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A novel approach for the cryodesiccated preservation of tissue-engineered skin substitutes with trehalose

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ABSTRACT

Tissue-engineered skin (TES) holds great promise for wound healing in the clinic. However, optimized preservation methods remain an obstacle for its wide application. In this experimental work, we developed a novel approach to preserve TES in the desiccated state with trehalose. The uptake of trehalose by fibroblasts under various conditions, including the trehalose concentration, incubation temperature and time, was studied. The cell viability was investigated by the MTT assay and CFSE/PI staining after cryodesiccation and rehydration. TES was then prepared and incubated with trehalose, and the wound healing effect was investigated after desiccated preservation. The results showed that the optimized conditions for trehalose uptake by fibroblasts were incubation in 200 mM trehalose at 37 °C for 8 h. Cryodesiccated cells and TES maintained 37.55% and 28.31% viabilities of controls, respectively. Furthermore, cryodesiccated TES exhibited a similar wound healing effect to normal TES. This novel approach enabled the preservation and transportation of TES at ambient temperature with a prolonged shelf time, which provides great advantages for the application of TES.

1. Introduction

Tissue-engineered skin (TES) is an optimized alternative for the treatment of skin defects resulting from trauma, inflammation and tumor excision [1]. The strategy involves the combination of keratinocytes or fibroblasts with certain scaffolds [2]. Cells seeded into the scaffold secrete various growth factors and extracellular matrix proteins, which could stimulate the proliferation and differentiation of adjacent epithelial tissue and accelerate skin defect repair [3]. Several TES products, including Apligraf® and Dermagraf®, have been authorized by the FDA [4–6].

To facilitate the application of TES in emergent situations, a relatively large storage of TES is needed, because the preparation of TES is time consuming. However, some TES products, such as Apligraf®, should be preserved at 4 °C and the shelf time is very short, usually ranging from 7 to 10 days because of the difficulty in keeping cell viability [7,8]. Furthermore, conditioned transportation is also necessary to deliver these living TES products [9]. Cryopreservation of cells in suspension has been demonstrated to be effective under the protection of DMSO [10,11]. However, the technique is not applicable for the cryopreservation of tissues or organs with relatively large volume due to their low thawing rate [12–15]. Therefore, it is necessary to develop novel approaches to preserve and deliver TES.

Some organisms have developed specialized adaptations to protect their cellular components from the damage caused by desiccation and rehydration. For example, the larvae of the sleeping chironomid can survive complete dehydration in a "cryptobiosis" or "anhydrobiosis" state [16]. Water bear, a tardigrade, can be revived after extreme desiccation for decades [17]. One mechanism, common to all such organisms, is the accumulation of disaccharides, especially trehalose, within cells and tissues at the onset of dehydration.

Trehalose is a non-reducing disaccharide that is primarily used for drying or freeze-drying of proteins and other biological compounds [18]. Trehalose could form hydrogen-bonds to polar groups on proteins and lipids and prevent the occurrence of fusion or denaturation [19–21]. Chen et al. [22] demonstrated that trehalose could significantly improve human keratinocyte viability in suspension and tissue-engineered cell sheets during cryopreservation. When transplanted into nude mice, trehalose-cryopreserved keratinocyte sheets repaired skin defects as efficiently as that of non-cryopreserved controls, indicating that trehalose could maintain the function of cryopreserved keratinocyte sheets.

Trehalose could also replace water molecules, thereby maintaining the structure of plasma membrane and preventing protein denaturation and aggregation during the process of desiccation [23]. Wolkers et al. [24] reported that trehalose could be rapidly taken up by human platelets, and 85% of freeze-dried platelets could be recovered after rehydration. Importantly, the function of the recovered platelets was almost identical to that of fresh platelets. McGinnis et al. [25] treated mouse sperm with trehalose and injected the desiccated sperms (stored at 4 °C for 1 month) into oocytes and found that the percentage of blastocyst formation was up to 63%. Gordon et al. [26] incubated

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human mesenchymal stem cells with trehalose, and then air dried the cells. Further tests showed that the cells maintained high viability and proliferation capacity, expressed mRNA for characteristic stromal factor, and responded to reagents known to induce differentiation.

To provide the best clinical outcome, TES must be processed and stored in a manner that maintains the viability and structural integrity until needed for transplantation. In the current study, we hypothesized that TES could be cryodessicated and functionally preserved in the dried state with trehalose. The ability to preserve and transport TES products in the dried state at ambient temperature provides a significant advantage for their application.

2. Materials and methods

2.1. Mouse fibroblast cell culture

Mouse fibroblast cells (L929 cell line) were purchased from the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences Institute of Cell Resource Center. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 1% non-essential amino acids (Hyclone, USA), and 100 U/100 μg penicillin/streptomycin (Hyclone, USA) at 37 °C with 5% CO_2. Cells were subcultured at a 1:4 split ratio by 0.25% trypsin when reached approximately 70–80% confluent for further experiments.

2.2. Trehalose loading and quantization

Approximately 4×10^4 fibroblasts in 200 μL cell culture medium were plated into each well of 96-well plates and cultured overnight for adhesion. To measure the trehalose uptake by fibroblasts, the medium was removed, and the cells were incubated in fresh medium with different conditions, including trehalose concentration, temperature and incubation time. For the concentration series, cells were cultured in medium containing 50, 100, 200, 300 or 400 mM of trehalose at 37 °C for 8 h ($n = 5$). For the temperature series, cells were incubated at 4 °C, 20 °C, 37 °C and 4–37 °C (4 °C for 10 min and then transferred to 37 °C) in medium with 200 mM trehalose for 8 h ($n = 5$). For the time course series, fibroblasts were incubated in medium containing 200 mM trehalose at 37 °C for 0.5, 1, 2, 4, 6 and 8 h ($n = 5$). Cell morphology was observed by phase contrast microscopy during the experiment.

Cells were collected by centrifugation at 1000 rpm for 10 min after digestion with trypsin. Cells were then mixed with 4 mL of 80% methanol and incubated at 85 °C for 1 h to extract the intracellular trehalose. After collecting the supernatant by centrifugation and evaporating in a vacuum drier, the dry residue was dissolved in 1 mL distilled water. Trehalose quantification was performed by the anthrone reaction [27]. Briefly, the samples were mixed with 3 mL of 2% anthrone reagent (Sigma-Aldrich, USA) and heated at 100 °C for 10 min. The absorbance at 630 nm was measured on a spectrophotometer (Thermo, Multiskan FC, USA) and compared with a standard curve ($n = 5$ for each group).

2.3. Cryodesiccation and rehydration

2.3.1. Cryodesiccation

Approximately 4×10^4 fibroblasts in 200 μL of medium were plated into each well of 96-well plates and cultured overnight for adhesion. The cells were then incubated in fresh medium containing the following: (1) 200 mM trehalose + 10% DMSO (Sigma-Aldrich, USA), (2) 200 mM trehalose, (3) 10% DMSO. Fresh cell culture medium acted as control ($n = 5$ for each group). After incubation at 37 °C for 8 h, the medium was removed, and the cells were transferred immediately into a programmed freezing container (Ruobilin, RBL-PA, China) and frozen at cooling speed of 1 °C/min to −50 °C. Cells were freeze-dried for 7 h at −50 °C with a LGJ-25 vacuum Freeze Drier (Xiangyi, China) and were stored in vacuum condition at room temperature for 1 week before use.

2.3.2. Rehydration

Samples were rehydrated 1 week after cryodesiccation. For the trehalose + DMSO group and the trehalose group, cells were rehydrated by adding 200 μL cell culture medium containing 200 mM trehalose and were incubated at 37 °C and 5% CO_2 for 45 min. While for the DMSO group and control group, cells were rehydrated by 200 μL normal culture medium.

2.4. Cell viability assay

2.4.1. Fluorescent labeling

The viability of the rehydrated cells after cryodesiccation was determined by 5- or 6-(N-succinimidyloxycarbonyl)-3′,6′-O,O′-diacetylfluorescein (CFSE, Sigma-Aldrich, USA)/fluorescent propidium iodide (PI, Sigma-Aldrich, USA) labeling, according to the manufacturer's protocol. Briefly, the cells were incubated in the medium with 20 μL PI solution at 37 °C. After 15 min, the cells were washed twice with PBS and were added by 2.5 μM CFSE prepared in PBS for 15 min incubation. The staining was stopped by replacing the medium with fresh DMEM and incubating for another 30 min. Cell fluorescence was then imaged by a fluorescent microscope (Olympus, FV1000, Japan).

2.4.2. MTT assay

The viability of the rehydrated cells was further determined by the MTT assay following the manufacturer's instruction. A 10 μL volume of MTT solution was added into each well and incubated at 37 °C for 4 h. Then, the medium was gently removed, and 200 μL of DMSO was added. All the wells were measured immediately by a microplate reader (Versa Max, USA) at 490 nm. Normal fibroblasts acted as control ($n = 5$ for each group).

2.5. Cryodesiccation of TES and wound healing effect

2.5.1. Mouse fibroblast isolation and culture

Mouse fibroblasts were isolated from newborn male BALB/c mice (2–3 days after birth). The sterilized skin samples were digested with 0.25% trypsin (Hyclone, USA) at 37 °C for 2 h and washed with PBS. Primary fibroblasts were suspended in DMEM supplemented with 10% FBS, 1% non-essential amino acids and 100 U/100 μg penicillin/streptomycin at 37 °C with 5% CO_2. Cells were subcultured at a 1:4 split ratio by 0.25% trypsin when reached approximately 70–80% confluent for further experiments. Cells of third passage were used for TES preparation.

2.5.2. Preparation of TES

TES samples were prepared as described previously [28]. A 150 μL volume of 10× DMEM was added into 1 mL of an ice cold collagen solution (4 mg/mL type I collagen from rat tail dissolved in 0.1% acetic acid). After neutralization with 500 μL 0.1% NaOH solution, 100 μL of cell suspension (5×10^6 cells) was then added and mixed immediately. The mixture was transferred into 48-well plates and incubated at 37 °C for gelling.

2.5.3. Cryodesiccation, rehydration and MTT assay

The prepared TES were incubated in medium containing 200 mM trehalose + 10% DMSO, 200 mM trehalose and 10% DMSO for 8 h respectively. The TES specimens were processed for cryodesiccation and rehydration as described for mouse fibroblasts above. Rehydrated samples were first examined by gross inspection and handling test. The MTT assay was further applied to test the viability of the specimens 4 weeks after storage in vacuum condition at room temperature. Normal TES without treatment acted as the control ($n = 5$ for each group).