·Basic Research·

Comparison of conventional and directional freezing for the cryopreservation of human umbilical vein endothelial cells

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Abstract

• AIM: To compare conventional slow equilibrium cooling and directional freezing (DF) by gauze package for cryopreservation of human umbilical vein endothelial cells (HUVECs).

• METHODS: HUVECs were randomly assigned to conventional freezing (CF) and DF by gauze package group. The two groups of HUVECs were incubated with a freezing liquid consisting of 10% dimethylsulfoxide (DMSO), 60% fetal bovine serum (FBS) and 30% Dulbecco's modified Eagle's medium (DMEM) and then put into cryopreserved tubes. CF group, slow equilibrium cooling was performed with the following program: precool in 4℃ for 30min, -20℃ for 1h, and then immersion in -80℃ refrigerator. DF group, the tubes were packaged with gauze and then directional freezing in -80℃ refrigerator straightly. One month later, the vitality of HUVECs were calculated between two groups.

• RESULTS: There was no significant difference in the survival rate and growth curve between CF and DF groups. The DF group was significantly better than CF group in adherent rates, morphological changes and proliferative ability.

• CONCLUSION: In the conventional cryopreserved method, cells are slow equilibrium cooling by steps (4℃, -20℃ and finally -80℃), which is a complicated and time-consuming process. But the improved DF by gauze package method is better than conventional method, for which is convenient and easy to operate.

• KEYWORDS: cryopreservation; human umbilical vein endothelial cells; slow equilibrium cooling; directional freezing
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INTRODUCTION

Human umbilical vein endothelial cells (HUVECs) are extensively used for vascular research. They are experimentally pliable and phenotypic plasticity ranging from formation of continuous monolayers to angiogenesis in three dimensional cultures [1]. Moreover, HUVECs continue to express signaling molecules which respond to inflammatory mediators, hypoxia and angiogenic growth factors such as vascular endothelial growth factor (VEGF)[2]. Thus, HUVEC was an useful tool to research the mechanism and treatment of neovascular eye disease.

Cryopreservation is a process where cells or whole tissues are preserved by cooling to sub-zero temperatures. At low enough temperatures, any chemical activity or enzymatic which might cause damage to the material in question is effectively stopped. Cryopreservation methods search to reach low temperatures without leading to additional damage caused by the formation of ice during freezing. Some authors[3,4] proved that there was no significant difference in the biological characteristics of cryopreservation before and after. Nevertheless, it is important to improve the efficiency

of cryopreserving HUVECs. At present, there is a main method for cryopreserving cells: conventional freezing (CF)[5]. CF operates on the principle of slow equilibrium cooling, which is complex and time-consuming. This paper will introduce a new method: directional freezing (DF) by gauze package. It's a new method of cryopreserved cell, which further improving the cumbersome aspects of CF cryopreserved process.

SUBJECTS AND METHODS

Subjects This study adhered to the tenets of Declaration of Helsinki and ethical approval was given by the medical ethics committee of Jinan University, and informed consent was obtained from patients. The HUVECs were supplied by Institution of Obstetrics and Gynecology, Jinan University. Dulbecco's modified Eagle's medium (DMEM) powder was purchased from Gibco (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from Hyclone (Shanghai, China). Dimethylsulfoxide (DMSO) was purchased from Puboxin Company (Beijing, China). Trypan blue powder was purchased from Sigma (St Louis, MO, USA). The methylthiazoletetrazolium bromide (MTT) and Annexin V/FITC assay were purchased from Kaiji company (Nanjing, China).

Methods

Method of obtaining culture of human umbilical vein endothelial cells HUVECs were obtained by means of enzymatic influence of collagenase on the inner surface of umbilical vein wall in a sterile environment, as described earlier[6].

Cryopreservation of human umbilical vein endothelial cells HUVECs were digested with trypsin, then terminate digestion with complete medium, and the supernatant were removed by centrifugation (1000 r/min, 5min). Put the suspensions of HUVECs adjust to the concentration $(1.0\text{-}2.0)\times10^9$/L, follow the steps, and ensure that you use the configured frozen stock solutions (the volume fraction of 10% DMSO, 60% FBS and 30% DMEM serum-free medium)[7]. HUVECs were randomly assigned to CF group and DF group by gauze package. The former using the principle of slow equilibrium cooling: precool in 4℃ for 30min, -20℃ for 1h, and then immersion in -80℃ refrigerator[8-11]. The latter using the method of gauze wrapped around 20 layers, and then put cryopreserved tube directly into -80℃ refrigerator.

Recovery of human umbilical vein endothelial cells[12,13] After a month cryopreservation in -80℃ refrigerator, the HUVECs were thawed in a 37℃ water bath for 1-2min. Oscillate the cryopreserved tube until cells suspension melt completely, then the cells suspension were diluted by ten times volumetric DMEM liquid, 900 r/min centrifugal for 5min and supernatant was discarded.

Survival rate of human umbilical vein endothelial cells Trypan blue cell counting method was used to verify the Survival rate of HUVECs [14]. In brief, HUVECs suspension was injected into the cell counting board under the inverted phase contrast microscope (Olympus, Japan) to observe. Followed by recording the ratio of not been blue-stained cell number and total cell count under the 100 times inverted microscope. Under normal circumstances, record cell numbers of four non-overlapping horizons and figure out its average, while this value is regarded as the cell survival rate.

Microscopic observation of human umbilical vein endothelial cells The cryopreserved HUVECs were cultured in a six-well plate at 2×10^5 cells per plate for 2, 12 and 24h. The morphology of HUVECs was observed from the culture flask under phase-contrast inverted microscope. Images of HUVECs were captured using a Cail Zeiss microscope (Cail Zeiss, Germany).

MTT assay Cell growth was determined using a MTT assay (Methylthiazoletetrazolium bromide, Kaiji). This assay is based on the reduction of MTT into a blue formazan dye by viable mitochondria. In brief, the two groups cryopreserved HUVECs were seeded into each well of 96-well plates with 5 replicates for each group at each time point. After stated incubation time, 20 mL MTT solution (5 mg/mL) was added to each well. After 4h of incubation, the medium was aspirated as completely as possible without disturbing the formazan crystals. Then, 150 mL DMSO was added to each well and placed on a plate shaker for 10min. The optical density values at 570 nm were then measured with a microplate reader equipped (Tecan Safire2, Switzerland).

Flow cytometry analysis on apoptosis cells To quantify apoptosis cells, Annexin V and propidium iodide staining were used. Briefly, cryopreserved HUVECs were grown on a six-well plate at 1×10^6 cells per plate for 24h. Thereafter, cells were collected and stained with Annexin V-FITC and PI in binding buffer. The samples were then measured by flow cytometry (BD FACSAriaTM, USA).

RESULTS

Survival Rate of Human Umbilical Vein Endothelial Cells There were no blue-stained cells between two groups (blue-stained rate was 0%, survival rate was 100%).

Morphological Changes of Human Umbilical Vein Endothelial Cells The anabiotic HUVECs were grown in

T25 culture bottle. There were fifty percent of the adherent cells in DF group, however only parts of the adherent cells in CF group for 2h (Figure 1). After 12h of incubation, the HUVECs grown to 80%-90% confluence in DF group, but only 50% confluence in CF group. After 24h incubation, the DF group cells had approximate 100% confluence. The morphology and growth feature of HUVECs were observed by inverted microscope every day between two groups. The result showed that there was no different between DF group and non-frozen cell. On the contrary, there was difference between DF and CF groups (Figure 2).

Growth Curve of Human Umbilical Vein Endothelial Cells After Recovery Graphics of two groups showed a typical S-shaped curve, almost perfect correspondence. Compared with non-cryopreserved group, HUVEC growth curve was no significant difference in the 2nd and 4th days. However, the visible difference from the first six days (slightly below the freezing group), as shown in Figure 3.

Apoptotic Rate of Human Umbilical Vein Endothelial Cells After Recovery The results showed that cell necrosis accounted for 0.20%, early apoptosis (3.45%), late apoptosis (1.78%) and cell survival (94.57%) in DF group; cell necrosis accounted for 0.43%, early apoptosis (2.25%), late apoptosis (2.01%) and cell survival (95.32%) in CF group, as shown in Figure 4.

DISCUSSION

Trypan blue staining method showed that there were no dyed blue cells between DF and CF groups. That is to say, HUVECs grown in normal living condition in two groups. Flow cytometry analysis showed that some of the cells have been apoptosis in early stage or late stage, and even necrosis at last. Traditional test methods of the cell vitality often use trypan blue staining, which only applicable for the detection of necrotic cells, but if the cell apoptosis, it's still able to reject trypan blue staining [15]. As our results showed that the anabiosis cells of both DF group and CF group were no being blue-stained, but contrast to flow cytometric analysis showed that some cells have already been apoptosis. The result was consistent with previous research [16]. Moreover, as time goes on, trypan blue staining will also happen in the surviving cells, so the resulting count difference is very great, and the trypan blue staining method was not be regarded as the appraisal standard of cell death [17]. Thus, the researchers observed the morphological changes of the resurgent cell in the experiment, and performed the apoptosis detection with flow cytometry and drew the growth curve with MTT method.

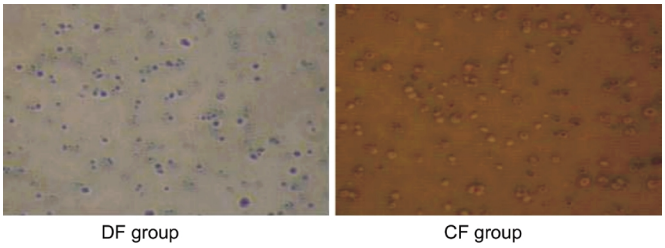

Figure 1 Morphological changes of HUVECs (2h) DF group: Directional freezing by gauze package; CF group: Conventional freezing method (×50).

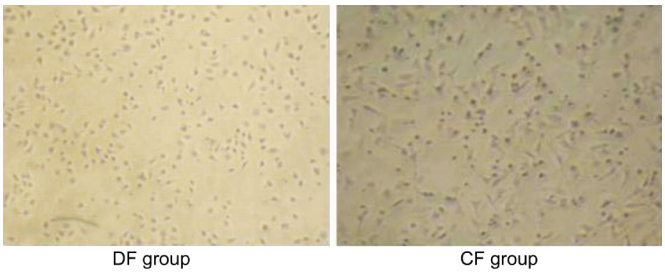

Figure 2 Morphological changes of HUVECs (12h) DF group: Directional freezing by gauze package; CF group: Conventional freezing method (×50).

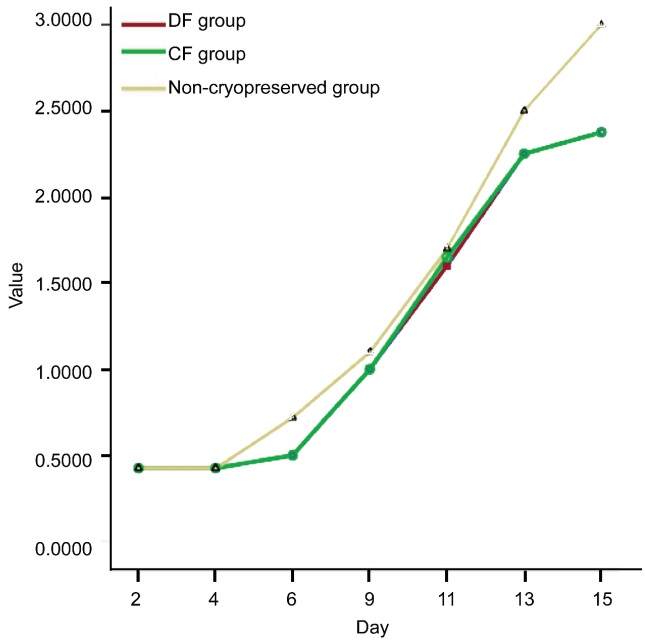

Figure 3 Growth curve of HUVECs in each group by MTT DF group: Directional freezing by gauze package; CF group: Conventional freezing method.

Flow cytometric testing results showed that there is no significant difference between the improved gauze wrapped DF method and the CF method for cryopreserving HUVECs. At this point, for the preservation of cells, the improved gauze wrapped DF method was superior to CF cryopreserved method. Cell adherent capacity and proliferation of DF method were much better than CF method, but MTT method showed that at the base of inoculating the same number of cells, the total activity of the cell detected by the two methods

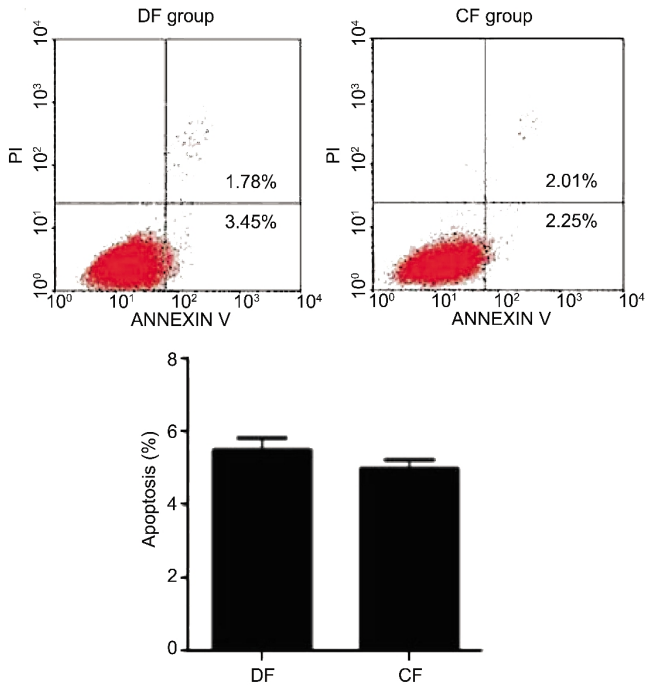

Figure 4 Apoptotic rate of HUVECs was detected by flow cytometry DF group: Directional freezing by gauze package; CF group: Conventional freezing method.

was similar. There was some possible reason to illuminate the phenomenon. Although cell adherent capacity of CF was inferior to the DF group, after a long time (96h) of culturing, there was no significant difference between the two groups. Kuczyński *et al* [18] found that frozen sperm technology is an effective process of screening, with which aging or deformity sperm can be eliminated. Hence, there is a bold hypothesis that the cryopreserved cells differentiation process can also be seen as a kind of natural selection, in which the cell viability with low level were eliminated, and only left those with high viability cells including membrane function stability metabolic exuberant and chromosome normal cells can endure low temperature to be preserved, so MTT experimental results of two groups showed no obvious difference. In terms of the growth curve drawn by MTT method, there was no significant difference between the two groups. Similarity was found in growth curve radian of the DF group and non-frozen ones, but the whole slightly lower than non-frozen group, this suggested that HUVECs for cryopreservation is feasible. The effect of improved cryopreservation method is not inferior to the existing cryopreservation ones, as the cell vitality, cell cryopreservation still can result in certain damage.

With the development of the technology of cell cryopreservation, many kinds of cells can be stored for a long time *in vitro* [19,20]. The growth state after recovery directly influences the effect of various biology experiments, it mainly relates to the temperature range.

The process of cryopreserved cells needs three phases, ice crystals form, dehydration and soluble substances concentration. The standard cryopreserved program is slow freeze-stored [21]. If the temperature decline slowly, every minute down 1℃, the intracellular don't appear ice crystal. Ice crystals mainly form in the extracellular and cell itself only gradually dehydrated, does not suffer injury [22]. If the cooling speed perform too fast, intracellular and extracellular simultaneously form ice crystal, which will cause damage to organelles, protein degeneration and nuclear chromosome space configuration change *etc*; and finally also lead to cell death [23]. Therefore, the "slow freezing" is the key factor to avoid the cells suffers from serious injury [24]. Ice crystal formation and concentration of electrolyte mainly occurs between -10℃ and -5℃. The cryopreserved cells of traditional method stay in the -20℃ to -4℃ for a long time, the temperature drop severely. Improved cryopreserved method that gauze wrapped cell freeze-stored tube, can obviously reduce the speed of the drop in temperature. The temperature of cells in the freeze-stored tube decline gently, as previously described "slow freezing" method, so it can effectively reduce the damage to cells in a low freezing temperature. Under the cryopreserved environment, cells biochemical reactions and energy metabolism are basically static, so freeze-stored time has no obvious or less significant effect on cells. Zhang *et al* [25] proved the correctness of this inference. Rigol *et al* [26] also indicated that freeze-stored time had no obvious effect on vascular function. To achieve good cryopreserved effect, the crucial points are preservation solution and the cooling rate.

In summary, our results revealed that improved gauze wrapped method is no significant difference compared with the traditional CF method, or even better than CF method such as adhesion rate and cell morphology, *etc*. Moreover, compared with CF method, the improved gauze wrapped DF method features concise in operation steps, less cost, and suitable to be popularized and used. In this study, we just only observe cryopreserved cells in -80℃, as to whether can be used in liquid nitrogen, still needs further experimental research.

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