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The viability change of pigskin *in vitro*

Liangpeng Ge[a], *Lihua Sun*[a], *Junyin Chen*[b], *Xiuli Mao*[a], *Yong Kong*[a], *Fei Xiong*[a], *Jun Wu*[c], *Hong Wei*[a,*]

[a] *Department of Laboratory Animal Science, College of Basic Medicine, Third Military Medical University, Chongqing, China*
[b] *Department of Pathology, 324 Hospital of the People's Liberation Army, Chongqing, China*
[c] *Institute of Burn Research, Southwestern Hospital, Third Military Medical University, Chongqing, China*

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ABSTRACT

Background: It is widely recognised that take of grafts is strongly influenced by tissue viability. Although porcine skin is currently the most widely used xenograft, the viability change of pigskin *in vitro* has not been extensively studied. The purpose of this study was to assess the change of the viability of Bama miniature pigskin after harvest and cryopreservation, and to set up a guideline for pigskin preservation and storage that would allow the skin to retain the highest viability after treatment and still be used in the clinical applications.

Methods: Harvested pigskin grafts were divided into five groups: normal saline medium/4 °C (group 1), Dulbecco's minimum essential medium (DMEM)/4 °C (group 2), normal saline medium/25 °C (group 3), DMEM/25 °C (group 4) and cryopreserved (group 5). In our experiment, the viability was investigated by 3-(4,5)-dimethylthiazol-2,5-diphenyl tetrasolium bromide (MTT) salt assay. We also evaluated the transplantation performance of preserved skin in different conditions by using a rat recipient model, in which primary take was evaluated by gross observation and predetermined histological criteria after 7 days.

Results: Skin stored at 4 °C showed a very slow viability decrease with time. The sample showed a viability decrease of about 70% after 3 days in normal saline and 4 days in DMEM medium. Nevertheless, skin stored in DMEM at 25 °C underwent a viability increase during the first 4 h and then decreased gradually to about 70% after 20 h, while the viability declined very quickly for skin grafts stored in normal saline medium at 25 °C, and maintained the same viability only within 6 h of preservation. On the other hand, cryopreserved skin has been shown to maintain a level of skin metabolism equal to 77% of the fresh sample when measured immediately after thawing, and the viability remained about 70% after 6 h at 25 °C and 2 days at 4 °C in DMEM. The graft performance of skin specimens with 70% viability of fresh skin stored in different conditions has not shown statistical significance compared with fresh pigskin.

Conclusions: Based on these results, we suggest that the conservation period of fresh pigskin should not exceed 72 or 96 h when stored in normal saline or DMEM at 4 °C, and should not exceed 6 or 18 h when stored in normal saline or DMEM at 25 °C. Cryopreserved pigskin should be stored in DMEM for a maximum period of 48 h at 4 °C and 6 h at 25 °C after thawing.

* *Corresponding author at*: Department of Laboratory Animal Science, College of Basic Medicine, Third Military Medical University, Gao-tan Yan Street Shapingba District, Chongqing 400038, China. Tel.: +86 23 68752051.
E-mail address: weihong63528@163.com (H. Wei).

Abbreviations: DMEM, Dulbecco's minimum essential medium; MTT, 3-(4,5)-dimethylthiazol-2,5-diphenyl tetrasolium bromide; DMSO, dimethyl sulphoxide; SFDA, State Food and Drug Administration of China.

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A major advance in optimising the care of the severely burnt victim is the clinical procedure of early excision and skin grafting [1]. The concept behind this procedure is the displacement of the necrotic tissue burden with a skin graft. It is a commonly accepted view that fresh cadaver allograft is the gold standard biologic dressing for closure of burns [2–4]. Unfortunately, the source of human skin and its application in treatment of burn victims is very limited due to skin shortage, possible transmission of infectious diseases, religious reason and other social factors. So it is important to set up pigskin banks to replace human skin banks because pigskin is cheap and readily available (pigs have large litters and are quick to mature) and its histological structure is supposedly similar to human skin; this is especially more so in those hospitals that do not have a human skin bank [5,6].

Viability assessment in graft skin is an essential step to ensure good-quality skin supply for clinical repair of wounds. It is widely recognised that take of grafts is strongly influenced by tissue viability [7–10]. Advantages of graft take with regard to wound healing include an immediate barrier to micro-organism invasion and minimal new tissue synthesis required to close the defect. Although pigskin is currently the most widely used xenograft [5,6], the viability change of pigskin *in vitro* has not been extensively studied. So, it is necessary to assess the influence of common operation and storage conditions on the viability of pigskin. Here, we also studied the viability change of pigskin after cryopreservation and thawing because skin is often stored in liquid nitrogen in the hospital, and we compared the results with other commonly used skin preservation methods at 25 or 4 °C in regular preservation medium. The purpose of the present study is to evaluate the viability of pigskin *in vitro* and to set up guidelines that allow for the retention of the highest skin viability after treatment and still can be used in clinical applications.

1. Materials and methods

1.1. Procurement of pigskin sample

According to our protocol, Bama miniature pigs (average age of 4.5 months, average weight of 13.8 kg) were sacrificed and shaved. The pig carcasses were cleaned with 10% povidone–iodine solution and then 0.3- to 0.6-mm partial-thickness skin from pigs' back were harvested with a dermatome. Immediately after the removal, the harvested skins were placed into sterile boxes in normal saline supplemented with penicillin/streptomycin and sent to the laboratory, then soaked in 0.1% benzalkonium bromide for 15 min to sterilise and rinsed thrice in normal saline supplemented with penicillin/streptomycin.

The harvested skin were stored at 4 and 25 °C or placed in a polythene bag for cryopreservation research.

1.2. Storage at 4 and 25 °C

After procurement, skin samples were held in sterile containers in DMEM, supplemented with penicillin (100 IU ml^{-1})/streptomycin (100 μg ml^{-1}), 10% newborn bovine serum (Sigma, USA), 1.6 mg ml^{-1} sodium bicarbonate (Sigma, USA) and 1.5 mg ml^{-1} HEPES (Sigma, USA) and normal saline supplemented with penicillin (100 IU ml^{-1})/streptomycin (100 μg ml^{-1}) at 4 or 37 °C. The medium was changed every 2 days. The first analysis with MTT salts was performed immediately after harvesting (positive controls). MTT tests were then performed every 2 h after skin harvest up to 24 h at 25 °C and then daily for the first week of preservation, then weekly at 4 °C.

1.3. Cryopreservation protocols

Skin samples were cryopreserved using 10% DMSO. Two different cryopreservation protocols (quick freezing/snap frozen and slow freezing) were performed in this study. In the first method, incubation with cryoprotectant was carried out at 4 °C for 20 min, skin samples were placed in a polythene bag and enclosed by heat-sealing machine, and then snap frozen and stored in liquid nitrogen until thawing. In the second method, the freezing process was performed in three stages: the first 1 h at −20 °C, the second at −80 °C for at least 24 h and the third in liquid nitrogen until use [8]. Storage time varied from 15 to 30 days before thawing and viability testing.

Cryopreserved skin samples were thawed rapidly while still inside their innermost sealed packets by immersion in a 40 °C water bath. When the frozen cryoprotectant solution had nearly all melted, the pouches were removed from the water bath and opened. The grafts were then washed with sterile NaCl 0.9% solution. Lastly, the grafts were divided into two groups: one was held at 4 °C in normal saline immediately after thawing and tested every day for 10 days to assess the retained viability of the samples, the other was put into DMEM and then processed at 25 °C; MTT tests were then performed every 2 h after skins were thawed up to 24 h.

1.4. Viability assay

Skin biopsies were assayed for viability by measuring tetrazolium reductase activity. Grafts were assayed one by one from each beaker. A skin disc of 6 mm diameter was obtained using a single-hole punch device (Eagle, China) and weighed in a 5-ml EP tube. One graft can make three skin discs. A single disc was incubated with 1 ml MTT salts (0.5 mg ml^{-1}) at 37 °C for 1 h, in an atmosphere of 5% CO_2. After removing the MTT salt, the skin disc was dissolved in 2.5 ml dimethyl sulphoxide (DMSO) with shaking at 100 r min^{-1} and 20 °C for 2 h. The dissolved skin solution was read on a spectrophotometer (570 nm). As negative control, skin discs were devitalised by boiling or repeatedly snap freezing and thawing using liquid nitrogen. The average measurement from the two devitalising methods was used as the negative control value; the negative control was afterwards treated as other samples and optical density was subtracted from each sample [8,10].

The viability of a skin sample was expressed as the ratio of its O.D. (570 nm) to its weight in mg:viability index (VI) = $\frac{\text{optical density (570 nm)}}{\text{weight (mg)}}$

The mean viability index was calculated from three skin discs of every graft. The percentage viability index of the sample is the mean viability normalised to that of the fresh skin (considered as 100%).