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Short-term storage of human testicular tissue: effect of storage temperature and tissue size

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Katrien Faes started her PhD in the research group Biology of the Testis at the Vrije Universiteit, Brussel in 2011 and successfully defended in May 2016. During this PhD, she focused on the short-term preservation of human testicular tissue and the translation of the spermatogonial stem cell transplantation towards a clinical application.

KEY MESSAGE

Adult human testicular tissue can be maintained at 4°C or room temperature for up to 3 days and before cryopreservation, without altering tissue morphology, Sertoli cell morphology, number of spermatogonia or number of apoptotic cells. The tissue does not need to be extensively dissected as tissue morphology is better preserved in larger fragments.

A B S T R A C T

During short-term storage, before cryopreservation, testicular tissue quality can be affected by storage medium, duration, temperature and tissue size. We previously established the best storage medium and time for short-term maintenance of tissue. In this study, three different storage temperatures (4°C, room temperature, 37°C) and four tissue sizes (~6 mm^3; ~ 15 mm^3; ~ 50 mm^3 or ~80 mm^3) were assessed over the course of a fixed period of 3 days. Storing human testicular tissue at 37°C caused a significant increase in the number of apoptotic cells per tubule ($P = 0.002$), compared with fresh control, but this was not the case at 4°C or room temperature. Temperature did not affect viability, tissue morphology or number of spermatogonia in samples. The morphology of the testicular tissue was optimally preserved when stored as large fragments (~50 mm^3: $P = 0.018$; ~ 80 mm^3: $P = 0.018$). Tissue size did not significantly affect viability, number of spermatogonia or apoptotic cells. Adult human testicular tissue can be preserved at 4°C or room temperature without altering tissue morphology, Sertoli cell morphology, number of spermatogonia or number of apoptotic cells. The tissue does not need to be extensively dissected as tissue morphology is better maintained in larger fragments.

Introduction

About 15 years ago, the Universitair Ziekenhuis (UZ) Brussel started to cryopreserve testicular tissue biopsies for prepubertal boys at risk of spermatogonial stem cell (SSC) loss, which can be caused either by genetic defects or by gonadotoxic treatments. To date, preservation is only carried out in a few centres worldwide (Picton et al., 2015), which implies that the site of testicular biopsy might not be the same as the site of long-term storage. The way that the testicular biopsy

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is transported is decisive in maintaining the best possible tissue quality before cryopreservation. Improper storage jeopardises the viability and functionality of SSCs after thawing and, as they are scarce (Phillips et al., 2010), this should be avoided.

Although hypothermic storage and cryopreservation ultimately have the same goal (cell or tissue preservation), hypothermic storage is limited to a few days or weeks, whereas cryopreservation can last years (Baust et al., 2015). Typically, for short-term storage, a hypothermic temperature is chosen as cooling prolongs in-vitro survival owing to the metabolism being slowed down, reduction in oxygen demand and conservation of chemical energy (Brockbank and Taylor, 2006). Hypothermic short-term storage is categorized as mild (32–35°C), moderate (27–32°C), profound (10–27°C) and ultra-profound (0–10°C) (Baust et al., 2015).

During short-term storage, tissue quality can be influenced by storage medium, duration, temperature and tissue size. In our previous study, the importance of storage medium and storage time was addressed for adult human testicular biopsies (Faes and Goossens, 2016). It was demonstrated that the biopsy was best preserved in Dulbecco's modified Eagles medium: Nutrient mixture F-12 (DMEM/F12) for no longer than 3 days. To date, storage temperature has remained at 4°C and size of testicular tissue about 6 mm^3, consistent with tissue size at cryopreservation (Baert et al., 2013).

Although porcine testicular cell suspensions are best preserved at 4°C (Yang et al., 2010) and a light and electron microscopy study has shown that spermatogonia are well preserved overnight in a humid chamber at 4°C (Feng and Holstein, 1990), the optimal short-term storage temperature has never been assessed for human testicular tissue.

Additionally, only a few studies have addressed tissue size during short-term storage of porcine testicular tissue. Yang et al. (2010) investigated three tissue sizes for preservation of porcine testicular tissue: 30 mg, 100 mg and intact testes, and did not observe any significant differences in cell survival or morphology when the tissue was stored in Hypothermasol Free of Radicals and Scavengers during 6 days at 4°C. Abrishami et al. (2010) investigated the hypothermic preservation of immature intact porcine testes up to 72 h in Dulbecco's phosphate buffered saline (DPBS). During the length of cooling, no reduction in viable cells was observed and structural integrity was not impaired. Xenografting of the cooled testicular tissue revealed that the proliferative potential was not harmed (Abrishami et al., 2010). To our knowledge, the optimal tissue size for short-term storage of human testicular tissue has not been assessed. If the biopsy can be kept whole, the tissue would need less manipulation and, therefore, the chance to recover a sufficient number of cells for further fertility preservation techniques might increase (Yang and Honaramooz, 2010).

In this study, storage at room temperature (~22°C) and body temperature (37°C) was investigated and compared with 4°C conditions. Furthermore, optimal tissue size for short-term storage of human testicular tissue was assessed.

Material and methods

Tissue source

All human samples were obtained from patients undergoing vasectomy reversal after written informed consent. All patients had a normal tubular structure as proven by histology. Immediately after biopsy, the tissue was transported on ice to the laboratory. The experiments were approved by the ethical committee of the UZ Brussel on 8 January 2014 (reference number: 2013/397).

Experimental design

An overview of the experimental design is given in **Figure 1**. During the first part of the experiment, the optimal storage temperature (4°C, room temperature or 37°C) was investigated and, in the second part of the experiment, the optimal tissue size was assessed. The largest size was chosen based on the average size of a whole testicular biopsy (~80 mm) in prepubertal boys (Ginsberg et al., 2010). Each experiment lasted 3 days.

Viability

After receiving the testicular biopsy, the tissue was washed in DMEM/F12 (31330-095; Invitrogen, Belgium) containing L-glutamine and 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, cut into desired fragment size and kept in DMEM/F12 for 3 days. Fragment evaluation is presented in **Figure 2**.

To measure cell viability of the day 0 controls and one-half of the fragments at the end of the experiments, testicular tissue was incubated with 10 mg/kg collagenase IV (C5138; Sigma-Aldrich, Belgium) for 30 min in a 37°C water bath. Next, the supernatant was removed after centrifugation at 600 g for 5 mins. The tissue was further digested by incubation with 4 mg/ml trypsin (T4665; Sigma, Belgium) and 2.2 mg/ml deoxyribonuclease (DNase; DN25; Sigma, Belgium) I for 10 min in a 37°C water bath. The reaction was stopped by adding 2.5 volumes of 10% human serum albumin (HSA; 20 mg/ml; 10064, Vitrolife, Sweden). After three washes with phosphate buffered saline, the cells were resuspended in DMEM/F12. To determine the number of viable cells, 100 µl of the cell suspension was incubated with 1 µl of dead cell red (A10786, Life Technologies, Belgium) for 3 min. After incubation, 25 µl of the total solution was added to each chamber of the Tali slide for counting by the cytometer.

Histology and immunohistochemistry

After fixation of the remaining fragments, the samples were embedded in paraffin and cut into 5-µm thick serial sections (**Figure 2**).

For histology and immunohistochemistry, per fragment (one for day 0; two per temperature and per tissue size) two sections, with a depth difference of 400 µm, were investigated. To evaluate the tissue size effect, two to five tissue depths were investigated, depending on the size of the tissue fragment. Care was taken to reach the centre of the fragment. Morphological appearance was assessed by haematoxylin/periodic acid–Schiff (H/PAS) staining. Per section, 10 randomly chosen tubules were evaluated for four parameters: structure of tubules, ruptures of the basement membrane, swelling of tubular cells and tubular cell loss. Each parameter was scored as stated in our published previously (Faes and Goossens, 2016) (**Figure 3A to D**). The best morphology was scored as 3 and the worst as 0.

To evaluate the appearance of the supporting Sertoli cells, vimentin (M0725; Dako, Belgium) staining was carried out. On each consecutive serial section, 10 tubules were scored to evaluate the number of spermatogonia per area (mm^2) by staining the sections for Melanoma Antigen Family A4 (MAGE-A4; monoclonal antibody, a murine immunoglobulin G, kindly provided by Dr Giulio Spagnoli, University

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