Vitamin E Pretreatment of Mesenchymal Stem Cells: The Interplay of Oxidative Stress and Inflammation

Shadi Mehrzad1†, Sepideh sadat Hosseini1†, Madjid Momeni-Moghaddam1, Moein Farshchian2, Halimeh Hassanzadeh2,3, Mahdi Mirahmadi2, Fatemeh Sadeghfir4†, Hamid Reza Bidkhoris2†

1Department of Biology, Faculty of science, Hakim Saeerni University, Saeerni, Iran
2Stem Cells and Regenerative Medicine Research Department, Academic Center for Education, Culture and Research (ACECR)-Khorasan Razavi, Mashhad, Iran
3Department of Biology, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran

Received 18 February 2020 Accepted 12 March 2020

Abstract

Oxidative stress occurs as a result of breaking down the balance between oxidants (e.g., reactive oxygen species (ROS)) and antioxidants in cells. Several studies have shown that there is a close relationship between oxidative stress and inflammation at the sites of injury. Mesenchymal stem cells (MSCs) are exposed to endogenous and exogenous oxidants generated during their ex vivo expansion or following in vivo transplantation. α-tocopherol (vitamin E) is a fat-soluble compound known for its anti-oxidant and anti-inflammatory properties. In many studies, the immunomodulatory effects of vitamin E have been observed in vivo. This study aimed to determine whether pretreatment of MSCs with antioxidants like vitamin E, will enhance the anti-inflammatory and immunomodulatory properties of these cells. For this purpose, adipose-derived MSCs (ASCs) were treated with vitamin E (600 µM) for 48 h. Quantitative PCR (qPCR) experiments were performed to evaluate the expression of genes related to inflammation (IL-1β, IL-6, IL-17, IL-10) or immunomodulation (TSG-6, COX-2, TDO2, TGF-β1). Results indicated that vitamin E significantly increased the expression of COX-2, TSG-6, and IL-1β genes at the mRNA level compared with the control group, while it significantly decreased IL-6 and TGF-β expressions. No effect was observed for IL-17, IL-10, and TDO2 genes. These results suggest that in vitro preconditioning of ASCs with vitamin E may allow the cells to improve their anti-inflammatory and immunoregulatory capacities. Vitamin E pretreatment could lead to the improvement of their therapeutic abilities in conditions that are influenced by oxidative stress.

Keywords: Mesenchymal Stem Cells, Vitamin E, Immunomodulation, Oxidative stress, Preconditioning

Introduction

Reactive oxygen species (ROS), which are generated during cellular metabolisms (Schieber and Chandel, 2014), are neutralized by antioxidants to gain a balance between oxidants and anti-oxidizing agents. Oxidative stress occurs as a result of excessive levels of ROS or low levels of antioxidants (Barrows et al., 2019). Oxidative stress as a pathophysiological condition is closely related to inflammation. ROS can initiate intracellular signal transductions and mediates the activation of various transcription factors (e.g., Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)) (Yuan et al., 2019). These transcription factors, in turn, raise the expression of pro-inflammatory genes and induce chronic inflammatory status (Biswas, 2016). Concurrently, inflammatory cells promote oxidative stress by releasing numerous reactive species at the sites of inflammation (Droge, 2002).

Antioxidant therapy seems to be a beneficial strategy to prevent or improve inflammatory diseases caused by oxidative stress. Nevertheless, some clinical studies were not promising (Kelly et al., 2008; Mahmood et al., 2018; Mishra et al., 2003). α-tocopherol (vitamin E) is the most effective lipid-soluble antioxidant that protects polyunsaturated fatty acids (PUFAs) of biological membranes (Azzi, 2007) and, is critical in the regulation of the immune response (Lee and Han, 2018).
Numerous studies have illustrated that vitamin E has a modulatory effect on the immune system. Xue et al. showed that this vitamin improved experimental autoimmune neuritis (EAN) in a rat model by suppressing the production of pro-inflammatory cytokines and inhibiting progressive oxidative damages (Kihara et al., 2019). It was also suggested that vitamin E modulates the phase conversion between naïve T cells and T helper1 (Th1) or T helper2 (Th2) cells, as a response to the stimulation of dendritic cells (Xue et al., 2016). The anti-inflammatory effects of vitamin E have also been reported in vivo, which seems to be independent of its antioxidant properties. Tahan et al., in 2011, found that vitamin E suppresses inflammatory cytokines and inhibits the acetic acid-induced chronic inflammation in a rat model (Tahan et al., 2011). Xue et al. in 2016, revealed that vitamin E decreases the number of inflammatory cells in lymph nodes and spleens of the animals in vivo, which inhibits the proliferation of stimulated splenocytes in vitro (Xue et al., 2016).

Stem cell-based therapy is a proper strategy for controlling the symptoms of inflammatory and immune-mediated diseases. Mesenchymal stem/stromal cells (MSCs) have been widely used for allogeneic cell therapy to treat autoimmune diseases (Rad et al., 2019), inflammation-mediated disorders (Francis et al., 2019; Zhao et al., 2019), and cardiovascular diseases (Yun and Lee, 2019). The successful isolation of MSCs from a variety of adult tissues, e.g., bone marrow and adipose tissues, has provided a powerful tool for applied biological research (Wei et al., 2013).

Many studies revealed that some environmental and pharmacological stimuli (e.g., small molecules) or preconditioning strategies could influence the functional properties of MSCs in the context of immunotherapy (Linares et al., 2016; Pittenger and Martin, 2004; Schaefer et al., 2016). Furthermore, endogenous and exogenous oxidants that MSCs may expose to them during ex vivo expansion or in vivo transplantation procedures are considered as significant bottlenecks in cell therapy experiments (Yang et al., 2015). High levels of ROS are harmful to preserve self-renewal, reparative, and immunoregulatory functions of MSCs (Denu and Hematti, 2016; Yang et al., 2015). ROS, as a metabolic side product, increases adipogenic differentiation, enhances senescence, diminishes osteogenic differentiation, and hinders the immunomodulatory properties of MSCs (Denu and Hematti, 2016). Moreover, inflammatory responses, in addition to the production of ROS at the ischemic target sites, lead to the loss of transplanted MSCs. Hence, it is vital to reduce ROS either by manipulating the cells or their target sites (Devine et al., 2001; Pittenger and Martin, 2004; Yang et al., 2015).

Accordingly, understanding the effects of ROS on MSCs biology could shed light on the immunomodulatory behaviors of the cells under inflammatory conditions.

To the best of our knowledge, this is the first study that evaluates the effects of vitamin E on immunomodulatory and anti-inflammatory properties of human ASCs. Considering the vitamin E’s evident antioxidant and anti-inflammatory properties, we anticipated that priming of ASCs with vitamin E could boost the beneficial effects of these therapeutically valuable cells.

Materials and Methods

Isolation and culture of human ASCs

Adipose tissues were obtained from three healthy donors undergoing elective liposuction at a private cosmetic day clinic in Mashhad, Iran. All three patients signed the informed consent form. The Academic Center for Education, Culture, and Research (ACECR) Biomedical Research Ethics Committee authorized all downstream protocols (IR.ACECR.JDM.REC.1398.009).

200 ml of adipose tissues were washed three times with phosphate-buffered saline (PBS) containing 0.1% penicillin-streptomycin (pen-strep) (Biowest, Canada) and incubated for one hour in constant-temperature bath at 37°C in the presence of 0.1% collagenase type I (Invitrogen, USA). Fetal bovine serum (FBS, Gibco, USA, 10%) was applied for collagenase I inactivation. Then, the mixture was centrifuged at 800 g for 10 min to remove adipose cell debris. In the following, pellets were suspended in Dulbecco’s Modified Eagle Medium (DMEM, Biowest, Canada) contained 10% FBS and 0.1% pen-strep. Then, the cells were transferred into cell culture vessels and kept in a 5% CO2 incubator at 37°C (Naderi-Meshkin et al., 2016). We changed the culture medium every three days. All the following experiments were conducted with the cells at passage number 3.

Characterization of human ASCs

Flowcytometric approach was applied for the identification of mesenchymal lineage-specific surface markers. A suspension of 10^6 single cells was transferred into the staining buffer contained PBS and 5% FBS. Then, anti-human monoclonal
antibodies (all from Cytognos, Spain) against clusters of differentiation 44 (CD44), CD90, CD73, CD13, CD14, CD34, and human leukocyte antigen− DR (HLA-DR) antigens were mixed with the cells and incubated for 45 min at 4 °C. FACS Calibur cytometer equipped with 488 nm argon laser (Bioscience, US) was used for data acquisitions. Data analysis was performed using FlowJo (7.6.1) software.

The capacity of the cells for differentiation toward osteogenic and adipogenic lineages was qualitatively determined based on the previously described alizarin red and oil red O staining methods, respectively (Naderi-Meshkin et al., 2016). Briefly, adipogenesis was induced through the culture of ASCs in the presence of DMEM supplemented by 10% FBS, 200 mM indomethacin, 10 mM β-glycerophosphate, and 1 mM dexamethasone. After 14 days, the cells were rinsed with PBS and fixed in 10% formalin solution. Then, they were stained with 0.5% Oil Red O (Sigma, Germany) for 15 min.

The osteogenic inductive medium was composed of DMEM, 10% FBS, 0.5 mM acid ascorbic, 10 mM β-glycerophosphate, and 1 mM dexamethasone. The cells were incubated in this medium for 21 days. Then, they were fixed and stained with alizarin red (Sigma, Germany) for 30 min to detect the mineralized matrix of the bone, secreted by differentiated cells.

Preconditioning of human ASCs with vitamin E

Human ASCs were cultured in DMEM supplemented with 10% FBS and 1% pen-strep. Upon reaching 80% confluency, the proper concentration of vitamin E (Sigma, Germany) was added to the cultures. Untreated ASCs or cells that had been cultured with ethanol-containing media were applied as controls.

MTT assay

MTT (2, 3-bis (2-methoxy-4-nitro-5-5 sulfoxyphenyl)-2H-tetrazolium) assay was carried out to evaluate the possible toxic effects of various concentrations of vitamin E against human ASCs. 10⁵ cells were seeded in 96-well plates, and after reaching 80% confluency, they were treated with 200, 400, 600, 800, and 1000 µM of vitamin E for 24 to 72 hours. Cells cultured in the presence of DMEM or DMEM supplemented by an equal volume of ethanol were used as blank and control groups, respectively. Cell viabilities were determined following the addition of MTT dye (5 mg/ml) to the wells, incubating the vessels at 37°C for 4 hours, and recording optical densities (ODs) at 540nm by NanoDrop spectrophotometer (NanoDrop, BIO-TEK, Winooski, VT).

RNA extraction and quantitative PCR (qPCR)

Total RNAs were extracted from ASCs after 48 hours of treatment with 600 µM of vitamin E and control cells using TRIzol protocol provided by the manufacturer (Roche, Germany). The integrity of RNA samples was indicated using 1% agarose gel, and their concentrations were assessed via a NanoDrop spectrophotometer (NanoDrop, BIO-TEK, Winooski, VT). One µg of DNase I-treated total RNA was used for cDNA synthesis in each case (Thermo Scientific, USA). cDNA synthesis steps were performed according to the kit instructions (Takara, Japan).

qPCR was accomplished by SYBR Green PCR Master Mix (amplicon, USA) according to the kit protocol with The CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Germany). Ribosomal protein lateral stalk subunit P (RPLP0) gene was used as an internal control (reference gene) to normalize the transcript level of tested genes. Primers were designed by AlleleID 6 software and are shown in table 1.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6. Data were expressed as mean of independent experiments±SEM. One way ANOVA and two samples T-test were used for statistical analysis. Events with p values less than 0.05 were considered significant.
Results

Characterization of human ASCs

Cultured ASCs were characterized through investigation of surface markers expression levels and their potential for multi-lineage differentiation. Flow cytometry analysis results indicated that >98% of these cells expressed ASC specific markers including CD13, CD44, CD90 and CD73 and <3% showed the expression of hematopoietic cell-specific markers including CD14, CD34, CD45, and HLA-DR (Figure 1).

The differentiation potential toward adipocytes and osteocytes was investigated using adipogenic and osteogenic differentiation media and staining with Oil Red O and Alizarin Red, respectively (figure 2 D, E & F).

Investigating the toxic effects of vitamin E on ASCs

The effects of various concentrations of vitamin E (200-1000 µM) on ASCs’ survival rate was explored by MTT assay. The results showed that after 24, 48, and 72 h, no significant difference (P<0.05) was observed between cell viabilities of control and sample groups among all concentrations and investigated time points (Figure 3).

Both lipid depositions and mineralization of the extracellular matrixes were visualized following the staining procedures, which confirmed the adipogenesis and osteogenesis of ASCs.

Figure 1. Immunophenotype characterization of sub-cultured MSCs at passage 3. Diagrams show flow cytometry data for MSC specific surface markers (CD13, CD44, CD73 & CD90) and hematopoietic markers (CD14, CD34, CD45 & HLA-DR). Data presents the percentages of the cells which were positive for each marker.

Figure 2. Characterization of human ASCs. Photomicrographs show the morphology and differentiation capacity of ASCs. A) Spindle-like morphology of human ASCs 8 days after harvesting from adipose tissues. B) Morphology of the control group after 48h. C) Morphology of ASCs preconditioned with 600 µM of vitamin E after 48 h. D) Oil Red O staining to detect adipogenic differentiation. E) Alizarin Red staining to measure osteogenic differentiation. F) Alkaline phosphatase assay to confirm osteogenic differentiation.

http://jcmr.um.ac.ir

102
Gene expression profiling of vitamin E stimulated ASCs

The expression levels of two categories of genes were investigated in this study: inflammatory-related genes including interleukin 1-beta (IL-1β), IL-6, IL-17, IL-10 and immunomodulatory genes such as TNF-stimulated gene 6 (TSG-6), cyclooxygenase-2 (COX-2), tryptophan 2,3-dioxygenase (TDO2) and transforming growth factor-beta (TGF-β). The qPCR results showed that pretreatment with vitamin E markedly enhanced the gene expression of TSG-6, IL-1β, and COX-2 at mRNA level and significantly (p<0.05) reduced the expressions of IL-6 and TGF-β compared with the control group (Figure 4). In contrast, ASCs pretreatment did not affect IL-10, IL-17, and TDO2 gene expressions compared to the control group.

Discussion

As a recommended supplement, vitamin E inhibits the production of ROS molecules and pro-inflammatory cytokines and depicts immunosuppressive properties (Lee and Han, 2018b). The findings of this study demonstrated that Vitamin E when applied as a small molecule for preconditioning of ASCs, altered the expression of some genes which are involved in immunomodulation and inflammation. Here, we argued that the pretreatment of stem cells with Vitamin E before cellular therapy could have beneficial effects on their immunoregulatory capacities.

ASCs are multipotent cells with a high capability for interacting with a variety of immune cells. These cells release various factors with immunomodulatory potential such as cytokines and chemokines, which make them a decent choice to treat numerous immune-mediated diseases accompanied by chronic inflammation (Baer et al., 2018). Priming MSCs with appropriate agents can promote the efficacy of some specific immunotherapeutic applications (Hu and Li, 2018; Silva et al., 2018; Tang et al., 2014; Wisel et al., 2009).

Vitamin E is recognized not only for its antioxidant properties but also for its regulatory effects on signaling pathways through the induction of gene expression modifications (Azzi, 2018; Sangiorgi et al., 2016; Zingg, 2015). We studied the consequences of vitamin E treatment at a high concentration (600 μM) on the cell proliferation rate and cytokine production status of the cells in vitro. Our findings showed that the preconditioning of MSCs by 600 μM of vitamin E significantly attenuated the expression of IL-6 at least by two folds and altered the expression of TGF-β slightly. We also observed a significant increase in the expression of COX-2, TSG6, and IL-1β.

Wang et al. found that rat bone marrow-derived MSCs could ameliorate peritoneal injury by repairing mesothelial cells. They also showed that MSCs lacking TSG-6 (TSG-6-siRNA MSCs) had no apparent effects on the peritoneal fibrosis. Thus, it was confirmed that the secretion of TSG-6 by MSCs made a significant contribution to their clinical outcomes (Wang et al., 2012). In line with their findings, Roddy et al. reported that
intravenous administration of human MSCs primed to express TSG-6 suppressed the inflammatory damages of the cornea following the induction of chemical injury in rats. Additionally, Roddy et al. demonstrated that the siRNA knockdown of TSG-6 impeded the anti-inflammatory effects of these cells on damaged corneal epithelial cells (Roddy et al., 2011). Given these observations, we suggest that preconditioning of MSCs with vitamin E could improve their immunomodulatory properties by enhancing the expression of TSG-6.

IL-6 is a pleiotropic and multifunctional cytokine involved in many physiological events, such as inflammation through NF-κB and signal transducers and activators of transcription (STAT) signaling pathways. It was shown that blockade of IL-6 prevents the progression of autoimmune-based diseases and tumor formation (Barnes et al., 2011; Schaper and Rose-John, 2015; Tanaka et al., 2014). The blockade of TGF-β1 also causes anti-tumor immunity and tumor regression (Mariathasan et al., 2018; Shangguan et al., 2012), which increases safety concerns in tumorigenesis. COX-2 is a crucial enzyme in prostaglandin E2 synthesis, which promotes the anti-inflammatory features of macrophages (M2) (Lu et al., 2017; Németh et al., 2009). The pro-inflammatory cytokine, IL-1β, is regulated through NF-κB and c-jun signaling pathways (Libby, 2017; Palomo et al., 2015; Rodriguez et al., 2019). In contrast, MSC pretreatment did not affect IL-10, IL-17, and TDO2 gene expressions in the current study. Their weak expression by naïve MSCs could explain it (Ben-Zwi et al., 2019).

There was a strong correlation between the changes in redox potential and the production of pro-inflammatory cytokines with the inflammatory pathways, e.g., NF-κB. NF-κB is a transcription factor thought to be modulated by oxidative stress (Behl et al., 1994; Lingappan, 2018). Antioxidants like vitamin E are believed to prevent the activation of NF-κB and other inflammatory pathways through the inhibition of lipid peroxidation (Saxena et al., 2019). Together, these findings support the notion that vitamin E improves the anti-inflammatory characteristics of ASCs. This effect could be due to inhibition of the activation of some inflammatory signaling pathways, such as NF-κB, in human MSCs that inhibits the production of pro-inflammatory cytokines.

Acknowledgments
This study was funded by ACECR-Khorasan Razavi. It was an Hakim Sabzevari University M.Sc thesis, Sabzevar, Iran. We are grateful to Dr. Naser Sanjar Mosavi for his kind contribution in collecting samples.

Conflict of interest
The authors declared no competing interest.

References


Current Opinion in Toxicology 7:81-86.


Mesenchymal stem cells and their potential as cardiac therapeutics. Circulation Research 95:9-20.


**Note added in proof**

*COVID-19 outbreak has been a challenging global issue recently, and the early outcome of Sarilumab and Tocilizumab clinical trials as monoclonal antibodies, that work by inhibiting the IL-6, were successful. Hence, our results regarding the downregulation of IL-6 by vitamin E could be considered as a therapeutic option that needs to be evaluated by further studies.*

Open Access Statement: This is an open access article distributed under the Creative Commons Attribution License (CC-BY), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

http://jcmr.um.ac.ir