Biomaterials 113 (2017) 56–67

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Topical administration of cryopreserved living micronized amnion accelerates wound healing in diabetic mice by modulating local microenvironment

Yongjun Zheng [1], Shizhao Ji[***,1], Haibin Wu [1], Song Tian, Yunqing Zhang, Li Wang, He Fang, Pengfei Luo, Xingtong Wang, Xiaoyan Hu, Shichu Xiao[**], Zhaofan Xia[*]

Burns Center of Changhai Hospital, The Second Military Medical University, Shanghai, China

ARTICLE INFO

Article history:
Received 29 July 2016
Received in revised form
20 October 2016
Accepted 20 October 2016
Available online 21 October 2016

Keywords:
Amnion
Cryopreservation
Wound healing
Macrophages
Neovascularization
Dermal substitute

ABSTRACT

Approximately 25% of diabetic patients suffer from diabetic lower-extremity ulcer throughout their lives and 7%–20% of patients will eventually need an amputation despite standard care treatment. The development of new therapies to treat diabetic wounds is urgent. In this study, we used cryopreserved living micronized amnion (300–600 μm) to treat wounds in diabetic mice. Post-thaw micronized amnion retained high cell viability, as well as intact cell morphology and membrane structure. When transplanted onto the wounds of db/db mice, the cryopreserved living micronized amnion greatly promoted wound healing in diabetic mice mainly by secreting growth, inflammation, and chemotaxis-related factors that regulated macrophage migration and phenotype switch, recruited $CD34^+$ progenitor cells, and increased neovascularization. In addition, the micronized amnion matrix can exist in the dermis and serve as a long-term dermal scaffold. These results demonstrated the potential of the cryopreserved living micronized amnion as a ready-to-use living dermal substitute that addresses multiple defective physiological processes of impaired wounds to treat diabetic ulcers and other chronic wounds in clinics.

1. Introduction

Diabetic foot ulcer is a serious complication of diabetes mellitus, and approximately 25% of diabetic patients suffer from diabetic lower-extremity ulcer throughout their lives [1]. Even with the standard therapies, including moist dressing, debridement, infection control, and wound offloading, these wounds still slowly heal, and 7%–20% of patients will eventually need an amputation [2]. The development of new therapies to treat diabetic wounds and prevent foot ulcers from leading to amputations is thus urgent.

For the past 100 years, the therapeutic potential of human amnion tissue grafts in wound healing has been well established by hundreds of published clinical papers [3–12]. Although the exact molecular and cellular mechanisms underlying clinical benefit of amniotic membrane have yet to be fully elucidated, several key mechanisms of action have been elucidated. Human amnion contains a variety of growth factors and cytokines, such as platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF), promoting human dermal fibroblast proliferation, mesenchymal stem cells recruitment, epithelialization and neovascularization [13–16]. Aside from being a source of growth factors and cytokines, amnion can also serve as a scaffold and substrate for the growth, migration and adhesion of keratinocytes and fibroblasts and thus find widespread applications in skin tissue engineering [17–20]. In addition, amnion could decrease the risk of infection because of its anti-microbial properties, as a result of not only its function as a biological barrier but also the expression of several antimicrobial molecules, such as beta3-defensin [21]. At the same time, human amniotic epithelial cells (HAECs) retain a high level of pluripotency [22,23] and amnion-derived cellular cytokine solution greatly improves wound healing in acute and chronic wound models [24,25]. Recently, Jin has also reported that HAECs

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: shizhaoji@aliyun.com (S. Ji), huangzhuoxiao@sohu.com (S. Xiao), xiazhaofan_smmu@163.com (Z. Xia).
[1] These authors contribute equally to this work.

http://dx.doi.org/10.1016/j.biomaterials.2016.10.031
0142-9612/

promote acute wound healing in mice [26]. Despite the great potential of amnion in treating various wounds, some drawbacks should be overcome to maximize its clinical potential. First, the present preservation techniques, such as freeze-drying, gamma sterilization or glycerol preservation, lead to the complete loss of HAECs viability of amnion. Second, human amnion is very thin with low mechanical strength, thus amnion in the form of large sheets does not easily completely flatten and adhere to the wound, and gradually dries, only used clinically as a temporary biological dressing. In addition, the therapeutic characteristics and mechanisms of HAECs directly used to treat chronic wounds, such as diabetic ulcers, are currently unknown.

In this study, we processed human fresh amnion into micronized (300–600 μm) amnion and further cryopreserved it in a serum-free stem cell cryopreservation medium for 6 months. The intact structure, cell viability and biologically active components of micronized amnion after cryopreservation were evaluated. Post-thawing micronized amnion was then transplanted to the wounds of db/db mice to evaluate its effect on repairing full-thickness skin defects. The mechanisms were further explored, mainly focusing on inflammation and neovascularization through in vivo and in vitro experiments, and the role of amniotic matrix as a long-term dermal scaffold.

2. Materials and methods

2.1. Preparation of living micronized amniotic membrane (LMAM) and decellularized micronized amniotic membrane (DMAM)

All study protocols were approved by the Ethics Committee of Changhai Hospital, Shanghai, China. After obtaining informed consent, we obtained human placentas (20 fetuses: 10 males, 10 females) directly from parturients who underwent cesarean section. Serological tests showed that all donors were serologically negative for human immunodeficiency virus types I and II, human hepatitis B and C, and syphilis. Each placenta was processed individually to avoid cross-contamination. All procedures were performed under sterile conditions. The amniotic membrane was peeled from the chorion and then rinsed several times with cold sterile phosphate-buffered saline (PBS) containing penicillin, streptomycin, neomycin, and amphotericin B to remove blood remnants. A self-made electric microskin cutter was made up of three fixed blades, four movable blades and an electric motor. The amnion was cut by the electric power driven self-made electric microskin cutter with rotary-cutting and then filtrated through a metal mesh filter to obtain 300–600 μm microparticles to be used as LMAM. DMAM was prepared through repetitive freeze–thawing cycles and DNA enzymatic digestion as described in our previous study [17].

2.2. Cryopreservation and resuscitation of LMAM and DMAM

The LMAM and DMAM from 3 cm^2 amnion were resuspended in STEM-CELLBANKER™ (Zenoaq, Fukushima, Japan), frozen in a cryotube, and then preserved in a liquid nitrogen tank for 6 months. For thawing, the samples were removed from liquid nitrogen and then immediately placed in a water bath at 37 °C until they were nearly completely thawed. The post-thaw LMAM was cultured with 10% fetal bovine serum (FBS) for further analysis.

2.3. LMAM viability assay

The cell viability of HAECs in LMAM was determined by using a fluorescent-based live/dead assay (Invitrogen Detection Technologies, USA) immediately after thawing in accordance with the manufacturer's instructions. Viable cells were stained with the green fluorescent dye calcein AM, whereas dead cells were stained with the red fluorescent dye Ethidium-1. The total number of dead and live cells was counted under a fluorescent microscope (Leica, Germany). Fresh LMAM was served as the control.

2.4. Animals, wound model, and treatment

All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Second Military Medical University, Shanghai, China. The male db/db mouse used in the experiment (C57BL/KsJ, leptin receptor-deficient diabetes, 8–12 weeks, purchased from Slac Laboratory Animal Co. Ltd., Shanghai, China) is a well-established type 2 diabetic animal model. The inclusion criterion was a blood glucose level of more than 300 mg/dL. The mice were intraperitoneally anesthetized with 1% sodium pentobarbital. Two full-thickness splinted wounds (12 mm diameter) were created on the back of the mice as previously described [27]. The mice were randomly and equally divided into three groups: LMAM, DMAM, and blank. Cryopreserved LMAM or DMAM was transplanted on wound surface of the mice from the LMAM and DMAM groups, respectively, and the outer layer was covered with vaseline gauze and sewn intermittently by 4-0 suture (Jinhuan, China). Animal behavior and bandage integrity were monitored throughout the experiment. Photographs were taken regularly and the wound healing rate was calculated using the Image-Pro Plus Software.

2.5. Flow cytometry

Peripheral blood was collected from a cardiac puncture into a syringe containing 100:1 heparin (1000 units/ml; Sigma-Aldrich). Mononuclear cells were isolated from blood with Histopaque 1083 (Sigma-Aldrich) density gradient centrifugation (400 g, 30 min). The mononuclear fraction was removed, centrifuged (250 g, 10 min), and red blood cells were lysed with 0.8% ammonium chloride solution (Stemcell Technologies), washed 3 times in PBS with 1% albumin, and then used for testing by flow cytometry. To count the number of EPCs (endothelial progenitor cells) subpopulation in peripheral blood, we analyzed the expression levels of CD133 (1:100, eBiosciences, USA) and VEGFR-2 (1:100, eBiosciences, USA) in peripheral blood as previously described [28]. Single cells from wound samples were prepared as previously described [29] and stained with the antibodies APC-F4/80 anti-mouse (BioLegend), PE-CCR7 anti-mouse (BioLegend), and PE/Cy7 anti-mouse CD206 (BioLegend) in accordance with the manufacturer's instructions. M1-like and M2-like macrophages were identified as $F4/80^+CCR7^+$ or $F4/80^+CD206^+$ cells, respectively.

2.6. Hematoxylin and eosin (HE) staining, immunohistochemical staining, and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay

The wounds, together with unwounded skin margins, were excised, fixed in 10% formaldehyde for at least 24 h and embedded in paraffin, transversely cut into 5 mm-thick sections, and then stained with hematoxylin and eosin. The sections were also incubated with the primary antibody against CD31 (1:50, ABcam, USA) to observe angiogenesis, CD34 (1:100, ABcam, USA) to calculate the number of hematopoietic stem cells in the wounds, and F4/80 (1:50, ABcam, USA) to evaluate the infiltration of macrophages in the wounds. To trace the fate of the HAECs in transplanted LMAM, we stained the sections with anti-human mitochondria antibody (1:200, ABcam, USA). Briefly, sections were deparaffinized, hydrated, and subjected to proteinase K treatment for antigen