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Effect of cryopreservation on viability and growth efficiency of stromal-epithelial cells derived from neonatal human thymus

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ABSTRACT

The thymus is the major site of T lymphocyte generation and so is critical for a functional adaptive immune system. Since, thymectomy is a component of neonatal surgery for congenital heart diseases, it provides great potential for collection and storage of thymic tissue for autologous transplantation. However, specific investigation into the optimum parameters for thymic tissue cryopreservation have not been conducted. In this research, we evaluated the effect of different cryoprotective media compositions, which included penetrating (Me_2SO, glycerol) and non-penetrating (dextran-40, sucrose, hydroxyethyl starch) components, on the viability and functionality of frozen-thawed human thymic samples to select an optimal cryoprotective medium suitable for long-term storage of thymic tissue and a stromal-epithelial enriched population. Our primary focus was on receiving, low-temperature storage, culturing and evaluation of thymic tissue samples from newborns and infants with congenital heart diseases, who had undergone thymectomy as a part of standard surgical procedure. Thus, this work builds the platform for autologous clinical intervention into the thymus-deficient patients with congenital heart diseases. From our data, we conclude that although there were no significant differences in efficiency of tested cryoprotective media compositions, the combination of Me_2SO and dextran-40 compounds was the most suitable for long-term storage both thymic cell suspensions and thymic fragments based on the viability of $CD326^+$ epithelial cells and stromal-epithelial cell monolayer formation.

1. Introduction

The thymus is one of the central organs of immune system; it is the major site of T lymphocyte generation and hence is critically important for a functional adaptive immune system. However, with aging, the thymus is among the first organs to degenerate in normal healthy individuals [12]. Thymus function is suppressed under stress and by influence of various cytotoxic effects, including some medical treatments [36]. While many functions of the adaptive immune system are established during infanthood, partial or total thymectomy is a part of some surgical procedures, particular at the congenital heart diseases, especially in newborns [5]. Some forms of severe combined immunodeficiency such as DiGeorge syndrome, Nezelof syndrome, Louis-Bar syndrome, Swiss syndrome are due to inherent thymic aplasia [8,20,32]. Impaired thymic function has a number of consequences for the immune system including increased susceptibility to infections and autoimmunity, high risk of cancer development as well as decreased response to vaccines with age [1,10,17,22,29,35]. Because of these, there is currently a great interest in developing strategies for boosting thymic function by either cell replacement or regenerative strategies. In this regard, the regulation of thymic recovery is extremely relevant and clinically significant for immune system rehabilitation especially in thymectomized newborns and infants.

Since, thymectomy is a component of neonatal surgery for congenital heart diseases [16,19,30], it provides great potential for collection and storage of thymic tissue and its use for autologous

Abbreviations: 7-AAD, 7-aminoactinomycin D; annexin V-EGFP, annexin V-enhanced green fluorescent protein; Abs, antibodies; CPM, cryoprotective medium; CM, culture medium; FBS, fetal bovine serum; FSP, fibroblast surface protein; FACS, fluorescein activated cell sorter; PI, propidium iodide; SCB, Stem-CellBanker; TESC, thymic epithelial stem cells.

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transplantation. Thus, development of validated protocols for storage and autologous transplantation of thymic tissue or stromal-epithelial cells, primarily thymic epithelial cells (TEC) can lead to the successful reconstitution of immune system functions as was shown on animal models and in some clinical investigations [3,4,9,15,23,24,27,28]. However, while the born marrow and cord blood are priority sources of hematopoietic stem cells, and cryopreservation protocols for these tissues were established, as have been protocols for cryopreservation of stromal cell preparations from adipose tissue and from bone marrow [2,7,13,33,37,38], the thymus as a potential source for stem cell therapy has received little attention. There are only a few reports of freezing thymus tissue, where protocols with dimethyl sulfoxide (Me_2SO) and fetal bovine serum (FBS) were used for cryopreservation of thymic tissue or thymic stromal cells [6,18,25]. No special investigations have been performed concerning thymic tissue cryopreservation. In current research, we have evaluated the effect of different cryoprotective media (CPM) compositions, which included penetrating (Me_2SO, glycerol) and non-penetrating (dextran-40, sucrose, hydroxyethyl starch) components [21] on the viability and functionality of frozen-thawed human thymic samples to select an optimal cryoprotective medium suitable for long-term storage of thymic tissue and stromal-epithelial population enriched by TEC prepared by different methods. Our primary focus was on thymic tissue samples obtained from newborns and infants with inherent heart diseases. Our data indicate that although there were no significant differences in efficiency of tested cryoprotective media compositions, the combination of Me_2SO and dextran-40 compounds was the most suitable for long-term storage both thymic cell suspensions and thymic fragments based on the viability of $CD326^+$ epithelial cells and stromal-epithelial cell monolayer formation.

2. Materials and methods

2.1. Thymus collection and evaluation

Human thymic samples were obtained from newborns and children under the age of 3 years as a part of standard operation procedure on the heart from patients with congenital heart diseases. Thymus sampling was performed under aseptic conditions in the Institute of Heart of the Ministry of Health of Ukraine at the Pediatric Department (Kyiv, Ukraine). Informed consents from the parents for thymic tissue collection and using for research aims within ThymiStem project were received before surgery and approved by Bioethics Committees of the Institute of Heart and the University “Ukraine”. According to the regulations of the Ministry of Health of Ukraine, before the surgical operation, patients were tested for Hepatitis B, Hepatitis C, Treponema Pallidum and HIV infections. Only thymic samples from patients free of these infections were collected for further research.

For transportation, thymic lobes were put into 100 ml sterile plastic tubes for tissue sampling with 25 ml of RPMI-1640 (Sigma, USA) or DPBS (ThermoFisher Scientific, USA) with antibiotics (streptomycin/penicillin) in concentration of 50 U/ml of penicillin and 50 μg/ml (33.55 IU) of streptomycin (both from ThermoFisher Scientific, USA) – transportation medium. The tubes were transported in an isothermal box on ice to the laboratory. Total time, including thymus removal and transportation to the laboratory, was about 2 h. Before preparation and freezing, thymic lobes were weighed in sterile conditions, and ranged from 3.15 g to 74.56 g (mean, 24.41 g). Eighty thymuses were collected, prepared and stored in liquid nitrogen from several days up to two years with use of different CPM compositions. Thymic samples were analyzed with a set of methods as described below.

2.2. Thymic tissue preparation

Thymic lobes were transferred from the transportation tubes into large Petri dishes (d = 10 cm, ABC Scientific, USA) with RPMI-1640 (Sigma, USA) or DPBS (ThermoFisher Scientific) transportation medium, and cut into fragments approximately of 0.8–1.0 cm^3 in size using sterile scissors. Cell suspensions were obtained where required by mechanical disaggregation or enzymatic digestion of thymic fragments, performed as described below.

For mechanical disaggregation, thymic fragments were homogenized in a transportation medium using a glass Dounce mechanical homogenizer, filtered through a sterile nylon filter (Falcon® 70 μm Cell Strainer, Fisher Scientific, USA) and washed 3 times in a transportation medium by centrifugation at 300*g* for 5 min. All procedures were performed on ice.

Enzymatic disaggregation was performed as described [14,26,34] with some modifications for human thymic tissue. Thymic lobes were cleaned from capsule and other non-thymic tissues under aseptic conditions. Then they were transferred into a Petri dish in transportation medium and cut with sterile scissors into 0.8–1 cm^3 pieces. These pieces were gently agitated to release T cells. Each thymic piece was then cut into as many small pieces as possible and agitated gently in a Petri dish with transportation medium to release remained T cells. For further removal of remaining T cells, thymic pieces were transferred into 50 ml centrifuge tubes containing 50 ml of transportation medium and agitated at room temperature for 30 min. The supernatant containing thymocytes was removed, and the remaining thymic pieces transferred to a conical tube containing 10 ml of transportation medium. The thymic fragments were pipetted and, after the thymic pieces had settled, supernatant was removed. This procedure was repeated two further times.

For further enzymatic isolation of thymic stromal-epithelial cells from these primary treated thymic fragments, the thymic pieces were incubated for 30 min at 37 °C in 5 ml of transportation medium with collagenase IV (0.125% w/v; Gibco, USA) and DNAse I (0.1% w/v; Gibco, USA) gently agitating with a pipette every 5 min. After the thymic pieces had settled, the supernatant that contained stromal-epithelial cells, was collected into a new 50 ml centrifuge tube with 50 ml of transportation medium, and was kept in CO_2 incubator until use. Fresh collagenase IV with DNAse I in transportation medium was then added to the tube containing the residual thymic pieces, and the incubation with agitation was repeated as described above. After the thymic pieces had settled, supernatant containing released stromal-epithelial cells was collected again, mixed with the cell portion collected before and centrifuged at 300 g for 5 min. The cells were resuspended in Ca^{++}/Mg^{++}- free DPBS with 5% FBS and 0.1% DNAse I (FACS buffer), centrifuged at 300 g for 5 min to pellet the cells. Finally, the cells were resuspended in the required cell culture medium, and the cell number and cell viability were evaluated. An aliquot of cells was resuspended in fresh FACS buffer for analysis of primary thymic cell composition by flow cytometry.

2.3. Thymic samples freezing and thawing procedures

The freezing procedures were carried out on thymic fragments (0.8–1 cm^3 in size), thymic cell suspensions after mechanical homogenization, thymic cell suspensions after enzymatic digestion, and cultured stromal-epithelial cell suspensions. Thymic samples were placed into 2 ml round bottom cryovials (Greiner Bio-One, Germany) with 1 ml of a cryoprotective medium (CPM). All procedures were performed on ice. The cryovials were then moved into a −80 °C freezer in a styrofoam box to provide gradual