The Effect of L-Ascorbic Acid-2-Phosphate on Human Adipose-Derived Stem Cells Population Doubling Time and Angiogenic Potency

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Abstract. Adipose-derived stem cells (ADSCs) have been developed as substitute cells for tissue engineering. However, in order to get a high number of cells, over-repassage leads the cells to become big in spread size indicating that senescence occurred. This study aimed to evaluate the potency of ascorbic acid to induce the proliferation and angiogenic potency of human ADSCs and inhibit the aging process of the cells. Human ADSCs were isolated by an enzymatic process. ADSCs were cultured in 10% fetal bovine serum (FBS) supplemented by ascorbic acid in various concentrations (1, 3, 6 μg/ml). The morphology, proliferation, population doubling time (PDT), and angiogenic potency were evaluated. Angiogenic potency analysis was performed using gene expression of vascular endothelial growth factor (VEGF) and its receptor (VEGFR). Ascorbic acid on 6 μg/mL suppressed the senescence proved by cell spread areas, growth curves, viability, PDT, and percentage of scratch width closure. No significant difference in vascular endothelial growth factor and its receptor expression to the control. However, the high concentration of ascorbic acid (6 μg/ml) expressed the highest VEGF and VEGFR. Our study showed that ADSCs with supplementation of ascorbic acid (6 μg/ml) promoted proliferation and angiogenic potency.

Keywords: ADSCs · L-ascorbic acid-2-phosphate · PDT · angiogenic potency

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1 Introduction

Stem cells are cells that can regenerate themselves and differentiate into many types of cells, such as adipocytes, chondrocytes, and osteocytes [1]. Based on their origin, stem cells divide into two types, embryonic stem cells, and adult stem cells [2]. Due to ethical problems, many studies that developed came from adult stem cells and the researcher together fixed the limitation of adult stem cells through nutrition and/or substrate manipulation and genetic engineering [3]. The research consent about stem cells is initiated by their potency to repair tissue and/or organ, and also could use as regenerative therapy since many studies reported that stem cells can cure degenerative diseases [3, 4]. To conduct regenerative stem cell therapy, culturing the cells to get pure stem cells should be conducted. However, in order to get a high number, we have to repassage the cells so the yield of cells increases and the outcome therapy to cure degenerative disease appears.

Repassage of stem cells initiated the unnormal spread of cell size indicated by large size [1]. It showed that the cells get senescence. Senescence cells decrease the growth rate of cells and also increase the time of population doubling time (PDT) and spread cell size [1, 3]. PDT is the time needed by cells from one cell to become two cells [5]. Due to the senescence condition, doubling cells take a long time [6].

Senescence cells also limited their differentiation potency. When the senescence cells are administered to the human body, the embolism chance to have happened is increased. So, the prevention of senescence cells due to the repassage of cells is part of increasing the yield process that should be fixed.

Vitamin C is a natural compound that has a high activity of antioxidants and proof that can inhibit senescence or the aging process of the human body [7, 8]. Based on the previous study, we suggested that vitamin C also plays a role in the aging process of stem cells and has the ability to suppress the senescence indicated by normal spread cell size [6]. This study aimed to evaluate the vitamin C potency towards adipose-derived stem cell (ADSCs) aging performed by population doubling time (PDT), growth curve, and wound model in vitro analysis.

2 Methods

2.1 Stem Cell Isolation

The stem cells were isolated from adipose tissue using an enzymatic process [3, 9]. After incubation through a shaker incubator for 60 min at 37oC, the cells were separated using a centrifuge. The pellet was stromal vascular fraction (SVF). SVF contains many types of cells such as pericytes, endothelial cells, pre-adipocytes, stem cells, and immune cells. Culturing the SVF were removed unattached cells. Adipose-derived stem cells proliferated due to the use of specific medium. The basal medium involved Dulbecco's modified Eagle's medium (DMEM), 1% antibiotics, and 10% fetal bovine serum (FBS). The medium should be changed every 3 days until the cells reach about 80% confluency.

2.2 Treament Groups

The ADSCs were divided into 4 groups including control (without Vitamin C or ascorbic acid supplementation). The treatment groups that supplemented by Vitamin C were divided into various concentrations involving 1, 3, and 6 μg/mL of ascorbic acid.

2.3 Cell Analysis

The ADSCs observation was conducted using OPTICA Laboratory microscopy (OPTIKA, ITALY; objective 10x). The images were processed by ImageJ to measure the size of the cells.

2.4 Growth Curve and Population Doubling Time

The growth curve was assessed by 2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The observation of the growth curve was on days 1, 3, 5, 7, 14, and 21. The assessed cells using MTT assay were applied using a solvent of dimethyl sulfoxide (DMSO). The intensity was measured using a microplate reader.

The ADSCs were evaluated for their PDT by calculating the cell number and viability. After the cell number has been recorded, the PDT was analyzed. The viability of cells was performed using the trypan blue staining method.

2.5 Wound Model Analysis

Wound models of cells were conducted using the scratch assay technique. The cells were scratched using tips followed by observation of cells for 24 h. The wound rate closure was analyzed through ImageJ software (National Institutes of Health, USA). The formula of wound as below

$$Wound\ area(mm2) = \frac{(area t = 0\,h) - (area t = \Delta h)}{(area t = 0\,h)}$$

Area t = 0 h is wound area after starch on 0 h.

Area t = Δ h is wound area on observation hours.

After 24 h of observation, the cells were harvested, and isolated their RNA to analyze the vascular endothelial growth factor (VEGF) and VEGFR as angiogenic markers.

2.6 Gene Expression Analysis

Isolation System (Promega, USA). Synthesized cDNA and qPCR analysis were performed GoTaq® 2-Step RT-qPCR System (Promega, USA), following manufacturer instructions. The reference gene that used is GAPDH glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The angiogenic markers were vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor (VEGFR). The primE design was shown on Table 1. The qPCR cycles were 40 cycles. The qPCR results were analyzed following the 2-ΔΔCT method described by Livak and Schmittgen [10].

Table 1. Primer Design

Gene		Nucleotide Base	Base Numb
GAPDH	Forward	CAAGAGCACAAGAGGAAGAGAG	22
	Reverse	CTACATGGCAACTGTGAGGAG	22
VEGF	Forward	GAGCTTCCTACAGCACAACA	20
	Reverse	CCAGGACTTATACCGGGATTTC	22
VEGFR	Forward	ATCTCTCCTGTGGATTCCTACC	22
	Reverse	CTCTTCCTCCAACTGCCAATAC	22

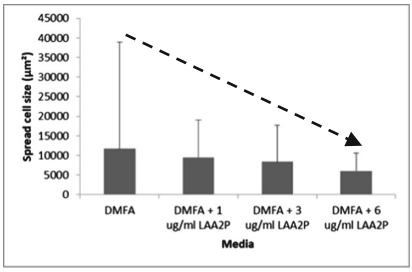

Fig. 1. Morphology of Stem Cells, without suplementation of Ascorbic Acid (A); 1µg/mL Ascorbic Acid (B) 3 µg/mL Ascorbic Acid (C); 6 µg/mL Ascorbic Acid (D); and Spread Cell Size (E)

3 Results and Discussion

3.1 Growth Curve and Population Doubling Time Analysis

The morphological analysis of stem cells showed in Fig. 1. The biggest cells are shown in the control group which is the group without supplementation of ascorbic acid. Supplementation of ascorbic acid in various concentrations kept the cells at the normal size indicating the aging process was suppressed. Interestingly, the high concentration of ascorbic acid leads to the spread size consistency on the normal level (Fig. 1E). The use of ascorbic acid on post-thawed stem cells indicated that the aging process was inhibited [3, 11].

3.2 Population Doubling Time and Growth Curve

The time needed by the cell to divide from one cell to two cells is population doubling time (PDT). PDT assay was conducted from passages 3 to 6 in order to evaluate the proliferation of cells after supplementation of ascorbic acid (Fig. 2A). PDT of stem cells showed that ascorbic acid plays role in the doubling time of cells. High concentrations of ascorbic acid (3 and 6 µg/mL) support proliferation cells indicated by PDT data. High concentrations of ascorbic acid provide a suitable microenvironment for cells to grow so

the time of doubling time increased faster than without ascorbic acid supplementation. The viability of cells for all groups showed more than 90% indicating no mistakes has been made when the cells were cultured in the laboratory (Fig. 2B).

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To evaluate the proliferation rate, not only PDT was conducted but also the growth curve using MTT assay. Overall, the cells gradually increased until day 21 of observation including control and 3 treatment groups. However, on day 21 of observation, the control group was the lowest rate of proliferation while the highest was on the high concentration of ascorbic acid (6 μg/mL) group (Fig. 3). Ascorbic acid has been reported that can enhance and promote proliferation of cardiac progenitor cell [13, 14]. Bone marrow stem cells (BMSCs) has similar response towards ascorbic acid supplementation. Ascobic acid promotes BMSCs proliferation and is particularly useful to expand the cells [14].

Wound rate closure analysis was conducted by the wound *in vitro* model technique. The cells cultured in the well plate were scratched. After 24 h of observation, the wound rate closure was analyzed. The highest wound rate closure was shown in the high concentration of the ascorbic acid group (6 μg/mL) while the lowest is in the control group (Fig. 4). Supplementation of platelet-rich plasma or ascorbic acid on human adipose-derived stem cells increased the wound closure rate [3, 15].

These results showed that ascorbic acid has a vital role not only in their proliferation but also the angiogenesis. To evaluate the angiogenic process, the cells were harvested followed by molecular analysis of mRNA expression. Vascular endothelial growth factor (VEGF) and its receptor (VEGFR) expression were evaluated.

Gene expression of VEGF and VEGFR was conducted in order to provide the molecular mechanism of how ascorbic acid increased the wound closure rate. The results are represented in Fig. 5A-B. As a control, when there was a scratch, the cell synthesized a high concentration of VEGF in order to increase the wound closure rate. Additional

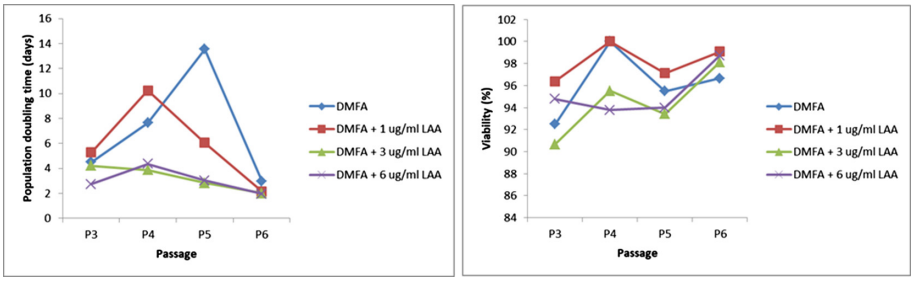

Fig. 2. Population Doubling Time of Adipose-Derived Stem Cells

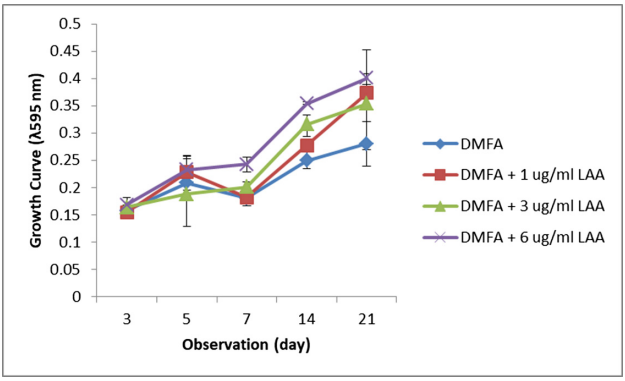

Fig. 3. Cell observation, growth curve (A); viability of cell (B)

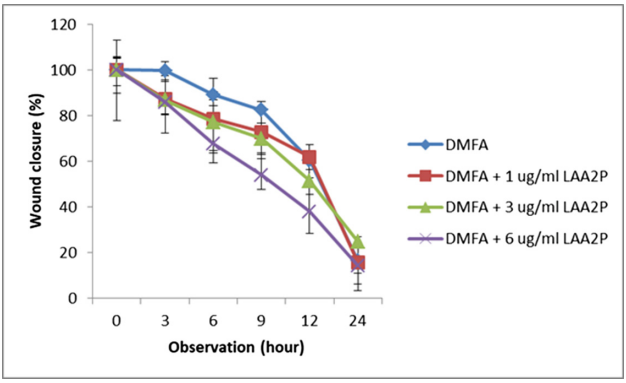

Fig. 4. Wound Closure Rate of Cells

ascorbic acid at 1 μg/mL increased the receptor of VEGF (VEGFR) while at 3 μg/mL concentration increased the VEGF protein. These results explain, unlike 1 μg/mL of ascorbic acid, 3 μg/mL has a similarity of closure rate for 24 h of observation. In contrast, 6 μg/mL of ascorbic acid increased not only VEGF but also VEGFR and explained why the wound closure rate was faster than others. To find the correlation between ascorbic acid and angiogenic marker pathway, Rosadi et al., (2022) [16] explained through in silico analysis. The ascorbic acid receptor is prolyl hydroxylase 2 (PHD2) protein. Molecular interaction analysis showed that the ascorbic acid and its receptor has indirect correlation pathway (Rosadi et al., 2022) [16].

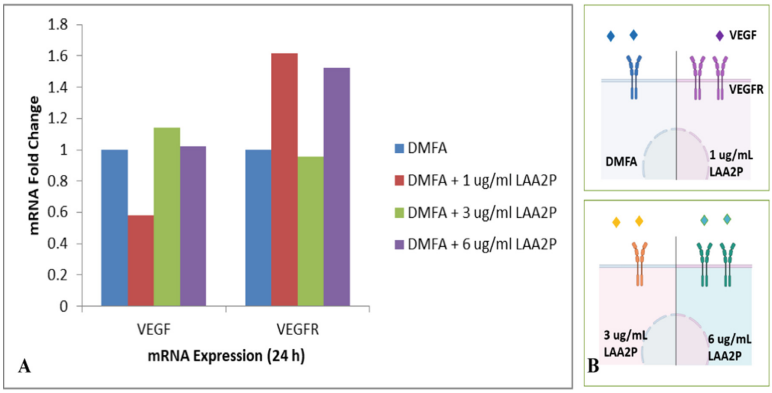

Fig. 5. Molecular Analysis of mRNA Expression; VEGF and VEGFR After 24 h of Observation (A); Illustration of Molecular Analysis (B)

4 Conclusion

Ascorbic acid increased the proliferation and population doubling time of ADSCs, and kept the spread cell size of ADSCs on normal size thus ascorbic acid has potency as anti-aging. Ascorbic acid also increased the wound healing closure time proven by increasing VEGF and VEGFR expression as two of the angiogenic marker. The best concentration of ascorbic acid to kept the cell size, increased proliferation, and PDT as well as angiogenic potential was 6 ug/mL.

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