Original Article

Stem Cell INVESTIGATION

Effect of ascorbic acid on morphology of post-thawed human adipose-derived stem cells

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Background: Ascorbic acid-2-phosphate has been reported to play a role in cell division and to suppress aging of cell. However, post-thawed cell morphology on various concentration of ascorbic acid is still unclear. In this study, we aimed to observe the morphology of post-thawed adipose-derived stem cells (ADSCs) in medium containing L-ascorbic acid-2-phosphate (LAA2P) (50 and 100 µg/mL).

Methods: The cells were isolated from adipose tissue. Isolated cells then cultured and cryopreserved in liquid nitrogen. We detected mRNA expression of type 1 collagen on day 5. Cell seeded in T25 flask using basal medium [Dulbecco's modified Eagle's medium (DMEM) only] as a control group, DMEM with 10% fetal bovine serum (FBS) and antibiotics as DMFA group, while DMFA with ascorbic acid (50 and 100 µg/mL) as ascorbic acid treatment group.

Results: The results showed that the cells cultured in DMEM only attached until 96 hours of observation while serum groups with or without ascorbic acid supplementation showed the proliferation until 240 hours of observation. The highest spread size of cell was in a serum group without ascorbic acid supplementation and the highest yield of cells showed in a group with 50 µg/mL of ascorbic acid supplementation. Reduced mRNA expression of type 1 collagen which related to aging was showed in cells cultured without ascorbic acid supplementation.

Conclusions: These results showed that ascorbic acid increased the cell division and suppressed the aging processes indicated by normal spread cell in size compared to cell cultured in DMFA without ascorbic acid supplementation.

Keywords: Ascorbic acid; stem cells; morphology; senescence; post-thawed

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Introduction

Mesenchymal stem cells (MSCs) have the ability to differentiate into several types of cells including adipocyte, chondrocyte, osteocytes and hold promise for application in regenerative medicine (1,2). The source of MSCs that many studied and reported is from bone marrow which well known as bone marrow stem cells (BMSCs) and/or lipoaspirate which is known as adipose-derived stem cells (ADSCs) (3,4). However, human adult BMSCs is known to be painful, invasive, and low cell number upon harvesting compared to ADSCs (5). ADSCs as a cell source support many studies to discover nature of adult stem cells including their stemness towards physical stress such as post freeze-thawed processes.

To use MSCs for regenerative treatment, usually the cells

Table 1 Primer design

Gene	Nucleotide base	Base number
GAPDH		
Forward	CAAGAGCACAAGAGGAAGAGAG	22
Reverse	CTACATGGCAACTGTGAGGAG	22
Type 1 collagen		
Forward	AGAGTGGAGCAGTGGTTACTA	21
Reverse	GATACAGGTTTCGCCAGTAGAG	22

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

are cryopreserved in liquid nitrogen (6-8). However, freeze-thawed cells decreased their viability and physiology of cells. To maintain their potential and hold aging processes, ascorbic acid might be as an alternative solution to suppress senescence due to their ability to suppress reactive oxidative stress (9). Ascorbic acid can increase the proliferation rate of cell during *in vitro* culture (10,11).

Human MSCs are unable to synthesize ascorbic acid which means ascorbic acid should be added in culture medium as supplementation. In order to make receipt of common culture medium, Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (DMFA). In this study, we added L-ascorbic acid-2-phosphate (LAA2P) (50 and 100 µg/mL) to DMFA and analyzed the morphology of ADSCs compared to the cells cultured without ascorbic acid supplementation. This study aimed to observe spread cell size towards ascorbic acid supplementation. Thus, this study aim was to evaluate the dose of ascorbic acid towards post-thawed cells on their morphologies and sizes.

Methods

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The liposuction protocol was approved by the Ethical committee of Dharma Nugraha Hospital (No. 001/SkeI-Kom/RSDN/I/2020) and informed consent was taken from the participant.

Cell culture

Subcutaneous adipose tissue from abdomen was obtained from one donor undergoing liposuction procedure with age of 46. Isolation of stromal vascular fraction was followed H-Remedy protocol (12) which involved enzymatic process, and incubation at 37 ℃, 300 rpm, 1 hour. Isolated cells then cultured to expand the cells using in house media then cryopreserved in several tubes. The cells that we used in this study were post-thawed cells. The cells were thawed and cultured in DMEM (Gibco, USA) only for control with seeding density 1,275 per cm^2, DMEM with 10% FBS (Biowest, USA), and 1% antibiotic-antimycotic (ABAM) (Gibco, USA) for DMFA group, and DMFA with 50 and 100 µg/mL of LAA2P for ascorbic acid groups. The cells were cultured at 37 ℃ in 5% CO_2, and the medium was changed every 3 days.

Cells spread morphology

In this study, we compared the effect of ascorbic acid supplementation towards post-thawed cells recovery. The control was DMEM only while DMEM with 10% FBS and 1% ABAM was DMFA group, while DMFA with 50 µg/mL LAA2P and DMFA with 100 µg/mL LAA2P were ascorbic acid groups. The observation was conducted at 1, 3, 6, 24, 48, 96, 144, 168, 240 h using inverted microscope (OPTICA microscope) (objective 10×). The cell spread size was calculated using ImageJ program.

Tryphan blue staining

Cells were seeded and cultured in T25 flask (1,275 cells per cm^2) up to 10 days. Cells were harvested using Tryple Select (Gibco, USA) for cells detachment then washed it. Viable cells were counted using Trypan blue staining method.

mRNA expression of type 1 collagen

The cells in treatment groups were harvested on day 5. RNA isolation, synthesis cDNA and mRNA expression analysis were performed by SV Total RNA Isolation System (Promega, USA) and GoTaq® 2-Step RT-qPCR System (Promega, USA) following manufacturer instruction. The mRNA expression was type 1 collagen and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as gene reference (*Table 1*). The cycle conditions were an initial denaturation step of 95 ℃ for 2 min, followed by 40 cycles of denaturation at 95 ℃ for 15 s, and annealing at 54.4 ℃ for 1 min, and a final extension at 60–95 ℃ for 5 s. The result of qPCR was analysed following the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen.

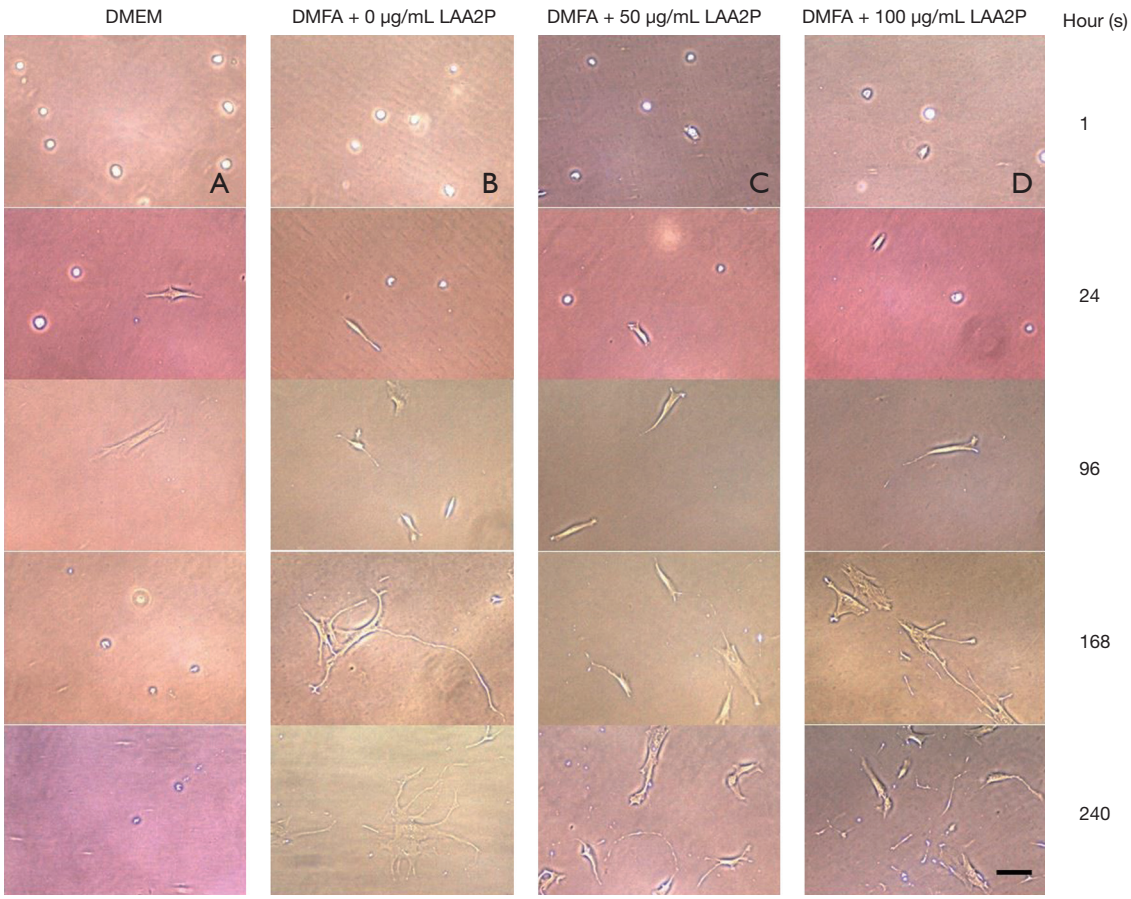

Figure 1 Post-thawed cell on morphology cultured in basal media (DMEM) (A), DMFA + 0 µg/mL LAA2P (B), DMFA + 50 µg/mL LAA2P (C), and DMFA + 100 µg/mL LAA2P (D), objective 10×, black bar =100 µm. DMEM, Dulbecco's modified Eagle's medium; LAA2P, L-ascorbic acid-2-phosphate.

Statistical analysis

Significance tests were done using individual *t*-test for normally distributed data and Mann-Whitney U for non-normally distributed data, with P value less than 0.05 considered as statistically significant.

Results

After 1-hour incubation at 37 ℃, the cells did not show spreading sign while after 24 hours of incubation, all of the group including control which cultured in basal medium (DMEM only) was spread (*Figure 1*). The maximum spreading of control group was shown after 96 hours of incubation then cells were detached. In contrast, medium supplemented by serum with or without ascorbic acid showed the cells proliferated. The cells cultured in basal medium (DMEM) showed spread curve of cells with the highest peak shown after 96 hours of observation then continued to sharply decreased indicated the cells died (*Figure 2*).

The spread size of cell without ascorbic acid supplementation showed had the largest spreading cell in average at 240 hours of observation (*Figure 3*). However, ascorbic acid maintains the proper size of the cells. Interestingly, the proper concentration of ascorbic acid also has a role in yield outcome (*Figure 4*).

The highest yield of cells reported when cells cultured with serum containing 50 µg/mL of ascorbic acid compared to other groups (*Figure 4*). It was related to the small size of cells which supplemented by 50 µg/mL of ascorbic acid. Supplementation of 50 and 100 µg/mL of ascorbic acid

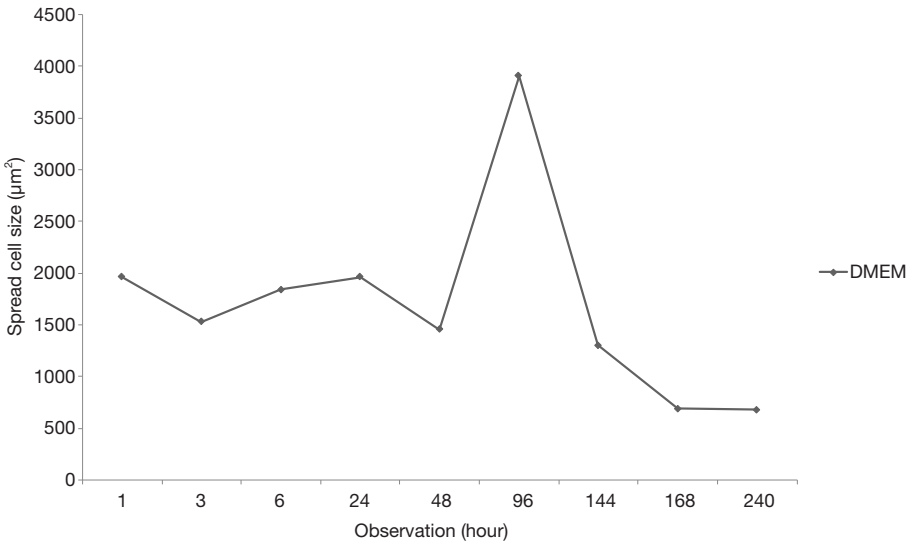

Figure 2 Cell spread size cultured in basal media (DMEM) showed the highest size was confirmed on hour 96 which be used as standard. DMEM, Dulbecco's modified Eagle's medium.

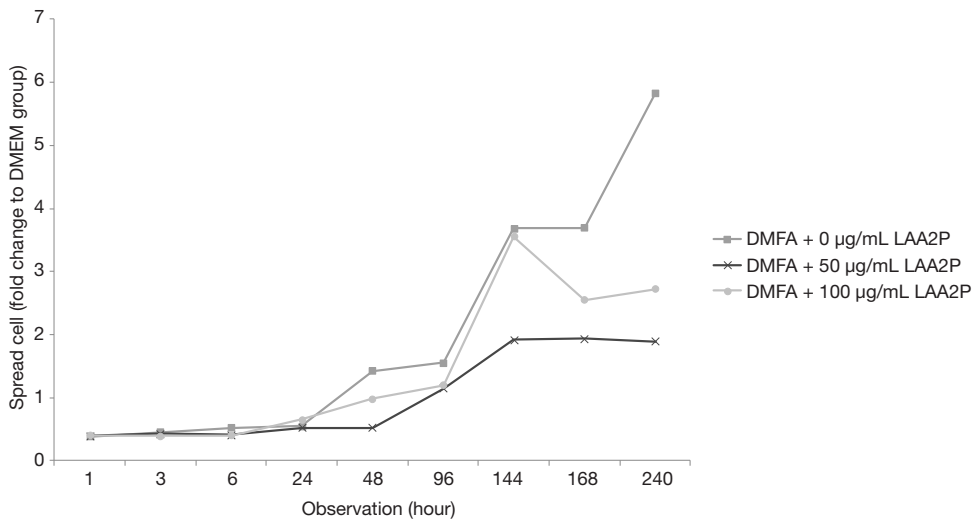

Figure 3 Cell size in various medium DMFA with or without LAA2P towards the highest cell size in basal media. LAA2P, L-ascorbic acid-2-phosphate; DMEM, Dulbecco's modified Eagle's medium.

also enhanced type 1 collagen (*Figure 5*). However, no significant difference in the collagen expression was seen for the different ascorbic acid concentrations. The illustration of cell attachment and spreading in this study showed in *Figure 6*. At first, cells attach to the substrate then spread together with cell division processes. However, the size of cell was getting bigger when cells were not supplemented with ascorbic acid.

Discussion

In this study, post-thawed cells exhibited fibroblast-like shape and adhered to the plastic culture flask. It was characterized by cell morphology with filopodia on the surface that depended on the cell adhesion mechanism (13). Cryopreservation does not affect MSCs morphology, surface marker expression, differentiation or proliferation potential (14). However, additional various concentrations of ascorbic

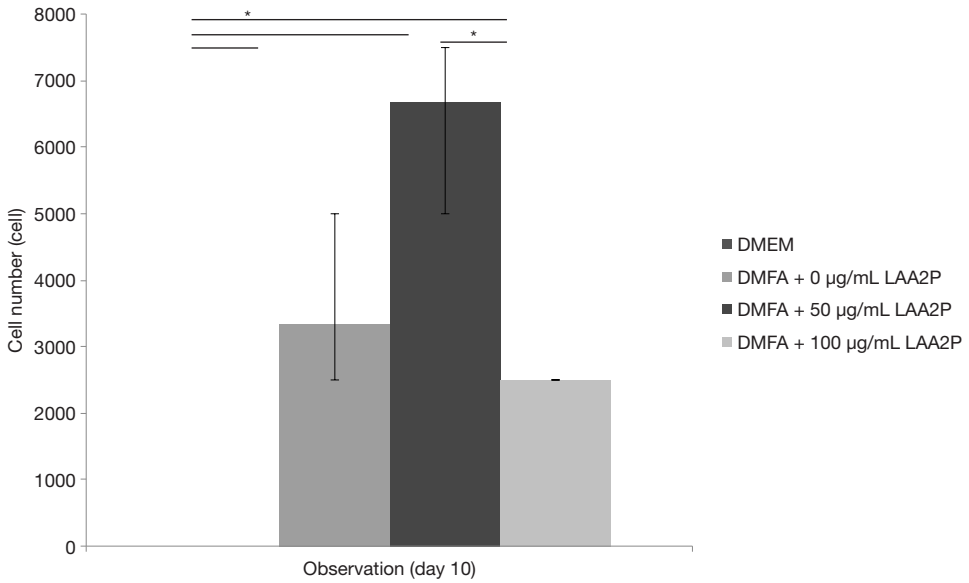

Figure 4 Cell number after 240 hours of observation, concentration of ascorbic acid has a role in proliferation of cells. *, P<0.05. DMEM, Dulbecco's modified Eagle's medium; LAA2P, L-ascorbic acid-2-phosphate.

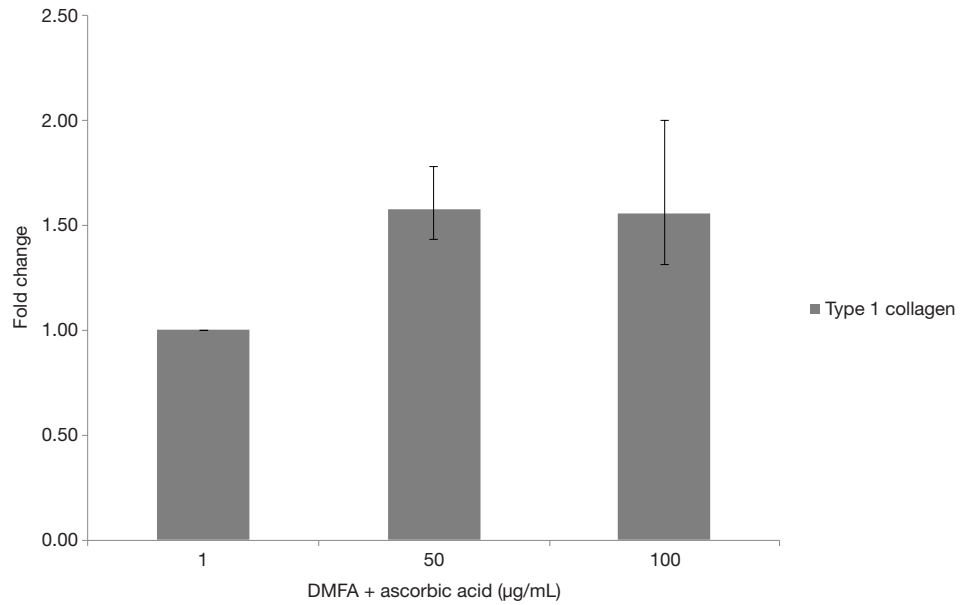

Figure 5 Type 1 collagen expression of cells cultured in various concentration of ascorbic acid after 120 hours of incubation.

acid in medium might show different results. Ascorbic acid could be used as an alternative cardiomyogenic inducing factor for human amniotic fluid MSCs (15). Ascorbic acid also supports BMSCs proliferation and promotes HIF1α breakdown as well as activates mitochondria, affecting cell proliferation and metabolism. A deficit of ascorbic acid in the culture medium decreases the activity of ascorbic acid-dependent enzymes (9).

The spreading size of cell in DMEM medium continuously increased during 1 hour of observation. After that, the highest spreading cell in size had been observed after 96 hours of incubation. This highest peak of spreading

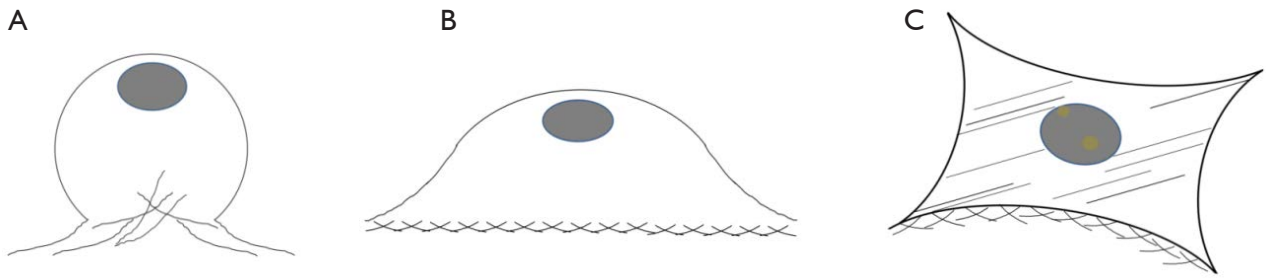

Figure 6 Illustration of cell attachment, less than 24 hours some cells were in a half spread (A); continues with spreading in average size compared to control (B), over spreading of cell was observed in group of DMFA without ascorbic acid supplementation (C).

cell was used as a standard to other groups which contained serum and ascorbic as well. Comparable effect of FBS only versus their combination with ascorbic acid was reported by Rosadi *et al.* (16) showed that in 1% ascorbic acid, the spread size of cells was smaller than control. The highest spread size of the cells and repeated cell division known as Hayflick phenomenon is a sign of senescence (17). Ascorbic acid has a role of multiple physiological functions involved in cell division and suppresses aging (18).

Medium supplementation with ascorbic acid increased proliferation rate and markedly increased number of cell doublings before reaching senescence, with a greater than 1,000-fold increase in total cell numbers compared with control cultures (19). In this study, there was increased yield in the cell with 50 µg/mL of ascorbic acid compared to other groups. In contrast, higher concentration of ascorbic acid (100 µg/mL) showed decreased in yield. It is suggested that ascorbic acid has negative feedback towards its concentration. Wu *et al.* (20) reported that the dose-dependent effect of ascorbic acid-induced oxidative cytotoxicity and its negative correlation with higher seeding density and smaller intercellular distance. In addition to the enhanced recruitment of anti-oxidative capabilities with more cells in high-density culture conditions, higher seeding density also exerted a defensive effect against the ascorbic acid-induced oxidative stress by enhancing the expression of anti-oxidative enzymes. Other studies reported that supplementing ascorbic acid in high concentration increased intracellular reactive oxygen species levels via the production of hydrogen peroxide (H_2O_2) (21,22). Thus, ascorbic acid can inhibit GAPDH and induce apoptosis (23,24). In addition, reduction of type 1 collagen expression increased ageing damages on human dermal fibroblast (25). In this study showed that ascorbic acid increased type 1 collagen on human ADSCs and suppressed ageing processes.

We demonstrated that adding ascorbic acid in proper concentration as supplementation might help cell to suppress their senescence processes and increase proliferation rate. Furthermore, the result of cell harvested demonstrated that ascorbic acid also increased the cell proliferation rate proved by highest yield counted. However, at present, the sample size and molecular side study is limited to be conducted. Therefore, further studies are required to increase sample sizes and conduct the molecular study to improve the results.

Conclusions

In conclusion, serum with ascorbic acid (50 µg/mL) could be considered as an effective dose to suppress aging on post-thawed cell morphology represented by the smaller spread cell size compared to control.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/sci-2020-011). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as

revised in 2013). The liposuction protocol was approved by the Ethical committee of Dharma Nugraha Hospital (No. 001/SkeI-Kom/RSDN/I/2020) and informed consent was taken from the participant.

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