Cryobiology 77 (2017) 14–18

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Methodology for reliable and reproducible cryopreservation of human cervical tissue

James M. Fox [a, *], Rebecca C. Wiggins [a], John W.J. Moore [a], Christine Brewer [b], Alison C. Andrew [b], Fabiola Martin [a, b]

[a] Centre for Immunology and Infection, Department of Biology and Hull York Medical School, University of York, UK
[b] York Teaching Hospital, NHS Foundation Trust, York, UK

ARTICLE INFO

Article history:
Received 13 March 2017
Received in revised form
5 June 2017
Accepted 8 June 2017
Available online 9 June 2017

Keywords:
Ex vivo
Cervix
Cervical tissue
Cryopreservation
Freezing
Thawing
Storage
Bio-banking

ABSTRACT

Background: In order to conduct laboratory studies on donated cervical tissue at suitable times an effective and reliable cryopreservation protocol for cervical tissue is required.
Methods: An active freezing approach was devised utilising 10% dimethyl sulfoxide in foetal bovine serum as a cryoprotective agent with a cooling rate of 1 °C/min to −50 °C then 10 °C/min to −120 °C; a related thawing protocol was also optimised which would allow for the bio-banking of cervical tissue. Viability of freshly harvested cervical tissue was compared to frozen-thawed samples utilising colorimetric MTT assay. In parallel, fresh and freeze-thawed samples were cultured and tested on days 1, 7 and 14 to determine whether bio-banking had detrimental effects on tissue viability over time.
Results: Repeat testing revealed that tissue viability between fresh and freeze-thawed samples was comparable at all four time points (days 0, 1, 7 and 14) with no apparent reductions of viability, thus demonstrating this method of cryopreserving cervical tissue is reliable and reproducible, without detrimental effects on live tissue culture. We believe this methodology creates the opportunity for bio-banking donated cervical tissues, which aids improved experimental design and reduces time pressures and wastage.

1. Introduction

Extensive scientific literature has reported the utilisation of cervical tissue organo-typical models to further our understanding of HIV transmission and to aid development of potential therapeutic interventions [12,15]. We are also aware of cervical explants being utilised to study mucosal epithelial cell differentiation and leukocyte infiltration [10], cytomegalovirus infection studies [6] and for vaccine research [2]. Almost exclusively, these studies and others were undertaken in fresh tissue transported without delay from hospitals to scientific laboratories for immediate use in experimental procedures. We aimed to conduct Human T-lymphotropic virus (HTLV)-1 *ex vivo* infection studies in donated cervical tissue from women who had undergone hysterectomies, to mimic physiological *in vivo* infection via the sexual contact route to extend our knowledge beyond more simple cell-cell infection studies; readily available tissue would be beneficial.

We utilised published protocols to establish an *ex vivo* organo-typical model for cervical tissue explants [3] aiming to co-culture HTLV-1 with live cervical explants. However, to facilitate reliable and reproducible studies of tissue co-cultures from the same donor at convenient time points and for purposes of long term storage of tissue for bio-banking, we sought to establish a protocol for cervical tissue cryopreservation akin to those validated for the bio-banking of other tissues, such as tonsils [11]. Here, we demonstrate that using the proposed protocol, cryopreserved cervical tissue can be thawed with minimal loss of tissue viability compared to fresh explants. We believe the development of this reproducible methodology permits long-term cervical tissue storage, thus reducing waste of precious tissue donations and, with relevant ethical approval in place, allows for cervical tissue bio-banking and tissue exploitation for a variety of studies.

2. Materials and methods

2.1. Ethics and tissue preparation

UK National Research Ethics Service (NRES 11/YH/0321),

* Corresponding author. Department of Biology, Wentworth Way, University of York, York, YO10 5DD, UK.
E-mail address: james.fox@york.ac.uk (J.M. Fox).

http://dx.doi.org/10.1016/j.cryobiol.2017.06.004

Research & Development at York Teaching Hospital NHS foundation Trust (YORA01992) and University of York ethically approved the study of *ex vivo* HTLV-1 cervical explant co-culture studies. Written informed consent was obtained from women with normal cervical smears who needed to undergo a planned hysterectomy for their own health and who were willing to donate cervical tissue. After the hysterectomy the uterus was transported to the histopathology department and reviewed macroscopically by the consultant histopathologist. Approximately 90% of the healthy cervix was released for research purposes.

Cervical tissue was immediately transferred into transportation medium (Leibovitz's L-15 medium containing heat-inactivated single-batch 10% foetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B [all from Invitrogen]) and cooled at 4 °C for transportation to research laboratories. All samples underwent processing within 4 h of surgery. Endo- and ecto-cervix were separated and all tissues were cut into approximately 1 cm^3 explants containing mucosal and submucosal tissue to maintain tissue architecture. Tissue explants were either immediately processed for laboratory studies and tissue viability assays on explant pieces defined as day 0 (d0), or cryopreserved for subsequent testing.

2.2. Tissue freezing

Cervical explants (~1 cm^3) that were not immediately studied were placed individually into 2 ml cryovials on ice containing 1 ml of pre-cooled (4 °C) freezing medium (90% FBS with 10% dimethyl sulfoxide (Me_2SO)) that was displaced by the explant ensuring cryoprotectant reached all the tissue. Cryovials were rapidly transferred to a control rate freezer pre-cooled to 4 °C (Planer KRYO560-16, Planer PLC, Sunbury-on-Thames, UK) and explants were cooled from 4 °C to −50 °C at 1 °C/min and then from −50 °C to −120 °C at 10 °C/min before transfer to liquid nitrogen for long term cryopreservation and bio-banking. Freezer chamber temperature data was recorded as evidence of successful cycle completion.

2.3. Tissue thawing

For this study, cervical explants were banked for an average of 189 days (range 119–236 days) before frozen tissue was thawed by cryovial retrieval from liquid nitrogen and immediate immersion in a water bath at 37 °C. As soon as freezing medium had started to thaw, the explant and thawed freezing medium was transferred, using forceps if necessary, to be completely submerged in 15 ml of pre-warmed (37 °C) culture medium (Roswell Park Memorial Institute 1640 medium containing heat-inactivated single-batch 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, all from Invitrogen) in 6-well plates in a sterile environment. Each explant was left undisturbed in this medium for 10 min at 37 °C in a humidified environment supplied with 5% CO_2 in air before three further successive transfers to 15 ml of fresh culture medium, incubating for 10 min in each aliquot of 15 ml medium for a total time of 40 min. Tissue viability testing was immediately performed on explant pieces and defined as d0.

2.4. Tissue viability testing

We tested the viability of fresh and frozen-thawed cervical explants on the defined day 0 and after 1, 7 and 14 days in culture. Each cervical explant was cut up into at least 12 roughly equal-sized small pieces; individual explant pieces were transferred to single wells on a 96 well plate and covered with 200 μl culture medium before immediate testing or culture. Medium was changed every 3 days by removal of 170 μl of medium and replacement with 200 μl of fresh culture medium; empty wells were filled with medium to reduce evaporation. An MTT assay was used to establish general tissue viability by measuring the intensity of purple colour produced through mitochondrial oxidoreductase reduction of the MTT tetrazolium dye to its insoluble formazan, as described previously [14]. A 50 ml solution of Thiazolyl Blue Tetrazolium Bromide (Sigma-Aldrich, UK) [MTT] was prepared at 250 μg/ml in Roswell Park Memorial Institute 1640 medium and filtered through a 0.2 μm filter; 1 ml per well of this solution was aliquoted into three wells of a 48 well plate at each time point. A piece of explant was incubated with the 1 ml of MTT for 3 h under standard tissue culture conditions, in triplicate. After this incubation, tissue was transferred to 1 ml of methanol for dye elution and incubated for 16 h. Explants were removed and left to dry for 24 h, to increase accuracy, before weighing. Two-hundred μl of the tissue eluate in methanol was transferred in triplicate to a 96 well plate before optical density (OD) readings were taken at 595 nm (VersaMax plate reader, Molecular Devices, Wokingham, UK). Triplicate OD readings for methanol alone were made in parallel before subtraction from each sample OD followed by averaging of the triplicate readings for each piece of tissue. Tissue pieces were dried overnight before weight corrected OD (wcOD) was calculated using the formula: average OD reading/dried tissue weight (grams). The percentage metabolic activity as a measure of tissue viability was calculated by dividing the wcOD of each sample at each time point (day 0, 1, 7 and 14) by the averaged wcOD of triplicate pieces of tissue at day 0. We compared the wcOD of 10 fresh and three freeze-thawed tissues at day 0, 1, 7 and 14 to establish a quality control protocol for the cryopreservation of cervical tissue.

2.5. Explant sectioning and staining

Cervical tissue (fresh or freeze-thawed) after 1 day in culture was snap frozen in O·C.T. (Agar Scientific, Stansted, UK) on specimen discs (Leica, Loughborough, UK) seated on dry-ice then transferred to a −80 °C freezer. The blocks were sectioned (5 μm sections) using an OTF5000 cryostat microtome (Bright Instruments Ltd. Luton, UK), and applied to poly-lysine coated glass slides (Fisher Scientific, Loughborough, UK). Air-dried sections were haematoxylin and eosin (H&E) stained by immersing into filtered Mayers haematoxylin (Sigma-Aldrich, Poole, UK) for 5 min then rinsed in running tap water for an additional 5 min before dipping twelve times in 0.5% Eosin (Sigma-Aldrich). Sections were washed again in distilled water then dehydrated by dipping in sequential ethanol baths (50%, 70%, 95% and 100% EtOH) for between 30 and 60 s in each bath. Slides were air-dried and mounted using minimal DePeX (Sigma-Aldrich), a toluene-xylene-based mounting compound, and a coverslip. Sections were tile-scan imaged using a 10× objective on an Olympus B×51 microscope and MagnaFire SP software (Olympus, Southend-on-Sea, UK) before composite images were constructed in Photoshop (Adobe, Maidenhead, UK).

3. Results

From 19 separate cervical donations we were able to obtain, on average, 14 ± 6 explants of cervical tissue measuring approximately 1 cm^3 that was identified by morphology as 1 ± 2 explants of endocervix and 13 ± 6 ectocervix explants (Supplementary Table 1). Explants surplus to experimental requirements were prepared and frozen as outlined and data recordings from the control rate freezer showed that the freezing process proceeded uninterrupted (data not shown). Using the protocols detailed above we performed viability assays at d0 on all cervical donations as well as on three randomly selected frozen/thawed-cervical tissue