region of 5 male Rattus rats. Oil red-O, alizarin red-S and toluidine blue staining were performed to evaluate the adipogenic, osteogenic and chondrogenic differentiation of rADSCs, respectively. Flow cytometric analysis was done for investigating the cell surface markers. The methyl thiazol tetrazolium (MTT) method was used to determine the cell proliferation of rADSCs following exposure to different concentrations of LC. rADSCs aging was evaluated by beta-galactosidase staining. The results showed significant proliferation of rADSCs 48 h after treatment with concentrations of 0.2 mM LC. In addition, in the presence of 0.2 mM LC, rADSCs appeared to be growing faster than control group and 0.2 mM LC supplementation could significantly decrease the population doubling time and

aging of rADSCs. It seems that LC would be a good antioxidant to improve lifespan of rADSCs due to the decrease in aging.

Keywords rADSCs · L-carnitine · Aging · Antioxidant

Introduction

Mesenchymal stem cells (MSCs) are multipotent stem cells that can be obtained from a variety of tissues such as bone marrow, adipose tissue, amniotic fluid, cord blood etc. and that can differentiate into diverse cell types, including adipocytes, osteoblasts, chondrocytes, myocytes, neuron-like cells, and so forth (Fathi and Farahzadi 2016a). MSCs have first been recognized by Friedenstein who have isolated and described bone precursor cells from rat bone marrow tissue (Friedenstein et al. 1974). The researcher considered some characteristics for these cells, including self-renewal, high proliferative activity in vitro and fibroblast-like cells (Parekkadan and Milwid 2010; Fathi and Farahzadi 2014a, b). MSCs are being explored to treat inflammation, cardiovascular disease and myocardial infarction (MI), brain and spinal cord injury, diabetes, cartilage and bone injury (Phinney and Prockop 2007; Ban et al. 2011; Gholizadeh-Ghalehaziz et al. 2015). Differentiation ability of MSCs into various cell lineages including cartilage, fat, bone etc., are so much affected by aging (Yu and Kang 2013). As a result, the use of MSCs from older donors is lower than younger donors. The effects of age on the MSCs are contradictory, probably due to differences in experimental parameters such as the type of donor, age, sex, cell division and cell culture protocols (Huibregtse et al. 2000). Asumda and Chase (2011) reported the significant differences in growth, morphology, population size and differentiation potential of the MSCs derived from bone marrow in

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young rats in comparison with old rats. They indicated that the young and old MSCs are fibroblast-like cells and broad form, respectively (Asumda and Chase 2011). Furthermore, proliferation and population doubling time in old MSCs slowed compared with the young MSCs ones. So it is crucial to maintain the proliferation and differentiation capacity of MSCs. It seems aging is the result of the accumulation of oxidative damage caused by free radicals generated as by-products during normal metabolism. The free radical theory of aging assumes that oxidative stress is one of the major causes of age-related cellular and molecular damage, and this phenomenon is implicated in the pathogenesis of a variety of human and animal diseases. The use of antioxidant compounds to prevent cellular aging is important (Syslová et al. 2014).

L-carnitine (LC) is one of powerful antioxidants that have an important role in the metabolism of fatty acids, consumption of ketone bodies, paroxysmal oxidation and reconstruction of erythrocyte membrane phospholipids. LC is responsible for decrease in lipid peroxidation, lipofuscin content during aging. LC is associated with a significant increase in the activities of antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, constitute a natural defense system against the activity of oxidants (Cao et al. 2011; Fathi and Farahzadi 2014a, b). LC can also act as a chelator by decreasing the concentration of cytosolic iron, which plays a very important role in free radical chemistry (Panjwani et al. 2007). LC has been investigated in maintenance of mental and physical function and reversal of decline with aging. In rats mitochondrial function declines with aging. Mitochondria of old rats have higher levels of products of oxidation of lipids, proteins and nucleic acids than do mitochondria of young rats (Rebouche 2012). In the heart, LC is the natural compound that, in comparison with other antioxidant molecules, displayed the largest inhibition in the expression of the transcriptional markers of aging, mimicking metabolic effects of caloric restriction and that it was similarly effective on the expression of biomarkers of cerebellum aging (Park et al. 2009).

The aim of this study is evaluate the effect of LC on the growth and survival of the valuable cells to create favorable conditions for the culture of rat adipose tissue derived mesenchymal stem cells (rADSCs) and decrease of oxidative damage as well as aging process.

Materials and methods

In this study, all materials were from Sigma (St. Louis, Mo., USA) and the media was from Gibco (Invitrogen, Carlsbad, Calif., USA), unless otherwise specified. All tissue culture plastic ware was from SPL Life Sciences Co., Ltd. All experimental procedures were repeated for three times.

Isolation and culturing of rADSCs

Inguinal adipose tissue was obtained from 5 male *Rattus* rats with 6–8 weeks age, under ethical guidelines of University of Tabriz, Iran. Fat tissue was collected in sterile tubes containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), carefully minced using a sterile scissors and washed extensively with phosphate buffer solution (PBS). Fat tissue was then enzymatically digested with 0.075% (w/v) collagenase type I (Invitrogen, UK) for 30 min at 37 °C. The specimen was added to the washed sample and the mixture was agitated at 37 °C for 1 h. The collagenase was inactivated with an equal volume of DMEM containing 10% (v/v) FBS and centrifuged at 800×g for 5 min. The cell pellet was resuspended in DMEM containing 10% FBS and 1% (v/v) penicillin/streptomycin solution. Culture flasks were maintained in a tissue culture incubator at 37 °C in humid air with 5% CO₂ and passaged with 0.25% trypsin (Gibco, UK) and 1 mM ethylenediaminetetraacetic acid (EDTA; Invitrogen, UK) when required.

Phenotypical characterization of rADSCs by flow cytometry

Flow cytometry was used to analyze cell surface markers, briefly, 10×10^5 rADSCs were collected from the passage 4 cultures and washed twice with PBS containing 3% FBS (solution buffer). After removing the supernatant, the cell pellet was incubated with an appropriate amount of fluorescein isothiocyanate (FITC) or FITC-conjugated mouse primary CD44, CD90, CD31 and CD34 antibodies (BD Pharmingen, San Diego, CA, USA) ($1 \mu\text{g}/10^6$ cells) in PBS supplemented with 1% (v/v) FBS for 30 min on ice. Cells were washed with solution buffer and centrifuged at 800×g for 5 min. 500 μl of solution buffer was added to cell pellet and the cells were transferred to flow cytometry tubes. Fluorescence activated cell sorter (FACS) analysis was also done using a FACScan (Becton Dickinson Franklin Lakes, USA) and data were analyzed with a FlowJo software (version 6.2).

Three-lineage differentiation potential

Adipogenic differentiation of rADSCs and oil red staining

Subconfluent cells at passage 4 were plated at a density of 5×10^4 cells in each well of 6-well plates and allowed to become 60–70% confluent. Adipogenesis was induced by the adipogenic differentiation medium composed of high-glucose DMEM with 10% FBS, 0.5 mM 1-methyl-3 isobutyl xanthine, 1 μM dexamethasone, 10 $\mu\text{g}/\text{ml}$ insulin, and 200 μM indomethacin. To confirm the successful adipogenic differentiation, after 21 days of culture, formalin-fixed cells were washed in 60% isopropanol and stained with Oil Red-O

for 15 min and lipid droplets observed by a light microscope (Aust et al. 2004).

Osteogenic differentiation of rADSCs and alizarin red staining

To promote osteogenic differentiation, the cells from passage 4 were plated at a density of 5×10^4 cells in each well of 6-well plates. When the culture was at approximately 60–70% confluency, the medium was replaced by osteogenic differentiation medium containing high-glucose DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.05 mM L-ascorbic 2-phosphate, 10 nM dexamethasone and 10 mM β-glycerol phosphate for 21 days. At the end of this period, calcium depositions were stained with alizarin red staining. Briefly, the cells were washed with PBS three times and fixed in a solution of 4% (v/v) paraformaldehyde. After 30 min, alizarin red (40 mM, pH 4.1) was added to each well. The plates were incubated at room temperature for 20 min and then they were washed with 2–3 times with PBS for 5 min to reduce nonspecific staining (Gregory et al. 2004).

Chondrogenic differentiation of rADSCs and toluidine blue staining

rADSCs were seeded the same as adipogenic and osteogenic differentiation. Chondrogenic differentiation medium composed of high-glucose DMEM with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 1.3 mM L-ascorbic 2-phosphate, 0.01 mM dexamethasone and ITS + 1 Liquid Media Supplement (100×) was used for 14 days. At the end of chondrogenesis, cells were fixed with 4% (v/v) paraformaldehyde for 30 min, washed with PBS for three times and proteoglycans were stained with 0.1% toluidine blue.

RNA extraction and reverse transcription (RT)-PCR analysis of adipose and bone tissue specific-genes expression.

Total RNA was isolated from the adipogenic, osteogenic and chondrogenic differentiated cells using Trizol reagent

(Invitrogen, UK). Extracted cellular RNA was dissolved in diethylphosphorocyanidate-treated water and treated with 1 U/µl of DNase I (RNase free, Roche) to remove any genomic DNA contamination. cDNA synthesis were performed using the RevertAid™ first strand cDNA synthesis kit (K1622; Fermentas, Germany). 2 µg RNA was used for the first strand cDNA synthesis in a total volume of 20 µL according to the manufacturer's instructions. The mRNA expressions of target genes in the rADSCs included peroxisome proliferator-activated receptor alpha and gamma (PPARα and PPARγ), alkaline phosphatase (ALP), osteocalcin (OCN), aggrecan and collagen II. The regimen for PCR was 15 min at 94 °C followed by the appropriate number of cycles of 1 min at 94 °C, 1 min at the proper annealing temperature for each primer pair (Table 1), and 1 min at 72 °C, with a final 10 min extension at 72 °C. Amplified PCR products were analyzed by ethidium bromide staining after 1.5% agarose gel electrophoresis (Eslaminejad et al. 2010).

Cell proliferation activity of rADSCs by MTT assay

The 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) test measures the mitochondrial activity in the cell culture, which reflects the number of viable cells. In brief, at either passage 3–6 cells were trypsinised, counted with hemocytometer and seeded at a density of 4×10^3 cells per well to a 96-well culture plate. Cells were maintained at subconfluent levels in a 37 °C incubator with 5% CO₂. LC was added to the wells at final concentrations of 0.1, 0.2 and 0.4 mM and incubated under the same culture conditions for 24, 48 and 72 h. Control wells were prepared by addition of corresponding medium. At the end of incubation period, the stock MTT dye solution (5 mg/mL) was added to each well. Following incubation for 4 h, the medium was removed and 200 µl of dimethyl sulfoxide was added to each well and incubated for 30 min at 37 °C to dissolve the insoluble purple formazan crystals formed by the reduction of MTT. The optical density of each well was measured in an ELISA Reader (Labsystems, Helsinki, Finland) at a wavelength of 570 nm.

Table 1 Primer sequences used for detection of the bone, adipose and cartilage tissue specific-genes

No.	Gene	Primer pair sequence (5'-3')	Product length (bp)
NM_013059.1	ALP	CCTTGAAAAATGCCCTGAAA CTTGGAGAGAGCCACAAAAGG	191
NM_001278484.2	OCN	GTCCACACAGCAACTGC CCAAAGGCTGAAGCTGCCG	219
NM_013196.1	PPAR-alpha	CCCTGCCTTCCCTGTGAACTGAC GGGACTCATCTGTACTGGTGGGGAC	387
NM_013124.3	PPAR-gamma	GGTGAAACTCTGGGAGATCC TGAGGGAGTTGAAGACTCTTC	400
NM_012929.1	Collagen	GGCTTAGGGCAGAGAGAGAAG TGGACAGTAGACGGAGGA AAGTC	475
NM_022190.1	Aggrecan	GGCAACCTCCTGGGTGTAAG TCGCACCACCAGGTCCTC	444
NM_017008.4	GAPDH	ATGACTCTACCCACGGCAAG CTGGAGATGGTGTGGGTT	88

Growth curve

To determine the growth pattern for rADSCs in the presences of LC, growth curves were plotted. For this purpose, 10^4 cells/cm² were seeded in 24-well culture plate. Three days after seeding, LC was added to the wells at final concentrations of 0.1, 0.2 and 0.4 mM for up to 48 h at 37 °C in 5% CO₂. During the culture time, cells were trypsinized daily and counted with hemocytometer and growth curve was plotted for each culture.

Calculation of population doubling time (PDT)

To compare the expansion rate of the rADSCs in the presence of different concentrations of LC, PDT (the time by which cell population doubles in number) was calculated. Population doubling number (PDN) was calculated according to the following equation: $PDT = CT/PDN$, where CT is the culture time and PDN the population doubling number. To determine the PDN, following formula was used: $PDN = \log(N1/N0) \times 3.31$. In this eq. N1 is the cell number at the end of cultivation period, N0 the cell number at culture initiation. CT (culture time) was the passage 1–3. To determine the CT, N and N0, passaged-4 cells were counted and plated at density of 5×10^4 cells in each well of 6-well plates for about 7 days. At the end of this time the cells were trypsinized and counted.

Senescence-associated Beta-galactosidase (SA-β-gal) staining

To determine the percentage of senescent cells in the presence of different concentrations of LC, SA-β-gal staining was used. The cells from passage 3, 5, 7, 9 and 11 treated with 0.1, 0.2 and 0.4 mM LC at 37 °C in 5% CO₂. Following treatment for 48 h, cells were washed twice with PBS for 30 s per wash, and fixed with 2% (vol/vol) formaldehyde and 0.2% (vol/vol) glutaraldehyde in PBS for 5 min at room temperature. The cells were then

washed with PBS as described prior, incubated with freshly staining solution containing 40 mM citric acid/Na phosphate buffer, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM sodium chloride, 2 mM magnesium chloride and 1 mg/ml 5-bromo-4-chloro-3-indolyl-D-β-galactosidase (X-gal) in distilled water for overnight (12–16 h) at 37 °C in the absence of CO₂ condition. After the incubation, wash the cells twice with PBS, and once with methanol and allow the dish to air dry. A minimum of 100 cells was counted by light microscopy in 10 random fields to determine the percentage of SA-β-gal-positive cells which were appeared as blue-stained cells (Debacq-Chainiaux et al. 2009).

Statistical analysis

The results were analyzed using the software program Graph Pad Prism version 6.01. One-way and two-way ANOVA was used to determine the significant difference among groups. The *p* value <0.05 was considered to be significant.

Results

Phenotypical characterization and three lineage differentiation of rADSCs

rADSCs had the capacity to adhere to plastic flasks in culture, and morphologically they appeared fibroblast-like appearance (Fig. 1a). Flow cytometric analysis showed that rADSCs had high levels of expression of CD44 (94%), CD90 (92.4%) and hematopoietic cell lineage-specific antigens, such as CD31 (0.03%) and CD34 (0.5%) were not detected in these cells (Fig. 1b). The multipotent capacity of rADSCs was determined with adipogenic, osteogenic and chondrogenic differentiation. Positive adipogenic differentiation was confirmed by Oil Red-O staining. Treated rADSCs with adipogenic

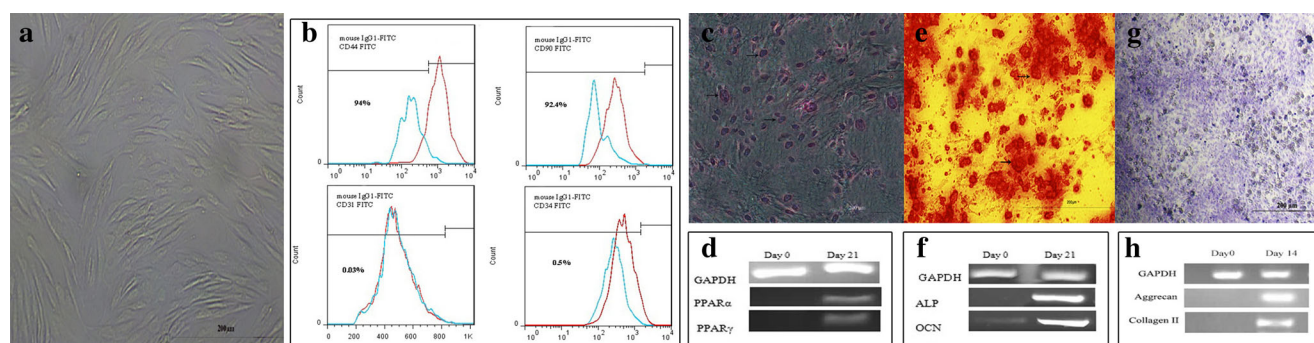


Fig. 1 **a** Culture of rADSCs in fourth passage, 7 day after seeding (bar = 200 μm); **b** Flow cytometric analysis of rADSCs. rADSCs are stained with monoclonal antibodies directed against either CD44, CD90, CD31 and CD34 coupled to fluorescein isothiocyanate (FITC). rADSCs were positive for CD44 and CD90 and negative for CD31, CD34 and CD56; **c** Differentiation into adipose cells. Arrows show lipid vacuoles generated after adipose differentiation (bar = 200 μm); **d**

Expression of PPAR-α and PPAR-γ as fat-specific genes; **e** Osteogenic differentiation and cell aggregates (were stained with alizarin red staining). Arrows show some of the mineralized cell aggregates (bar = 200 μm); **f** RT-PCR analysis and detection of two bone specific genes ALP and OCN; **g** differentiation of rADSCs to chondrocytes, Proteoglycan aggregates stain with toluidine blue. **h** RT-PCR analysis of aggrecan and collagen II as chondrocyte-specific gene (bar = 200 μm)

differentiation medium stained lipid droplets (Fig. 1c). The expression of PPAR- α and PPAR- γ as adipocyte-specific genes were detected by RT-PCR analysis (Fig. 1d). Also, after induction in osteogenic medium for 21 days, redness of the nodules confirmed by alizarin red S staining (Fig. 1e). RT-PCR analysis confirmed the expression of bone-specific genes ALP and OCN in the treated cells (Fig. 1f). Toluidine blue was used to staining the aggrecan aggregates as a key molecule within the cartilage matrix (Fig. 1g). Gene expression analysis was also performed to chondrogenesis confirmation (Fig. 1h). These results confirmed that our isolated cells were rADSCs.

The effect of LC on the proliferation, growth curve and population doubling time (PDT) of rADSCs

Cell proliferation was examined by MTT assay in the presence of different concentrations of the LC for 24, 48 and 72 h. As shown in Fig. 2a, rADSCs after 48 h treatment in the presence of 0.2 mM of LC showed significantly more rapid growth than the 24 and 72 h treatment ($P < 0.01$). Therefore, the most suitable time and concentration for LC treatment of the rADSCs was in the 48 h of incubation in the presence of 0.2 mM LC. According to the plotted curve, the cells in both culture conditions (control and 0.2 mM LC treated cells), started proliferating immediately after being plated. The culture reached plateau in approximately 6 days after initiation (Fig. 2b). As shown in Fig. 2c, PDT value was 19.5 h for rADSCs while this value was recorded as 14.5 h after treatment with 0.2 mM LC. The difference was significant ($P < 0.05$).

SA- β -gal staining

Senescent cells were not present at the passages 1 and 2 of rADSCs both culture conditions (control and 0.2 mM LC treated cells). Stained cells were first appeared at passage 3 and increased with advancing the passage number. As shown in Fig. 3, LC in the 3, 5, 7 and 9 cell passages to reduce the number of stained cells, while the reduction in passage 7 and 9

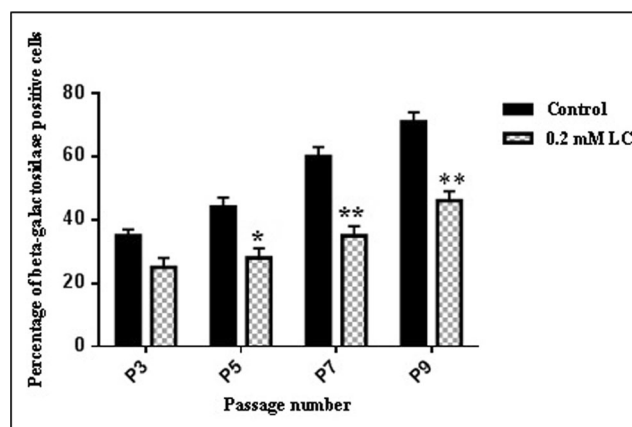


Fig. 3 The presence of senescent cells at culture. Senescent cells were first observed at passage 3 and their number was increased as the passage number advanced. (* $P < 0.05$ and ** $P < 0.01$ compared with control group)

is more. There were statistical significant differences at passage 5 ($P < 0.05$), and at passages 7 and 9 ($P < 0.01$).

Discussion

MSCs have recently received widespread attention because of their ability to renew themselves and to differentiate into many different cell types in the body. MSCs are also able to use in organ transplantation and repair damaged tissues (Fathi and Farahzadi 2016b). Similar to normal somatic cells, adult stem cells are exposed to stressors during the life span, leading to an age-dependent decline in their number and function. Many studies have constantly considered a senescent tendency of MSCs upon expansion (Tsai et al. 2011). A decline in stem cell number or activity may, therefore, lead to compromised organ and tissue function that is characteristic of aging (Wang and Jones 2011). It has been reported that the aged MSCs show a decline in differentiation potential as well as in proliferation rate. Various studies have indicated that the MSCs transplants from older donors appears to be less effective than

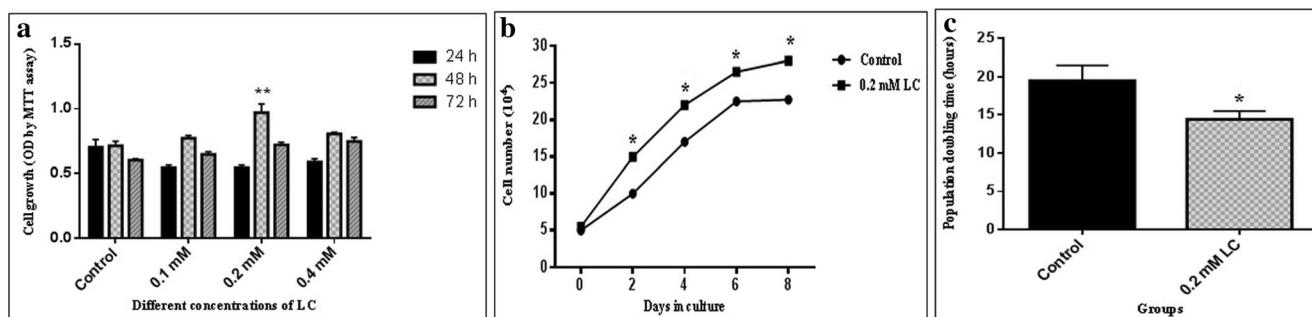


Fig. 2 a Proliferation rates of rADSCs in the presence of different concentrations of LC for 24, 48 and 72 h, using MTT assay; b Growth curve plotted for rADSCs in the presence and absence of 0.2 mM LC. The cells, started proliferation immediately after being plated (* $P < 0.05$

compared with control group); c Population doubling time (PDT), * indicates significant short PDT of 0.2 mM LC-treated cells compared with that of untreated cells ($P < 0.05$)

application of their younger counterparts (Rauscher et al. 2003; Li et al. 2014; Farahzadi et al. 2016). Furthermore, the difference in stem cell properties and the senescence encountered during the expansion hinder the clinical applications of MSCs. Thus, evaluating stem cell aging status is essential for the successful use of stem cells in clinical practices. As reported by Stolzing et al., MSCs isolated from 56 week-old Wistar rats increased levels of apoptosis and reduced proliferation than that isolated from 8 to 12 week-old rats (Stolzing and Scutt 2006).

According to the previous reports, antioxidants are usually beneficial for prevention of MSCs aging. A prolonged lifespan and an enhanced growth rate are observed in human MSCs cultures supplemented with antioxidants (Kasper et al. 2009; Fusco et al. 2007). Of all the antioxidants, exogenous/endogenous compounds, LC works as an endogenous with no adverse effects (Huang and Owen 2012). Lenzi et al. (2004) demonstrated that carnitine may protect DNA and cell membranes from damage caused by oxygen free radicals. In addition, studies have shown that acetyl-LC, one of the short-chain acyl esters, enhances learning capacity in aging animals, and improves the symptoms of nerve-degenerative disorders such as Alzheimer's disease (Pettegrew et al. 2000) and attenuates the neurological damage seen following brain ischemia and reperfusion (Ando et al. 2001; Pettegrew et al. 2000). Gulcin (2006) showed that carnitine can loss of superoxide radicals and hydrogen peroxide, also LC can prevent lipid peroxidation.

Based on these effects of LC, we tried to plan a project to use LC to improve the lifespan of rADSCs. According to our findings, we found that the 0.2 mM of LC had the most effect on the cell proliferation in the time-dependent manner. Our results indicated that in the presence of 0.2 mM LC, rADSCs appeared to be growing faster than control group. In addition, population doubling time and percentage of senescent cells were significantly decreased in LC-treated rADSCs as compared with untreated cells.

As mentioned above, LC at the concentration of 0.2 mM would be a good antioxidant to improve lifespan of rADSCs due to the decrease in population doubling time and aging. Although, the detailed mechanism of its effects is still unknown and further in vitro and in vivo investigations need to be carried out.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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