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Cryopreservation of tissue-engineered dermal replacement in Me_2SO: Toxicity study and effects of concentration and cooling rates on cell viability ☆

Xin Wang [a], Tse-Chao Hua [a,*], Da-Wen Sun [b,*], Baolin Liu [a], Guanghui Yang [c], Yilin Cao [c]

[a] *Institute of Cryomedicine, Shanghai University of Science and Technology, Shanghai 200093, China*
[b] *Biosystems Engineering, University College Dublin, National University of Ireland, Earlsfort Terrace, Dublin 2, Ireland*
[c] *Shanghai Research & Development Center of Tissue Engineering, Shanghai 200235, China*

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Abstract

Cryopreservation of tissue-engineered human dermal replacement plays an important role in skin tissue engineering and skin banking. With the inspection of electronic scanning microscope and viability evaluation by Trypan Blue staining assay and the tetrazolium salt, MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, this study investigated the toxicity of Me_2SO to dermal fibroblasts and effects of cryoprotectant concentration and cooling rate on the viability of dermal replacement. The results demonstrated that the Me_2SO toxicity to fibroblasts was affected by the exposure time, temperature, and concentration. Furthermore adding cryoprotectant solution at low temperature of 4 °C significantly reduced the toxic effect on the tissue-engineered dermal equivalent. An optimal cryopreservation protocol consisting of cooling rate at 1 °C min^{-1} in 10% (V/V) Me_2SO was derived, with the viability of studied dermal equivalent treated by this protocol being 75% of that of fresh control. The micrograph obtained by electronic scanning microscope also confirmed this result.

Keywords: Cryopreservation; Dermal equivalent; Dermal replacement; Tissue engineering; Allograft; Skin graft; Me_2SO

Based on the revolutionary development in biotechnology, tissue engineering is emerging as a promising technique for solving the problem of organ and tissue deficiencies and for providing the next generation of medical implants [8,16,19]. Tissue-engineered skin equivalents are the first in a long pipeline of products being developed, and a wide variety of engineered skins are in different stages of development, some of which have recently reached the market [1,2,15,17,24]. Unlike traditional split thickness skin grafts or allografts, tissue-engineered skin replacements can maintain biointeractive after implantation, thereby offering structure as well as the physiologic functions of the replaced damaged or diseased skin. Additional advantages of using normal human skin produced by in vitro culture techniques include safety assurance, product efficacy, ease of use, and off-the-shelf and consistent tissue supply.

However due to the long production cycle, for example, 2–3 weeks for skin replacement and the limited shelf-life of the "fresh" constructs (about 72 h), preservation of the product is critical to ensure the off-the-shelf availability to clinicians. For long-term preservation, cryopreservation is likely the optimal choice. While cryopreservation of isolated human foreskin fibroblasts has successfully obtained a high cell survival rate of over 95% [7], the cryopreservation of tissue-engineered skin equivalent would be one of the major obstacles for the commercialization of engineered skin products [14,17,23]. The local environmental changes of the cells at different locations in the tissue,

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* Corresponding authors.
E-mail addresses: tucha@sh163.net (T.-C. Hua), dawen.sun@ucd.ie (D.-W. Sun).
URLs: www.ucd.ie/refrig, www.ucd.ie/sun (D.-W. Sun).

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i.e., the change of concentration of cryoprotectant and temperature, during the addition/removal of cryoprotectant and cooling/warming, would have significant effect on cell viability, proliferation and differentiation, and even the cell to cell/scaffold junctions. Thus the selection of an optimal cryopreservation protocol to achieve maximum cell viability and tissue functionality for the tissue-engineered skin equivalent is very important.

For a variety of cells, optimal cell recovery depends on the type of cryoprotectant, the concentration and exposure time/temperature to cryoprotectant, freezing and warming protocols, and even the adding and removing procedures of cryoprotectant solutions [6,13,21,25]. Despite the above studies, the toxicity of Me_2SO to dermal fibroblasts and the effects of cryoprotectant concentration and the cooling rate on the viability of dermal replacement were not evaluated, therefore this paper studied the effects of above-mentioned factors on the cell viability of tissue-engineered dermal replacement with dimethyl sulfoxide (Me_2SO) as the cryoprotectant.

Materials and methods

Preparation of the dermal fibroblasts and the skin replacement

Human neonatal dermal fibroblasts were provided by Shanghai Tissue Engineering R&D Center (Shanghai, China). A polyglycolic acid (PGA) scaffolding (Shanghai Tissue Engineering R&D Center, Shanghai, China) was used to support the growth of the dermal fibroblasts and the formation of a dermal equivalent. Normal human neonatal dermal fibroblasts were grown for 5 days with fibroblast culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA). The harvested fibroblasts were seeded onto the surface of the sterilized scaffolding at a density of 3×10^6 cells/cm^2. The dermal replacement was cultured at 37 °C and a 5% CO_2 atmosphere for several days with medium changes every other day. Then the tissue was cut into small slices ($10 \times 3 \times 2$ mm) for subsequent cryopreservation.

Two groups of samples were defined—(1) fresh control, and (2) experimental group. The fresh control was immersed in DMEM for 1 h before cell viability assessment.

Cryoprotectant toxicity: exposure to dimethyl sulfoxide

In order to determine the cryopreservation conditions, including cryoprotectant concentration, keeping temperatures and holding time, for subsequent experiment, the toxicity of Me_2SO to dermal fibroblasts was investigated by exposing cells to different concentrations of Me_2SO. Cells were exposed for 10–30 min at 4, 25, or 37 °C to one of the following Me_2SO target concentrations (V/V): 5%, 10%, 15%, 20%, 25%, and 30%, and these cells-cryoprotectant solution systems were diluted with equal volume DMEM solution, respectively, and centrifuged at 1000 rpm for 5 min. The Me_2SO was dissolved in the DMEM solution. Every test was repeated three times and the average data were reported. The fresh cells' viability was taken as control.

Cryopreservation of dermal equivalent

Based on the results of the above cryoprotectant toxicity experiment, further experiment were conducted to investigate the effects of Me_2SO concentration and cooling rate on the cell viability of freeze-thawed tissue-engineered dermal replacement. The dermal slices were placed into sealed polyvinyl chloride (PVC) bags (35×60 mm) (Shanghai Blood Center, Shanghai, China), which contained 1mL of the Me_2SO solution. Then cooling was carried out in a computer-controlled programmable cooler described specifically elsewhere [4] at designed cooling rate from 4 to −60 °C and then plunged into the liquid nitrogen Dewar (University Shanghai of Science and Technology, Shanghai, China) immediately and stored for 24 h.

As a preliminary test, the effect of cryoprotectant concentration on the cell viability was studied in a much wider concentration range of 5–30% at a cooling rate of 2 °C min^{-1} in order to identify suitable concentration range, with a dermal replacement cultured for 9 days being used. Then based on this preliminary experiment, the following further experiments were conducted to investigate the effects of cryoprotectant concentration in a much narrower concentration range of 10–20%, and the effect of cooling rate on the cell viability, with a dermal replacement cultured for 14 days being used.

After 24 h of cryopreservation, the samples were removed from liquid nitrogen Dewar and placed at room temperature in air for 0.5–1 min, in order to avoid fracture, prior to submergence in a water bath at 37 °C for thawing. The bags were cut open and the tissue slices were transferred to culture dishes, and rinsed in the solution of DMEM with gentle agitation for 5 min. Each experiment was repeated three times and the average data were reported.

Cell viability assessment

Survival of dermal fibroblasts was determined after exposure to different concentrations of Me_2SO solutions using Trypan Blue staining assay [20]. And the viability of dermal equivalents was assessed after cryopreservation and incubated 24 h post-thawing using the modified MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay [23].

For comparison, the fresh dermal fibroblasts and the tissue-engineered dermal equivalent were assayed, respectively, for viability prior to cryopreservation, and the