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Original Article

Alpha-Tocopherol increases the proliferation of induced pluripotent stem cell derived neural progenitor cells

Mahboobeh Malakoutikhah[1], Leila Satarian[2], Sahar Kiani[2], Mohammad Javan[1,2*]

1. Department of Physiology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
2. Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

Abstract

In addition to its antioxidant effect, Vitamin E or α–tocopherol is suggested to enhance remyelination in the animal model of non-inflammatory demyelination. In this study, the possible proliferative effect of vitamin E on human- induced pluripotent stem cell-derived neural progenitors (hiPS-NPs) and the underlying mechanisms were investigated in vitro. NPs were induced from iPS cells via 3 steps within 18 days and then characterized for NPs markers NESTIN, SOX1 and OTX2. MTT assay was used to compare cell populations. LY294002, U0126 and PP2 were used for selective inhibition of enzymes PI3K, MEK and Src-kinase, respectively. Vitamin E increased hiPS-NPs proliferation after 24 and 48 h exposure. The inhibition of both PI3K/Akt and Src-kinase signaling pathways counteracted the effect of vitamin E of NPs. Our data suggest that vitamin E may enhance NPs proliferation via activating PI3K and Src-kinase and may enhance myelin repair following demyelinating Injuries.

Keywords:
Neural progenitor cells;
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Vitamin E;
α –tocopherol;
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***Correspondence to:**
M. Javan
Tel: +98 21 82884522
Fax: +98 21 82884528
Email:
mjavan@modares.ac.ir

Introduction

Demyelination as the main hallmark of multiple sclerosis (MS) is caused by immune cells activation against the myelin of the central nervous system (CNS). Demyelination disrupts the nerve signal transmission and eventually results in axonal degeneration (Radtke et al., 2007). Myelin repair or remyelination occurs in almost newly affected patients as a spontaneous process (Patrikios et al., 2006), but as the disease progresses, the process fails and leads to progressive disability in the patients (Martino et al., 2010, Tripathi et al., 2010). A variety of endogenous cells including neural stem/ progenitor cells (NS/PCs) and oligodendrocyte precursor cells (OPCs) participate in the processes of remyelination. NS/PCs are scattered within the brain parenchyma but they are main ly located within the subventricular zone (SVZ) of lateral ventricles and the subgranular zone of dentate gyrus of hippocampus (Miller and Gauthier-Fisher, 2009). The OPCs are scattered throughout the white and gray matters of brain (Pluchino and Martino, 2008, Zhao et al., 2008). Evidence shows that

although NS/PCs possess moderate capacity for remyelination compared to OPCs (Zhao et al., 2008), yet these cells release immunomodulatory molecules such as anti-inflammatory cytokines and neurotrophic factors such as NGF, FGF-2, CNTF, BDNF (Pluchino and Martino, 2008, Feng and Gao, 2012, Jadasz et al., 2012). There are two strategies for increasing remyelination rate in affected patients: stimulation of endogenous neural stem and progenitor cells (NSCs/NPs) and/or oligodendrocyte progenitor cells (OPCs) for proliferation and differentiation to myelinating cells in the CNS, and transplantation of myelinating cells prepared in the laboratory (Blakemore, 2008, Huang and Franklin, 2011, Feng and Gao, 2012). Due to the diffused nature of lesions of MS, it seems that the strategy of stimulating and supporting of endogenous NSCs/NPs and OPCs for proliferation, migration and efficient differentiation is more feasible (Zhao et al., 2008, Huang and Franklin, 2011). Furthermore, any therapeutic method that supports the endogenous cells may also support the transplanted cells prepared in vitro.

Vitamin E is an effective antioxidant which has been introduced as a free radical scavenger. This vitamin has eight isoforms, including tocopherols with α, β, γ, δ isoforms and tocoterienols with α, β, γ, δ isoforms. α- tocopherol is the most abundant and active isoform of vitamins E in vivo. α-tocopherol prevents oxidative damage in a dose-dependent manner (Yoshida et al., 2007). Besides its anti-oxidant effects, other properties of vitamin E such as its effects on cell signaling and gene expression have also been demonstrated (Azzi et al., 2003, De Pascale et al., 2006, Azzi, 2007). Reduced levels of α-tocopherol in the brain have been associated with neural dysfunction, from mood disorders to neurodegeneration (Crouzin et al., 2011). Some studies showed the decreased level of vitamin E in patients suffering from MS (Salemi et al., 2010). In rat model of experimental demyelination, vitamin E caused reduced demyelination and interestingly accelerated remyelination phase (Goudarzvand et al., 2010). In the current study, in vitro produced NPs which previously were used for remyelinating the chemically demyelinated axons of rat optic chiasm (Pouya et al., 2011, Satarian et al., 2013) were used to evaluate the effect of α-tocopherol on proliferation and differentiation of NPs. In addition, the possible role of the signaling pathways PI3K/Akt, MAPK/Erk and Src-kinase in the proliferating effect of vitamin E was evaluated.

Materials and methods

Culture and induction of pluripotent stem cells to NPs

Human induced pluripotent stem cell (hiPSCs) line Royan-hiPSC8 (Royan Institute, Iran) (Pakzad *et al.*, 2010, Totonchi *et al.*, 2010) was cultured and differentiated as mentioned earlier (Satarian *et al.*, 2013). Briefly, hiPSC colonies were expanded and passaged in feeder-free culture conditions in a pluripotent stem cell medium as previously described (Pakzad *et al.*, 2010). We induced hiPSCs to NPs five days after passaging the cells and performed it over a three-week period. Differentiation medium included DMEM/F12 (Gibco-Invitrogen, 21331-020) supplemented with 5% knockout serum replacement (KOSR, Life Technologies # 10828010), 2 mM L-glutamine (Gibco-Invitrogen, 25030-024), 1% nonessential amino acids (Gibco-Invitrogen, 11140), 0.1 mM β-mercaptoethanol, 2% N2 supplement (Gibco-Invitrogen, 17502-048), and 300 ng/ml bFGF (Royan Institute, Iran). Neural induction was performed within 18 days (Fig. 1A). During the first step (6 days) of neural induction, Noggin (100 ng/ml, R&D, 1967-NG) was added to the medium for neural ectoderm induction. All-trans retinoic acid (RA; Sigma-Aldrich, R2625) 2 µM was administered in the second step (6 days) in conjunction with Noggin 250 ng/ml. During the 3rd step (6 days), Noggin was eliminated but RA 2 µM was continued. Then, the neural tubes were manually separated and re-plated on poly-L-ornithine (Sigma-Aldrich, P4707) and laminin (Sigma-Aldrich, L2020)-coated tissue plates. Expansion medium consisted of basal medium supplemented with human epidermal growth factor (EGF, 20 ng/ml, Sigma-Aldrich, E9644) and ascorbic acid (0.2 mM, Sigma-Aldrich, A8960). hiPSC derived-NPs (hiPS-Nps) were checked for the expression of Nestin, SOX1 and OTX2 to confirm NP differentiation (Fig. 1B).

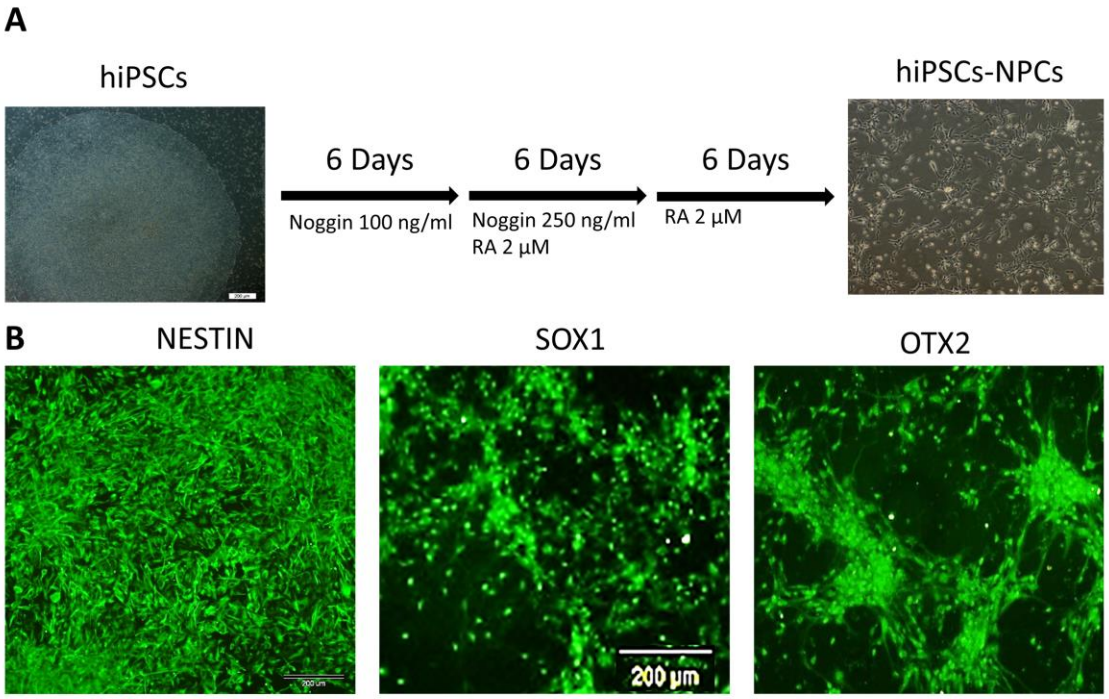

Fig 1. Neural progenitor cells were induced from human induced pluripotent stem cells (hiPSCs) in 3 steps (A). The characteristics of induced NPs were confirmed by immunocyto- fluorescent studies against NESTIN, SOX1 and OTX2. RA: retinoic acid.

Subculturing of hiPS-NPs and interventions

hiPS-NPCs were cultured in 6 cm tissue culture dishes coated with Poly L-Ornithine/Laminin. The cells were expanded in maintaining medium containing DMEM/F12 (Invitrogen, 21331-020) with 5% knock out serum (KOSR), 2 mM L-glutamine (Invitrogen, 25030-024), 0.1 mM nonessential amino acid, 1% Penicilin/Stereptomycin, 2% N2 supplement, 0.001% B27 supplement (Invitrogen, 17504-044), 0.1 mM β-mercaptoethanol, 0.2 mM Ascorbic acid, 100 ng/ml bFGF (Royan institute, Iran), 20 ng/ml EGF (Royan institute) in 37 ˚C, 5% CO2 and under humidified atmosphere. Cell culture medium was changed every other day. After 6 days, when the confluency appeared to be 80-90%, cells were re-plated. Cells at passages 12-16 were used to investigate the effect of drug treatment. Cells were subcultured using 1 ml 0.05% Trypsin/EDTA, pipetting and transferred with a ratio of 1:2 to new plates.

To study the effect of α-tocopherol (vitamin E), a 50 mM stock solution was prepared in absolute ethanol. For each experiment, a fresh stock of vitamin E was prepared and dose-response studies were done after 24 or 48 h of exposure to the vitamin. For studying the role of signaling pathways, the specific inhibitors LY294002 (Sigma-Aldrich L9908) as inhibitor of PI3K, U0126 (Sigma-Aldrich U120) as inhibitor of MEK and PP2 (Sigma-Aldrich P0042) as inhibitor of Src, were prepared as stock solutions (30 mM) in dimethyl sulfoxide (DMSO). Before plating the cells for drug treatment, cells were counted and seeded in $5 X10^5$ cells/well.

MTT proliferation assay

MTT assay is widely used to quantify cells based on metabolic activity. The enzyme Mitochondrial dehydrogenase converts a water-soluble tetrazolium salt into formazan crystals insoluble in water, but soluble in DMSO. The intensity of the color, which is quantified spectrophotometrically, is directly proportional to the number of viable cells. For MTT assay the cell medium was discarded and 200 µl of MTT solution 5 mg/ml was added per well and 800 µl medium was added and the cells were incubated for 1-

2 hours. Afterward, the MTT solution and medium were discarded and 500 µl of DMSO was added per well and after several pipetting rounds, formazan optical density was read at 540 nm wavelength using a microplate reader machine. To avoid variation in reading of absorption between the plates, MTT assay data was expressed as percentage of control wells absorption.

Immunocytofluorescent study

Immunofluorescence staining was used to study the expression of specific markers such as NESTIN, SOX1 and OTX2, in hiPS-NPs. The cells were fixed with paraformaldehyde 4% for 10 min at room temperature, and then washed twice for 5 min with washing buffer containing PBS (phosphate buffered saline) and 0.05% Tween. The cells were permeablized with 0.5% Triton to make the intracellular markers available for antibodies and blocked with PBS containing 10% goat serum and 2% BSA for 45 min. Primary antibodies including mouse monoclonal anti-NESTIN (Chemicon, MAB5326), rabbit monoclonal anti-SOX1 (Sigma-Aldrich, S8318), rabbit monoclonal anti-OTX2 (Sigma-Aldrich, HPA000633), were added and incubated for 3 h at room temperature. The cells were washed with washing buffer for three times and then incubated with secondary antibodies including goat anti-mouse FITC-conjugated (SC-2010), goat anti-mouse FITC-conjugated (F9259), goat anti-rabbit FITC-conjugated (Sigma-Aldrich, F1262) for 45 min at room temperature and washed with washing buffer for three times. The fluorescent dye 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, D-8417) was used for nuclei staining. The preparations were observed and photographed using a Olympus fluorescence microscope LX71. The images were taken randomly from all regions of the well and from each well more than 500 cells were counted to calculate the ratio of positive cells to total cells. For each marker, each experiment was performed 3 times.

Statistical Analysis

One way-ANOVA and Tukey post-test were performed to compare the results of different groups. The least significance level was considered as $p< 0.05$. Data are reported as mean±SD.

Results

Culture and Differentiation of hiPSCs to neural progenitors

Self-renewing NPs were produced from hiPSC8 line cultured under feeder free conditions. As it was shown in Figure 1A, hiPSCs were induced for neural differentiation in adherent culture in the presence of Noggin (100 ng/ml) for 6 days followed by additional 6 days culture in the same medium with increased concentrations of Noggin and RA supplementation.. Culturing continued by removal of Noggin for an additional 6 days. The first step led to the appearance of the columnar cells, the second step led to formation of rosette structures, and the final step produced neural tube-like structures. A pipette was used to harvest neural tube-like structures under phase contrast microscopy for replating in the presence of bFGF and EGF on poly-L-ornithine/laminin-coated plates. Under these conditions, the cells generated a monolayer of morphologically homogeneous cells which expressed neural progenitor markers Nestin, SOX1 and OTX2 (Fig. 1B).

Effects of vitamin E on proliferation of iPSC-NPCs

Effect of different concentration of vitamin E on proliferation of NPCs after 24 and 48 h exposure was studied using MTT assay (Fig. 2). Following 24 h exposure, vitamin E at doses 25, 50 and 100 µM increased cell proliferation ($p<0.05$, $p<0.001$, $p<0.001$, respectively). When vitamin E was added to the cells medium over an 48 h period with doses ranging from 50 and 100 µM, it increased cell proliferation ($p<0.001$ and $p<0.01$, respectively).

Role of signaling pathways in proliferative effect of vitamin E

To study the possible role of PI3K in proliferative effect of α-tocopherol, LY294002 was used to inhibit PI3K signaling pathway. Figure 3A shows the effect of different concentration of LY294002 on cell proliferation. Twenty four hours exposure to LY294002

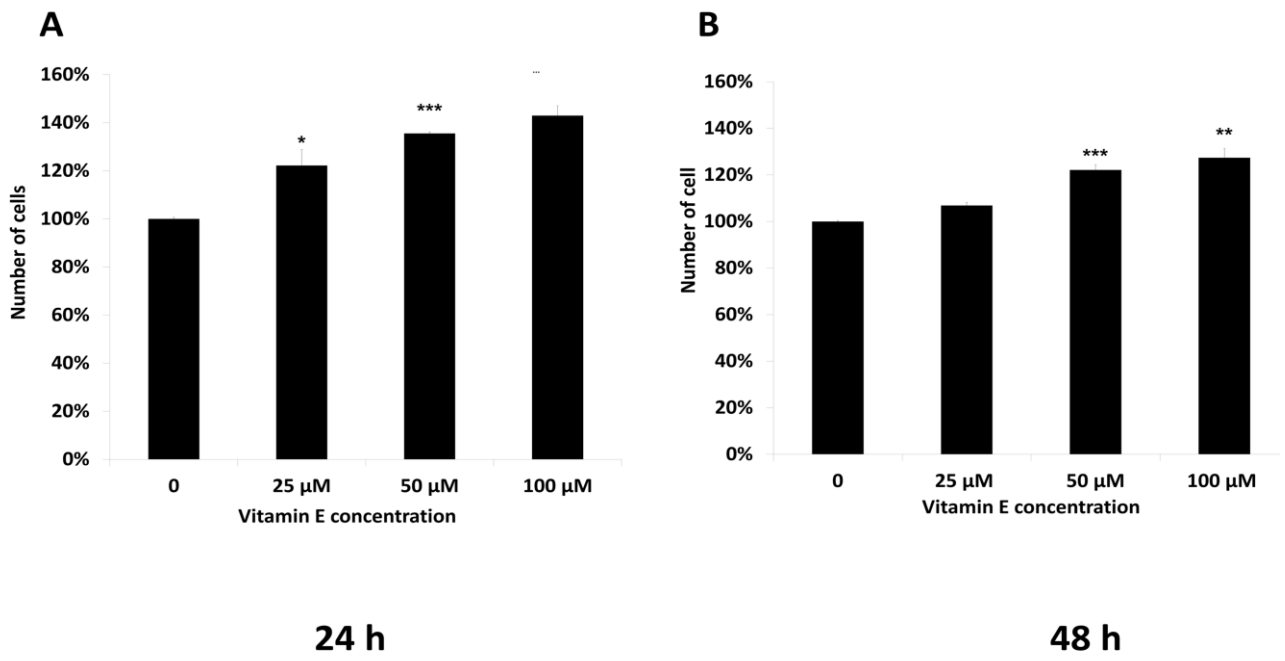

Fig. 2. Effect of vitamin E in concentrations of 25, 50 and 100 µM on the proliferation of neural progenitor cells. A. Proliferation of neural progenitor cells after 24 h. B. Proliferation of neural progenitor cells after 48 h. MTT test was used to compare the cell populations. *p< 0.05, **p<0.01 and ***p<0.001 compared to control (dose 0). The experiments were done triplicated.

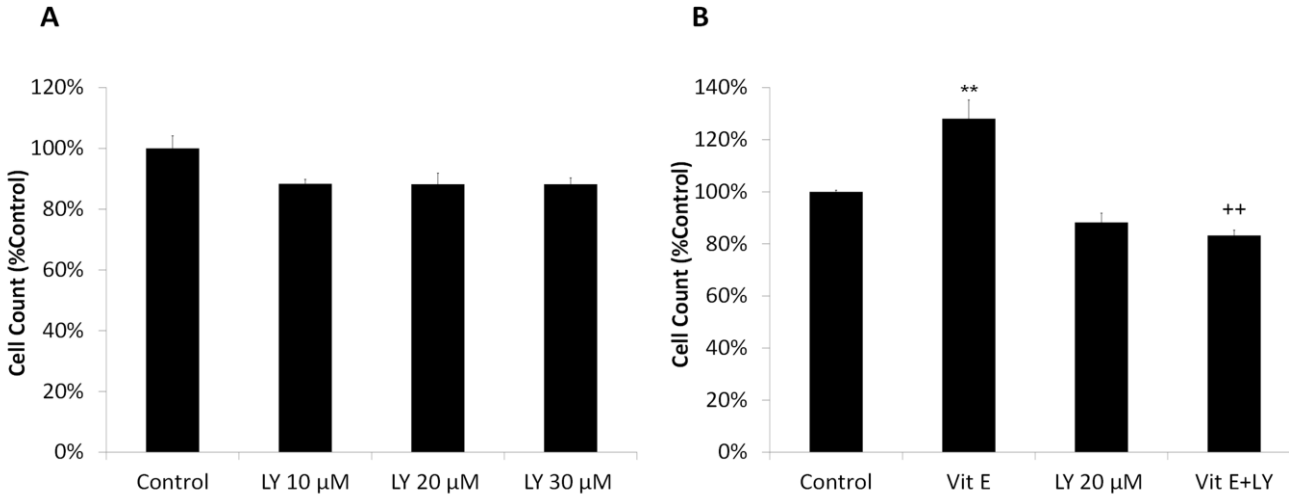

Fig. 3. Effect of inhibition of PI3K/Akt pathway with LY294002 on vitamin E induced NPs proliferation. A. different doses of LY294002 did not change cell proliferation significantly. B. LY294002 inhibited proliferative effect of vitamin E. **p< 0.01 vs control; ++p<0.01 vs vitamin E treated group. The experiments were done triplicated.

at doses 10, 20 and 30 µM did not exert significant effect on cell proliferation. Then we studied the proliferative effect of vitamin E at dose 100 µM in the presence of LY294002 20 µM (Fig. 3B). In the presence of LY294002, vitamin E did not increase cell proliferation. The difference between Vitamin E and LY+Vitamin E treated groups was statistically significant ($p<0.01$).

To study the possible role of Src kinase in proliferative effects of vitamin E, PP2 was used to inhibit Src kinase. As it is shown in Figure 4A, when PP2 was administrated alone at doses 1, 5 and 10 µM, it did not exert any effect on iPSC-NPs proliferation. As Figure 4B shows, Vitamin E alone increased iPSC-NPs

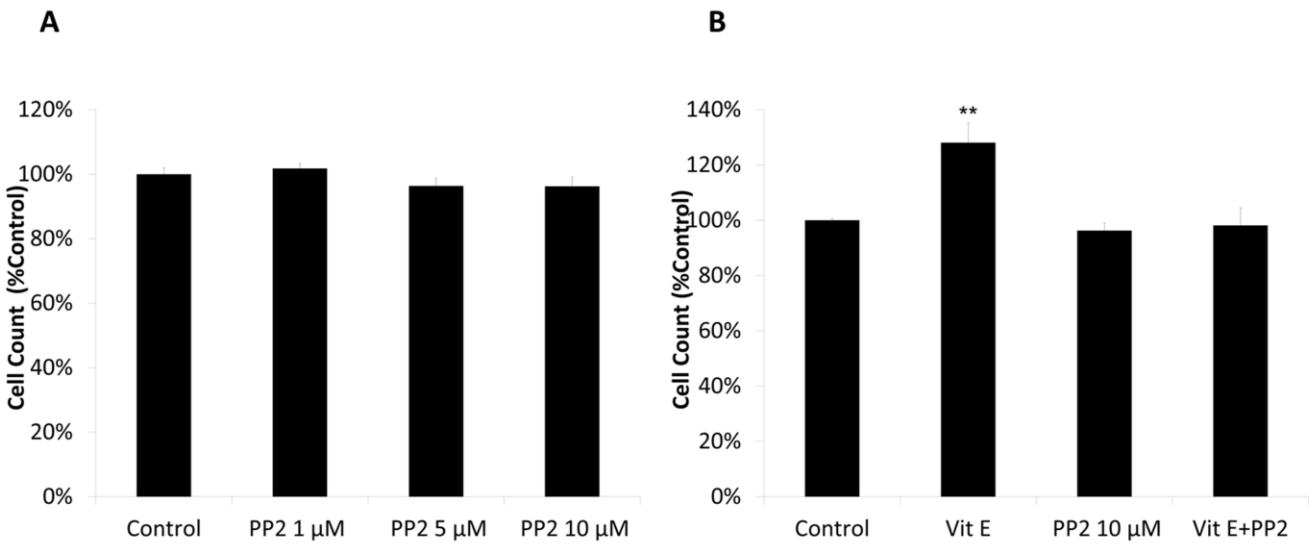

Fig. 4. Effect of inhibition of Src-kinase pathway with PP2 on vitamin E induced NPs proliferation. A. different doses of PP2 did not change cell proliferation significantly. B. PP2 inhibited proliferative effect of vitamin E. **p< 0.01 vs control. The experiments were done triplicated.

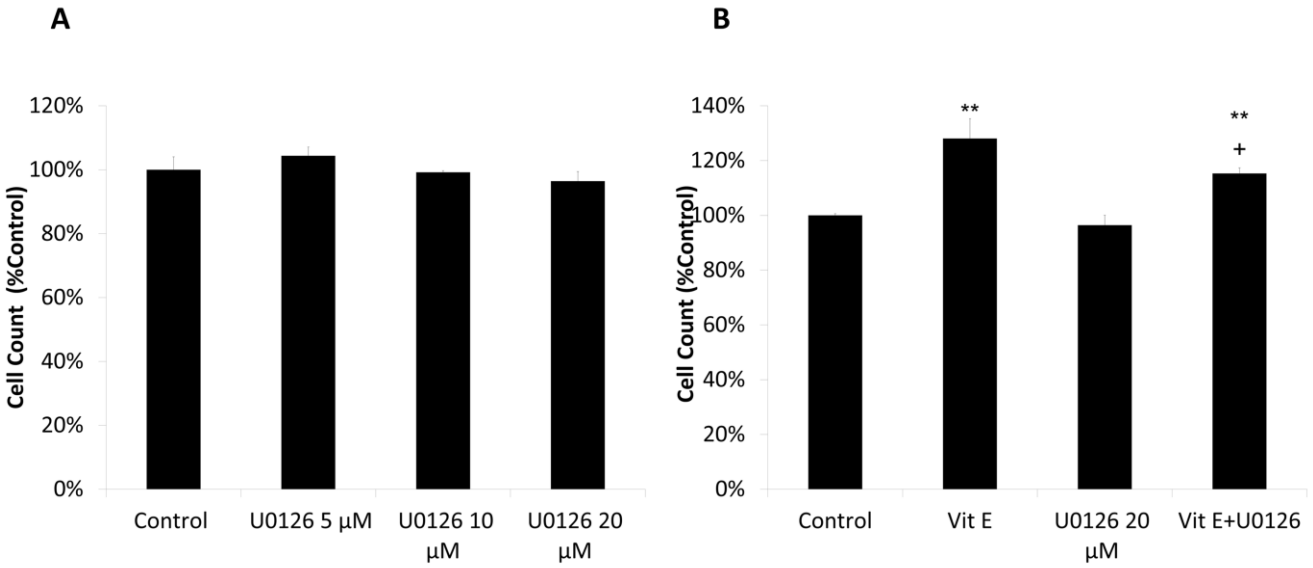

Fig. 5. Effect of inhibition of MAPK/Erk pathway with U0126 on vitamin E induced NPs proliferation. A. different doses of U0126 did not change cell proliferation significantly. B. U0126 did not inhibit proliferative effect of vitamin E. **p< 0.01 vs control; +p<0.05 vs vitamin E treated group. The experiments were done triplicated.

proliferation but in the presence of PP2 10 µM, it did not changed the rate of proliferation of neural progenitors, a finding which implies a role for of Src kinase in vitamin E induced iPSC-NPs proliferation. The difference between Vitamin E and Vitamin E+PP2 groups was statistically significant ($p<0.05$).

Figure 5 shows the results of our study on the role of MAP kinase signaling pathways in the proliferative effect of vitamin E on neural progenitor cells. U0126 as the inhibitor of MEK, at doses 5, 10 and 20 did not show any effect on neural progenitors proliferation (Fig. 5A). Cell proliferation in the presence of vitamin E+U0126 was similar to the Vitamin E group but significantly higher than Control and U0126 treated groups ($p<0.01$ and $p<0.05$, respectively; Fig. 5B). It seems that MAP kinase pathway does not play a

significant role in proliferative effect of vitamin E .

Discussion

Neural progenitor cells have great potential for treatment of neurodegenerative diseases such as multiple sclerosis (Pluchino *et al.*, 2003, Yun *et al.*, 2010, Pouya *et al.*, 2011). Stem cells may be used for regenerative purposes in two different strategies; first, neural progenitor cells preparation in the lab and transplantation for repairing the neural lesions, and second, stimulation of endogenous stem cells present in certain regions of the brain such as subventricular zone and dentate gyrus of hippocampus. Following transplantation, neural progenitors may either directly differentiate to neural cells to replace the lost cells or they may release trophic factors such as NGF, FGF2, CNTF and BDNF to enhance endogenous repair (Pluchino *et al.*, 2005, Feng and Gao, 2012, Satarian *et al.*, 2013). Endogenous neural progenitors differentiate to OPCs and then to oligodendrocytes and remyelinate the lesions (Mozafari *et al.*, 2010, Mozafari *et al.*, 2011, Dehghan *et al.*, 2012, Pazhoohan *et al.*, 2014).

Vitamin E is an antioxidant, but its effects on signaling pathways and changes in gene expression have also been demonstrated (Nogueira-Pedro *et al.*, 2011). Some studies show decreased levels of Vitamin E in multiple sclerosis patients (Langemann *et al.*, 1992, Karg *et al.*, 1999). In 2010 Goudarzvand and colleagues found that following ethidium bromide-induced demyelination in rats brain, vitamin E decreased demyelination and accelerated the process of remyelination; moreover, besides preventing apoptosis due to its antioxidant properties, vitamin E likely enhances proliferation, migration or differentiation of endogenous neural progenitors to myelinating cells (Goudarzvand *et al.*, 2010). To confirm the proliferative effect of vitamin E on neural progenitors, we performed the current study and investigated the effect of vitamin E on neural progenitor cells derived from human induced pluripotent stem cells. in vitro. MTT assay showed that vitamin E increased neural progenitor cell proliferation in a dose dependent manner. Neural progenitor cells were exposed to Vitamin E for 24 and 48 h in micro-molar concentrations of 25, 50, 100. MTT assay results showed that vitamin E in both 24 and 48 h exposure times increased the progenitor cell proliferation. In scientific literature, the effect of vitamin E on cell proliferation seems controversial and varies from cell to cell lines. In a previous report ,Vitamin E was found to stimulate the growth in culture of an epidermoid carcinoma cell line derived from chemically induced tumors of hamster buccal pouch (Odukoya *et al.*, 1986). In a controversial report, vitamin E inhibited cell proliferation and activation of the Erk cascade during promotion of urethane-induced lung tumorigenesis in mice; the effect was independent of its antioxidative effect (Yano *et al.*, 2000).

In addition to the proliferative effect of vitamin E, we studied the possible role of signaling pathways in the proliferative effect of vitamin E. Following inhibition of the signaling pathway PI3K/Akt with LY294002, vitamin E could not increase proliferation of neural progenitor cells which confirms the role of PI3K/Akt pathway in the proliferative effect of Vitamin E. In a previous mechanistic study, it was showed that the anticancer effects of γ-tocotrienol were associated with a suppression in PI3K/Akt signaling (Sylvester and Ayoub).

Furthermore, the blockage of Src-kinase with inhibitor PP2, inhibited the proliferative effect sof vitamin E on neural progenitor cells. When the inhibition of MAPK/Erk signaling pathway was achieved by U0126, vitamin E was effective in enhancing the proliferation of neural progenitor cells. This finding rules out any significant role of MAPK in vitamin E induced cell proliferation in the current study.

The results of our study indicated that vitamin E may be used as a supplement in the treatment of multiple sclerosis to increase the number of endogenous progenitors. PI3K and Src-kinase seems to be involved in the proliferative effect of vitamin E on hiPS-NPS.

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Conflict of interest

The authors declare that they have no conflict of

interest.

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