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Original Article

Acceleration of diabetic wound healing by a cryopreserved living dermal substitute created by micronized amnion seeded with fibroblasts

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Abstract: Bioengineered dermal substitutes have been used for the treatment of diabetic ulcers in clinics and achieved satisfactory results. However, constructing traditional tissue engineered dermal substitutes with two-step method is high-cost, time-consuming and greatly decreases fibroblast proliferative activity because of repeated trypsinization. Inthisstudy, we created a 3D micronized amniotic membrane (mAM) and used it as a natural microcarrier for ex vivo culture and amplification of human dermal fibroblasts (HDF) combined with the rotary cell culture system (RCCS). This one-step mAM-RCCS method couldamplify HDF quickly and construct a dermal substitute HDF-mAM simultaneously. To facilitate the clinical application of mAM-RCCS, anoptimized storage method was used. Post-thawing HDF-mAM retained high cell viability, intact cell morphology and active peptide secretion. When transplanted to the wounds of db/db mice, cryopreserved HDF-mAM promoted vascularization and diabetic wound healing significantly. These results demonstrate the potential application of cryopreserved HDF-mAM as a living dermal substitutefor treating diabetic ulcers and other chronic wounds in clinics.

Keywords: Amniotic membrane, microcarrier, fibroblasts, dermal substitute, cryopreservation, diabetes, wound healing

Introduction

One of the most common and serious complications of diabetes is diabetic foot ulcer. Approximately 25% of diabetic patients suffer from diabetic lower-extremity ulcer throughout their lives [1]. These wounds often heal slowly and reoccur frequently, there by increasing the risk for infection and the need for amputation. At present, moist dressings, debridement, infection control, and wound offloading are standard therapies to treat diabetic ulcers. Even with those conservative treatments, these wounds are still notably slow to heal, and 7%-20% of patients will subsequently need an amputation despite standard care treatment [2]. Exploiting new treatment for diabetic woundsis urgently needed to prevent foot ulcers from leading to amputation.

Advanced therapies, such as bioengineered skin grafts, have been shown to accelerate wound closure, thereby resulting in a more consistent and faster diabetic ulcer healing compared with standard treatments [3, 4]. Until now, Dermagraft (Organogenesis, Inc., Canton, Massachusetts, USA) is the only bioengineered dermal substitute approved by the US Food and Drug Administration for diabetic foot ulcer treatment. It is a sterile, cryopreserved, and human fibroblast-derived dermal substitute generated by seeding neonatal dermal fibroblasts onto a bioabsorbable polyglactin mesh scaffold.Although Dermagrafthas been used for the treatment of diabetic ulcers in clinics and achieved satisfactory results, its inherent shortcomings limit its widespread application [5]. As with other traditional tissue engineered-skin substitutes, Dermagraft is constructed intwo steps: First, human fibroblasts are amplified in a 2D environment, such as in a culture plate or flask. Then, they are seededto apolyglactin mesh scaffold. Fibroblasts are likely to age because of repeated trypsinization, thereby

decreasing their proliferative activity. In addition, a long period of ex vivo construction makes obtaining fibroblasts in time for transplantation impossible. Optimizing construction processes and reducing construction costsare critically needed to develop novel skin substitutes for treating diabetic ulcers.

In our previous study, we found that micronized amniotic membrane (mAM) possessed an intact basement membrane structure and bioactivities. Compared with traditional 2D culture, bioengineered Mam provided an ideal microenvironment and a large surface area for cell adhesion and growth, and amplified epidermal stem cells quickly in combination with rotary cell culture system (RCCS). Along with good biocompatibility, mAMcan beused as anideal dermal scaffold in constructing a skin substitutethat contain sepidermal stem cells for repair of full-thickness skin defects [6]. On the basis of this previous study, we continued to create a 3D mAM and used it as a natural microcarrier for ex vivo culture and amplification of human dermal fibroblasts (HDF) in combination with RCCS. We hope to use this one-step mAM-RCCS method to amplify HDF quickly and construct a new dermal substitute HDF-mAM simultaneously. The efficacy of amplification was evaluated. To meetclinical translational requirements, we cryo preserved HDF-mAM, analyzed the post-thawing viability and structure of HDF-mAM, and detected secretion of active peptides. Post-thawing HDF-mAM was further transplanted to the wounds of db/db mice to evaluate the feasibility of using it as a living dermal substitute to repair full-thickness skin defects.

Materials and methods

Preparation of mAM

All the study protocols were approved by the Ethics Committee of Changhai Hospital, Shanghai, China. mAM wasprepared as described in our previous study [6]. Briefly, placenta tissues (10 fetus: 5 males, 5 females) were acquired from parturients who under went cesarean section after obtaining informed consent; and all donors were serologically negative for HIV, HBV, HCV, and syphilis. We separated the amniotic membrane from the underlying chorion by blunt dissection. All cells on the amniotic membrane, including epithelial cells and fibroblasts, were removed by repeated freezing and thawing in combination with DNase (1 mg/ml, Gibco, USA) digestion. Then, amniotic membrane patches were homogenized into microparticles with a macrohomogenizer (Tuohejidian, Shanghai, China), freeze-dried under a closed-vacuum condition, and finally filtrated through a metal mesh filter (Yibaiju, China) to obtain 300-600 μm microparticles.

Isolation of human fibroblasts

Neonatal human foreskin fibroblast was isolated after obtaining informed consent and cultured for fibroblasts as described previously [7]. Briefly, the circumcised foreskin was digestedovernight in 0.25% dispase (Gibco, Life Technologies) at 4°C to separate the dermis, which was then digestedin 1% collagenase (Gibco, Life Technologies) for 1 h at 37°C, filtrated, and centrifuged to collect cells. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (10% FBS) in a humidified atmosphere of 5% CO_2 and 95% air.

Culture of human fibroblasts on acellular amniotic membrane

Cell culture dish (CCD) (Corning, Corning, NY, USA) was separated into two equal parts. One half was covered with acellular amniotic membrane, and the other half was used as control. Acellular amniotic membrane was stabilized with a stainless steel ring. Passage 3 human fibroblasts were seeded onto CCD at 2×10^4 cells/cm^2 andcultured with 10% FBS. Cell morphology and proliferation were observed under a light microscope (Leica, Germany) after 24, 48, 72, and 96 h, and photomicrographs were taken.

Amplification of fibroblasts and construction of HDF-mAM

A total of 10 ml 5×10^4 cells/ml HDF and 100 mg mAM were added to the RCCS (Synthecon, USA) and cultured in a 37°C 5% CO_2 incubator with the revolution rate controlled at 15-20 r/min, and 50% of the medium was changed every other day. The sample was harvested regularly and stained with Hoechst 33342 (0.5 μg/ml, Beyotime, China), and observed under a fluorescent microscope for adhesion and proliferation of the fibroblasts.

To compare the proliferation of fibroblasts with the conventional 2D plate culture, a 12-well plate was used as control. Each well was added with 1 ml fibroblast suspension (5×10^4 cells/ml). Cell proliferation was measured at days 1, 3, 5, 7, 9, 11, and 14 with the CCK-8 kit (Beyotime, China). Briefly, 1 ml HDF-mAM was drawn from the RCCS and placed in the 12-well plate. Each well was added with 100 μl CCK-8 solution and cultured for 3 h, from which 100 μl was transferred to a new 96-well plate to measure absorbance at 450 nm with a multi-plate reader (Biotek, USA) for comparison of relative cell viability.

Cryopreservation and resuscitation of HDF-mAM

HDF-mAM constructed with mAM-RCCS method was harvested at day 9, resuspended with STEM-CELLBANKER™ (Zenoaq, Fukushima, Japan), and frozen ina cryotube in aliquid nitrogen tank for 6 months. During thawing, samples were removed from liquid nitrogen and immediately placed into a 37°C water bath until they were thawed almost completely. HDF-mAM was added to RCCS and cultured as described above for further analysis.

Cell viability assay

Cell viability of fibroblasts in HDF-mAM was determined by using a fluorescent-based live/dead assay (Invitrogen Detection Technologies, USA) immediately after thawing following the manufacturer's instructions. Viable cells were stained with green fluorescent dye calcein AM and dead cells with red fluorescent dye Ethidium-1. The total number of dead and live cells was counted under a fluorescent microscope (Leica, Germany) and percentage of viable cells was calculated by dividing the number of live cells with the total number of cells in the same field. Non-cryopreserved HDF-mAM was used as control.

Histological and scanning electronic microscopy (SEM) observation of HDF-mAM

The HDF-mAM surface with attached fibroblasts was immediately visualized afterthawing. Some HDF-mAM were routinely fixed, dehydrated, paraffin waxed, and sliced, and hematoxylin and eosin (HE) (Yuanye, China) were stained for microscopic observation. The remaining HDF-mAM were fixed, dehydrated, and observed under a field emission scanning electron microscope (SEM) (QUANTA FEG 450, USA). Non-cryopreserved HDF-mAM was used as control.

Detection of active peptide secretion from HDF-mAM

After thawing and continuousculturing with RCCS at days 1 and 3, HDF-mAM was washed with PBS and continued to culture for 24 h by addition of theserum-free medium. The supernatant was centrifuged and stored at -20°C. IL-6, IL-8, TGF-β1, and bFGF were determined by using an ELISA reagent kit (R&D Systems, USA) according to the manufacturer's instruction. Laminin and hyaluronicacid were detected by using an RIA reagent kit (Biotechnology Center of Shanghai Institute of Navy Medical Sciences, China) following the manufacturer's instruction. Supernatant of non-cryopreserved HDF-mAM was used as control.

Transplantation of cryopreserved HDF-mAM to full-thickness skin defects

All animal procedures were performed according tothe guidelines of the Institutional Animal Careand Use Committee of the Second Military Medical University, Shanghai, China. The male db/db mouse (C57BL/KsJ, leptin receptor-deficient diabetes, 8-12 weeks, purchased from SlacLaboratory Animal Co. Ltd, Shanghai, China) was a well-established type 2 diabetic animal model with continuous hyperinsulinemia and high-plasma glucose levels. The criterion for inclusion was a blood glucose level of more than 300 mg/dl. The mice were anesthetized with 1% sodium pentobarbital intraperitoneally. Two full-thickness splinted wounds (10 mm diameter) were created on the backs of the miceas previously described [8]. The mice were randomizedequally into three groups: cryopreserved HDF-mAM group, cryopreserved mAM group, and blank group. After thawing and culturing with RCCS overnight, the cryopreserved HDF-mAM was smeared on the wound surface of the corresponding groups; the outer layer was covered with vaseline gauze and sewed up intermittently with a 4-0 suture (Jinhuan, China). Animal behavior and bandage integrity were monitored throughout the experiment. Photographs were taken regularly from a

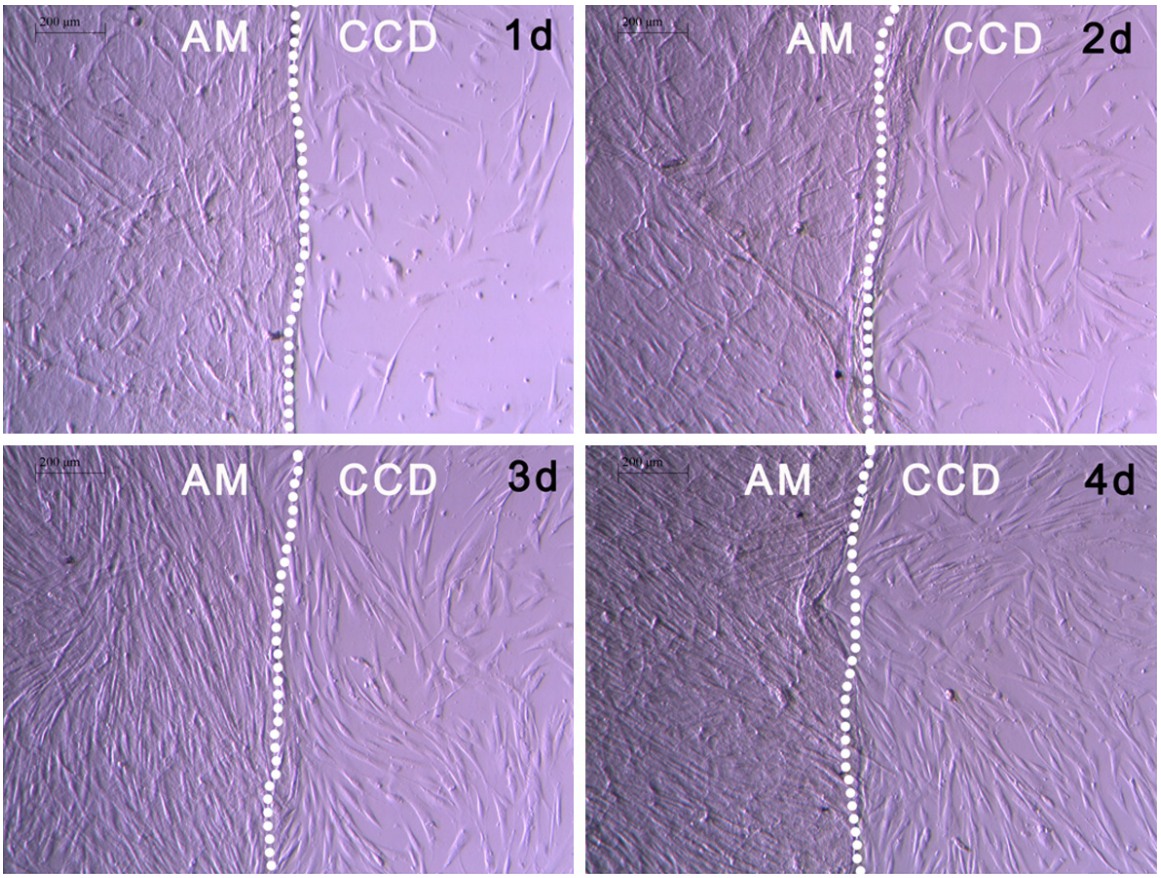

Figure 1. Fibroblast morphology and proliferation cultured on acellular amniotic membrane. CCD was equally separated into two parts with a dashed line. Fibroblasts were cultured on acellular amniotic membrane (AM, left) or CCD (right). Morphology and proliferation of fibroblasts were observed at days 1, 2, 3, and 4. Fibroblasts exhibited a similar spindle-shaped morphology cultured on the acellular amniotic membrane and cell culture dish; however, acellular amniotic membrane greatly promoted proliferation of fibroblasts from day 2. Scale bar: 200 μm.

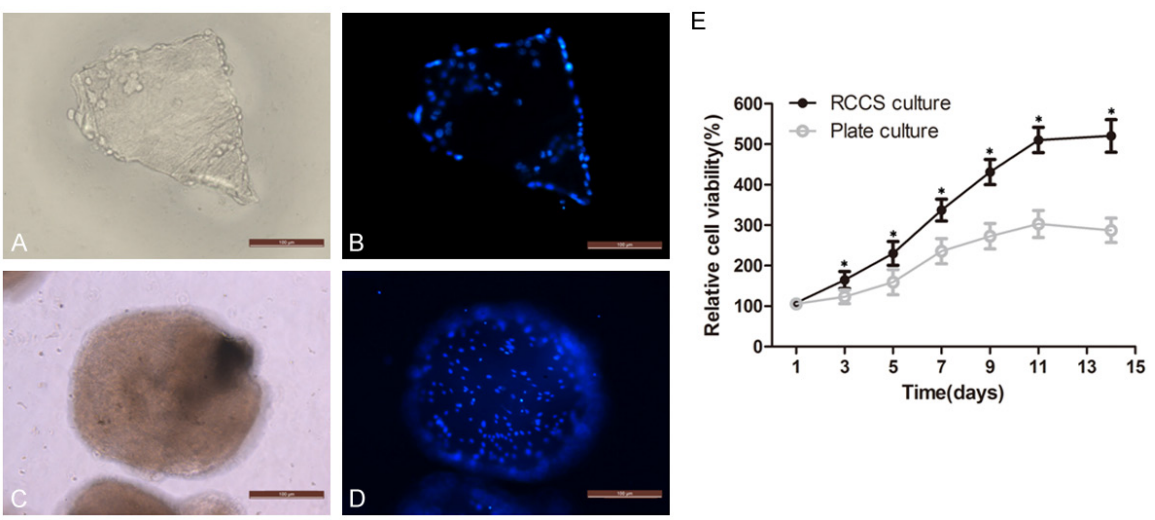

Figure 2. mAM-RCCS amplification of fibroblasts. (A and C) and (B and D): Observation of fibroblast amplification under a phase contrast microscope and a fluorescent microscope after Hoechst staining. (A and B) and (C and D): Showed that fibroblasts adhered to mAM and grew in a 3D manner at days 1 and 4 after culture. (E) Comparison of the proliferative activity between mAM-RCCS culture and conventional 2D culture. Fibroblast proliferative activity in mAM-RCCS culture was significantly higher than that in conventional 2D culture from day 3. All data represent means ± SD; n = 6; *P<0.05; scale bar: 100 μm.

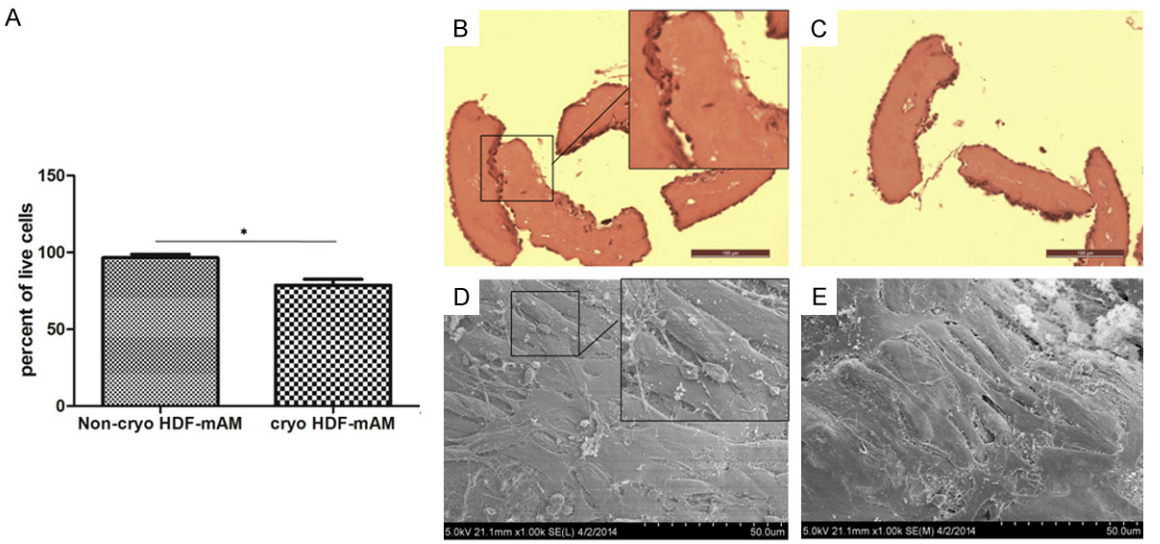

Figure 3. Cryopreservation effect on HDF-mAM. (A) The viability of fibroblast was determined immediately after thawing using live/dead assay, and viable cells were counted under a fluorescent microscope. Quantitative analysis of the percentage of viable fibroblasts was shown and non-cryopreserved HDF-mAM was used as control. (B-E) HE staining and SEM showed that the arrangement, morphology, and membrane structure of fibroblast in cryopreserved HDF-mAM group (B, D) were similar to those in the non-cryopreserved HDF-mAM group (C, E). No pores were seen on the fibroblast membrane of cryopreserved HDF-mAM after thawing (D). All data represent means ± SD; n = 6; *P<0.05; Scale bars: 100 μm (B, C); 50 μm (D, E).

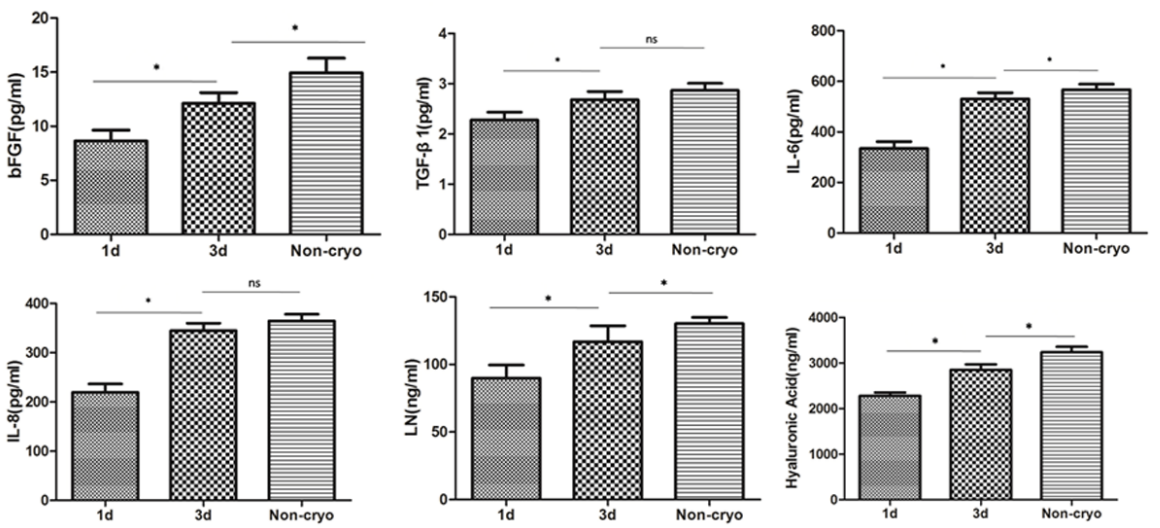

Figure 4. Detection of active peptides in the supernatant of HDF-mAM after thawing. Secretion of various active peptides from cryopreserved HDF-mAM at days 1 and 3 after thawing was measured. Active peptides secretion from non-cryopreserved HDF-mAM was shown as control. All data represent means ± SD; n = 6; *P<0.05; n.s., no significance.

certain distance after treatment. The wound healing rate was calculated using the Image-Pro Plus Software. Wound specimens were taken at day 28 after wounding and stained with hematoxylin and eosin. Angiogenesis at day 14 after wounding was observed by immunohistochemistry using CD31 (1:200, Santa Cruz, USA) as the primary antibody and developed by DAB (Thermo, USA).

Statistical analysis

Data statistical analysis was performed by SPSS16.0, and the results were expressed as

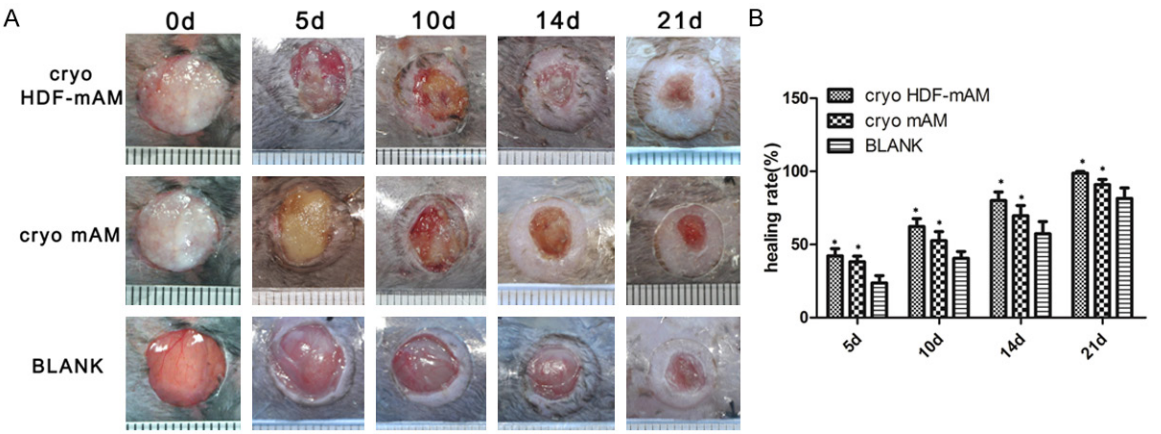

Figure 5. Wound healing of full-thickness skin defects in db/db mice. A. Full-thickness skin wounds were created on the backs of db/db mice, treated with cryopreserved HDF-mAM, cryopreserved mAM or without any treatment, and examined 0, 5, 10, 14, and 21 days after wounding. Wounds were digitally photographed. B. Quantitative analysis of wound healing rate, showing faster wound healing rate in cryopreserved HDF-mAM group than in cryopreserved mAM and blank groups. All data represent means ± SD; n = 6; *P<0.05 compared with blank group.

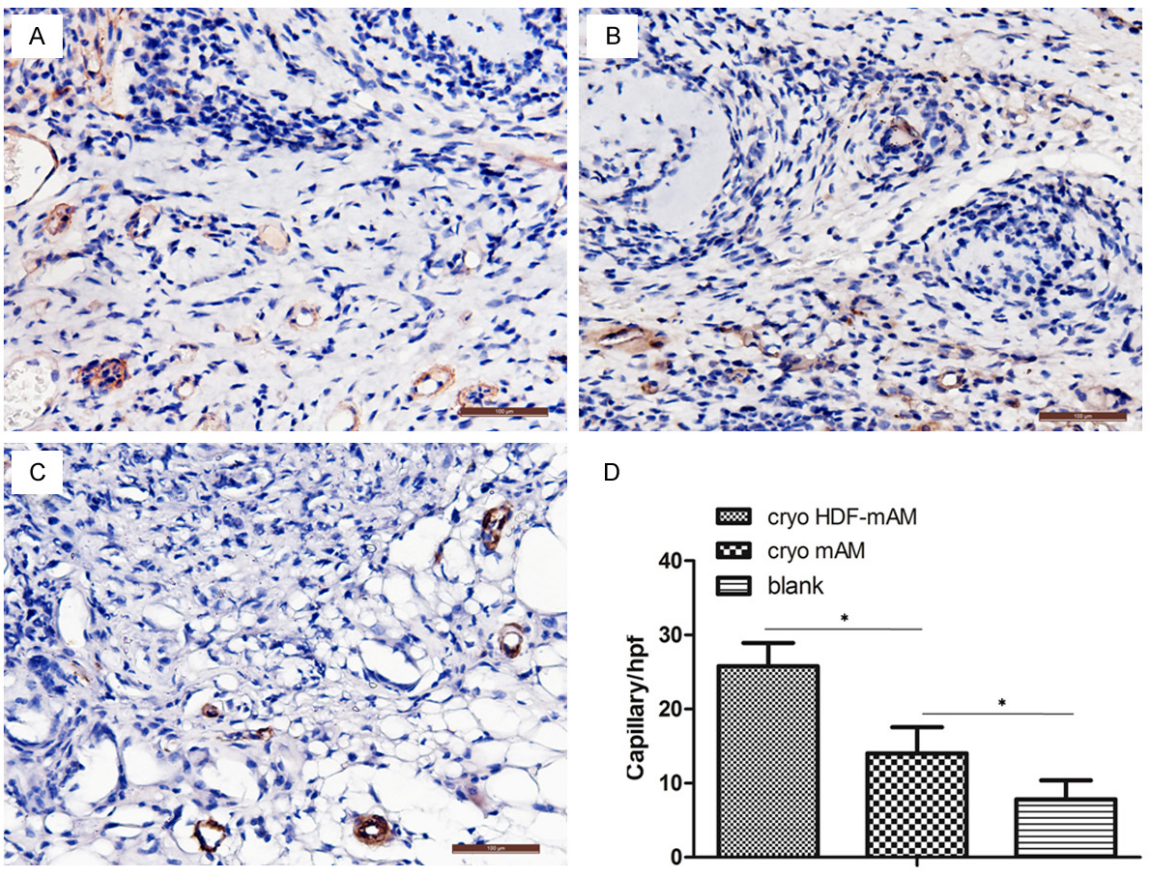

Figure 6. Wound angiogenesis after transplantation. (A-C) Specimens from day 14 wounds were stained with CD31, a blood vessel endothelium cell marker. Representative pictures of newly formed vessels in cryopreserved HDF-mAM group (A), cryopreserved mAM group (B), and blank group (C) were shown. (D) Quantitative analysis of neovascularization, showing that the number of the newly formed vessels in cryopreserved HDF-mAM group was significantly higher than that in cryopreserved mAM and blank groups. All data represent means ± SD; n = 6; *P<0.05; scale bar: 100 μm.

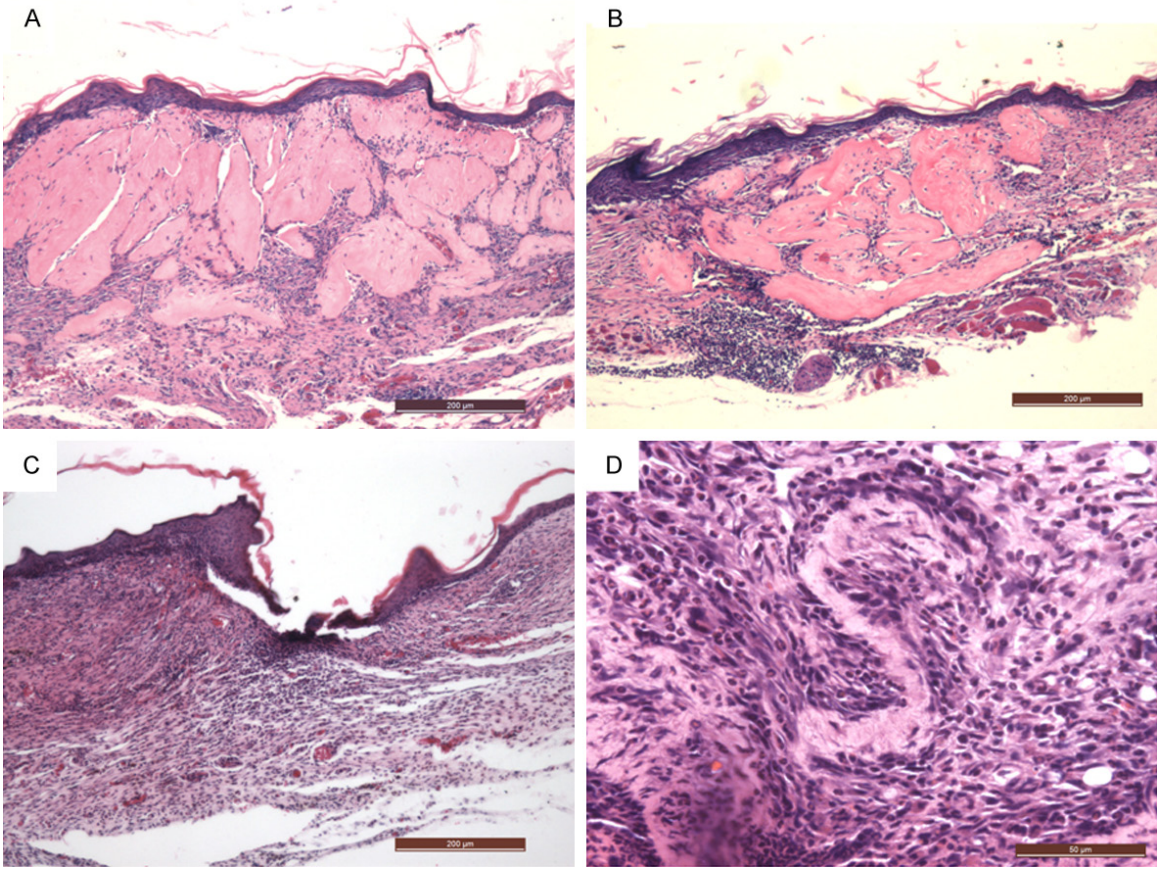

Figure 7. Histological sections after transplantation. (A-C) HE staining of sections at day 28 after transplantation, showing that the wounds had completely healed and the dermal matrix was filled with microparticles in cryopreserved HDF-mAM group (A) and cryopreserved mAM group (B), while re-epithelialization was still incomplete in the blank group with a large number of inflammatory cells infiltration in the dermis (C). (D) Two months after grafting, HE staining showed that mAM had grossly degraded and newly formed vessels disappeared gradually in the cryopreserved HDF-mAM group. Scale bars: 100 μm (A-C); 50 μm (D).

mean ± SD. Statistical analysis was conducted using paired 2-tailed Student's t-test or one-way ANOVA. A p-value<0.05 was regarded as significant.

Results

Morphology and proliferation of fibroblasts cultured on acellular amniotic membrane

CCD was separated into two equal parts and one half was covered with acellular amniotic membrane. In **Figure 1**, fibroblasts grew evenly throughout the amniotic membrane and exhibited a well-defined spindle-shaped morphology. No significant difference in the morphology of fibroblasts was observed between the CCD and acellular amniotic membrane as a substratum for culturing fibroblasts. However, fibroblasts cultured on acellular amniotic membrane showed faster proliferation from day 2 compared with the CCD.

Amplification of HDF by mAM

HDFs adhered to the surface of mAM 1 h after seeding. From day 1 of culture with RCCS, fibroblastsspreadon the surface of mAM gradually. At day 4 of culture with RCCS, fibroblasts spread throughout the mAM surface. The structure of the microsphere was gradually formed as microflake-like mAM edge coiled from one side to the opposite side (**Figure 2A-D**). To compare the difference in HDF amplification between mAM-RCCS culture and the conventional 2D plate culture, proliferation of fibroblasts was measured by CCK-8. In **Figure 2E**, relative proliferative cell activity in RCCS was significantly higher than that in the 2D plate from day 3 and reached 337±27% and

510±34% by days 7 and 11 respectively; these cell activities were much higher than those in the conventional plate culture (236±31%, 303±33%, P<0.05).

Cryopreservation effect on HDF-mAM

To evaluate fibroblast viability of cryopreserved HDF-mAM after thawing, we used live/dead assayto determine the percentage of viablefibroblasts and compared the result with that of the non-cryopreserved HDF-mAM. In **Figure 3A**, the cell viability assay showed that 96.62±2.19% of fibroblasts were viable in the non-cryopreserved HDF-mAM group and 78.64±3.98% in the cryopreserved HDF-mAM group.In **Figure 3B**, **3C**, HE staining revealed that the surface of cryopreserved HDF-mAM was evenly surrounded by fibroblasts, and most of the fibroblasts on the surface were arranged in a single-layer immediately post-thawing similar tothat of non-cryopreserved HDF-mAM. However, few fibroblasts detached from the surface of the cryopreserved HDF-mAM immediatelypost-thawing (**Figure 3B**). Further SEM analysis showed that the membrane structureof fibroblast in the cryopreserved HDF-mAM group remained intact immediately after thawing and was similar to that inthe non-cryopreserved HDF-mAM group (**Figure 3D**, **3E**). No pores were seen on the fibroblast membraneinthecryopreserved HDF-mAM group after thawing (**Figure 3D**).

Quantitative analysis ofactive peptides secretion from cryopreserved HDF-mAM

After thawing and continuous culturing with RCCS at days 1 and 3, various active peptides secreted from HDF-mAM, including IL-6, IL-8, TGF-β1, bFGF, laminin, and hyaluronic acid, were determined. Non-cryopreserved HDF-mAM was used as control. In **Figure 4**, levels of all active peptides secreted fromcryopreserved HDF-mAM were significantly lower than that from non-cryopreserved HDF-mAM at day 1 after thawing. The expression of active peptides increased gradually and reached asimilar level with non-cryopreserved HDF-mAM at day 3 after thawing.

Cryopreserved HDF-mAM promoted vascularization and diabetic wound healing

Full-thickness skin wounds were created on the backs of db/db mice, treated with cryopreserved HDF-mAM, cryopreserved mAM, or without any treatment, and examined 0, 5, 10, 14, and 21 days after wounding (**Figure 5**). At day 5 after wounding, cryopreserved HDF-mAM survived well, and the wound was moist and ruddy. The percentage of wound closure was significantly greater in mice treated with cryopreserved HDF-mAM (42.24±4.90%) than those treated with cryopreserved mAM (38.18±3.92%, p<0.05) and blank group (23.70±4.96%, p<0.05). The gap of wound healing rate between these three groups gradually became bigger. At day 21 after wounding, most ofthe wounds in HDF-mAM group healedcompletely; theaverage wound healing rate was 98.87±1.05%, which was significantly greater than that in the cryopreserved mAM group (90.90±3.54%, p<0.05) and blank group (81.51±7.22%, p<0.05). Immunohistochemistry of CD31 showed that vascularization incryopreserved HDF-mAM group was more evident than that in the other groups at day 14. Capillary density in cryopreserved HDF-mAM group (25.8±3.1/HP) was significantly higher than that in the cryopreserved mAM group (14.0±3.5/HP, p<0.05) and blank group (7.8±2.6/HP, P<0.05) (**Figure 6**). Four weeks after transplantation, HE staining of the sections showed that the wounds had healed completelyand the dermal matrix was filled with microparticles in cryopreserved HDF-mAM and cryopreserved mAM groups. In addition, mAM was mainly surrounded by fibroblasts and newlyformed vessels, without obvious inflammatory cells infiltration. By contrast, re-epithelialization was still incomplete in the blank group, with a large number of inflammatory cell infiltrations in the dermis. To further observe dermal remodeling after wound healing, we obtained samples from thecryopreserved HDF-mAM group two months after grafting. HE staining showed that mAM had grossly degraded, and newly formed vessels gradually disappearedin the dermis (**Figure 7**).

Discussion

Fibroblasts in chronic ulcers have reduced collagen synthetic capacity, a lack of response to stimulatory TGF-β1, altered morphology, reduced proliferative capacity, and an increased proportion of senescent fibroblasts [9-12]. Dysfunction of fibroblasts is one of the main mechanisms for delayed and poor wound healing in diabetic ulcers. Bioengineered skin sub-

stitutes that contain normal human fibroblasts could provide a new method for treating diabetic ulcers. In this study, we used 3D micronized AM as a natural microcarrier for ex vivo culture and amplification of fibroblasts, and simultaneously constructeda living dermal substitute HDF-mAM with the one-step mAM-RCCS method. We thencryopreserved HDF-mAM with a commercial and serum-free cryoprotectant, and post-thawing HDF-mAM was further utilized to heal the chronic full-thickness skin defects in a db/db mice wound model.

The rationale behind the application of amniotic membrane as microcarriers is based on theintrinsic biological properties of amniotic membrane. Amniotic membrane exhibits good biocompatibility and high affinity tohuman allogeneic cells, and has been used as a cell amplification carrier to construct tissue-engineered products and can maintain morphology and phenotype of human allogeneic cells [13-16]. In our previous study, we showed that mAM not only possessed the characteristics of microcarrier but completely retained the basement membrane structure and abundant active substances, such as NGF, HGF, KGF, bFGF, TGF-β1, and EGF, in the amniotic matrix, thereby providing an ideal niche microenvironment to amplify epidermal stem cells quickly [6]. Similar to those previously reported studies, in this study, we showed that human fibroblasts exhibited a well-defined spindle-shaped morphology and fastproliferation when cultured on a patch of acellular amniotic membranecompared with a CCD. We then used the one-step mAM-RCCS method to simultaneously construct HDF-mAM during the expansion of human fibroblasts. Traditionally, bioengineered dermal substitutes, such as Dermagraft, are constructed intwo steps, namely, amplifying human fibroblasts in a 2D environment and then seeding them ontoadermal scaffold. Ex vivo construction takes a long time to complete and repeated trypsinization promotes aging of fibroblast. In thisstudy, constructing HDF-mAM withtheone-step mAM-RCCS methodnot only shortened the time of the fabricated dermal substitute but also avoided possible damage to fibroblasts due torepeated trypsinizationand maintained the proliferative activity of fibroblasts. The relative cell viability in RCCS culture at days 7 and 11 was significantly higher than that in the conventional 2D plate culture (337±27% and 510±34% vs. 236±31% and 303±33%).

To facilitate bioengineered dermal substitute to treat wounds in clinics, such as burns, ulcers, and cosmetic repairs, cryopreservation technique that yields the optimal viability of fibroblasts is highly advantageous for providing ready-to-use living dermal substitutes in clinics. Dermagraft is normally obtained as 5×7.5 cm sheets and maintains about 60% cell viability post-thawing [17]. In the present study, we showed that micronized HDF-mAM retained higher cell viability (78.64±3.98%) andmaintained intact morphology and membrane structure of fibroblast immediately post-thawing. In addition, various active peptides secreted from HDF-mAM after thawing were well preserved and reached asimilar level as the non-cryopreserved HDF-mAM at day 3 after thawing and continuous culturing with RCCS. STEM-CELLBANKER is a novel commercial, serum and animal-free mediumthat contains dimethylsulfoxide and anhydrous dextrose, which areboth permeating and non-permeating cryoprotectants [18]. It was recently reported as a favorable cryoprotectant to freeze adipose and bone marrow-derived MSCs, human hepatocytes, human embryos, and induced-pluripotent stem cells [18-21]. Here, we cryopreserved HDF-mAM with STEM-CELLBANKER and agreed thatit is feasible, safe, and efficient for clinical use to cryopreserve HDF-mAMin the future.

Finally, cryopreserved HDF-mAM was further utilized to heal the chronic full-thickness skin defect in a db/db mice wound model. Our results showed that topical application of cryopreserved HDF-mAM accelerated wound healing in diabetic micesignificantly. Wound closure was significantly faster in wounds treated with cryopreserved HDF-mAM than those treated with cryopreserved mAM or without any treatment at day 5 after wounding; this trend was maintained during the entire healing period. In addition, capillary density in cryopreserved HDF-mAM group was significantly higher than in other groups. Many factors account for increased neovascularization in the cryopreserved HDF-mAM group. Various active peptides secreted from HDF-mAM, such as IL-6, IL-8, TGF-β1, bFGF, laminin, and hyaluronic acid, are all factors that stimulate the proliferation, adhesion, and growth of endothelial cells [22,

23]. Interactions between fibroblasts and endothelial cells can also promote angiogenesis.

Moreover, HDF-mAM is convenient and flexible for treating a variety of wounds in clinics. HDF-mAM could pass through a 20-gauge needle, there by enabling irregularly shaped defects, such as diabetic ulcers and some deep degree wounds, to be filled easily and for implantation to be contoured precisely according to the patient's needs. Unlike alarge sheet of traditional skin substitutes, such as Dermagraft, HDF-mAM may be applied to a recipient site by way of injection, spraying, layering, or packing, thereby avoiding the need for incisions and surgical dissections.

One limitation of this study is that we did not follow up the survival of fibroblasts after transplantation. Fibroblasts do not express MHC-II-like antigens and possess weak immunogenicity; thus, we suggest that fibroblasts would not induce significant rejection after transplantation and could survive in wounds. Hansbrough [24] seeded allogenous fibroblasts onto PGA or PGL mesh and found that fibroblasts survive well in the wound and 82% of the cells were transplanted with fibroblasts rather than the invaded host fibroblasts 9 days after transplanting. One month after transplantation, 60-64% of the transplanted fibroblasts still exist. Although the above findings suggest that allogenous fibroblasts can survive in wounds for a long period of time, further studies are still necessary to determine the outcomeof fibroblasts after transplantation onto diabetic wounds.

Another limitation of this study is that we cryopreserved HDF-mAM with serum and animal-free cryoprotectant, but cultured and amplified fibroblasts with animal serum, there byincreasing the risk of disease transmission. Further studies are necessary to develop new methods of culturing human fibroblasts without animal product contamination.

In conclusion, we used the one-step mAM-RCCS method in combination with STEM-CELLBANKER cryopreservation to construct a time-saving, cost-effective and ready-to-use living dermal substitute to promote diabetic wound healing and neovascularization. This method may be used to treatdiabetic ulcers and other chronic wounds in the clinic.

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Disclosure of conflict of interest

None.

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