European Review for Medical and Pharmacological Sciences 2014; 18: 1625-1631

Lycopene protects bone marrow mesenchymal stem cells against ischemia-induced apoptosis *in vitro*

Y. LI, F. XUE, S.-Z. XU[1], X.-W. WANG[1], X. TONG[1], X.-J. LIN[1]

Department of Orthopaedic, Fengxian Center Hospital of the Sixth Hospital of Shanghai, Shanghai, China
[1]Department of Orthopaedics, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang, China

Abstract. – OBJECTIVE: Bone marrow mesenchymal stem cells (MSCs) have been identified to have the potential to differentiate into multiple types of cells. And the therapy based on transplantation of MSCs in some solid organs has been suggested in recent years. However, the rejection reaction often occurs in the transplantation of MSCs and could induce cell death. Ischemia has been reported to one of the main causes of MSCs death during transplantation. The primary objective of this study was to verify whether a natural antioxidant, lycopene, could protect MSCs from ischemia-induced apoptosis *in vitro*.

MATERIALS AND METHODS: MSCs were isolated from bone marrow in mice femoral bone marrow. The effect of lycopene on MSCs during oxygen-glucose-serum deprivation was investigated.

RESULTS: We found that lycopene protected MSCs from serum deprivation- and hypoxia-induced apoptosis. The reactive oxygen species (ROS) generation and inducible nitric oxide synthetase expression were also inhibited dose-dependently by lycopene. Further investigation revealed that lycopene could activate phosphoinositide-3 kinase (PI3K)/Akt. Pretreatment with PI3K/Akt inhibitors prevented the protective effect of lycopene on MSCs.

CONCLUSIONS: Lycopene could protect MSCs from ischemia-induced apoptosis through reducing ROS generation; therefore, lycopene could be useful in MSC transplantation.

Key Words:
Bone marrow mesenchymal stem cells, Lycopene, Reactive oxygen species, PI3K/Akt pathway.

Introduction

Hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) are both known as multipotent adult stem cells. Bone marrow mesenchymal stem cells have been proved to have the potential to differentiate into multiple types of cells[1-3]. Including neurons, hepatocytes, cardiomyocytes and epithelial cells, transplantation of MSCs have been used in the treatment of some tissue injuries, such as ischemic heart failure[4], hind limb ischemia[5] and liver tranplatation[6]. However, after transplantation, poor survival of the implanted cells often occurs in the progress of stem cell therapy. Also the immunomodulatory properties of cultured MSCs make it possible for cell therapy[7], the implanted MSCs often fall into rapid cell death after transplantation[8].

However, the mechanism of the high implanted MSCs apoptotic rate still remains unknown. It has been noticed that in the transplantation of MSCs into ischemia-induced heart injury, over 99% implanted MSCs generated cell death in only 4 days[8]. Thus, ischemia might be involved in the process of implanted MSCs apoptosis. In recently researches on MSCs transplantation, especially in ischemia-induced heart injury, an expensive cells death of transplanted MSCs could be observed[9-12]. Therefore, ischemia could be one of important causes that induce implanted MSCs death. Protection of MSCs from ischemia-induced apoptosis might provide benefit for cell therapy.

The aim of this study was to investigate whether one antioxidant, lycopene, could protect primary cultured MSCs from ischemia-induced apoptosis. We found that lycopene could reduce ischemia-induced MSCs apoptosis in a dose-dependent manner. Meanwhile, lycopene decreased the reactive oxygen species (ROS) generation and inducible nitric oxide synthetase expression. Inhibition of Akt phosphorylation could restore the anti-apoptotic effect of lycopene, which suggested that lycopene could protect MSCs from ischemia-induced cell death through reducing the ROS generation via activation of PI3K/Akt pathway.

Corresponding Author: Feng Xue, MD; e-mail: lxiangjin@yeah.net

Materials and Methods

Isolation and Culture of MSCs

MSCs were isolated from the marrow of six-week-old male C57BL6/J mice as described by Tropel et al with minor modification[13]. Briefly, the femurs and tibias of mice were removed, and all the connective tissues were cleaned. Both epiphyseal extremities were cut. Then the marrow was flushed out with cultured medium (Dulbecco's Modified Eagle's medium (DMEM)/F12 containing 10% inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 g/mL streptomycin, GIBCO, Carlsbad, CA, USA) at 37°C with 5% CO_2 in a humidified tissue culture incubator. Hematopoietic and nonadherent cells were removed by medium changing after 24 h. Then MSCs were cultured and used for experiments between the third and sixth passage.

To evaluate the purity of MSCs, MSCs were incubated with rabbit monoclonal anti-mice CD34, CD105, CD45 and CD90. Then flow cytometry was performed to identify the surface antigens of MSCs.

Induced Ischemia of MSCs

The hypoxia of cell was induced as previously reported[9]. Briefly, the cells were pretreated with different concentrations of lycopene for 1 h in complete cultured medium, then the medium were removed and the MSCs were washed with phosphate buffered saline (PBS) for twice. The cells were then exposed to 5% CO_2 and 95% N_2 in a hypoxia gas chamber (Ruskin, Biotrace, Bridgend, UK) in serum-free medium for 6 h in the presence of lycopene with/without 20 μM LY294002 (PI3K/Akt inhibitors, Calbiochem, San Diego, CA, USA). Control group was treated as ischemia-treated cells in the absence of lycopene. For normoxic control, the cells were cultured in normal condition in a humidified tissue culture incubator for 6 h.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium Bromide (MTT) Assay and Determination of ROS Generation

MTT assay were used to investigate the cell viability of MSCs[11]. MSCs were planted in 96-well plates (10,000 cells per well) and treated as mentioned above. After treatment, the MTT reagents (0.5 mg/mL dissolved in culture medium) were added to each well and cultured for 4 h. MTTwas then converted to purple formazan by active cells. Then the insoluble formazan were dissolved by dimethyl sulfoxide (DMSO). The absorbance at 490 nm was measured by MK3 multiskan (Thermo Fisher, Hong Kong, China).

ROS generation was also determined in each group of treated cells by a ROS detection kit (Beyotime, Shangai, China) following its protocol. Briefly, ROS could convert 2',7'-dichlorofluorescein diacetate (DCFH-DA) to fluorescent dichlorofluorescein (DCF), the DCF production could be was measured by Flexstation 3 (Molecular Device, Silicon Valley, CA, USA) at a wavelength of 488 nm excitation and 535 nm emission.

Terminal Deoxynucleotidyl Transferase dUTP Nick end Labeling (TUNEL) Assay and Western Blot Analysis

To determine the apoptosis ratio, TUNEL staining was performed following the protocol (Roche, Indianapolis, IN, USA). MSCs were seeded in six-well plates and were treated as above described. Then the apoptotic cells were stained with green fluorescence (TUNEL), all the cells were stained with Hoechst33342. The ratio of apoptotic was calculated as the apoptotic cells divided by the total cells.

After treatment as described above, RIPA lysis buffer (Beyotime, China) was used to lysis the cells according to its protocol. Western blot was performed as described with some modification[14]. All primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), the secondary antibody (Goat anti rabbit IgG, 1: 10000) were from the Jackson Laboratory (Bar Harbor, ME, USA). The dilution of the primary antibodies was: caspase-3 (1: 1000), NOS2 (1:500), Akt (1:500), β-actin (1: 1000), respectively.

Statistical Analysis

All values were expressed as mean ± SD. One-way ANOVA analysis were carried out to calculate the difference between each group. Significance was defined as a value of $p < 0.05$.

Results

Primary Culture and Identification of MSCs

The MSCs were isolated from the long bones of C57BL6/J mice and cultured *in vitro*. At passage 3, MSCs could generate colonies as

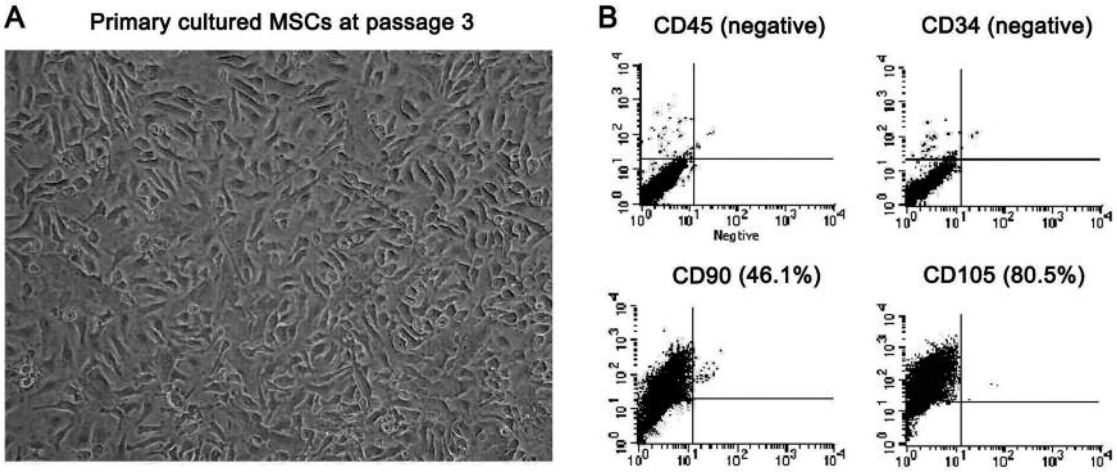

Figure 1. ***A,*** Primary cultured MSCs at passage 3. ***B,*** The flow cytometry analysis of CD45, CD90, CD34 and CD105.

fibroblast like cells and increased gradually, while the heterogeneous cells reduced. Most of the cells were spindle-shaped (Figure 1A) as Ji et al described[15].

Then the primary cultured MSCs at passage 3 were characterized by flow cytometry. The endothelial marker CD34 and the hematopoietic cell marker CD45 were negative in the MSCs at passage 3 (Figure 1B). However, as reported[16], CD105 and CD90, which is not expressed in murine hematopoietic cells, were positive stained in MSCs. The ratio of CD105- and CD90-positive cells was 80.5% and 46.1%, respectively (Figure 1B).

Lycopene Protected MSCs from Ischemia-Induced Apoptosis

The cells were pretreated with different concentrations of lycopene (0, 1, 2, 5, 10, 20, 50 μM, Figure 2A) for 1 h and then exposed to ischemia for 6 h. Cell viability was measured by MTT. It was found that lycopene could dose-dependently restore the ischemia and hypoxia-induced decreasing of cell viability. At a dose of 20 μM, lycopene rescued cell viability significantly (Figure 2B). Meanwhile, lycopene had no effect on normal MSCs (Figure 2C). Therefore, the subsequent experiments were performed with 20 μM lycopene.

To further investigate if lycopene could reduce ischemia-induced cell apoptosis, TUNEL staining was carried out. It was found that 20 μM lycopene could significantly reduce cell apoptosis compared to ischemia and hypoxia treated group (Figure 2D).

Lycopene Protected MSCs from Ischemia and Hypoxia-induced Apoptosis Via Inhibition of ROS Generation

ROS could induce cell apoptosis via oxidative stress[17]. During the process of ischemia, ROS production increased (Figure 3A) compared to normal cells. However, lycopene significantly inhibition ROS generation at a dose of 20 μM according to the DCFH oxidation assay (Figure 3A). NOS2 expression was also significantly reduced by lycopene (Figure 3B). With the decreased level of ROS, the caspase-3 expression level was also reduced (Figure 3B), which prompted that lycopene reduced cell apoptosis via inhibition of oxidative stress.

Lycopene Activates the PI3K/Akt Pathway to Inhibit ROS Generation and Protect MSCs from Ischemia-induced Cell Apoptosis

PI3K/Akt plays an important role in the process of cell survival under stimulations[18]. Whether the effect of lycopene on the MSCs was associated with the PI3K/Akt pathway was further investigated. We found that lycopene could increase Akt phosphorylation after the cells were treated under hypoxia condition in a time-dependent manner (Figure 4A). An Akt inhibitor, LY294002 (20 μM) could inhibit lycopene-stimulated Akt phosphorylation (Figure 4B). Moreover, LY294002 could also reverse the decrease of ROS by lycopene (Figure 4C) and protective effect of lycopene (Figure 4D) in ischemia-treated MSCs.

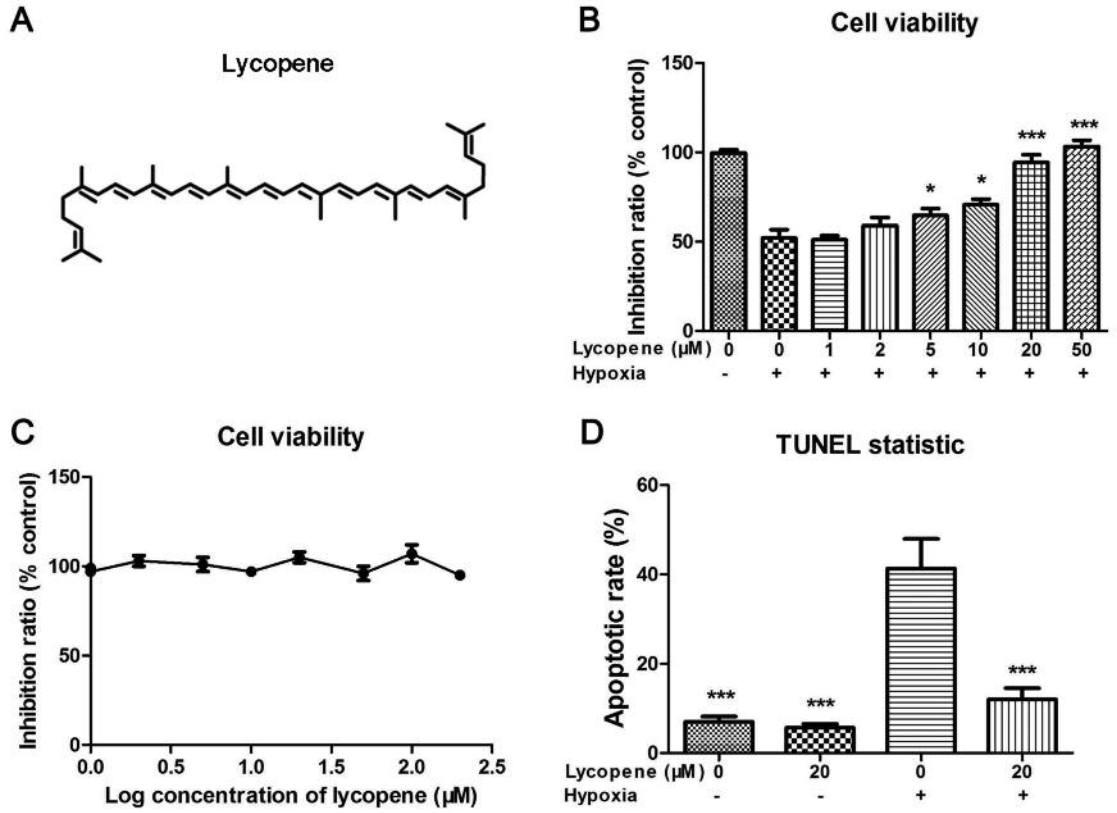

Figure 2. A, The structure of lycopene. ***B,*** Dose-dependently lycopene (0, 1, 2, 5, 10, 20, 50 μM) treated MSCs during hypoxia stimulation for 6 h. The cell viability was expressed as inhibition ratio (% of control). * = $p < 0.5$ and *** = $p < 0.001$ compared hypoxia-treated alone group. ***C,*** Dose-dependently lycopene (0, 1, 2, 5, 10, 20, 50 μM) treated alone in MSCs. The cell viability was expressed as inhibition ratio (% of control). ***D,*** TUNEL statistic. The apoptotic rate was calculated as TUNEL-positive cells divided by total cells. *** = $p < 0.001$ compared hypoxia-treated alone group.

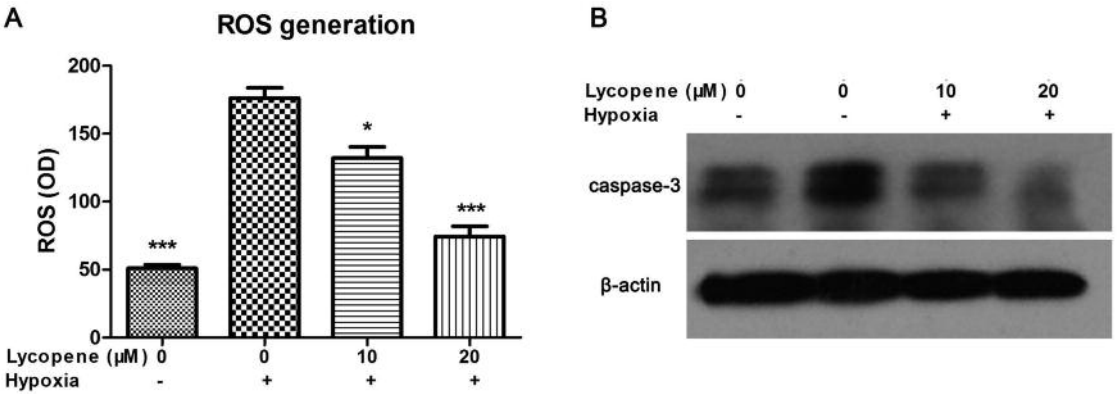

Figure 3. A, ROS generation in each group. After treatment, the ROS generation was measured by OD of DCFH. * = $p < 0.05$ and *** = $p < 0.001$ compared hypoxia-treated alone group. ***B,*** The caspase-3 expression of each group. The expression level of caspase-3 was higher in hypoxia-treated alone group than control group, and the lycopene treatment reduce the caspase-3 expression.

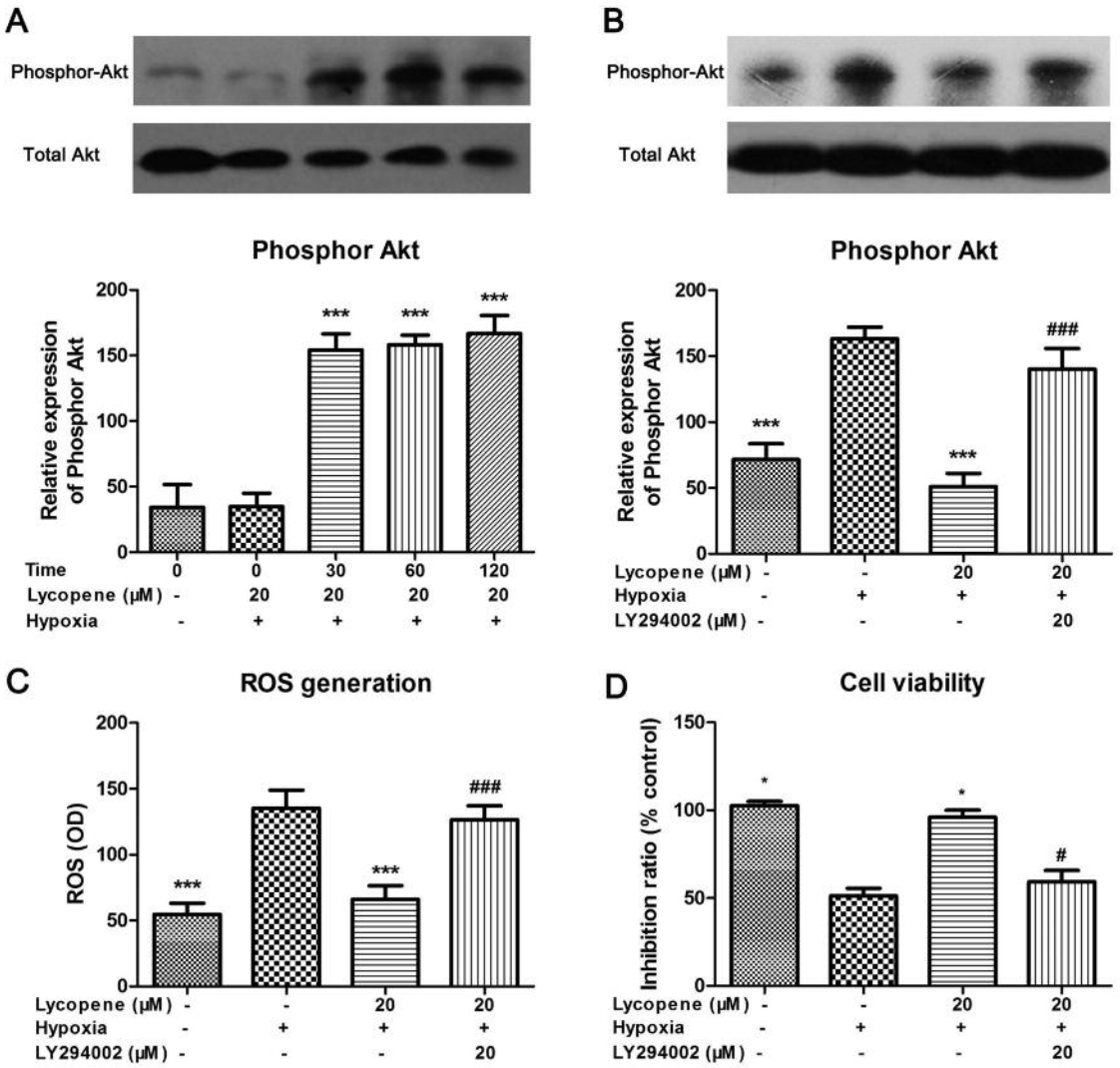

Figure 4. ***A,*** The phosphor-Akt level in each group. 20 μM lycopene could significantly activate Akt phosphorylation in a dose-dependently manner. *** = $p < 0.001$ compared to hypoxia and lycopene co-treated at 0 time. ***B,*** The phosphor-Akt level in each group. After 60 min treatment, the phosphor-Akt level in each group was analyzed. *** = $p < 0.001$ compared to hypoxia-treated alone group, ### = $p < 0.001$ compared to lycopene and hypoxia co-treated group. ***C,*** ROS generation in each group after 60 min treatment. *** = $p < 0.001$ compared to hypoxia-treated alone group, ### = $p < 0.001$ compared to lycopene and hypoxia co-treated group. ***D,*** Cell viability in each group after 60 min treatment. *** = $p < 0.001$ compared to hypoxia-treated alone group, ### = $p < 0.001$ compared to lycopene and hypoxia co-treated group.

Discussion

Nowadays, transplantation of MSCs have been used and exploited widely in variety of diseases therapy[19]. The protocol of primary culturing MSCs have been a well-established procedure and used for various researches. As Bourzac et al[20] and Friedenstein et al[21] reported, in our experiment, primary cultured MSCs has been defiend by hematopoietic cell markers and stem cell-specifically markers. However, the using of MSCs for transplantation is limited by the high percentage of the donor cells death rapidly[22]. It has been proved that the ischemia environment could induce MSCs apoptosis both *in vitro* and *in vivo*[9,11,19]. We also found that hypoxia-treated cells could generate apoptosis significantly. Thus, protect MSCs from ischemia-induced apoptosis might be crucial in the cell therapy based on MSCs transplantation.

Hypoxic or ischemic conditions have been proved important during the process of cell

apoptosis as these conditions could induce ROS generation[22-24]. ROS could be generated under stimulations including cytokines, ischemia, inflammatory signals and so on[25]. Multiple pathways are involved in ROS activated signal transduction, such as mitogen-activated protein serine/threonine kinases (MAPKs) and PI3K/Akt pathways[26]. Increased level of ROS could cause cell apoptosis and inhibit proliferation. Meanwhile, excessive ROS could injury cellular functions directly[27]. We found that ROS level of hypoxia-treated MSCs were significantly higher than control group. Meanwhile, the expression level of caspase-3, typical marker of execution-phase of cell apoptosis[28], was as higher as ROS.

Lycopene is one kind of carotenoids that belongs to a series of pigments[29]. Carotenoids are naturally occurring products widespread in vegetables and fruits[30,31]. It has been reported to be one of the most potent antioxidant among all carotenoids[32]. Furthermore, lycopene could restore the free radicals, such as ROS or other peroxy radicals, induced reactions[32,33]. Thus, whether lycopene could protect MSCs from ischemia-induced apoptosis was studied. We found that lycopene dose-dependently restored the injury cell viability induced by ischemia in MSCs. Moreover, lycopene blocked the generation of ROS in ischemia-treated MSCs, suggested that lycopene might exert antiapoptotic activities by inhibition of ROS generation.

PI3K/Akt signal pathway is important in cell process including cell metabolism, proliferation, migration and survival[34]. PI3K/Akt could reduce cell death by inhibition of some apoptotic factors like Bad and caspase-9[35]. In the current study, it was observed that during hypoxia-induced MSCs apoptosis, lycopene could increase the level of Akt phosphorylation time-dependently. One PI3K/Akt inhibitor, LY294002, could block lycopene-induced Akt phosphorylation. Moreover, the lycopene-reduced ROS level in hypoxia-treated MSCs was also increased by LY293002 pretreated. The protective effect of lycopene was suppressed by the Akt phosphorylation inhibitor. Taken together, PI3K/Akt pathway could be important in the protective effect of lycopene during hypoxia/ischemia-induced MSCs apoptosis.

Conclusions

Lycopene could protect primary cultured MSCs from ischemia-induced apoptosis through inhibiting ROS generation possibly via PI3K/Akt pathway, indicating that lycopene might provide benefit for the transplantation of MSCs in cell therapy.

Conflict of Interest

All authors declare that there are no conflicts of interest and agree with the contents of the manuscript for publication and support open access publishing to allow unlimited access and high publicity of the published paper.

References

1) Pereira RF, Halford KW, O'Hara MD, Leeper DB, Sokolov BP, Pollard MD, Bagasra O, Prockop DJ. Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc Natl Acad Sci U S A 1995; 92: 4857-4861.
2) Azizi SA, Stokes D, Augelli BJ, DiGirolamo C, Prockop DJ. Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats—similarities to astrocyte grafts. Proc Natl Acad Sci U S A 1998; 95: 3908-3913.
3) Ferrari G, Cusella-De AG, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F. Muscle regeneration by bone marrow-derived myogenic progenitors. Science 1998; 279: 1528-1530.
4) Rosova I, Dao M, Capoccia B, Link D, Nolta JA. Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. Stem Cells 2008; 26: 2173-2182.
5) Iwase T, Nagaya N, Fujii T, Itoh T, Murakami S, Matsumoto T, Kangawa K, Kitamura S. Comparison of angiogenic potency between mesenchymal stem cells and mononuclear cells in a rat model of hindlimb ischemia. Cardiovasc Res 2005; 66: 543-551.
6) Yu Y, Yao AH, Chen N, Pu LY, Fan Y, Lv L, Sun BC, Li GQ, Wang XH. Mesenchymal stem cells over-expressing hepatocyte growth factor improve small-for-size liver grafts regeneration. Mol Ther 2007; 15: 1382-1389.
7) Pons J, Huang Y, Arakawa-Hoyt J, Washko D, Takagawa J, Ye J, Grossman W, Su H. VEGF improves survival of mesenchymal stem cells in infarcted hearts. Biochem Biophys Res Commun 2008; 376: 419-422.
8) Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation 2002; 105: 93-98.
9) Zhu W, Chen J, Cong X, Hu S, Chen X. Hypoxia and serum deprivation-induced apoptosis in mesenchymal stem cells. Stem Cells 2006; 24: 416-425.
10) Matsui T, Li L, Del M, Fukui Y, Franke TF, Hajjar RJ, Rosenzweig A. Adenoviral gene transfer of activated phosphatidylinositol 3'-kinase and Akt inhibits apoptosis of hypoxic cardiomyocytes in vitro. Circulation 1999; 100: 2373-2379.

11) Chen J, Baydoun AR, Xu R, Deng L, Liu X, Zhu W, Shi L, Cong X, Hu S, Chen X. Lysophosphatidic acid protects mesenchymal stem cells against hypoxia and serum deprivation-induced apoptosis. Stem Cells 2008; 26: 135-145.

12) Xu Z, Yan L, Ge Y, Zhang Q, Yang N, Zhang M, Zhao Y, Sun P, Gao J, Tao Z, Yang Z. Effect of the calcium sensing receptor on rat bone marrow-derived mesenchymal stem cell proliferation through the ERK1/2 pathway. Mol Biol Rep 2012; 39: 7271-7279.

13) Tropel P, Noel D, Platet N, Legrand P, Benabid AL, Berger F. Isolation and characterisation of mesenchymal stem cells from adult mouse bone marrow. Exp Cell Res 2004; 295: 395-406.

14) Meng ZX, Nie J, Ling JJ, Sun JX, Zhu YX, Gao L, Lv JH, Zhu DY, Sun YJ, Han X. Activation of liver X receptors inhibits pancreatic islet beta cell proliferation through cell cycle arrest. Diabetologia 2009; 52: 125-135.

15) Ji JF, He BP, Dheen ST, Tay SS. Interactions of chemokines and chemokine receptors mediate the migration of mesenchymal stem cells to the impaired site in the brain after hypoglossal nerve injury. Stem Cells 2004; 22: 415-427.

16) Da SML, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci 2006; 119: 2204-2213.

17) Tan S, Sagara Y, Liu Y, Maher P, Schubert D. The regulation of reactive oxygen species production during programmed cell death. J Cell Biol 1998; 141: 1423-1432.

18) Yamaguchi H, Wang HG. The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change. Oncogene 2001; 20: 7779-7786.

19) Haider H, Ashraf M. Strategies to promote donor cell survival: combining preconditioning approach with stem cell transplantation. J Mol Cell Cardiol 2008; 45: 554-566.

20) Bourzac C, Smith LC, Vincent P, Beauchamp G, Lavoie JP, Laverty S. Isolation of equine bone marrow-derived mesenchymal stem cells: a comparison between three protocols. Equine Vet J 2010; 42: 519-527.

21) Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP. Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation 1968; 6: 230-247.

22) Robey TE, Saiget MK, Reinecke H, Murry CE. Systems approaches to preventing transplanted cell death in cardiac repair. J Mol Cell Cardiol 2008; 45: 567-581.

23) Cao YJ, Shibata T, Rainov NG. Hypoxia-inducible transgene expression in differentiated human NT2N neurons—a cell culture model for gene therapy of postischemic neuronal loss. Gene Ther 2001; 8: 1357-1362.

24) Wang G, Hazra TK, Mitra S, Lee HM, Englander EW. Mitochondrial DNA damage and a hypoxic response are induced by CoCl(2) in rat neuronal PC12 cells. Nucleic Acids Res 2000; 28: 2135-2140.

25) Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007; 39: 44-84.

26) Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M. Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell 2005; 120: 649-661.

27) Droge W. Free radicals in the physiological control of cell function. Physiol Rev 2002; 82: 47-95.

28) Harrington HA, Ho KL, Ghosh S, Tung KC. Construction and analysis of a modular model of caspase activation in apoptosis. Theor Biol Med Model 2008; 5: 26.

29) Holzapfel NP, Holzapfel BM, Champ S, Feldthusen J, Clements J, Hutmacher DW. The potential role of lycopene for the prevention and therapy of prostate cancer: from molecular mechanisms to clinical evidence. Int J Mol Sci 2013; 14: 14620-14646.

30) Engelmann NJ, Clinton SK, Erdman JJ. Nutritional aspects of phytoene and phytofluene, carotenoid precursors to lycopene. Adv Nutr 2011; 2: 51-61.

31) Shi J, Le Maguer M. Lycopene in tomatoes: chemical and physical properties affected by food processing. Crit Rev Food Sci Nutr 2000; 40: 1-42.

32) Krinsky NI. The antioxidant and biological properties of the carotenoids. Ann N Y Acad Sci 1998; 854: 443-447.

33) Di Mascio P, Kaiser S, Sies H. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. Arch Biochem Biophys 1989; 274: 532-538.

34) Stambolic V, Woodgett JR. Functional distinctions of protein kinase B/Akt isoforms defined by their influence on cell migration. Trends Cell Biol 2006; 16: 461-466.

35) Downward J. PI 3-kinase, Akt and cell survival. Semin Cell Dev Biol 2004; 15: 177-182.