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Human umbilical cord stem cell encapsulation in novel macroporous and injectable fibrin for muscle tissue engineering

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Abstract

There has been little research on the seeding of human umbilical cord mesenchymal stem cells (hUCMSCs) in three-dimensional scaffolds for muscle tissue engineering. The objectives of this study were: (i) to seed hUCMSCs in a fibrin hydrogel containing fast-degradable microbeads (dMBs) to create macropores to enhance cell viability; and (ii) to investigate the encapsulated cell proliferation and myogenic differentiation for muscle tissue engineering. Mass fractions of 0–80% of dMBs were tested, and 35% of dMBs in fibrin was shown to avoid fibrin shrinkage while creating macropores and promoting cell viability. This construct was referred to as “dMB35”. Fibrin without dMBs was termed “dMB0”. Microbead degradation created macropores in fibrin and improved cell viability. The percentage of live cells in dMB35 reached 91% at 16 days, higher than the 81% in dMB0 ($p < 0.05$). Live cell density in dMB35 was 1.6-fold that of dMB0 ($p < 0.05$). The encapsulated hUCMSCs proliferated, increasing the cell density by 2.6 times in dMB35 from 1 to 16 days. MTT activity for dMB35 was substantially higher than that for dMB0 at 16 days ($p < 0.05$). hUCMSCs in dMB35 had high gene expressions of myotube markers of myosin heavy chain 1 (MYH1) and alpha-actinin 3 (ACTN3). Elongated, multinucleated cells were formed with positive staining of myogenic specific proteins including myogenin, MYH, ACTN and actin alpha 1. Moreover, a significant increase in cell fusion was detected with myogenic induction. In conclusion, hUCMSCs were encapsulated in fibrin with degradable microbeads for the first time, achieving greatly enhanced cell viability and successful myogenic differentiation with formation of multinucleated myotubes. The injectable and macroporous fibrin–dMB–hUCMSC construct may be promising for muscle tissue engineering applications.

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Keywords

Fibrin hydrogel; Fast-degradable microbeads; Human umbilical cord mesenchymal stem; cells; Injectable cell delivery; Macroporous hydrogel

1. Introduction

Tissue engineering approaches offer a promising solution for many challenging diseases, such as critical-sized tissue and organ dysfunction [1,2]. Muscle tissue engineering is of crucial importance because muscle tissues comprise the largest part of the human body, including the critical organs or tissues such as heart and face. However, to date, much less attention has been paid to muscle engineering research, compared to bone and cartilage engineering [3]. Human umbilical cord mesenchymal stem cells (hUCMSCs) were recently shown to be promising for regenerative medicine applications [4,5]. Several studies investigated hUCMSCs for muscle engineering by using tissue culture plastic or injecting hUCMSCs in vivo without scaffolds [6,7]. A literature search revealed no report on hUCMSC encapsulation in three-dimensional (3-D) macroporous scaffolds for muscle tissue engineering, except our recent study [8].

Injectable scaffolds are promising to deliver progenitor cells for tissue engineering in a minimally invasive manner [9–12]. Several biodegradable hydrogels were investigated as cell delivery systems in the form of an injectable liquid/paste, such as fibrin, alginate, collagen, Matrigel and synthetic hydrogels [13–16]. Among them, fibrin, a polymer of fibrinogen, is a promising natural extracellular matrix (ECM) for cell delivery, allowing adequate cell attachment, spreading, migration and proliferation [13–15,17,18]. With these advantages, fibrin has been investigated for muscle tissue engineering applications [17,19,20].

However, fibrin gels have only a microporous structure, without macropores. For the repair of critical-sized defects, macroporous constructs can provide better nutrient and waste exchange and thus successful tissue regeneration [21–24]. Several methods have been used to fabricate porous scaffolds for tissue engineering, including solid freeform fabrication (SFF), freeze-drying, porogen leaching, gas-foaming and soft lithography [20,22,25–27]. Most of the techniques, however, only allow for fabrication of preformed scaffolds, which are not injectable. It is highly desirable to develop a macroporous and injectable fibrin. Recently, gelatin beads several hundred micrometers in diameter were used as a porogen for generating pores within cell-encapsulated alginate and agarose hydrogel [28,29]. Beads were formed by gelation of gelatin at a low temperature (4 °C), and their subsequent dissolution led to the creation of pores. However, low temperature fabrication could have an adverse effect on cell survival.

Our recent study developed novel fast-degradable microbeads (dMBs) which were composed of partially oxidized alginate and fibrin [30]. These dMBs had diameters of a few hundred micrometers suitable for injection. The dMBs started to degrade at 4 days in culture media, while the traditional alginate microbeads did not degrade in 4 weeks [30]. Alginate hydrogels have a good mechanical stiffness to maintain room for tissue regeneration [8,31,32]. However, when cells were encapsulated in alginate, their spreading was restricted and their survival was not satisfactory [8,32,33]. In the present study, fibrin was used as a matrix to encapsulate hUCMSCs, because fibrin could enhance cell spreading and survival [17,18,33]. To further improve cell functions, the present study incorporated dMBs into the fibrin matrix to create macropores.

Therefore, the objectives of this study were: (i) to encapsulate hUCMSCs in an injectable fibrin hydrogel containing dMBs to create macropores for the first time; and (ii) to investigate the encapsulated hUCMSC proliferation and myogenic differentiation for muscle tissue engineering. The following hypotheses were tested: (i) a macroporous fibrin construct with hUCMSC encapsulation will yield much better cell viability, proliferation and spreading than the usual fibrin control without macropores; and (ii) hUCMSCs inside the macroporous fibrin construct will differentiate into the myogenic lineage with elevated myogenic gene expressions and the formation of fused multinucleated myotubes.

2. Methods and materials

2.1. Expansion of hUCMSCs

The use of hUCMSCs was approved by the University of Maryland. hUCMSCs harvested from the Wharton's Jelly in umbilical cords of healthy babies (ScienCell, Carlsbad, CA) were expanded in growth medium composed of low-glucose Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Grand Island, NY), 15% hMSC screened fetal bovine serum (FBS) (Thermo Fisher, Logan, UT) and 1% penicillin/streptomycin/glutamine (Life Technologies), as previously described [34]. A recent study characterized the expression of surface markers of the hUCMSCs using flow cytometry [35]. The hUCMSCs were found to express high levels of adhesion markers (CD29 and CD44) and MSC-specific antigen CD105. The hUCMSCs were positive for HLA-class I (HLA-ABC) and negative for HLA-class II (HLA-DR), which are characteristic for MSCs. The hUCMSCs did not express endothelial marker (CD31) or hematopoietic lineage markers (CD34 and CD45). It was concluded that the hUCMSCs expressed surface markers characteristic of MSCs, and were negative for typical hematopoietic and endothelial cell markers [35]. Passage 6 hUCMSCs were used in the present study.

2.2. Fabrication of hUCMSC constructs

Bovine plasma fibrinogen (Type I-S, 65–85% protein; Sigma–Aldrich, St Louis, MO) was dissolved in calcium-free DMEM (Life Technologies) at 37 °C to obtain a final concentration of 10 mg ml⁻¹ [17]. hUCMSCs were suspended in the fibrinogen solution at a concentration of 4 × 10⁶ cells ml⁻¹. dMBs were made of 7.5% oxidized alginate (UP LVG, ProNova, FMC, Sandvika, Norway) and fibrin, as previously described [30]. dMBs were collected using a 40 µm nylon cell strainer (BD Falcon, Franklin Lakes, NJ), washed with sterilized ddH₂O three times and added into the fibrinogen solution. The dMB mass fractions in the fibrinogen solution were 0%, 20%, 35%, 50%, 65% and 80%. The resulting fibrin–dMB constructs were referred to as dMB0, dMB20, dMB35, dMB50, dMB65 and dMB80, respectively.

For dMB0, 150 mg of fibrinogen solution was pipetted into each well (9.8 mm in diameter) of 48-well tissue culture polystyrene (TCPS) plates (BD, Franklin Lakes, NJ). For the other constructs, 150 mg of dMB–fibrinogen solution was placed into the well, followed by a thorough mix. Gelation was initiated by adding 150 µl of CaCl₂ solution (40 mM, Sigma–Aldrich) containing thrombin (5 NIH units ml⁻¹, Sigma–Aldrich) [17]. The solution was mixed by gently shaking the molds, which were then left undisturbed to gel for 5 min at 37 °C. Fig. 1A and B shows schematically the fibrin construct without or with dMBs, respectively.

2.3. Thiazolyl blue tetrazolium bromide assay

The constructs were washed with DMEM and cultured in 1 ml of growth medium for 1, 4, 8 and 16 days. The medium was changed every 2 days. The viability of the encapsulated hUCMSCs in fibrin gel was assessed by thiazolyl blue tetrazolium bromide (MTT, Sigma–

Aldrich) absorption. Briefly, 900 μl of fresh growth medium and 90 μl of MTT solution (5 mg ml^{-1} stock in Dulbecco's phosphate-buffered saline) were added into each well and incubated for 4 h at 37 °C. The crystals formed were dissolved by using 1 ml of dimethyl sulfoxide (DMSO, Sigma–Aldrich) per disk. The absorbance of 100 μl of the solubilized product was analyzed using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA) at 570 nm.

2.4. Live/dead assay

The results of Section 2.3 showed that among all dMB-containing constructs, dMB35 showed the best cell proliferation rate. Therefore, dMB35 was selected for further cell experiments. dMB35 was tested using a live/dead assay (Life Technologies). The dMB0 construct, which used the usual fibrin without dMBs, served as a control. Constructs were incubated with Dulbecco's phosphate-buffered saline (D-PBS) containing 4 μM calcein AM and 8 μM EthD-1 at 37 °C for 45 min, washed with D-PBS and examined via a Zeiss LSM 510 Meta/Axioplan 2 laser scanning confocal microscope equipped with argon and HeNe lasers (Carl Zeiss, Thornwood, NY).

2.5. Cryo-SEM examination of construct microstructure

The dMB35 and dMB0 constructs were cultured in growth medium for 1, 4, 8 and 16 days. The microstructure of the constructs was determined using cryo-scanning electron microscopy (cryo- SEM), which can maintain the hydrogel structure in the natural state [36]. The samples were washed with D-PBS and frozen in liquid nitrogen. The samples were freeze-fractured and then the surface was etched by a vacuum at -100 °C for 30 min to remove the ice produced during freezing. The samples were examined immediately on a cryo-stage using SEM (Quanta 200, FEI, Hillsboro, OR) without coating treatment [37].

2.6. Myogenic differentiation

Because hUCMSCs in dMB35 had much better viability than dMB0, myogenic induction in dMB35 was performed following previous methods [6,38–40]. 5-Azacytidine (5-Aza), a DNA demethylating agent has previously been utilized to induce mature skeletal and cardiac myogenesis in vitro [6,38–40]. It was used in conjunction with horse serum (HS) to test the in vitro myogenesis of hUCMSCs encapsulated in dMB35 [6]. Briefly, after culturing in growth medium overnight for cell recovery and expansion, the constructs were cultured in myogenic inductive medium for 2 days. The myogenic inductive medium consisted of high-glucose DMEM, 20% FBS, 1% penicillin-streptomycin-glutamine and 10 μM 5-Aza (Sigma–Aldrich). The constructs were then cultured in the myogenic proliferative medium from 3 to 16 days. The myogenic proliferative medium consisted of high-glucose DMEM, 20% FBS, 1% penicillin-streptomycin-glutamine, 10% HS (Life Technologies) and 1% chick embryo extract (Accurate Chemicals, Westbury, NY) [6]. The medium was changed every 3 days.

2.7. Quantitative RT-PCR

Myogenic differentiation was induced in dMB35 and the expression of myogenic markers was evaluated by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR, 7900HT, Applied Biosystems, Foster City, CA) using the $2^{-\Delta\Delta C_t}$ method. This test included the myosin heavy chain 1 (MYH1) and alpha-actinin 3 (ACTN3). MYH1 and ACTN3 are two key genes associated with the differentiation of myotubes and were measured in previous studies [41]. Briefly, total RNA was extracted from homogenized constructs using the TRIzol reagent and the PureLink RNA Mini Kit (Life Technologies). Purified RNA was then reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). The PCR primers and TaqMan probes

(Taqman Gene Expression Assay Mix, Life Technologies) were as follows: MYH1 (Assay ID: Hs00947164_g1), ACTN3 (Assay ID: Hs00153812_m1), and 18S rRNA (used as reference gene; Assay ID: Hs99999901_s1). The final volume of each reaction mixture was 20 μ l, containing 3 μ l of cDNA template, 900 nM each primer, 250 nM TaqMan probe, 10 μ l of TaqMan Fast Universal PCR Master Mix with No AmpErase UNG (Life Technologies) and 6 μ l of diethylpyrocarbonate (DEPC, American Bioanalytical, Natick, MA)-treated water. hUCMSCs cultured on six-well TCPS in the growth medium for 1 day served as the calibrator sample [34].

2.8. Immunostaining

The purpose for immunostaining was to detect the hUCMSC expression of myogenic marker proteins and the morphological phenotype of cell fusion. The dMB35 constructs were collected at 1, 4, 8 and 16 days of myogenic induction and fixed using 4% paraformaldehyde (Sigma–Aldrich) overnight at 4 °C. The constructs were permeabilized with 0.15% Triton X-100 (Sigma–Aldrich). After blocking with 1% bovine serum albumin (BSA, Sigma–Aldrich) in PBT (0.2% Triton X-100 in PBS) for 2 h, the constructs were stained with the primary antibodies overnight at room temperature, including mouse anti-myogenin (1:80, clone F5D, BD Pharmingen, San Diego, CA), mouse anti-sarcomeric alpha-actinin (ACTN, 1:80, clone EA-53, Sigma–Aldrich), mouse anti-myosin heavy chain (MYH, 1:80, clone A4.1025, Millipore, Billerica, MA), and rabbit polyclonal anti-actin alpha 1 (ACTA1, 1:80, Novus, Littleton, CO). The samples were stained using Alexa Fluor 488 goat anti-mouse IgG (1:100, Life Technologies) or plus Alexa Fluor 594 goat anti-rabbit IgG (1:100) for double-labeling immunofluorescence staining for 4 h at room temperature. The constructs were washed with PBS and then stained with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI, 2 μ gml⁻¹ in PBS, Roche, Germany) as nuclear dye for 15 min. Immunofluorescence was detected using a Zeiss LSM 510 Meta/Axioplan 2 laser scanning confocal microscope (Carl Zeiss, Thornwood, NY). The fusion index F was used to quantify myoblast fusion and myotube formation, calculated as $F = N_F/N_T$, where N_F is the number of nuclei incorporated in the multinucleated cells which contain two or more nuclei, and N_T is the total number of nuclei in each image [42,43]. For calculation of the fusion index, the cell nuclei were visualized with DAPI staining.

For evaluation of significant effects of the variables, one-way and two-way ANOVA were performed, and Tukey's multiple comparison tests were used with a p value of 0.05.

3. Results

The fibrin constructs tend to shrink because of contractile forces within the construct [44]. When the mass fraction of dMB increased, the construct attachment to the bottom surface of the well weakened and the gel shrank. Therefore, the mass fraction of dMBs needed to be limited to avoid gel shrinkage. Fibrin gel shrinkage did not happen in dMB0, dMB20 and dMB35. Fig. 1C shows a representative image of dMB35 cultured in growth medium at 16 days. In contrast, shrinkage occurred in dMB50, dMB65 and dMB80. Fig. 1D shows an image of dMB80 with shrinkage at 16 days. Therefore, dMB mass fraction of 35% in fibrin was used to create macropores without fibrin shrinkage. Fig. 1E plots the MTT results. The data were normalized to MTT of dMB0 at 1 day. From 8 to 16 days, dMB35 showed the best MTT activity among all constructs ($p < 0.05$). For dMB35, the increasing trend of MTT at 16 days suggests a good cell proliferation and viability. Higher mass fractions (50%, 65% and 80%) of dMBs in fibrin lowered the cell viability, likely because there was not enough fibrin matrix with limited living space for the cells, as well as gel shrinkage, which could harm cell growth. These results show that 35% of dMBs in fibrin was the best to promote cell viability.

Fig. 2 shows representative live/dead images for dMB0 (A–C) and dMB35 (D–F) with examples at 1 day and 8 days. At 1 day, cells had a healthy spreading morphology in dMB35, compared to rounded cells in dMB0. At 8 days, there were noticeably more live cells in dMB35 due to cell proliferation, than in dMB0. In C and F, the dead cells were stained as red dots and there were relatively few dead cells in both dMB0 and dMB35.

Fig. 3 plots the percentage of live cells (A), and the live cell density (B). The percentage of live cells in dMB35 increased from 85% to 91% at 16 days. In dMB0, the live cell percentage decreased from 1 to 8 days and then remained nearly the same from 8 days (79%) to 16 days (81%). dMB35 had higher percentages of live cells than dMB0 at 4, 8 and 16 days ($p < 0.05$). In dMB35, cells proliferated and the live cell density was 1.6-fold that of dMB0 at 16 days.

Cryo-SEM was used to determine the dMB35 and dMB0 constructs (Fig. 4). Neither constructs had macropores at 1 day and 4 days. However, macropores were created in dMB35 with the degradation of the dMBs. Representative macropores “P” are shown in Fig. 4A and B for dMB35 at 8 days and 16 days, respectively. In contrast, dMB0 had no macropores from 1 day to 16 days, with an example shown in Fig. 4C at 16 days. dMB0 had a mesh structure which is typical for fibrin without macropores. In addition, the microstructure of the fibrin matrix was examined at higher magnifications, with an example shown in Fig. 4D for dMB35 at 4 days. The fibrin matrix consisted of a network of fibrin fibers, with arrows indicating examples of thick fibers and thin fibers. Similar microstructures were observed in the fibrin matrices for both dMB0 and dMB35.

The gene expressions of myogenic differentiation were determined using RT-PCR for dMB35 in Fig. 5. Both MYH1 and ACTN3 expressions were up-regulated. MYH1 expression at 16 days was significantly ($p < 0.05$) higher than that of the control at 1 day, which was set at a value of 1. The ACTN expression peaked at 16 days, and the ACTN values at 8 days and 16 days were significantly higher than that of the control at 1 day ($p < 0.05$). These results indicate that the hUCMSCs encapsulated in dMB35 successfully differentiated into the myogenic lineage.

Myogenesis was further examined using immunostaining (Fig. 6). In Fig. 6A–C at 8 days, more than 90% of the cells were stained positive for myogenin (green), an early marker of muscle differentiation [45]. At 16 days, MYH (green) staining (Fig. 6D–F), and double staining of ACTN (green) and ACTA1 (red) (Fig. 6G–I) were detected. More than 90% of the cells were positive for MYH and ACTN, and ~50% of the cells were positive for ACTA1 staining. Fused cells, with each cell having two or more nuclei (blue), were observed with the specific myogenic markers, as indicated by the arrows in Fig. 6C, F and I. In Fig. 6I, a typical example of a myotube was shown containing three nuclei, and it expressed muscle specific proteins of ACTN and ACTA1. Both the number and the size of the multinucleated myotubes increased with time. As plotted in Fig. 6J, the fusion index increased with the myogenesis time ($p < 0.05$). These results demonstrated the successful myogenesis of the encapsulated hUCMSCs in the macroporous fibrin construct.

4. Discussion

hUCMSCs are advantageous because umbilical cords can be collected at a low cost to provide an inexhaustible stem cell source, without an invasive procedure, which is required for bone marrow MSCs. The present study developed hUCMSC-encapsulating macroporous fibrin construct for the first time, yielding excellent cell viability, proliferation and myogenic differentiation. Comparing with the usual method of cell encapsulation in fibrin without macropores, the novel macroporous fibrin–microbead method greatly increased cell viability. The live cell density of dMB35 at 16 days was increased by 60% compared to that

without macropores. High cell density not only indicates good survival but also promotes cell fusion during myogenesis [46]. The percentage of live cells in dMB35 reached 91% at 16 days. In a previous study, myoblasts were seeded in a macroporous alginate scaffold and growth factors were added to enhance cell viability for muscle tissue engineering [33]. The percentage of live cells in alginate hydrogel was ~60% [33]. In another study, cold gelatin microbeads were used as a porogen in alginate hydrogel and the live cell percentage was 67–84% [28]. In a recent study, hUCMSCs were seeded in alginate–fibrin microbeads, and the microbeads were then packed into a RGD-modified alginate matrix and subjected to myogenic induction [8]. However, the released cells from the degradable microbeads did not survive well inside the alginate matrix, with the percentage of live cells being only 67% or less [8]. Therefore, the present study developed a novel macroporous fibrin matrix, which greatly enhanced the survival of the encapsulated cells, yielding a percentage of live cells of 91% for dMB35 at 16 days. Furthermore, the hUCMSCs encapsulated in dMB35 were successfully induced to form myotubes in the myogenic medium for 16 days. The formation of myotubes was supported by the following evidence: (1) The hUCMSCs fused into multinucleated cells and the fusion index substantially increased with culture time; and (2) the cells expressed muscle specific proteins, including MYH, ACTN and ACTA1. These two criteria were similarly used to verify the formation of myotubes using bone marrow MSCs in previous studies [39,47].

The good cell viability was likely because the dMBs in the fibrin could start to degrade as early as 4 days [30]. Macropores were formed in fibrin containing hUCMSCs. Macropores not only enhanced nutrient/waste exchange, but also provided enough space for cell spreading, migration and growth. In addition, it has been demonstrated that cells along the edge of the hydrogel, and not those in the bulk of hydrogel matrix, exhibited superior proliferative activities, which were termed the “edge flourish” (EF) phenomenon [29,48]. Previous studies attributed this as a biomechanical response between hydrogel and the encapsulated cell colonies based on real-time microscopy examination, finite-element modeling (FEM) analysis and multiple-particle tracking assay [29,48]. These studies showed that the EF phenomenon was induced by the oriented outgrowth of encapsulated cells located at the edge of the hydrogel. The outgrowth of cells subsequently caused significant surface tension at the interface of the hydrogel and medium, which then contributed to the dynamic outgrowth of cells from the hydrogel bulk to the surface [48]. In the present study, the macropores in the fibrin created a large number of fibrin–culture medium interfaces. Consistent with the EF phenomenon, the encapsulated cells would tend to migrate into the macropores and attach at the fibrin–culture medium interface with enhanced cell viability. Hence, macroporous hydrogels with stem cell encapsulation is expected to have a higher tissue regeneration efficacy than non-macroporous counterparts. The novel macroporous fibrin–dMB–hUCMSC construct is not only promising for muscle tissue engineering, but may be also applicable to other tissue engineering applications for soft and hard tissues.

Several previous studies investigated the effects of fibrinogen and thrombin concentrations on the behavior of encapsulated cells. Fibrinogen solutions at concentrations of 5–50 mg ml⁻¹ were shown to play an important role in cell growth [49], with dilute fibrinogen solutions (5–10 mg ml⁻¹) yielding better cell proliferation [17,49]. Thrombin might inhibit myogenesis [50], hence a lower concentration of thrombin is preferred in muscle tissue engineering constructs. Therefore, the present study selected relatively low concentrations of fibrinogen (10 mg ml⁻¹) and thrombin (5 U ml⁻¹), following a recent study investigating fibrinogen for muscle tissue engineering [17]. Fibrin scaffolds made from bovine fibrinogen were used in the present pilot study to prove the concept, because bovine fibrinogen was much less expensive than human fibrinogen in commercial sources. In addition, the use of bovine fibrinogen was consistent with previous studies [51,52]. However, further study

should apply the novel macroporous fibrin scaffold in muscle defect repair using human fibrinogen instead of bovine fibrinogen.

Another important parameter in myogenic tissue engineering is the cell seeding density. A low cell seeding density may adversely affect myogenesis due to the lack of cell–cell interactions, while a higher cell seeding density can help promote cell fusion to form myotubes [46]. However, if the cell density is too high, the cell spreading could be suppressed by contact inhibition. Cell spreading is important for myogenic differentiation [53]. Previous studies using fibrin for muscle tissue engineering investigated a wide range of cell seeding density, from 2.5×10^5 to 1.5×10^7 cells ml^{-1} [13,17]. In order to determine a suitable cell seeding density, the present study tested several cell seeding densities in preliminary experiments, ranging from 1×10^6 to 1×10^7 cells ml^{-1} . The preliminary results suggested that 4×10^6 cells ml^{-1} was a suitable cell seeding density, which yielded an appropriate cell density and distribution in the hydrogel constructs, and avoided the cell density being too low, while achieving a healthy cell spreading morphology. Therefore, a seeding density of 4×10^6 cells ml^{-1} was used in the present study.

One drawback of fibrin is that it tends to shrink. If a scaffold shrinks, it cannot maintain adequate room for defect repair. In addition, the growth and spreading of the encapsulated cells are restricted. The prevention of fibrin shrinkage is therefore essential for tissue engineering [44]. Poly-L-lysine had been used in the culture well to fix the gel [44]. In the present study, TCPS was used to enhance gel fixation. The addition of dMBs weakened the gel fixation due to the reduced contact area between fibrin and TCPS. To prevent shrinkage, 35% mass fraction of dMBs in fibrin gel was suitable to maximize the macropore formation, without fibrin shrinkage. For in vivo usage of fibrin, attempts have been made to develop long-term stable fibrin gel to prevent this disadvantage [54]. While the novel macroporous fibrin-microbead construct was advantageous for hUCMSC viability and proliferation without shrinkage at 35% dMBs, further study needs to evaluate the shape stability of the macroporous fibrin construct in vivo.

The degradation products from tissue engineering scaffolds should not adversely affect the cells [55]. In the present study, dMBs were made of oxidized alginate and fibrin, both of which are biologically safe and have been shown to not produce harmful degradation products [56]. Previous studies showed that cells survived well when encapsulated in dMBs [30], indicating that the degradation products had no adverse effect on cell viability. This is consistent with the present study, which showed that the cell viability increased in dMB35 with increasing culture time, while the dMBs degraded and created macropores in the fibrin matrix.

Regarding the degradation of the constructs, it has been reported that the degradation time for fibrin constructs ranged from several days to several weeks in vivo, and from 3 to 5 weeks in vitro [57,58]. The difference in degradation time may result from the different degrees of fibrin crosslinking. Calcium chloride contributes to the crosslinking and subsequent stability of fibrin gel. Furthermore, the addition of protease inhibitors, such as aprotinin, can decrease the degradation rate. In the present study, no obvious fibrin degradation was observed in dMB0 and dMB35 during the 16 days of culture. Cells were observed to grow in a 3-D manner inside the construct. Cells were not observed to grow on a two-dimensional (2-D) surface for both types of constructs during the 16 day period. Further study is needed using longer culture times to determine when fibrin degrades fully, and if the cells will be growing on 2-D or synthesize their own 3-D matrix, both in vitro and in vivo.

Hydrogel–cell constructs without macropores were previously used for muscle tissue engineering. The commonly used hydrogels included fibrin and collagen [17,19,59,60]. Guo et al. incorporated bone-marrow-derived cardiac stem cells (MCSCs) in fibrin hydrogel for treatment of rat myocardial infarction [17]. They demonstrated that fibrin provided a good microenvironment for cell survival and migration and promoted the cardiomyogenic differentiation of MCSCs. Other studies investigated hydrogel–cell constructs with macropores due to their superiority for mass transport [33]. Macroporous alginate fabricated using wire porogens greatly promoted both the viability and migration of seeded myoblasts compared to micro- or nanoporous alginate scaffolds [33]. In the present study, hUCMSCs encapsulated in the novel macroporous fibrin– dMB construct were successfully differentiated into multinucleated myotubes. The results of myogenic differentiation suggested that the injectable macroporous fibrin fabricated by the addition of dMBs may be promising for muscle tissue engineering. Further studies are required to evaluate its potential in animal models.

5. Conclusions

hUCMSCs were encapsulated in macroporous fibrin–microbead constructs for muscle tissue engineering for the first time, yielding excellent cell viability, proliferation and myogenic differentiation. The fast-degradable microbeads in the fibrin hydrogel matrix successfully created macropores which substantially enhanced the cell functions, compared to the usual method of cell encapsulation in fibrin without the microbeads. When seeded in the novel macroporous fibrin construct, the hUCMSCs differentiated into the myogenic lineage and formed multinucleated myotubes. The injectable and macroporous fibrin–microbead–hUCMSC construct is promising for muscle tissue engineering. The method of incorporation of fast-degradable microbeads into hydrogels along with stem cells may have a wide applicability to other tissue engineering applications.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 1–6, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at <http://dx.doi.org/10.1016/j.actbio.2012.08.009>.

Fig. 1. (A, B) Schematic of a fibrin construct and a degradable microbead (dMB)-containing fibrin (fibrin-dMB) construct. (C, D) Representative images of dMB35 and dMB80 cultured in growth medium at 16 days. Shrinkage did not happen in dMB0, dMB20 and dMB35 over 16 days, similar to (C). In contrast, shrinkage was observed in some dMB50 and dMB65 constructs, and most dMB80 constructs at 16 days, similar to (D). (E) Relative thiazolyl blue tetrazolium bromide (MTT) absorbance of the encapsulated stem cells. For each experiment, all data were normalized to the MTT absorbance of dMB0 at 1 day (mean $\pm$ SD; $n = 5$).

Fig. 2. Confocal microscope images of live and dead assay of hUCMSCs in dMB0 and dMB35. Live cells were stained green and dead cells were stained red. Representative images were shown at 1 day and 8 days as examples. Cells were primarily alive. Dead cells were relatively few, with examples shown in (C) and (F).

Fig. 3. Quantification of the percentages of live cells (A) and live cell density (B) in dMB0 and dMB35. Five randomly chosen fluorescence images for each sample were analyzed for four constructs, yielding 20 images per group. Each value is mean $\pm$ SD; $n = 4$.

Fig. 4.

Representative cryo-SEM images of fibrin-based constructs. dMB0 and dMB35 without cells were cultured in growth medium for up to 16 days. (A, B) Macropores were formed in dMB35 as indicated by “P”. In contrast, dMB0 had no macropores, with an example shown in (C) at 16 days. In (D), the fibrin matrix consisting of a network of fibrin fibers includes thick fibers and thin fibers. The fibrin matrices for both dMB0 and dMB35 had similar microstructures.

Fig. 5.

Gene expression assay of myogenic differentiation in dMB35 using real-time RT-PCR. Myosin heavy chain 1 (MYH1) and alpha-actinin 3 (ACTN3) are two key genes associated with the differentiation of muscle fibers. Both (A) MYH1 and (B) ACTN3 expression were significantly up-regulated. hUCMSCs cultured on tissue culture polystyrene (TCPS) in the growth media for 1 day served as calibrator with a value set at 1. Data were reported as mean $\pm$ SD ($n = 4$). The results indicated that hUCMSCs in the dMB35 differentiated into the myogenic lineage.

Fig. 6. (A–I) Immunostaining of dMB35 treated with myogenic medium culture using confocal microscopy. Cells were positive for myogenin (green) staining at 8 days (A–C), myosin heavy chain (MYH; green) staining (D–F), and double staining of sarcomeric alpha-actinin (ACTN; green) and actin alpha 1 (ACTA1; red) (G–I) at 16 days. Nuclei were stained with DAPI (blue). Arrows indicates the multiple nuclei. In (I), an example of a myotube is shown with three nuclei, expressing muscle specific proteins ACTN and ACTA1. (J) The fusion index (mean $\pm$ SD; $n = 4$) increased with time. Values with dissimilar letters are different ($p < 0.05$).