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RESEARCH ARTICLE

Amniotic growth factors enhanced human pre-adipocyte cell viability and differentiation under hypoxia

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Abstract

One of the major drawbacks associated with autologous fat grafting is unpredictable graft retention. Various efforts to improve the survivability of these cells have been explored, but these methods are time-consuming, complex, and demand significant technical skill. In our study, we examine the use of cryopreserved amniotic membrane as a source of exogenous growth factors to improve adipocyte survivability under normal and hypoxic conditions. Human primary preadipocytes were cultured in a gelatin-ferulic acid (Gtn-FA) hydrogel with variable oxygen concentration and treated with amniotic membrane-derived condition medium (CM) for 7 days. This hydrogel provides a hypoxic environment and also creates a 3D cell culture to better mimic recipient site conditions. The O_2 concentration in the hydrogel was measured by electron paramagnetic resonance oxygen imaging (EPROI). The conjugation of FA was confirmed by FTIR and NMR spectroscopy. The cell viability and adipocyte differentiation were analyzed by alamarBlue™ assay, Oil Red O staining, and RT-qPCR. The expression of genes: Pref-1, C/EBP β, C/EBP α, PPAR-γ, SLC2A4, and VEGF-A were quantified. The cell viability results show that the 50% CM showed significantly higher cell pre-adipocyte cell viability. In addition, compared to normal conditions, hypoxia/CM provided higher PPAR-γ ($p < .05$), SLC2A4, and VEGF-A ($p < .05$) (early and terminal differentiating markers) mRNA expression. This finding demonstrates the efficacy of amniotic CM supplementation as a novel way to promote adipocyte survival and retention via the expression of key gene markers for differentiation and angiogenesis.

KEYWORDS
adipocytes, amniotic growth factors, fat grafting, hypoxia, synergism

1 | INTRODUCTION

Recent advancements in plastic surgery have led to the widespread use of autologous fat grafting for both aesthetic and reconstructive surgeries.[1] Fat grafting is the act of harvesting fat from a donor site (typically the abdomen and thighs), processing or washing the aspirate to varying degrees, and then reinjecting this fat into a recipient site. Initial methods and theory suggested that the fat should be injected only in thin

aliquots, no more than 1 mm wide, in order to optimize potential revascularization. However, the current pervasive and clinically successful practice of high volume fat grafting (up to 2 L in one setting), such as in the BBL (bilateral buttock augmentation), suggests a significant fundamental knowledge gap exists between theory and practice.[2]

The application of fat grafting extends from tissue augmentation, tissue reconstruction, fibrosis and scar tissue, radiation damage, hand contractures, scleroderma/systemic sclerosis, and facial rejuvenation. Other potential uses of fat grafting are the treatment of neuroma pain, arthritis, erectile dysfunction, and chronic wounds.[3–8] Despite its widespread use, fat grafting is not a predictable technique. There are many hurdles to success. One significant hurdle is the loss of volume due to adipocyte death. Grafted fat can lose from 20% to 80% of its initial volume after transplantation.[9] It has become clear in recent years that failure of grafted fat stems from a lack of vascularization.[3] As fat is placed at the recipient site, it is exposed to a hypoxic environment where oxygen diffusion determines the fate of adipocyte survivability. After the initial hypoxic shock, it is believed that adipocytes begin to regenerate due to environmental cues such as growth factors, cytokines, and neighboring adipocyte stem cells (ASCs).[10,11] With the large increase in clinical applications of fat grafting methods to increase the predictability and success of the grafted fat tissue bear investigations.

Recent advancements showed the therapeutic potential of growth factors, cells, and contents of the placental membrane. There have been over 100 clinical trials recorded on the NIH Clinical Trials website evaluating placental cells and the placental membrane with applications such as chronic wounds, dental, ophthalmic, surgical, spine injuries, and scars.[12] In contrast to drugs and other devices, these placental membranes do not require premarket approval, allowing a faster regulatory pathway to development. The amniotic membrane has already shown its potential as an allograft because of the availability of amino acids, growth factors, and other nutrients, which promote cell migration and repair.[13–16] Considering the anti-inflammatory and pro-angiogenesis properties, which enhance wound healing, their potential application in fat grafting outcomes is worth studying. Therefore, the research question addressed in this study is this. Do amniotic growth factors have the potential to enhance fat cell viability and functionality? This project aims to examine the effects of placental growth factors on fat cell proliferation using a 3D tissue-engineering scaffold. In the current study, we aim to develop a novel method with the amniotic growth factors to assist the success of fat grafts by supplementing essential growth factors to promote cell proliferation, cell migration, stimulate stem cell activity, promote angiogenesis, and modulate inflammation. We hypothesize that adipocytes in a fat graft supplemented with amniotic growth factors will have higher survivability and proliferative rate than adipocytes without supplementation.

2 | MATERIALS AND METHODS

2.1 | Materials

The materials utilized in this specific aim include Human Preadipocytes (HPADs) (Cat No. 802S-05A), HPAD growth media (Cat No. 811–500), HPAD differentiation media (Cat No. 811D-250), all purchased from Cell Applications Inc., (San Diego, CA). TRIzol reagent was purchased from Thermo Scientific (Cat No. 15596026), High-Capacity cDNA Reverse Transcription Kit was bought from Thermo Scientific (Cat No. 4368814), DreamTaq Green PCR Master Mix (2×) was purchased from Thermo Scientific (Cat No. K1081), SYBR Green PCR Master Mix was purchased from Thermo Scientific (Cat No. 4344463), primers were custom made and ordered from Integrated DNA Technologies. The cryopreserved placental membrane was donated by Smith and Nephew. Gelatin, type A from porcine skin <300 bloom (Cat. No. G2500), Laccase, lyophilized powder from mushroom, ≥4.0 units per mg from Agaricus bisporus (Cat. No. 40452), 3-Methoxy-4-hydroxycinnamic acid (ferulic acid [FA]; Cat. No. 90034), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC; Cat. No. E6383), N-hydroxysuccinimide (NHS; Cat. No. 130672), DMSO (Cat. No. D8418), Deuterium oxide (D2O; Cat. No. 151882), Cell proliferation Kit I (MTT) (Cat. No. 11465007001), and $CoCl_2$ (Cat. No C8661) was purchased from Sigma Aldrich. Dialysis membrane, molecular weight cutoff = 3500 Da, (Spectrum Laboratories, Cat. No. 132724), Syringe filter (Millipore, Cat. No. SLGV013SL), and Syringe filter unit with PES membrane, green (Millipore, Cat. No. SLGP033RS).

2.2 | Methods

2.2.1 | Placental membrane conditioned medium

Briefly, the preparation of this conditioned media (CM) is as follows: The membrane was cut to 1 cm^2 pieces per 1 ml of minimum essential medium (MEM) to extract the growth factors from the cryopreserved membrane. For example, 20 pieces of the membrane were incubated with 20 ml of media for 5 days at 4°C. After the incubation period, the media was filtered through a 0.2 μm filter and was used within 2 months. Prior to use, the media was warmed to 37°C in a water bath.

2.2.2 | Cell culture of human preadipocytes

The cells were cultured in human preadipocytes (HPAD) Growth Media (Cell Applications Inc., USA) without antibiotics or additional growth factors. Seeding density of 1×10^6 cells/ml was maintained in a T-75 flask. The cells were sub-cultured when they reached 80%–90% confluence by trypsinization using Trypsin–EDTA and neutralization with Trypsin inhibitor (Sigma). The cells were maintained at 37°C with 5% CO_2 with three passages every week. The cells used in all the experiments were between passage number 3 to 6.

2.2.3 | Pre-adipocyte cell viability

For adipocyte viability, HPAD cells (15,000 cell/cm^2 seeding density) were cultured in 96 well plates. After 24 h, the cells were treated with 10, 25, and 50% of CM with a growth medium and incubated for

7 days. The cell viability after Day1, 7, and 14 were evaluated using alamarBlue assay. Briefly, media was removed, and 10% alamarBlue solution was added to each well. The plate was incubated for 4 h at 37°C. After incubation, the absorbance was read at 570 and 600 nm.

2.2.4 | Adipocyte differentiation

Oil Red O staining

HPAD cells (5×10^4 cells/ well) were seeded onto a 24 well plate and incubated for 24 h. After 24 h the cells were treated with adipocyte differentiation medium for 14 days with routine media change every 2 days. After 7 and 14 days, the media was removed and 4% paraformaldehyde in PBS was added for fixation for an hour and stained with the Oil red O working solution (six parts of 0.5% Oil Red O stock, four parts $_dH_2O$) for 10 min, washed with deionized water four times, and dried. The images were taken using an Olympus light microscope.

Gene expression

Quantitative evaluation of adipocyte-specific gene markers was analyzed using qRT-PCR. The total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The purity of the RNA was analyzed by 260/280 ratio using nanodrop in a plate reader (Biorad). cDNA was prepared using a High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific). Gene expression was performed using quantitative RT-PCR using SYBR Green with primer against Preadipocyte factor-1 (Pref-1, also called DLK1/FA1), CCAAT/enhancer-binding protein-ß (CEBP ß), CCAAT/enhancer-binding protein-α (CEBPα), Peroxisome proliferator-activated receptor-γ (PPAR-γ), and Solute Carrier Family 2 Member 4 (SLC2A4) (Table 1). To assess the effects of placental membrane supplementation in the angiogenic response, VEGF-A expression was analyzed. The results were quantified by evaluating the relative mRNA expression (2^{-dCT}) normalized with GAPDH expression levels of each condition. The data was presented as mRNA expression relative to GAPDH. RT-qPCR was performed using the Applied Biosciences by Life Technologies qPCR QuantStudio™.

TABLE 1 Custom-made primer sequences

Primer	Sequence
F′ Pref-1	TTGCTCCTGCTGGCTTTC
R′ Pref-1	TTGTCATCCTCGCAGAATCC
F′ PPAR-Y	AAGAGTACCAAAGTGCAATCAA
F′ C/EBP B	TCCAAACCAACCGCACAT
R′ C/EBP B	AGAGGGAGAAGCAGAGAGTTTA
F′ C/EBP a	CCCGGCAACTCTAGTATTTAGG
R′ C/EBP a	AATGACAAGGCACGATTTGC
F′ SLC2A4	TCCTGATGACTGTGGCTCT
R′ SLC2A4	T ATGCCACGAAGCCAAAGA
F′ VEGF-A	TCTCGAGCTTGGCTGTCT
R′ VEGF-A	GTCCATCTTTCTTTCCCTCTCC

2.3 | Gelatin-ferulic acid hydrogel synthesis

Gelatin-ferulic acid (Gtn-FA) hydrogel was prepared based on the established protocol.[17,18] Briefly, Gelatin was added to 50 ml of cosolvent of DMSO and dH20 in a 1:1 volume ratio. The gelatin was kept at 40°C in an oil bath with constant stirring until dissolved. FA was added to 20 ml of the cosolvent until dissolved. Then EDC (N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride) and NHS (N-hydroxysuccinimide) was added and incubated at room temperature for 15 min to activate the terminal carboxyl groups of FA. After, the FA and gelatin solutions are combined and conjugated for 24 h at 40°C. After the conjugation, the solution was dialyzed (MW cut-off = 3.5 KDa) for 5 days against deionized water at 40°C with frequent water changes. Once the solution becomes transparent, filtered through a 0.2 μm-pore-size membrane to remove gelatin aggregates and other impurities and lyophilized, stored at 4°C until use. The lyophilized samples were characterized using ^{1}HNMR and FTIR spectroscopy.

2.4 | Vial tilting method

Gtn-FA polymer solution and laccase stocks were prepared by dissolving in DPBS. The stock solutions were kept at −20°C and incubated at 37°C for 15 min before use. The Gtn-FA hydrogel was prepared by mixing Gtn–FA and laccase stock solutions at a 3:1 (Gtn–FA/laccase) ratio; 200 μl of the hydrogel solution was prepared at a time in a clear 1 ml glass vial, and they were pipetted 20 times gently to initiate the crosslinking reaction. The vial was placed in a 37°C water bath, and every minute the glass was inverted to observe the gelation state. Gelation was measured as the time point after the vial was inverted, and the solution had no flow for 3 min. Various concentrations of the Gtn-FA polymer and laccase were tested until a favorable gelation time was achieved.

2.5 | Electron paramagnetic resonance oxygen imaging

Electron paramagnetic resonance oxygen imaging (EPROI) is a novel technique that enables noninvasive measurement and mapping of partial oxygen pressure (pO_2) map in vitro and in vivo.[19–23] EPR spectroscopy provides average pO_2 concentration for the volume of measurement. Briefly, EPROI manipulates unpaired electron spins with the help of magnetic field and magnetic field gradients to generate images of spin magnetization. If the relaxation of the spin probe is oxygen-dependent, then EPROI can provide a map of local oxygen pressure at the site of inquiry.[24] The oxygen sensing molecule of choice is Trityl OX071 (also known as OX063-D24) since its relaxation time is strongly influenced by pO_2, its non-toxic, and small non-polar O_2 molecules can efficiently reach each radical. Briefly, 10 mm sample tube and rubber stopper were sterilized using Sporklenz for 40 min. Then, samples were prepared with 34.4 and 33.6 mg of

lyophilized Gtn-Fa polymer. Gtn-FA polymer cross-linked with 112.3 μl of laccase was prepared in a 10 mm tube with 332 μl of DPBS used to reconstitute the polymer. Homogenization of samples was done by vortexing. Both samples were placed into a 37°C water bath to gel. Trityl was added at 500 μM concentration 30 min into gelation. The gelation time was more than 2 h. The first sample was completely gelled within the resonator at 3 h Deoxygenation was performed over 4 h by placing the tubes in the EPROI closed chamber.

2.6 | Cell encapsulation in Gelatin-FA hydrogel and $CoCl_2$ treatment to induce hypoxia

A 3:1 polymer/laccase solution was prepared and added to the cell pellet after removing the media, mixed well, and transferred to a 37°C water bath with continuous swirling. The solution was then swirled until gelation was apparent. Then, a minute was subtracted from that time, and this was marked as the "pre-incubation time" for the polymer. After this, the solution was immediately transferred to a 96-well plate, and 50 μl was added for nonhypoxic gels and 100 μl for hypoxic gels. Then, the plate was placed in the incubator for 20 min, and then the media was added (100 μl for nonhypoxic gels and 200 μl for hypoxic gels). The media conditions include control media, 25%CM and 50%CM. In addition, hypoxia was induced in 2D culture conditions using 100 μM $CoCl_2$ for 24 h to compare the results in the 3D culture condition. After this, the media change was performed every other day, and cell viability and gene expression analysis (qRT-PCR) were performed after day 1 and 7.

2.7 | Statistics

All statistical analyses were performed in GraphPad Prism version 9.0. The experiments were repeated three times with triplicates. The values were expressed as the mean ± SD. Statistical significance was evaluated using unpaired Student's *t* test and one way-ANOVA between the group as well as with control. $p < .05$ was reported as significant.

3 | RESULTS

3.1 | Adipocyte differentiation

The HPAD cells (Passage 3) were differentiated to fully mature adipocytes which were confirmed by oil red o staining (Figure 1A[i]) and gene expression (Figure 1A[ii]). It was observed that after 7 days of differentiation, there was a significantly higher expression of C/EBP β (3.8 ± 0.3 fold, $p < .05$), PPAR-γ (38.2 ± 5.5 fold $p < .05$), C/EBP α (367 ± 55 fold, $p < .05$) and SLC2A4 (2140 ± 755 fold, $p < .05$) compared with pre-adipocytes cells. In contrast, Pref-1 was significantly downregulated (0.1 ± 0.05 fold) compared to pre-adipocytes.

3.2 | Amniotic growth factor-induced adipocyte viability

As can be seen in Figure 1B, it was found that 50% of the conditioned media (CM), was optimal. In fact, when compared to MEM (basal minimal essential medium without FBS), at day 1 there was a 20.04% ($p < .05$) increase in viability in 10% ACM, 30.93% ($p < .05$) increase in 25% CM, and 43.45% ($p < .05$) increase in 50% CM. On day 4, there was a 38.13% ($p < .05$) increase in 50% CM. At day 7 there was a 28.23% ($p < .05$) increase in viability in 10% CM, 46.63% (p$p < .05$) increase in 25% CM, and 65.39% ($p < .05$) increase in 50% CM. After day 7 there were no marked differences between different conditions.

3.3 | Adipocyte differentiation in the presence of amniotic growth factors

After 1, 4, and 7 days of culture, Oil Red O stain was performed and from Figure 2A, it is evident that 50% CM supplementation had a greater differentiation effect on the HPADs compared to control at day 7 as well as the 25% experimental group. It was observed from Figure 2B, that there is a marked difference in gene expression between HPADs supplemented with differentiation medium versus the control, which consisted of HPADs supplemented with regular growth media. Figure 2B shows that even though there is a significant increase in the Pref-1 expression in CM-treated cells, amongst 25 (13.39 ± 1.8-fold) and 50% CM (4.8 ± 0.67-fold) groups, 50% CM shows a significant reduction in expression ($p < .05$). Even though there is no significant difference in expression of C/EBP β between 25% and 50% CM, the expression was 2–3-fold higher compared to control. Moreover, SLCA4 was also significantly higher with an increase in expression at 50%CM than 25% CM treated cells. No marked variation in expression in terminal differentiation genes C/EBP α and PPAR γ compared to control. However, both the genes were significantly elevated in CM treated conditions. Specifically, in the differentiation medium, the SLC2A4, and PPAR γ expression was increased and genes involved in a preadipocyte state, Pref-1 showed less expression compared to control. This data serves as a positive control to assess how effective the CM treatment is to the cultured HPADs.

3.4 | Characterization of Gelatin-FA hydrogel

Figure 3A(i) revealed the two characteristic peaks of FA (black) around 1600 cm^{-1}, which corresponds to the same peak in Gtn-FA (red) hydrogel confirming the successful conjugation of FA to gelatin. Further Raman spectrum also revealed the characteristic peak of FA in Gtn-FA polymer at 6.5 ppm (Figure 3A(ii). Gelation time was assessed via the vial tilting method. Figure 3A(iii) shows that the best gelation characteristics of Gtn-FA polymer (between 30 and 60 min) were achieved in the presence of lacasse. In this experiment, as evident from Figure 3B (with inset of pO_2 map) the oxygen levels drop steeply

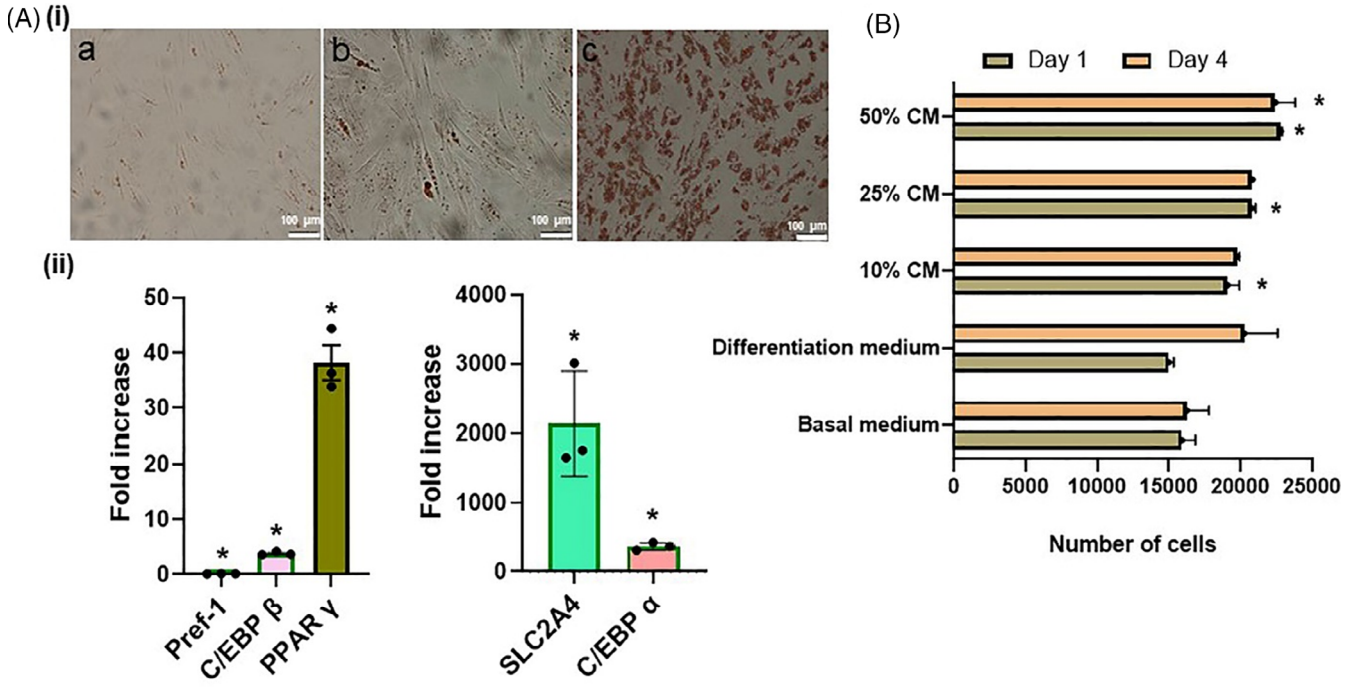

FIGURE 1 (A) Differentiation of HPADs: (i) Oil Red O stain was used to qualitatively assess the state of differentiation of HPADs under commercial differentiation medium after day 1 (a), day 7(b), and day 14 (c) (ii) Gene expression of fully differentiated HPADs. HPADs were given a differentiation medium to assess the gene expression. Statistical significance was established by comparison of the experimental group (differentiation medium) and the internal control (preadipocyte medium). (B) Cell Viability assessment of HPADs cultured with CM HPADs viability was assessed via alamarBlue for 7 days while supplementing with and without amniotic CM at various concentrations. An unpaired *t* test was performed to establish a significant difference between experimental groups and internal control (MEM). (* indicates $p < .05$ *p*-value)

from around 45-min. Further, after 2 h, greater than 60% hydrogel was hypoxic with less than 20 torr pO_2 as indicated in the graph and evident in the inset figure where dark blue color shows ≤20 torr.

The cytotoxicity of the Gtn-FA polymer demonstrated that there is no significant toxicity on adipocyte cells up to 25 mg/ml concentrations studied compared to control (Figure 4). The cytotoxicity of laccase increases as its concentration increases (Figure 4B). However, despite its toxicity in its free-form at higher experimental concentrations, the concentration will be greatly reduced once crosslinked to the Gtn-FA polymer.

3.5 | Synergistic role of hypoxia and amniotic growth factors in pre-adipocyte viability and differentiation

The cell viability assay (Figure 4B) suggests that after 7 days of culture CM supplementation can significantly increase the viability of HPADs cultured in both normal and hypoxic environments. On day 4, condition media supplementation assists in significantly higher cell viability under hypoxic conditions compared to normal culture. The hypoxic groups show much greater viability than control and normal conditions which could be caused by a synergistic effect between CM and a highly hypoxic environment.

Further gene expression analysis shows that PPARy had a higher relative expression at day 7 as cells were able to differentiate over time and were slightly suppressed under hypoxic conditions (Figure 5B). Also, VEGF-A had a much higher relative expression after 7 days of culture in hypoxic conditions supplemented with CM; this denotes that CM supplementation can trigger angiogenesis under hypoxia, unlike nontreated cells (Figure 5d) as suggested by Park et al.[25] Pref-1 remained relatively stable in the two groups denoting that there must have been a small number of cells that maintained their preadipocyte state, which is expected since cells were only cultured for 7 days. Lastly, SLC2A4 was expressed relatively higher in both normal and hypoxic groups after 7 days which could be explained by the fact that both hypoxia and CM supplementation can cause HPADs differentiation (Figure 5C).

To validate the relative mRNA results obtained a different method, where hypoxia was induced by blocking the degradation of HIF-1α using 100 µM of $CoCl_2$, was performed.[26] Results show that Pref-1expression was a several fold higher in the CM/HYP group compared to the other groups studied (Figure 6A). PPAR y expression was significantly elevated in the CM/HYP group compared to the other groups (13-fold), suggesting the CM supplementation and hypoxic condition caused differentiation to a higher degree compared to the other groups (Figure 6B). Both VEGF A and SLC2A4 were elevated in normal and hypoxic conditions with CM than hypoxic basal medium. A statistically significant elevated expression of SCL2A4 (Figure 6C) and VEGF-A (Figure 6D) was observed compared to control.

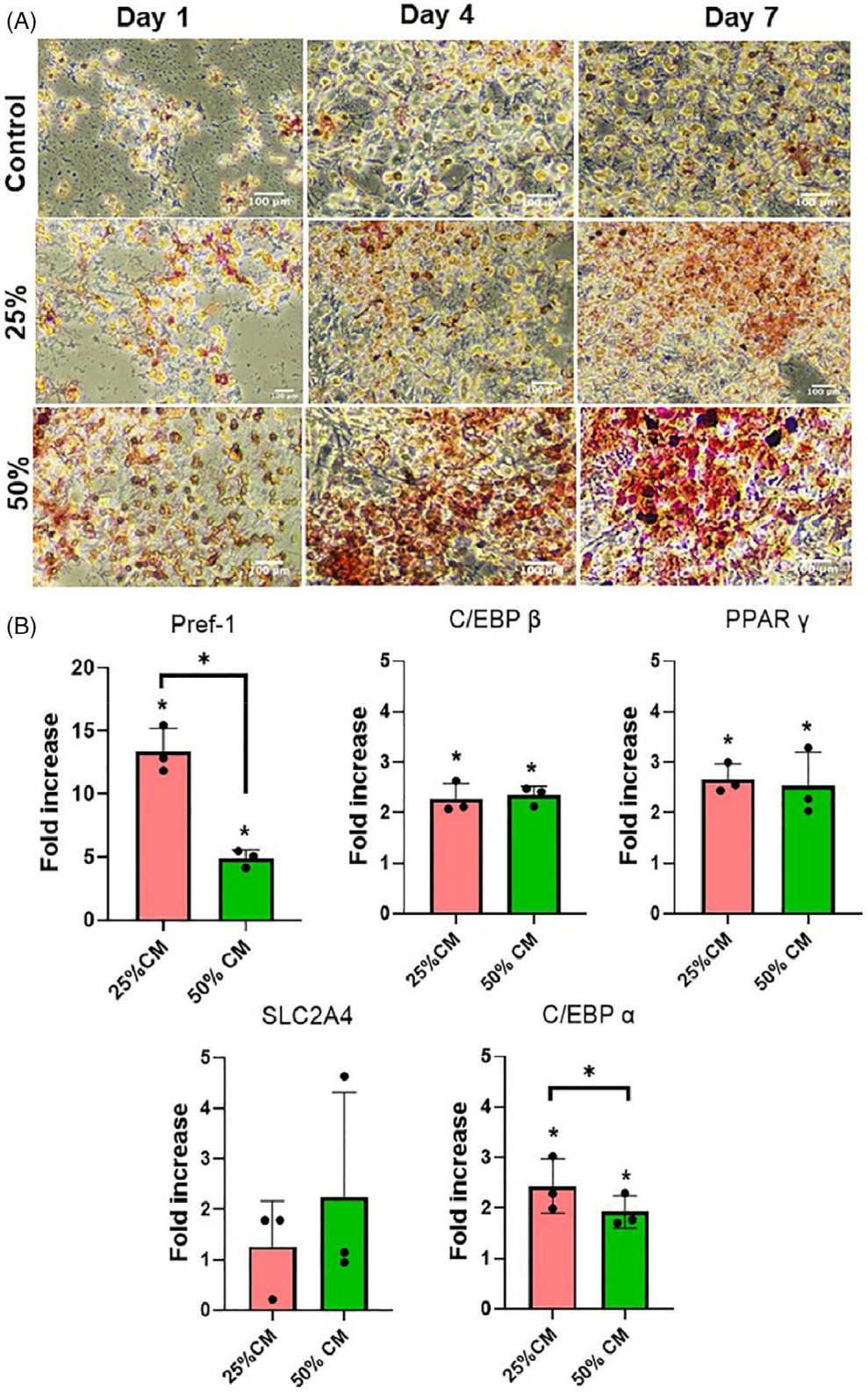

FIGURE 2 (A) Differentiation of HPADs after supplementation with CM Oil Red O stain was used to qualitatively assess the state of differentiation of HPADs under supplementation of amniotic growth factors (B) Gene expression analysis after CM supplementation. HPADs were treated with preadipocyte media growth media, 25% CM, and 50% CM, and RNA was collected after 7 days. Statistical significance was established between the comparison of the experimental group and internal control (preadipocyte medium) (* indicates $p < .05$ p-value)

4 | DISCUSSION

The use of cryopreserved placental membrane-conditioned media to supplement human preadipocytes is a novel combination that was hypothesized to enhance the viability of these cells and thus potentially translate to higher fat retention in a clinical setting. Due to this being a novel alternative method to aid in volume loss of fat grafting applications a "proof of concept" study was imperative. The type and concentration of growth factors in human placental tissue have been previously demonstrated by Irvin et al.[27] The growth

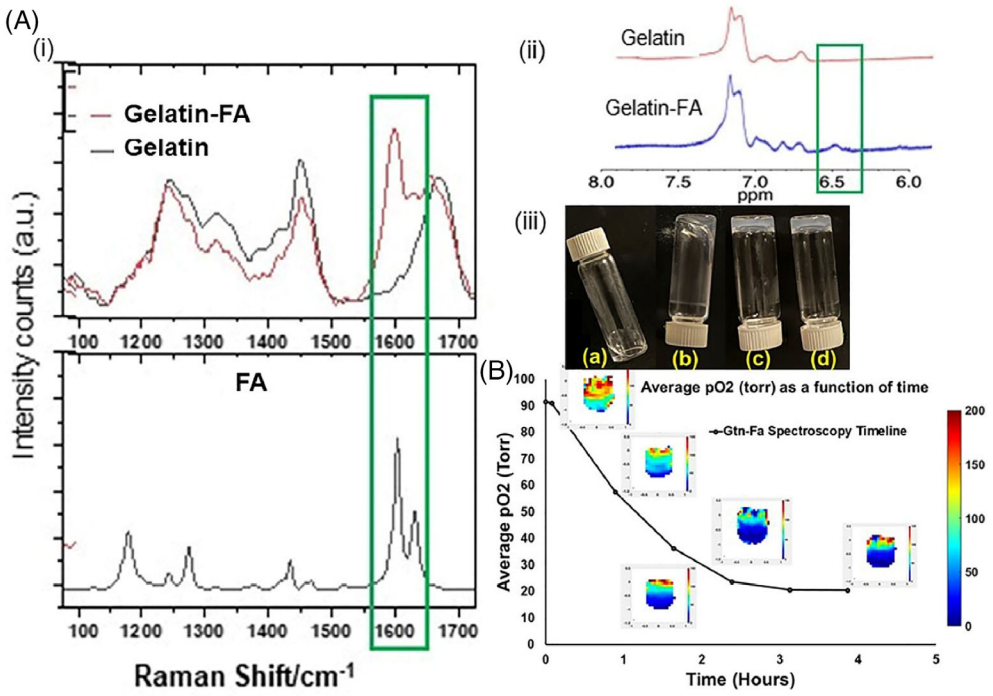

FIGURE 3 (A) (i) FTIR Spectrum of Gelatin, Ferulic acid (FA), and Gelatin-FA demonstrating the characteristic peaks of FA in Gelatin-FA hydrogel. (ii) Raman spectrum confirming the characteristic peak of FA in Gelatin-FA at 6.1–6.5 ppm compared with Gelatin (ii) showing the gelating of hydrogel after (a) 0, (b) 10, (c) 30, and (d) 60 min of incubation and 37°C. (B) Oxygen spectroscopy against timeline was demonstrated with corresponding oxygen images as a function of time in torr (0–200 torr)

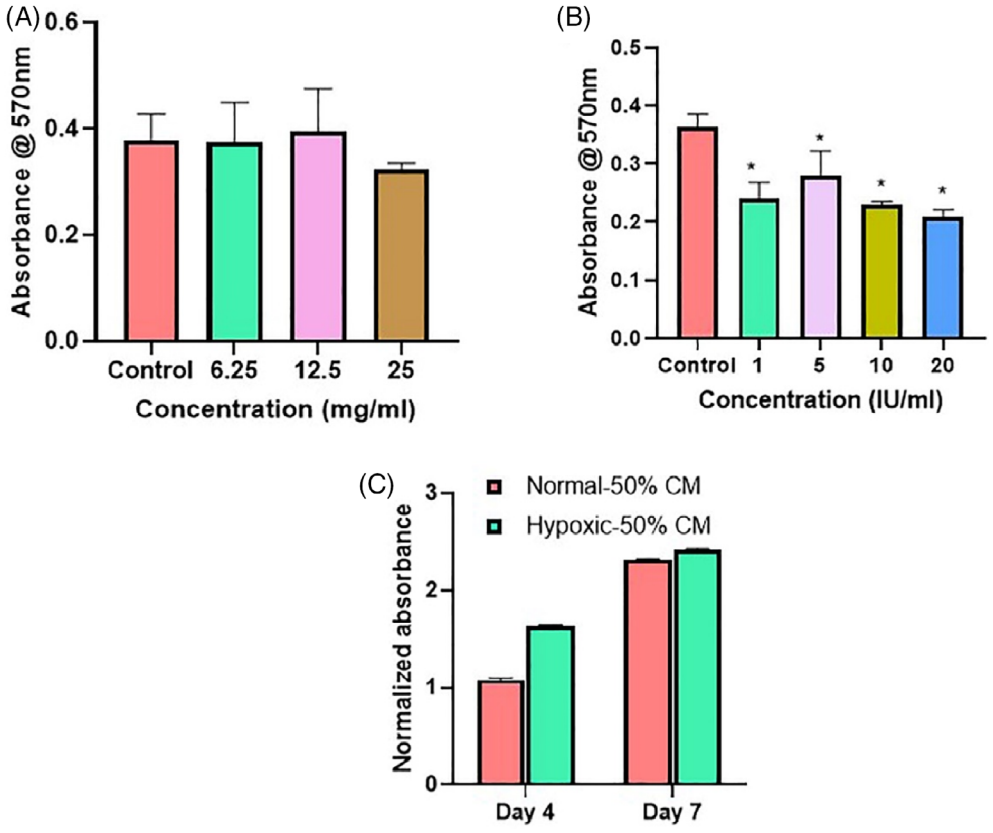

FIGURE 4 Cytotoxicity of (A) Gelatin-FA hydrogel and (B) Laccase after 24 incubation. (C) graph showing the normalized absorbance of HPADs under the normal and hypoxic conditions from the initial cell number in the presence of CM after 4 and 7 days of culture. (* indicates $p < .05$ p-value)

factors include PDGF, bFGF, EGF, KGF, PIGF, IL-4, TGF-β, VEGF, and TIMPs.[27] As hypothesized, the viability of HPADs was greatly increased when compared to control after 7 days of culture. After day 7 there were no marked differences between different conditions. It was established that the stagnation of cell proliferation after a week occurred as the cell population became 100% confluent. This is an established model in literature where after an exponential growth phase, cell division plateaus as the cell population becomes fully confluent.[28] Moreover, not only was the viability of HPADs increased after extended culture, but the number of mature adipocytes was also

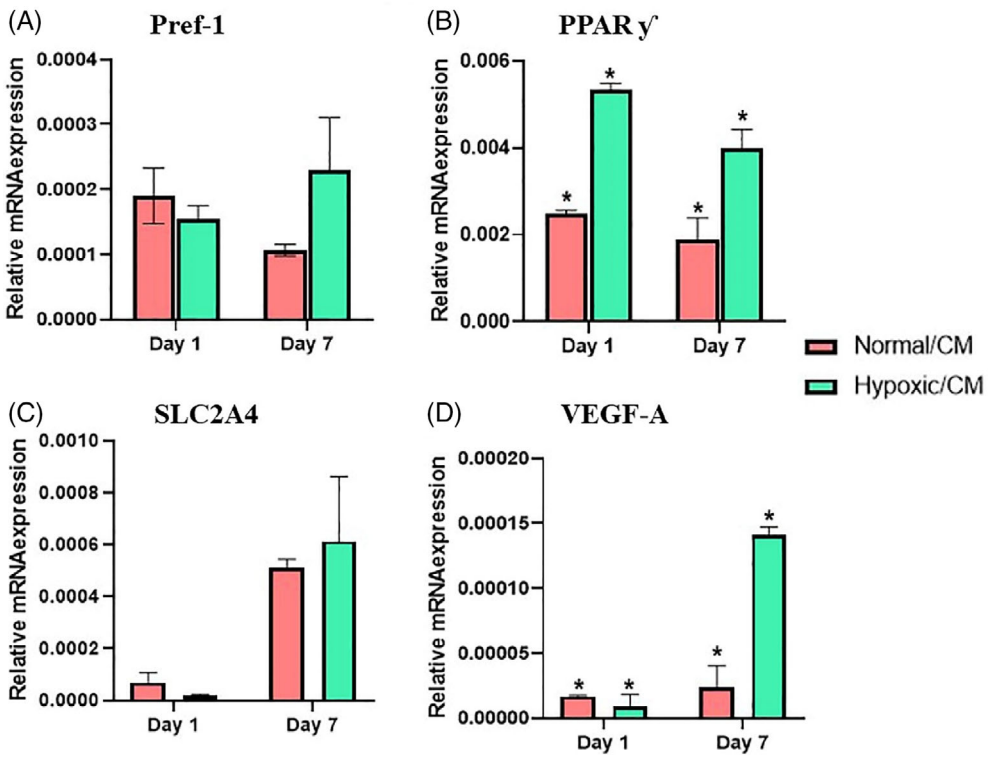

FIGURE 5 Graphs showing the relative mRNA expression of (A) Pref-1, (B) PPAR γ, (C) SLC2A4, and (D) VEGF-A by adipocytes after day 1 and 7 incubation in the presence of normal and hypoxic condition supplemented with CM

higher when compared to control via confirmation with Oil Red O staining (Figure 2A). Further, the gene expression study was performed to confirm the differentiation of pre-adipocytes under CM. The genes evaluated were Pref-1, C/EBP α, C/EBP β, PPAR γ, and SLC2A4. Pref-1 is the gatekeeper of adipogenesis.[29] As such, this key gene was chosen to quantify the number of adipocytes still in a preadipocyte state. Moreover, C/EBP α and β were also chosen to be analyzed as these leucine zipper transcription factors also serve a crucial role in adipocyte differentation.[30] From work by Cao et al.,[31] it was shown that C/EBP α and β work in conjunction in the early stages of adipogenesis. More specifically, C/EBP β is induced in the earlier stages and promptly trans-activates PPAR γ and C/EBP α later on as the adipocyte reaches a terminal mature adipocyte fate.[30] Lastly, SLC2A4 was chosen as a marker gene to assess the mature state of adipocytes as this gene is only found on the surface of mature adipose tissue, and it serves the purpose of facilitating the diffusion of circulating glucose down its concentration gradient into muscle and fat cell, the key functionality of adipose cells.[32]

From Figure 2B, it was clear that Pref-1 is expressed higher in the 25% CM treatment group compared to the 50% group. This would be expected since more adipocytes are in a preadipocyte state when supplemented with a lesser amount of amniotic CM. Further, the expression of SLC2A4 in the 50% CM treated group is upregulated, as is expected since more adipocytes have reached a mature adipocyte state. In the case of other target genes, C/EBP α and β, and PPAR γ, there is not much difference between 25% and 50% CM treated groups. Our positive control of the adipocytes treated with differentiated media showed almost no increase in Pref-1, but a multi-fold increase in key genes for differentiation as C/EBP β and PPAR γ as well as SLC2A4 demonstrating that the majority of adipocytes collected were fully mature and also functional.

To further understand their functional response, generating a hypoxic microenvironment is a key feature.[33] This is due to the clinical setting where a rapid accumulation of adipocytes injected into the recipient site creates zones where oxygen diffusion is absent, and adipocytes become necrotic.[34] The presence of hypoxia may be the leading cause of volume loss in fat grafting procedures. In high volume clinical grafting settings, reinjecting fat without carefully placing each tract as 1 mm aliquots very well-spaced out,[3] can prove to be extremely difficult. Although attempts have been made to try to negate the volume loss of adipocytes after a fat grafting procedure as previously discussed with cell-assisted lipotransfer and platelet-rich plasma,[35,36] these studies have not focused on studying the hypoxic microenvironment itself. The use of hypoxia-inducible hydrogels to mimic the hypoxic recipient site environment of a fat grafting procedure is a novel method to imitate clinically relevant conditions to study the effects of CM on cultured HPADs.

Through an adaption of previously published work on 0_2 controllable hydrogels, we have synthesized a hydrogel where HPADs can be encapsulated to study their response to placental membrane supplementation and a hypoxic environment.[18] Our results show that we were able to produce a hydrogel of similar characteristics to previously published work.[18] Moreover, EPROI[24] method provided a clear image of the oxygen gradient non-invasively. The gene expression data showed an interesting finding that CM combined with hypoxia exposure seemed to provide a synergistic effect as it relates to HPAD viability and increase in the expression of genes PPAR γ and VEGF-A (Figure 5). This was a fascinating finding as it is known that hypoxia or

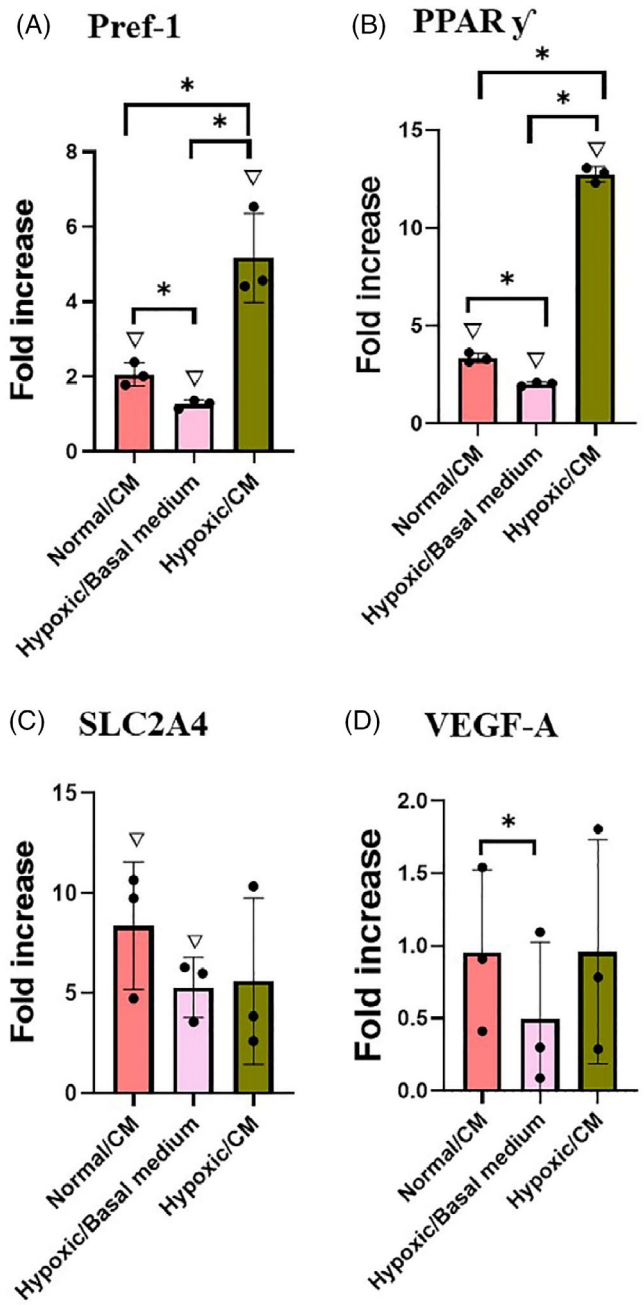

FIGURE 6 Gene expression analysis of adipocyte cells under $CoCl_2$ hypoxic conditions HPADs was seeded in a 24 well plate and after 24 h of normal culture 100 μM of $CoCl_2$ was given to induce hypoxia in two experimental groups. Cells were cultured for 7 days. Statistical significance was established by comparison of the treatment group to the control, which was preadipocyte medium (* = <.05 *p*-value)

any other stressful environment tends to induce a stress response to the cells.[37] In this case, HPADs seemed to react to the stress of hypoxia by differentiating and promoting angiogenesis. Even though CM supplementation alone also leads HPADs to react similarly, CM/HYP increases the viability and fold change of key gene markers significantly. We believe the finding is important given the hypoxia clinically present in the initial stages of fat grafting procedures. Thus supplementation of CM in a clinical setting could lead to very positive outcomes because adipocytes are encouraged to maintain a mature adipocyte state and promote angiogenesis to enhance survival as more oxygen would reach over time. In addition, it was observed that Pref-1 expression is a greater fold change in the CM/HYP group compared with the other groups indicating a higher viable pre-adipocyte population which is contradictory to what one would expect since the hypoxic conditions and CM addition should cause more of these pre-adipocytes to differentiate. However, this could be explained because CM and hypoxia leads to increased proliferation of the preadipocytes than when exposed to only normal media and CM. Moreover, the PPAR y expression in the CM/HYP group has a much greater fold change than the other groups (13-fold) as expected since the CM supplementation and hypoxic condition should cause differentiation to a higher degree than the other groups. The VEGF-A expression in the MEM/HYP group is expressed less fold as expected due to the lack of growth factors. Lastly, the SLC2A4 expression shows fold change across all groups is rather similar, which can be explained by the fact that there was a small amount of cell population that reached a degree of mature adipocyte state across all groups.

4.1 | Limitations and future scope

The focus of this study was to improve the viability of HPADs after exposure to a hypoxic microenvironment by encapsulating cells in a synthesized hypoxic hydrogel to mimic clinically relevant conditions. However, this study has certain limitations. In this study, pre-adipocytes were used to study the effects of amniotic supplementation; clinically mature adipocytes have been used in fat grafting procedures. However, the results of this study can still serve as a "proof of concept" to enable future experiments. The potential of angiogenesis by amniotic growth factors is explained based on the VEGF-A activation. However, further studies are required to confirm this effect. To overcome the limitations discussed previously, future aspects of this project are to perform ex-vivo experiments with isolated human adipocytes supplemented with amniotic membrane conditioned media. The current study only used HPADs in vitro to assess the effect of CM supplementation. To make the study even more clinically relevant, mature adipocytes should be used to show how these mature cells will react to hypoxia and CM supplementation. In addition, long-term studies under hypoxia using 3D printed scaffolds together with a bioreactor system would also provide an interesting perspective.

5 | CONCLUSIONS

Based on the results of this study, it can be concluded that Gtn-FA hydrogel crosslinked with laccase effectively produces a hypoxic environment as validated by EPROI. After exposure to a hypoxic environment, amniotic membrane supplementation significantly increased

viability and key gene markers for adipocyte differentiation and functionality of cultured preadipocytes.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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