

L-ascorbic acid-2-phosphate and Fibrin Substrate Enhances the Chondrogenic Differentiation of Human Adipose-derived Stem Cells

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ABSTRACT

Background: Adipose-derived stem cells (ASCs) have been developed as a substitute for chondrocytes in cartilage tissue engineering. This study aimed to evaluate the potency of ascorbic acid to induce the chondrogenic differentiation of human ASCs cultured on fibrin substrate. **Methods:** Human ASCs were isolated by an enzymatic process and characterized. The fibrin scaffold was fabricated from human blood as a natural scaffold. The ASCs were cultured in 10% fetal bovine serum (FBS) supplemented by ascorbic acid in various concentrations (1, 3, 6 $\mu\text{g/ml}$) and a control without an ascorbic acid supplementation. The chondrogenic differentiation of the ASCs was evaluated involving glycosaminoglycan analysis, and the mRNA expression of type 2 collagen, aggrecan and type 1 collagen. **Results:** Chondrogenic differentiation occurred when the ASCs were supplemented by 3 $\mu\text{g/ml}$ of ascorbic acid and cultured on a fibrin scaffold confirmed by glycosaminoglycan (GAG) analysis, and the mRNA expression of type 2 collagen and aggrecan. The expression of type 1 collagen, which is an osteogenic marker, was low. **Conclusions:** ASCs with a supplementation of 3 $\mu\text{g/ml}$ of ascorbic acid cultured on a fibrin substrate promotes chondrogenic differentiation.

Key words: ASCs, L-ascorbic acid-2-phosphate, chondrogenesis, fibrin, differentiation

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent cells and they have an important role in regenerative medicine therapy¹. The most common source of MSCs are the bone marrow and adipose tissue^{2,3}. However, due to the invasive risk of cells being harvested and the low yields from bone marrow, adipose tissue is more studied nowadays⁴. The MSCs isolated from adipose tissue are called adipose-derived stem cells (ASCs). ASCs were first reported by Zuk *et al.* along with their ability to differentiate into several different types of cell^{5,6}.

The proliferation and differentiation of ASCs depends on their niche (microenvironment) and substrate⁷. In low oxygen tension ($< 1\% \text{ pO}_2$) for two days, the ASCs morphology changed and the expression of SRY-box containing gene 9, aggrecan and collagen type II mRNA levels was enhanced⁸. In addition, the supplementation of vitamin C (ascorbic acid) also supports the proliferation of ASCs⁹, preventing the cells from aging because the ascorbic is antioxidant¹⁰, alongside induced cell differentiation into chondrogenic or osteogenic^{9,11}. Chondrogenic differentiation leads to the cells expressing glycosaminoglycan,

type 2 collagen and aggrecan while osteogenic differentiation leads the cells to express type 1 collagen¹². Ascorbic acid itself is a cofactor that can increase collagen production and synthesize the extracellular matrix (ECM) of cells in an optimal concentration¹³. The elevated concentration of ascorbic acid in a cell culture medium inhibits cell proliferation and induces cell apoptosis^{14,15}. The optimal concentration of ascorbic acid needs to be evaluated as a result. Fibrin substrate is a natural material substrate that is collected from human blood^{16,17}. Its potency to support cell attachment plays a role in the proliferation and differentiation of cells¹⁸.

Fibrin as substrate secretes protein that affects the cellular activity. It can be fabricated for the purpose of constructing tissue engineered cartilage^{16,19}. However, the combination of ascorbic acid and fibrin substrate to support proliferation and the chondrogenic differentiation of human ASCs is still unclear. In this study, we evaluate the effect of ascorbic acid on the chondrogenic differentiation of ASCs cultured on fibrin substrate.

METHODS

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Isolation and culture stromal vascular fraction

The stromal vascular fraction (SVF) cells were isolated from the lipoaspirate of one donor using an enzymatic method followed by the previous method^{20,21}. The protocol was approved by the Ethical committee of Dharma Nugraha Hospital (No.: 001/SkeI-Kom/RSDN/I/2020) and informed consent was taken from all individual participants. As much as 10% H-Remedy enzyme was added to the lipoaspirates and they were incubated for 1 hour at 37 °C at 300 rpm. To inactivate the enzyme digestion process, low-glucose (1 g/L) Dulbecco's modified Eagle's medium (DMEM) containing 4mM L-glutamine (Gibco, USA) was added to the lipoaspirates followed by centrifugation for 5 min at 600×g. The supernatant was discarded and the pellet SVF was then diluted in a saline solution. The cell number and viability were counted using the trypan blue staining methods. Isolated cells were cultured in a basic growth medium (DMFA) containing low glucose (1 g/L) DMEM with L-glutamine (4 mM) (Gibco, USA), fetal bovine serum, and 100X antibiotic-antimycotic (Gibco, USA). They were then incubated at 37 °C, 5% CO₂. After reaching 80 — 90% confluency, the cells were sub-cultured and expanded to passages 2 and 3 to be used in further assays.

Cell morphology

To investigate the effect of L-ascorbic acid 2-phosphate (Sigma-Aldrich, USA) on ASCs, cells were cultured in DMFA only as a control, and in DMFA with L-ascorbic acid 2-phosphate (1 µg/ml, 3 µg/ml, and 6 µg/ml). Passage 3 of cells (5 x 10³ cells) were then cultured in a 24-well plate. After 9 days of culture, the images were captured (Optica, Italy).

Fabrication of the fibrin substrate

The fibrin was obtained from Klinik Hayandra as a waste product of platelet-rich plasma. The fibrin was fabricated according to Kobayashi *et al.*,²². The weight of the freshly prepared, compression and overnight incubation at 37°C of fibrin was measured. The incubated fibrin was then cut into 5 x 5 mm as a substrate cultured in a 96-well plate (Figure 1).

In vitro degradation of fibrin

The fibrin was cut into 5 mm x 5 mm and the fibrin weight was measured. The fibrin was immersed in PBS (pH 7.0) (Lonza, USA) as a control and in Tryp select 1X (Gibco, USA) as a treatment group. It was then incubated at 37°C for 21 days. After 21 days of incubation, the fibrin weight was measured.

Chondrogenic differentiation assays

The ADSCs at passage 3 were cultured on fibrin (1 x 10⁵) in various media (DMFA, DMFA with 1 µg/ml, 3 µg/ml, and 6 µg/ml of ascorbic acid). The cells were analyzed on day 21 for the GAGs levels and the mRNA expression of type 2 collagen, aggrecan, and type 1 collagen.

Glycosaminoglycans (GAG) analysis

GAG analysis was conducted using alcian blue staining to stain the acidic polysaccharides, such as GAG. The cells were fixed in 4% paraformaldehyde for 15 min. The 4% paraformaldehyde was then removed and 1% alcian blue in 3% acetic acid was added to the fixed cells for 30 min. The alcian blue was then removed and washed twice with PBS (Lonza, USA). The deionizing solution was then added and incubated for 2 min. The washed cells were then mixed with 100 µl of 1% sodium dodecyl sulphate (SDS) and mixed in a shaker at 200 rpm for 30min. The GAG content was determined based on the absorbance value of the solution at a 595 nm wavelength on a microplate reader (iMark Bio-Rad).

mRNA expression of the chondrogenic and osteogenic markers

The cells of all groups were harvested on day 21. RNA isolation was performed using the SV Total RNA Isolation System (Promega, USA) while the synthesis of cDNA and mRNA expression analysis were performed using the GoTaq[®] 2-Step qPCR System (Promega, USA) according to the manufacturer. The amount of total RNA used to perform the reverse-transcription and the cDNA synthesis was 67 ng. Gene amplification and the software used for the data analysis were performed by the CFX96 Touch Real-Time PCR Detection System — Bio-Rad. The final amplification volume was 25 µL with 1 µL volume of cDNA used as the template.

The mRNA expressions analyzed were type 2 collagen, aggrecan, and type 1 collagen with GAPDH as the gene reference (Table 1). The cycle conditions were an initial denaturation step of 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 54.4 °C for 1 min, and a final extension at 60 — 95 °C for 5 s. The result of the qPCR was analyzed following the 2^{-ΔΔCT} method described by Livak *et al.* (2001)²³.

Statistical analysis

The data from each parameter was presented as average ± standard deviation (SD) in the tables and charts.

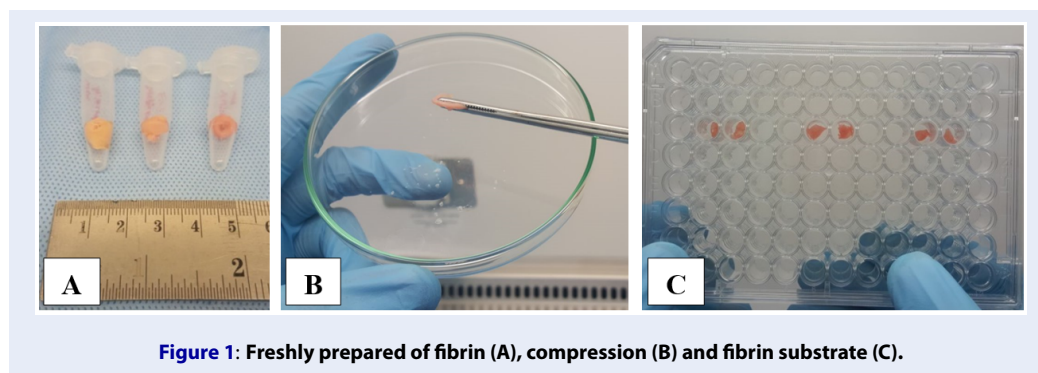


Figure 1: Freshly prepared of fibrin (A), compression (B) and fibrin substrate (C).

Table 1: Primer design

Gene		Nucleotide base	Base number	Amplicon length (bp)
GAPDH	forward	CAAGAGCACAAGAGGAAGAGAG	22	102
	reverse	CTACATGGCAACTGTGAGGAG	22	
Type 2 collagen	forward	GAACCCAGAAACAACACAATCC	22	86
	reverse	CATTCAGTGCAGAGTCCTAGAG	21	
Aggrecan	forward	CAGAATGGGAACCAGCCTATAC	22	104
	reverse	GCCTTCTGTACTTTCCTCTGTT	22	
Type 1 collagen	forward	AGAGTGGAGCAGTGGTTACTA	21	100
	reverse	GATACAGGTTTCGCCAGTAGAG	22	

The differences were analyzed ($p < 0.05$) using a parametric statistical test analysis of variance (ANOVA).

RESULTS

Cell morphology

There was slight difference in the cell morphology between the control group and the ascorbic acid supplementation groups. The cells were spread and attached tightly to the polystyrene substrate. The cell shape is represented on day 9 of the observation in **Figure 2**.

Biodegradability of fibrin

The decrease in fibrin weight when compressed and incubated shows that the fibrin substrate was successfully fabricated. This result was also previously reported by Kobayashi²². Compared to the freshly prepared wet weight, the compression and incubation was significantly reduced (**Figure 3**). The degradation of fibrin is shown in **Figure 4**. Fibrin that was incubated with PBS for 21 days showed no significant change while the fibrin that was incubated with Tryple Select 1X for 21 days was significantly reduced. Tryple degraded the fibrin substrate into small pieces. The

percentage of degradation of the PBS group was 20% of the weight while for Tryple, it was more than 90% of the weight.

Chondrogenesis of ASCs

GAGs content was observed in the ASCs cultured on the fibrin substrate (**Figure 5**). The groups with ascorbic acid supplementation that expressed the GAGs level abundantly had a blue color of an intensity compared to the control. The concentrations of 3 $\mu\text{g/ml}$ and 1 $\mu\text{g/ml}$ of ascorbic acid were the highest groups that expressed GAGs content followed by 6 $\mu\text{g/ml}$ and the control.

mRNA Expression

Ascorbic acid induced type 2 collagen and aggrecan expression depending on its concentration. The highest level of type 2 collagen and aggrecan was present in the group of 3 $\mu\text{g/ml}$ (5.11 ± 0.31 and 1.93 ± 0.67) followed by 1 $\mu\text{g/ml}$ (3.61 ± 0.13 and 1.31 ± 1.24), the control and 6 $\mu\text{g/ml}$ (1.15 ± 0.23 and 0.40 ± 0.29), respectively (**Figure 6**). There were significant differences in the type 2 collagen expression in the cells supplemented with 3 $\mu\text{g/ml}$ and those supplemented

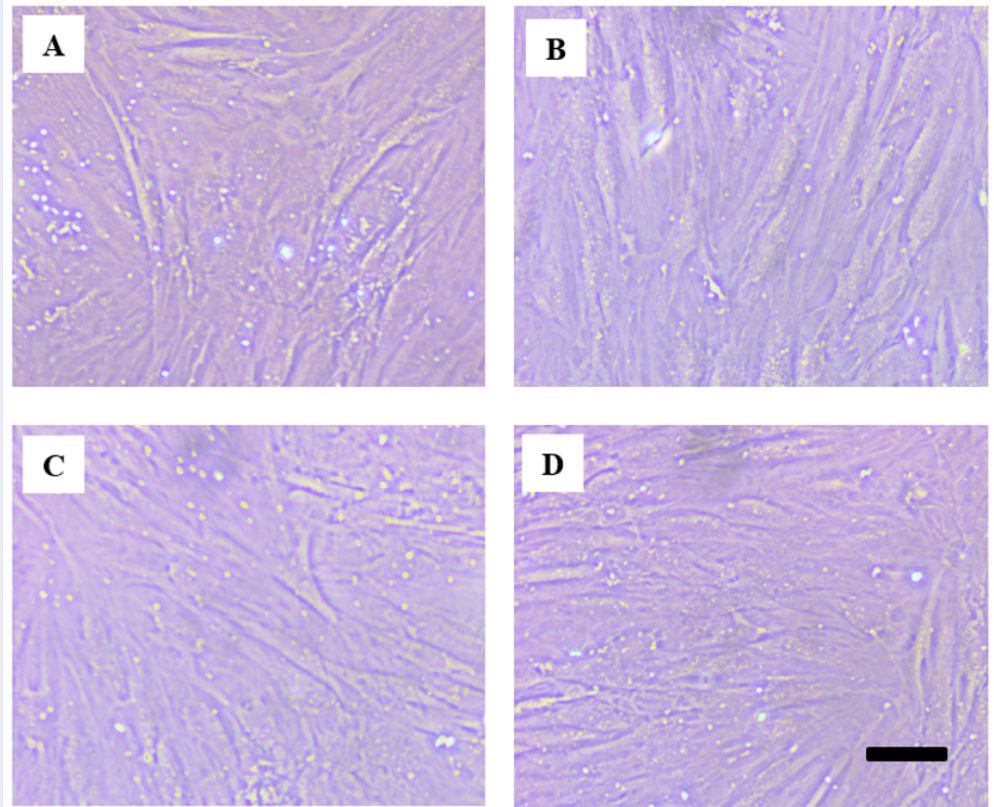


Figure 2: Morphology of cells with/without ascorbic acid on day 9 of observation, control (A); 1 µg/ml of ascorbic acid; 3 µg/ml of ascorbic acid; 6 µg/ml of ascorbic acid; black bar = 100 µm

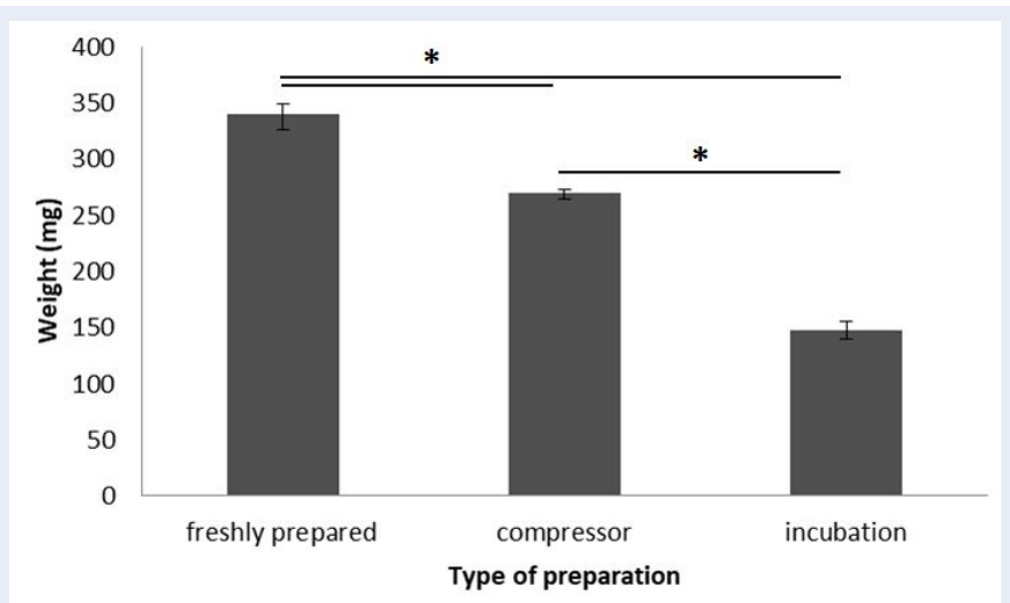
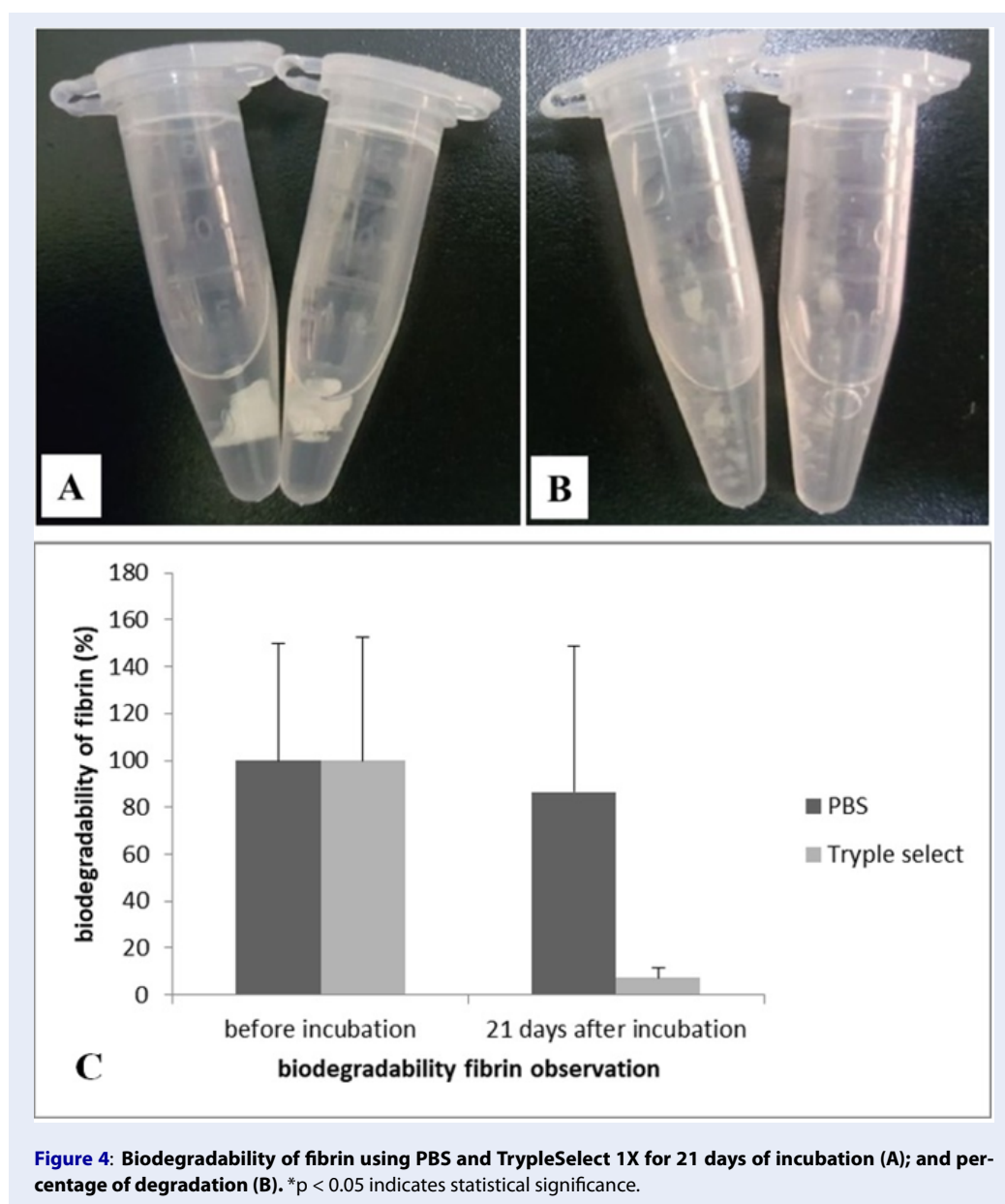


Figure 3: The decrease of wet weight of fibrin during compressor and incubation overnight (37 °C). *p < 0.05 indicates statistical significance.



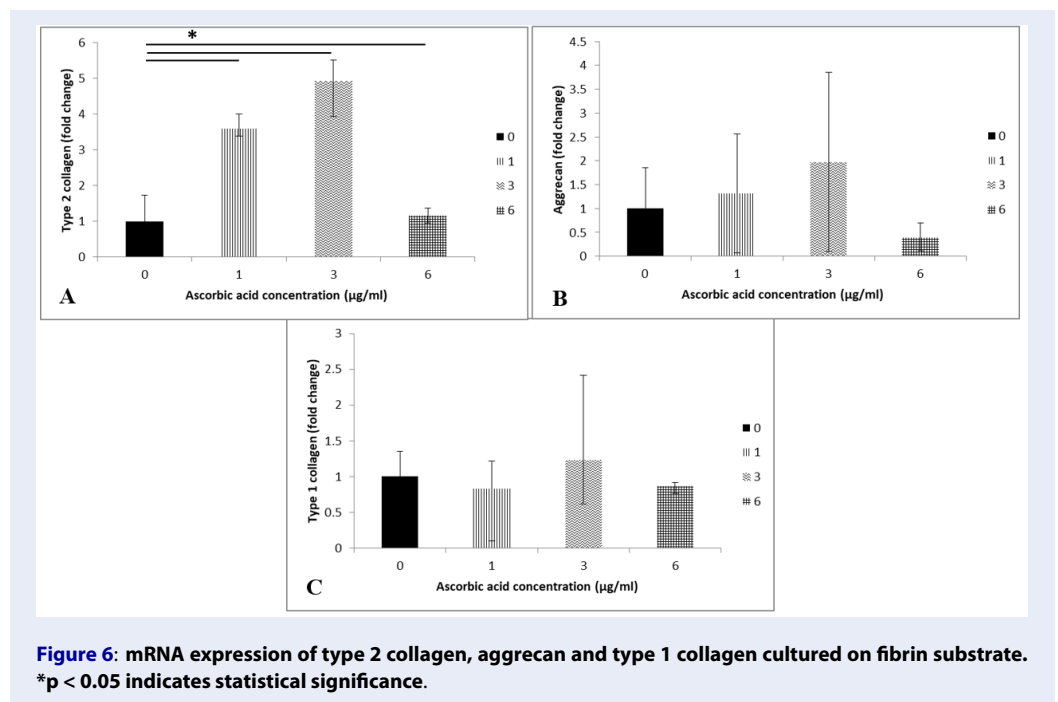
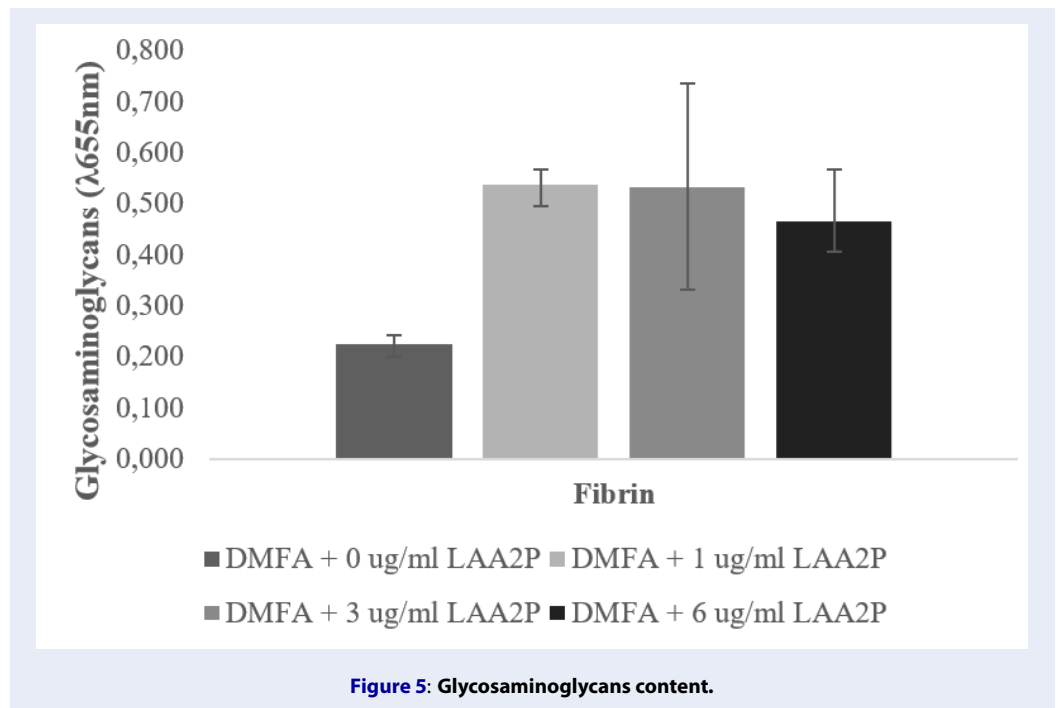
with 1 $\mu\text{g/ml}$ of ascorbic acid compared to the control. There was no significant difference between the aggrecan and type 1 collagen and the control. This study shows that 3 $\mu\text{g/ml}$ of ascorbic acid was the optimal concentration to induce the chondrogenesis of ASCs.

DISCUSSION

Our results show that the ASCs had a plastic adherence capacity with a similar shape of cells between the groups (fibroblast-like). We therefore continued to investigate the effect of ascorbic acid on the chondrogenic nature of human ASCs cultured in a human fib-

rin scaffold. According to the results, the fibrin substrate along with ascorbic acid has potency regarding cell attachment and supporting chondrogenic differentiation.

Ascorbic acid is often combined with serum as a micronutrient to increase the proliferation rate of ASCs²⁴. Fibrin was fabricated as a scaffold for tissue engineering purposes since fibrin contains a growth factor to initiate cell proliferation. Kobayashi *et al.*,²⁵ evaluated the angiogenesis protein in platelet-rich fibrin (PRF) and found that fibrin contains a vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF-BB)²⁵. Another study reported



that rabbit PRF stimulated cellular migration and the proliferation of meniscocytes²⁶. The combination of ascorbic acid supplementation and fibrin substrate may act synergistically to improve cell migration, proliferation and chondrogenic differentiation.

Regarding chondrogenic differentiation, collagen, aggrecan and GAG are the main components of the extracellular matrix (ECM) involved in both cell proliferation and the differentiation of chondrocytes. Chondrogenesis involves the condensation and deposition of ECM formation²⁷. Ascorbic acid induces ATDC5 cell differentiation by promoting the formation of a collagenous matrix mediated by the activation of the ERK signaling pathway¹³. Our study shows that the ASCs cultured on fibrin substrate supplemented by 3 $\mu\text{g/ml}$ of ascorbic acid had a higher expression of type 2 collagen, aggrecan, and GAG, indicating that the proper concentration of ascorbic acid and fibrin had an effect in terms of supporting the ASCs chondrogenesis. It has been reported that PRF contains transforming growth factor beta (TGF- β)²⁸. TGF- β has an important role in regulating chondrogenic differentiation¹². TGF- β secreted by fibrin activates the Smad signaling pathway while ascorbic acid activates the ERK signaling pathway. Both mechanisms lead to the chondrogenic differentiation of human ASCs. Up-regulated type 2 collagen and GAG of chondrocyte cultured on fibrin were also reported by Chien *et al.*,²⁸. Another study showed that human PRF stimulated proliferation and induced the aggrecan synthesis of ASCs²⁹. The presence of GAGs in the cells confirmed chondrogenic differentiation^{12,30}. In contrast, an osteogenic marker, specifically type 1 collagen, was expressed in a low concentration, indicating that osteogenic differentiation did not occur.

This report shows that ascorbic acid also has the potential to induce chondrogenesis in the ASCs cultured on the fibrin substrate. Although the pathway of ascorbic acid and fibrin to induce chondrogenesis in this study was unclear, there were previous studies explaining the role of both pathways. In addition, the results need to be evaluated due to our study having many limitations including a small sample size and no evaluation of the protein level. Regardless, these results indicate the potential of ascorbic acid and fibrin substrate in the differentiation of human ASCs for cartilage tissue engineering application.

CONCLUSIONS

It can be concluded that the supplementation of 3 $\mu\text{g/ml}$ of ascorbic acid for human adipose-derived stem cells cultured on fibrin substrate promotes type 2

collagen expression is optimal. However, it is too early to state that the cells lead to chondrogenic differentiation since the aggrecan expression is not significantly increased.

ABBREVIATIONS

None.

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AUTHOR'S CONTRIBUTIONS

(I) Conception and design: Imam Rosadi; Karina Karina; (II) Administrative support: Imam Rosadi; Karina Karina; Viol Dhea Kharisma; (III) Provision of study materials or patients: Karina Karina; (IV) Collection and assembly of data: Imam Rosadi; Arif Nur Muhammad Ansori; (V) Data analysis and interpretation: Imam Rosadi; (VI) Manuscript writing: Imam Rosadi; Viol Dhea Kharisma, Arif Nur Muhammad Ansori; (VII) Final approval of manuscript: All authors.

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None.

AVAILABILITY OF DATA AND MATERIALS

Data and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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