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Ascorbic Acid Enhances the Metabolic Activity, Growth and Collagen Production of Human Dermal Fibroblasts Growing in Three-dimensional (3D) Culture

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Highlights

- Ascorbic acid-2-phosphate (AA2P) increases the metabolic activity of human dermal fibroblasts (HDFs).
- AA2P stimulates the collagen deposition of HDFs in 3D culture.
- AA2P addition can be used to improve the culture efficiency of HDFs on a 3D synthetic biomaterial.

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Abstract

Tissue engineering (TE) enables the development of functional synthetic substitutes to be replaced with damaged tissues and organs instead of the use of auto or allografts. A wide range of biomaterials is currently in use as TE scaffolds. Among these materials, naturally sourced ones are favorable due to being highly biocompatible and supporting cell growth and function, whereas synthetic ones are advantageous because of the high tunability on mechanical and physical properties as well as being easy to process. Alongside the advantages of synthetic polymers, they mostly show hydrophobic behavior that limits biomaterial-cell interaction and, consequently, the functioning of the developed TE constructs. In this study, we assessed the impact of L-Ascorbic acid 2-phosphate (AA2P) on improving the culture conditions of human dermal fibroblasts (HDFs) growing on a three-dimensional (3D) scaffold made of polycaprolactone (PCL) using emulsion templating. Our results demonstrated that AA2P enhances the metabolic activity and growth of HDFs as well as collagen deposition by them when supplemented in their growth medium at 50 µg/mL concentration. It showed a great potential to be used as a growth medium supplement to circumvent the disadvantages of culturing human cells on a synthetic biomaterial that is not favored in default. AA2P's potential to improve cell growth and collagen deposition may prove an effective way to culture human cells on 3D PCL PolyHIPE scaffolds for various TE applications.

1. INTRODUCTION

Failure or loss of tissues and organs brings a major challenge to human health, and tissue engineering (TE) aims to circumvent these problems by developing functional synthetic substitutes for damaged tissues instead of the use of auto or allografts [1]. To date, a large variety of biomaterials have been proposed to be used as TE scaffolds. Among them, natural biomaterials are preferable due to their recognizable side groups that supports cell adhesion and growth, whereas synthetic biomaterials are mostly attractive because of providing high control on physical and mechanical properties as well as their geometries [2]. However, the main drawback of using synthetic biomaterials is that they mostly show hydrophobic behaviour that limits biomaterial-cell interaction and consequently impairs the functioning of the developed TE construct [3, 4].

Polycaprolactone (PCL) is an U.S. Food and Drug Administration (FDA) approved polymer for the manufacturing of surgical sutures and drug delivery systems, and it is demonstrated as a biocompatible and biodegradable synthetic polymer [5]. PCL has been reported widely as a promising biomaterial for its use in both hard and soft TE studies [6], and scaffolds made of PCL have been fabricated using various scaffold

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manufacturing techniques, including electrospinning [7], additive manufacturing [8], porogen leaching [9], solvent casting [10], and most recently, emulsion templating [11–15].

Emulsion templating gained particular attention in recent years for the fabrication of TE constructs because it enables the production of highly porous and interconnected scaffolds via having almost full control on both properties. It is briefly based on creating an emulsion from two immiscible liquids, an internal phase (the water) and a continuous phase (the polymer). When the internal volume phase percentage in the emulsion exceeds 74.05%, it is referred as a high internal phase emulsion (HIPE). Following the polymerization of the continuous phase, the resultant highly porous and interconnected template is called polymerized HIPE (PolyHIPE) [14]. To date, PolyHIPE scaffolds made of PCL have been shown to be applicable in many applications, including hard and soft TE [15, 16], dentistry [17], wound management [13], and *in vitro* models [11].

We have previously demonstrated various approaches to overcome the undesired surface properties of synthetic scaffolds and improve the culture of cells in 3D TE scaffold systems. These methods include surface plasma treatment [17, 18], surface coating with gelatin [7, 19], functionalization of the construct via *in vitro* produced extracellular matrix (ECM) [15], and co-culturing the cells of interest with other cells [7].

Accordingly, in this study, we fabricated PCL PolyHIPE scaffolds via emulsion templating and assessed (i) their suitability for culturing human dermal fibroblasts (HDFs) in 3D and (ii) how L-Ascorbic acid 2-phosphate (AA2P) affects the cellular activity, growth, localization and collagen synthesis of HDFs. L-ascorbic acid has previously been demonstrated to have a key role in collagen synthesis [13, 20] by taking a role in collagen production steps both at molecular [21] and gene levels [22, 23]. Thus, the research question of this study was could exogenous AA2P supplementation be a useful approach to improve the cellular activity of HDFs and their collagen deposition and consequently to support cell culture under 3D culture conditions?

2. MATERIALS

Epsilon (ε) caprolactone, pentaerythritol, tin (II) 2-ethylhexanoate, triethylamine (TEA), photo initiator (2,4,6-Trimethylbenzoyl Phosphine Oxide/2-Hydroxy-2-Methylpropiophenone blend) methacrylic anhydride (MAA), penicillin/streptomycin (PS), fetal calf serum (FCS), trypsin EDTA, L-glutamine, 25% glutaraldehyde, 37% formaldehyde, ethanol, glacial acetic acid, picric acid, hexamethyldisilazane (HMDS), L-ascorbic acid 2-phosphate (AA2P), eosin Y solution aqueous, Harris hematoxylin solution, Polydimethylsiloxane (PDMS), Triton X100, Dimethyl sulphoxide (DMSO), Alamar Blue® Metabolic Activity Assay, Sirius Red (Direct Red 80), and sodium hydroxide (NaOH) were purchased from Sigma Aldrich. Dichloromethane (DCM), methanol, chloroform, and toluene were purchased from Fisher Scientific. Hypermer B246 was a product sample sent by Croda. Human Dermal Fibroblast cells and fibroblast culture medium were acquired from Lonza.

3. METHODS

3.1. Fabrication of the PCL PolyHIPE Scaffolds

PCL PolyHIPEs were fabricated as described previously [18]. The 4-arm PCL methacrylate (4PCLMA) used in this study is synthesized in-house, and its protocol is described elsewhere [5]. After the polymer synthesis, 0.4 g of 4PCLMA and the Hypermer B246 (10% w/w) were mixed in a container and heated at around 40°C using a heat gun. Chloroform/toluene solvent blend (80% chloroform (w/w), 20% toluene (w/w)) and photoinitiator (10% (w/w)) were added, and it was stirred at 380 rpm for 60 seconds on a magnetic stirrer at room temperature (RT). Once it was homogeneous, the internal phase volume was adjusted to 85% (v/v) by adding 2.5 mL of water in 2 minutes dropwise. Finally, the emulsion was stirred for another 2 minutes.

Once the PCL HIPE was prepared, it was then transferred into silicon moulds. An ultraviolet (UV) curer (Omnicure Series 1000) was used to cure the emulsion from both sides for 3 minutes. The cross-linked scaffolds were undergone serial methanol washes (100%, 50% for 24 hours each) and were then kept in deionized (DI) water for 24 hours. The scaffolds were then removed from the DI water and placed at -80°C for 1 hour prior to transferring them into a vacuum oven. The parts were kept under vacuum for 24 hours for complete freeze-drying. Finally, a 5-mm biopsy punch was used to prepare circular scaffolds for further experiments. Before the cell culture experiments, the PCL PolyHIPE scaffolds was functionalized using air plasma (Diener Electronic, Ebhausen, Germany) from their cell attachment side as described previously [18, 24].

A scanning electron microscope (Philips/FEI XL-20 SEM, Cambridge, UK) was employed to investigate the porosity and interconnectivity of the PCL PolyHIPs. Briefly, scaffolds were cut and mounted on carbon tabs, and a gold sputter was used at 15 kV for 2.5 minutes. SEM images were captured at 5 kV acceleration power, and they were used to demonstrate the pore and window size distributions as described previously [15]. Briefly, randomly selected ninety pores and ninety windows from at least three different areas of three different samples were analyzed using ImageJ software. Following the measurements, a correction factor of $2/\sqrt{3}$ was applied to them, considering the uneven sectioning [5, 25].

3.2. Assessing the Changes in the Metabolic Activity of HDFs Growing on PCL PolyHIPE Scaffolds in Response to AA2P Treatment

To understand how AA2P affects the metabolic activities of HDFs, cells (between passages 2-8) stored in liquid nitrogen were thawed in a water bath and centrifuged for 5 minutes at 1000 rpm. The HDFs were then resuspended in fibroblast growth medium (FGM) (supplemented with 10% FBS and BulletKit) and expanded in 75 cm^2 tissue culture plastic at 37°C, 5% CO_2 until they reached confluency.

PCL PolyHIPE scaffolds were fabricated as described in Section 3.1. Before cell culture experiments, they were washed with 100% ethanol and disinfected in 70% ethanol (around 2 hours) before washing them with sterile phosphate buffer saline (PBS) four times. 4 x 10^4 HDFs were trypsinized, centrifuged and resuspended in 15-20 µl of FGM and pipetted onto the circular PCL PolyHIPE scaffolds. They were incubated at 37 °C for around 2 hours before adding FGM with and without 50 µg/mL AA2P supplement. The medium was replenished every 2-3 days throughout the experiments. Resazurin reduction assay was used to evaluate the cellular activity of cells over 14 days in response to AA2P treatment as described previously [19, 26]. Briefly, 100 µM AlamarBlue working solution was prepared in FGM. The culture medium was aspirated, and the HDFs were rinsed with sterile PBS. Then, HDFs were treated with 1 mL of resazurin reduction working solution for 4 hours at 37°C in a cell culture incubator. Then, approximately 200 µL of the AlamarBlue solution was pipetted into a 96-well microplate. On days 1, 7 and 14, a multimode microplate reader (Varioskan, Thermo Scientific) was used to measure the fluorescence at 540 nm (excitation wavelength) and 635 nm (emission wavelength).

3.3. Assessing the Collagen Deposition of HDFs on PCL PolyHIPE in Response to AA2P Treatment

Sirius Red (Direct Red 80) staining was used to assess how AA2P affects the collagen deposition by HDFs over 14 days, as described previously [13]. Briefly, HDFs were trypsinized and seeded onto PCL PolyHIPE constructs as described in Section 3.2. Scaffolds were cultured with or without the inclusion of 50 µg/mL of AA2P in the growth medium. First, Sirius Red powder was dissolved in picric acid (1 w/v%) and filtered to remove any remnants. For the evaluation of collagen deposition, the growth medium of HDFs was aspirated, and the scaffolds were washed with sterile PBS. Then 1 mL of Sirius red solution was added to each well, and the cells were incubated for around 1 hour at RT. Then, the solution was removed, and the scaffolds were rinsed with DI water on an orbital shaker. The water was replenished every five minutes until the water was finally clear. The scaffolds were then submerged into 0.2 M of NaOH:methanol (MeOH) (1:1) solution and incubated on an orbital shaker for 30 minutes at RT for destaining. Finally, approximately 150 µL of the Sirius Red solution was pipetted into a microplate and absorbance was read at 405 nm with a multimode microplate reader (Varioskan, Thermo Scientific).

3.4. Scanning Electron Microscopy Images of Biohybrid PCL PolyHIPE Scaffolds

SEM images were taken as described elsewhere [27,28] for the investigation of the growth of HDFs on the scaffolds. Briefly, on days 7 and 14, the culture medium was aspirated, and the PCL PolyHIPE constructs were washed with sterile PBS prior to fixation in 2.5% glutaraldehyde for 1 hour at RT. Samples were transferred into PBS for 45 minutes (PBS was replenished every 15 minutes) and submerged into DI water for 5 minutes. The scaffolds were then undergone serial ethanol washes (35%, 60%, 80%, 90%, and 100% for 15 minutes for each concentration) were applied. Following the dehydration steps, a chemical drying agent hexamethyldisilazane (HMDS) (1 hour in 50% HMDS (vol/vol in ethanol) and 5 minutes in 100% HMDS) was used to dry the samples. Finally, samples were kept at RT overnight in a fume hood for air-drying, and the SEM samples were prepared as described in section 3.1 prior to taking SEM images.

3.5. Histological Assessments of the PCL PolyHIPE Scaffolds

The growth medium was aspirated, and the PCL PolyHIPE scaffolds cultured with HDFs (with and without AA2P supplement) were washed with PBS once prior to fixation in 3.7% formaldehyde on days 7 and 14. Cryomolds were filled with optimum cutting temperature (OCT) freezing media, and the scaffolds were submerged into it, taking care of the bubble formation. Cryomolds were then incubated at -80°C for 15-20 minutes to freeze, and the 6–10 μm of sections of the scaffolds were taken on slides using a cryostat (Leica CM1860 UV, Milton Keynes, UK) [15, 29].

For the histological evaluation of the HDFs within the scaffolds, hematoxylin and eosin (H&E) staining was used as described elsewhere [19, 30]. Briefly, sections taken with cryostat were submerged into DI water for the removal of remnant freezing media and incubated in hematoxylin for 1.5 minutes and then washed gently under running water for 4 minutes. Then the slides were stained with eosin for 5 minutes and washed with DI water. Finally, samples were dehydrated with serial ethanol washes, dunked into xylene and mounted with Dibutylphthalate Polystyrene Xylene (DPX) mountant. Histological images were taken using a light microscope.

A modified version of the Sirius Red staining protocol for the tissue sections was performed as described elsewhere [31]. Briefly, Sirius Red solution was added to the slides and incubated for 60 minutes at RT. Then, Sirius red solution was aspirated, and the sections were undergone DI water washing steps until they were clear. The excess stain was removed by washing the slides with acidified water (0.5% glacial acetic acid). Excess water was then removed using a filter paper, and the slides were dehydrated in 100% ethanol prior to dunking into xylene and mounting with a DPX mountant.

3.6. Statistical Analysis

Statistical tests (one-way and two-way analysis of variance (ANOVA)) were performed using GraphPad Prism software (California, USA). Error bars given in the graphs indicate standard deviation (SD), and the n values are given in the Figure captions where relevant.

4. RESULTS AND DISCUSSION

4.1. The Results of the PCL PolyHIPE Scaffold Fabrication

The SEM images of the PCL PolyHIPE scaffolds demonstrated that they were highly porous and interconnected. Open porous structure was determined by the observation of interconnects (windows) on the inner surface of the pores. The pore size distribution of the scaffolds showed a range from 5.5 to 150 μm with an average pore size of 36.4±26.8 μm, and more than 90% of the pores were found to be lying at 20-60 μm intervals. The window sizes ranged between 2.6 to 22.0 μm, and the mean window size was calculated as 7.7±3.8 μm. SEM micrographs, pore and window size distribution of PCL PolyHIPE constructs are presented in Figure 1.

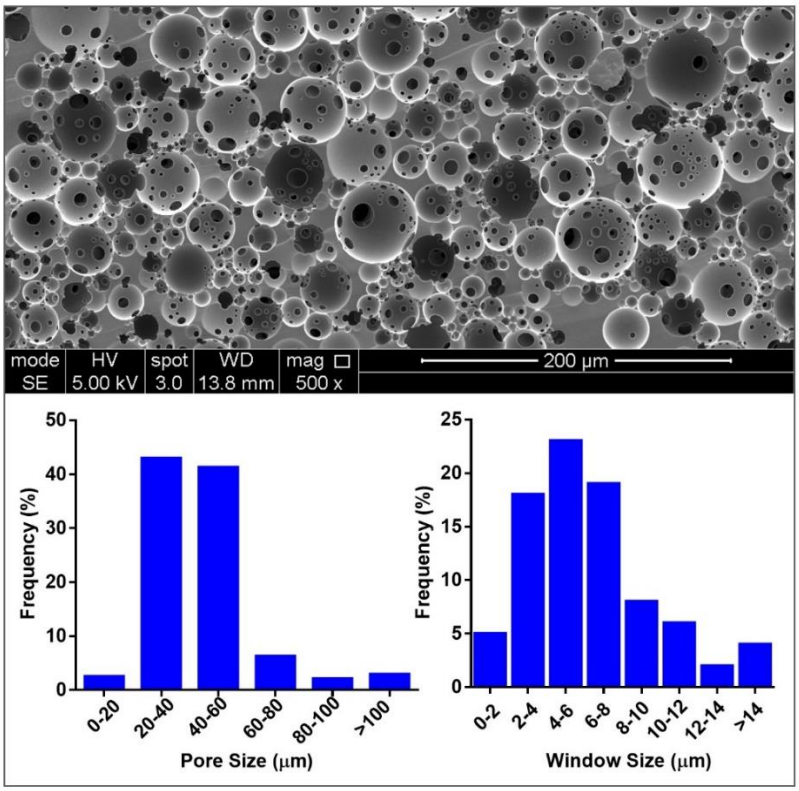

Figure 1. *The SEM micrograph of the PCL PolyHIPE shows the highly porous and interconnected architecture of the fabricated scaffold. The graphs given on the bottom show the pore size and window size distribution of the scaffolds*

4.2. AA2P Increases the Activity of HDFs Cultured on PCL PolyHIPE Scaffolds

The results of the Alamar Blue assay showed that the treatment of HDFs with 50 µg/mL AA2P increased their metabolic activity approximately 1.5-fold on day 14. Although a slight increase in the activity of HDFs was observed both on days 1 and 7, this increase was found to be not statistically meaningful. This metabolic activity increase was found in coherence with the previous reports where L-ascorbic acid has been demonstrated to improve cellular activity and proliferation of several cell types, including human dermal fibroblast [16, 20], human mesenchymal stem cells [32], human adipose-derived stem cells [33], and human gamma delta T cells [34]. Its positive impact on the collagen production, proliferation, and formation of a 3D tissue-like structure by fibroblast cells has previously been shown by many groups [35–37]. Ascorbic acid is critical for the healthy growth of fibroblast cells. Schafer et al. showed that fibroblasts growing in a medium containing less than 1 µg/mL of AA2P developed morphological alterations as symptoms of ascorbic acid deficiency compared to cells cultured in a medium including 50 µg/mL. They also reported that these alterations could be reversed by the addition of ascorbic acid to the media [38]. Hata et al. reported the positive impact of AA2P on skin fibroblast in terms of proliferation and collagen synthesis for up to 8 weeks. They further assessed if this increase in proliferation is due to the increase in the synthesis of collagen or not. In the following experiments, they showed that both collagen synthesis and proliferation of cells were affected negatively when an inhibitor of collagen synthesis, azetidine 2-carboxylic acid, was included in the growth medium of fibroblasts. Their results suggested that AA2P promotes cell proliferation by activating collagen synthesis [39].

Our metabolic activity results were also coherent with the SEM micrographs of the HDFs growing on PCL PolyHIPE taken on days 7 and 14. The SEM micrographs showed a better coverage of the surface by HDFs on both days when cells were cultured in AA2P supplemented growth media. The surface of the PCL PolyHIPE scaffolds was covered completely by HDFs on day 14 when cultured in the presence of AA2P. In contrast, acellular areas on the surface of PCL PolyHIPE were observed when HDFs were cultivated in the control growth medium non-supplemented with AA2P.

The Alamar Blue results and the SEM micrographs of HDFs are given in Figure 2.

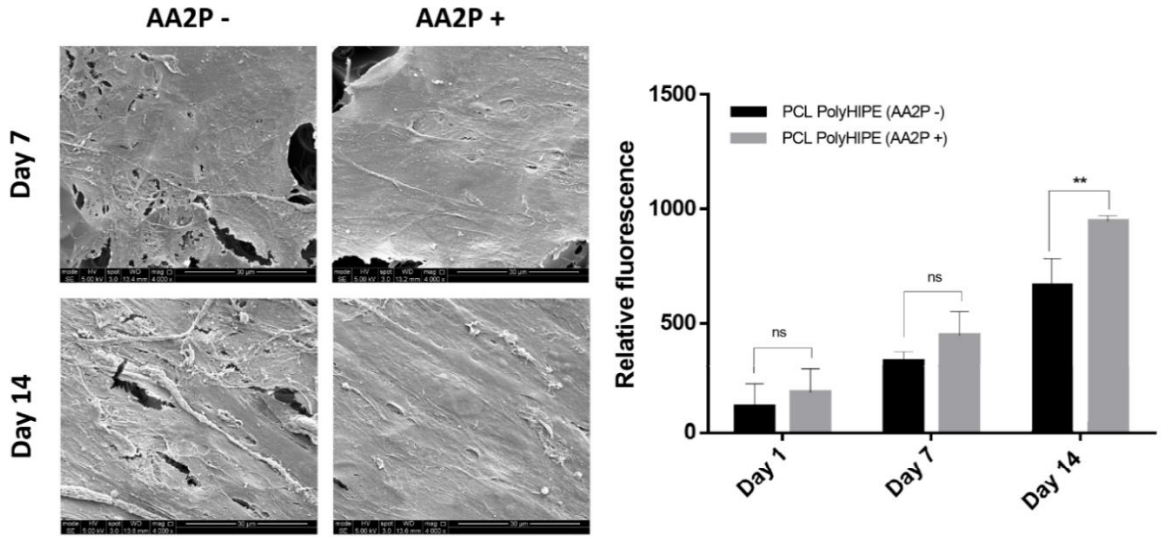

Figure 2. *The SEM micrographs show the 7 and 14-day culture of HDFs on PCL PolyHIPE scaffolds with or without AA2P supplement. The graph shows the metabolic activity change of HDFs over 14 days in response to AA2P treatment. ** $p < 0.01$, ns not significant, $p > 0.05$, $n = 3 \pm SD$. Scale bars represent 30 µm*

4.3. AA2P Stimulates the Collagen Deposition by HDFs Cultured on PCL PolyHIPE

Similar to the metabolic activity of HDFs, AA2P treatment significantly enhanced the collagen deposition of HDFs significantly on day 14 but not on day 7. These results verify previously conducted studies reporting the increased collagen production *in vitro* when the growth medium is supplemented with different derivatives of L-ascorbic acid [35, 40–43].

The quantitative assessment of collagen production showed that although HDFs were found to produce approximately 2.5-fold more collagen when treated with AA2P, the difference was not statistically meaningful on day 7. However, on day 14, the collagen production by HDFs cultured with AA2P was increased significantly by 2.3-fold compared to controls. The macro photos of the Sirius Red-stained PCL PolyHIPE scaffolds and the results of the Sirius Red assay are given in Figure 3.

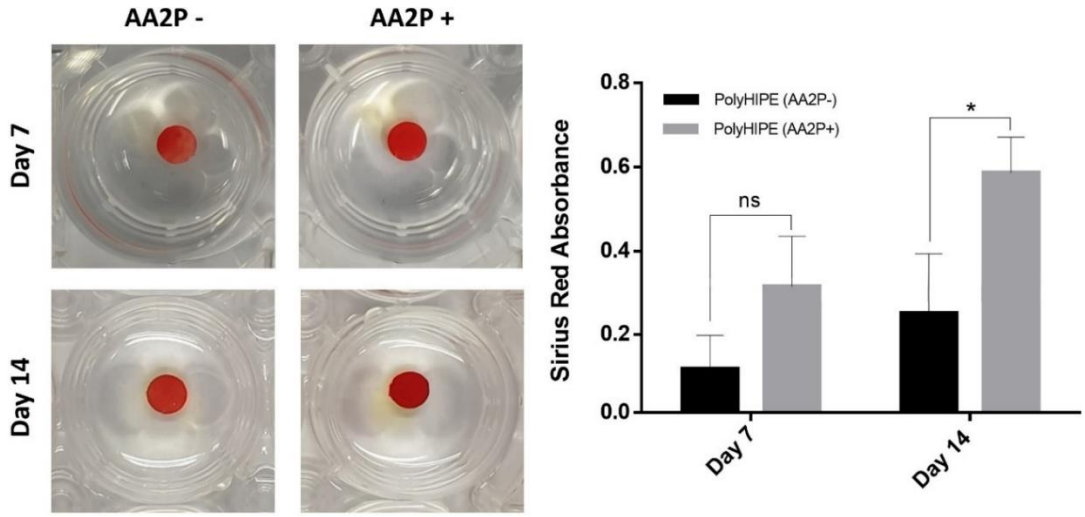

Figure 3. *The macro images on the left show the Sirius Red-stained PCL PolyHIPE scaffolds on which HDFs were cultured over 14 days with or without AA2P supplement. The graph shows the results of the Sirius Red assay that shows the collagen production by HDFs over 14 days in response to the AA2P supplement. * $p < 0.05$, ns: not significant, $p > 0.05$, $n = 3 \pm SD$*

Ascorbic acid has previously been demonstrated to have a key role in collagen synthesis [13, 20]. One of the possible reasons is that it takes a role as a cofactor for the enzyme (prolyl hydroxylase), responsible for the cross-linking of collagen fibrils and creation of triple helix [21], that plays a key role in the hydroxylation of proline and lysine residues in collagen [44]. Secondly, it has previously been shown that ascorbic acid augments the transcription of procollagen genes and enhances the stability of procollagen mRNA [22, 23, 45, 46]. In addition, ascorbic acid plays a key role in the modulation of the growth properties of human cells [47–50].

The abovementioned studies clearly show the importance of ascorbic acid on both the growth and collagen production of fibroblasts, but when it is dissolved, it is very unstable particularly under regular cell culture conditions at 37°C and at neutral pH [51]. Thus although L-ascorbic acid is the commonly found form of ascorbic acid in nature due to its low chemical stability [52], AA2P is the more stable form of L-ascorbic acid and is used effectively in cell culture and TE applications [35].

4.4. The Results of the Sirius Red and H&E Staining of the Sections

The results of the Alamar Blue and the Sirius Red assays were verified further with the H&E and Sirius Red staining of the sections taken from PCL PolyHIPE scaffolds.

The results of the Sirius Red assay demonstrated that AA2P supplementation to the growth media increased the collagen deposition by HDFs on both days 7 and 14. On day 14, the increase in collagen production was statistically meaningful. However, it is important to note that although the deposited collagen was clearly observable in the Sirius Red stained sections on day 7, the Sirius Red assay readings revealed that this increase was not statistically meaningful compared to controls. One possible explanation for that may be the insufficient recovery of the dye from the highly porous scaffolds on day 7. Although long washing steps were applied, unlike the two-dimensional tissue culture plates, a trace of dye may be entrapped within the pores and interconnects of the TE scaffolds. That is why it is important to evaluate the collagen deposition qualitatively alongside the quantitative assessment. That is why the collagen accumulation within the scaffolds was evaluated histologically by H&E staining of the sections.

The proliferation and localization of HDFs within the scaffolds were further assessed histologically to correlate with the metabolic activity measurements. The results of the H&E staining of the sections also showed that AA2P treatment dramatically improved the number of proliferated cells in the scaffolds on day 14. However, no such difference was observed in the overall appearance of the H&E-stained sections on day 7 between AA2P+ and AA2P- groups. Histological evaluations were found in coherence with the metabolic activity results, which also indicated a significant increase on day 14 but not on day 7. The findings of the H&E and Sirius Red-stained sections are given in Figure 4.

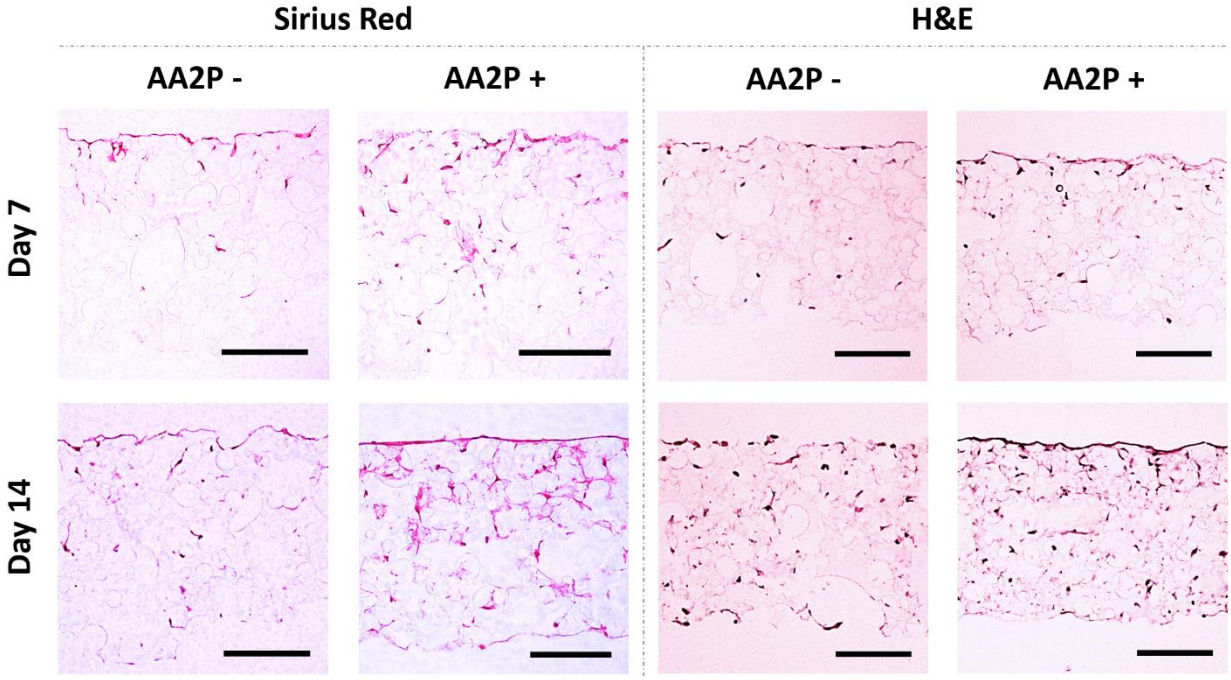

Figure 4. *Sirius Red (left) and H&E-stained (right) sections of the PCL PolyHIPE scaffolds, cultured with HDFs over 7 and 14 days either in the presence or absence of AA2P. Scale bars represent 100 µm*

The SEM micrographs of the cross-sections of the PCL PolyHIPE scaffolds cultured with HDF (day 14) with or without AA2P supplement are given in Figure 5. The images demonstrated that although HDFs were found to infiltrate and grow towards the inner sections of the scaffolds, the infiltration through the intersects of PCL PolyHIPE, and the overall ingrowth and localization of HDFs were more significant in the AA2P-supplemented group (AA2P+) compared to controls. One thing to note is that the effect of AA2P on HDFs growing on PCL PolyHIPE might not look very different when assessed only from the surface (Figure 1), but its positive impact on cell proliferation, localization, and infiltration is more obvious when the cross-sectional images were evaluated.

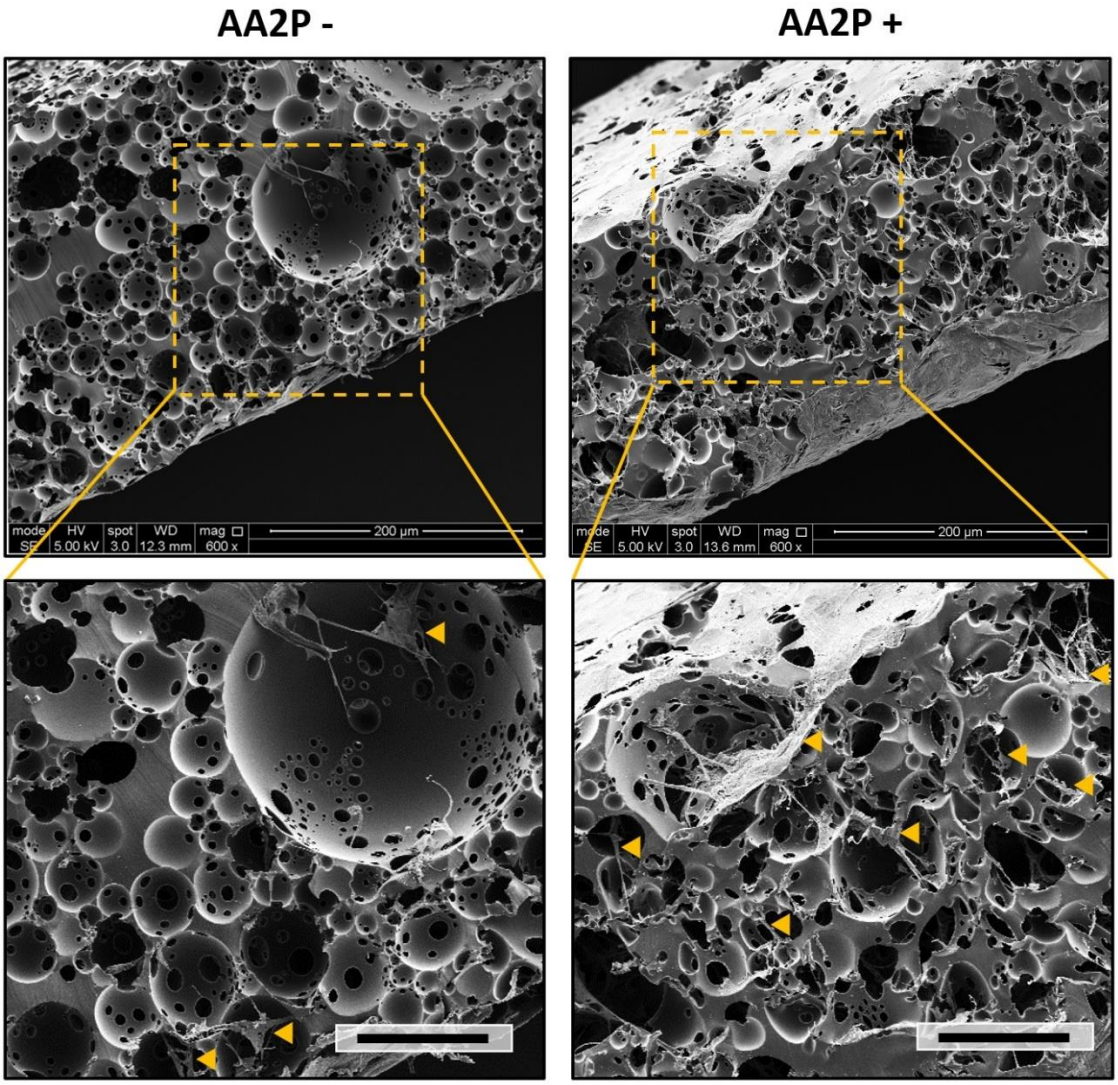

Figure 5. *The cross-sectional SEM micrographs of the PCL PolyHIPE scaffolds on which HDFs were cultured for 14 days show the highly porous and interconnected ultrastructure of PCL PolyHIPE, which allows cells to attach, and proliferate on it and infiltrate through the interconnects. Scale bars represent 200 µm and 50 µm, respectively, for lower and higher magnification images. Yellow arrows indicate the localization of HDFs within the pores and windows of the PCL PolyHIPE scaffold*

5. CONCLUSION

In conclusion, highly porous and interconnected PCL PolyHIPE scaffolds were manufactured successfully via emulsion templating. HDFs were capable of growing and depositing collagen onto PCL PolyHIPE without AA2P inclusion in their growth media. However, the addition of AA2P has been found to significantly enhance the metabolic activity and proliferation of HDFs and stimulate collagen deposition by them over 14 days in 3D culture. The results demonstrated that AA2P has a great potential to be used as a growth medium additive to circumvent the disadvantages of culturing human cells on a synthetic biomaterial that is not favoured by cells in default. Its potential to improve cell growth and collagen deposition may prove an effective way to culture human cells on 3D PCL PolyHIPE scaffolds for TE applications.

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CONFLICT OF INTEREST

No conflict of interest was declared by the author.

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