Two novel techniques to detect follicles in human ovarian cortical tissue

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BACKGROUND: Ovarian tissue cryopreservation and transplantation are becoming increasingly important issues for preserving female fertility as shown by recent successes in restoring ovarian activity and even fertility. Primordial follicle content before transplantation is a key issue for success. We investigated two novel methods to detect primordial follicles in human ovarian cortical tissue strips. METHODS: The first method used the fluorescent mitochondrial stain rhodamine 123 in combination with laser scanning confocal microscopy (LSCM). The second used a simple stereomicroscopic method with glass-bottom dishes for detecting primordial follicles in ovarian cortical tissue strips. RESULTS: Follicles were visible as white spots in thin cortical strips using LSCM in single and fast scanning at low magnification, allowing a fair estimation of the number of primordial follicles present. Using the second method, ovarian follicles were also visible using glass-bottom dishes under the stereomicroscope, although tissue thickness and density were limiting factors of its success. DISCUSSION: Follicles can be visualized in human cortical ovarian strips with R123 in combination with LSCM. Stereomicroscopy using glass-bottom dishes and transmitted illumination is a simple alternative method and has the advantage of allowing further safe clinical use of the analysed tissue.

Key words: cryopreservation/follicle visualization/ovarian transplants/rhodamine 123

Introduction

Recent successes in transplantation of the ovary and of cryopreserved human ovarian cortical tissue show its importance in restoring fertility in patients with extraneous premature ovarian failure (Donnez et al., 2004; Smits and Cortvrindt, 2004; Meirow et al., 2005; Silber et al., 2005). Human ovarian cortical tissue contains a pool of primordial follicles, which is the source of future oocytes (Newton et al., 1996; Van den Broecke et al., 2001). Assessment of the number of follicles present in the cryopreserved tissue before transplantation has major prognostic value, although quantification without affecting the safe clinical use of the analysed tissue is a daunting task. Cortvrindt and Smits (2001) reported that histological counting is reliable but has a long turnaround time; therefore they introduced fluorescent methods using Calcein AM and Picogreen, giving information on follicular density within 1 h, but both methods are cytotoxic and therefore prohibit the clinical use of the analysed tissues (Jonsson et al., 1996).

We investigated two novel methods of detecting primordial follicles in ovarian cortical tissue strips before transplantation while allowing re-use of the tissue. One method is based on rhodamine 123 (R123) vital staining in combination with laser scanning confocal microscopy (LSCM). R123 is a lipophilic cationic fluorescent dye that directly and selectively accumulates in the inner mitochondrial membrane and stains mitochondria of living cells (Johnson et al., 1981; Chen et al., 1982; Davis et al., 1985; Emaus et al., 1986; Chen, 1988). The second proposed method is simple stereomicroscopic visualization of follicles in ovarian cortical strips using glass-bottom dishes and transillumination.

Materials and methods

Source of human ovarian tissue

Cryopreserved human ovarian tissue was obtained from a consenting 22-year-old female-to-male transsexual and was kept frozen for about 3 years. Cryopreservation had been done with the method described previously by Van den Broecke et al. (2001). The use of this tissue for the current study was approved by the Ghent University Hospital Ethical Committee.

Staining of ovarian cortical tissue strips

A stock solution of 1 μg/μl R123 (R302, Invitrogen, Molecular Probes, Merelbeke, Belgium) dissolved in methanol was prepared and
kept at −20°C. Staining was done by adding 1 μl of the stock solution to 1 ml of medium before incubation. Vials containing thin human ovarian tissue strips were thawed in a water bath at room temperature. Tissue strips were washed five times with KSOM-Hepes (Potassium Simplex Optimized Medium + Hepes) buffered medium in 60 mm tissue culture dishes (Falcon; BD 35-3002, VWR, Leuven Belgium) to remove the cryoprotectant dimethylsulphoxide (Sigma, D5879, Bornem, Belgium). Tissue strips were cut to small pieces of about 0.5–1 mm² with surgical blades (NO 24) and were washed again in 5 ml KSOM-Hepes buffered medium. These mini strips were then incubated in 35 mm tissue culture dishes (Falcon; BD 35-3001) containing 2 ml of MCDB 105 (Sigma, M-6395); M199 (Sigma, M-2154) 1 : 1 with 1 ng/ml R123 for 60 min in a 5% CO₂ incubator.

Conventional fluorescence microscopic observation of human ovarian cortical tissue strips
Standard fluorescent images of human ovarian cortical tissue strips were obtained from stained tissue with R123 using glass-bottom dishes (MatTek, Ashland, USA) with a Nikon TE2000-Eclipse epifluorescence microscope equipped with a custom Nikon DS-U1 colour camera. This was done to inspect if it could be useful in visualizing the follicles in ovarian strips.

Visualization of follicles in human ovarian cortical tissue strips by LSCM
Visualization of stained tissue by R123 using glass-bottom dishes was done with a Nikon Eclipse TE300 epifluorescence microscope equipped with a Biorad Radiance 2000 confocal system. R123 was excited with a 514 nm Argon laser and detected with a photomultiplier tube (PMT) through a 590/70 nm HQ BP filter. Average duration of visualization was 5 min. The microscope objectives used were a ×100 plan oil immersion objective with a numerical aperture (NA) of 1.3, a ×40 oil objective (NA = 1.3), a ×10 dry objective (NA = 0.45) and a ×4 dry objective (NA = 0.13). Digital images were acquired with Lasersharp 2000 software under Windows 2000. Scanned tissues were fixed by buffered formaldehyde (10% v/v, Mallinckrodt Baker B.V., Deventer, The Netherlands) overnight at room temperature and processed for paraﬁn embedding and H&E staining. Histological examination was performed microscopically after serial sectioning to 4 μm thickness. To prevent recounting, a follicle was only counted once at the time that the dark-stained nucleus was seen. Follicles were classiﬁed as primordial (oocytes surrounded by one layer of flattened pregranulosa cells), primary (surrounded by one layer of cuboidal granulosa cells), pre-antral (with two or more layers of granulosa cells without antrum), antral (with an antral cavity), ovulated (MII oocytes found in a cavity formed at the grafting site) and corpora lutea.

Age-matched ovarian grafts that had not been exposed to rhodamine 123 and LSCM before transplantation served as controls.

Visualization of follicles in human ovarian cortical tissue strips using stereomicroscopy and glass bottom dishes
Human ovarian cortical mini strips were thawed and washed as described above. Tissue strips of approximately 0.5 mm (476 ± 176 μm; mean ± SD) (thickness) × 1 mm × 1 mm were then placed in pre-warmed KSOM–Hepes medium in a glass-bottom dish (without R123). Visualization was done under transillumination using an Olympus SZ-60 stereomicroscope.

Statistical methods
Statistic analysis was performed by chi-square test with Graphpad Prism software. P < 0.05 was considered significant.

Results
Fluorescence observation of human ovarian tissue strips
Using fluorescent microscopy, ovarian tissue stained as a large bright object, although it was not possible to discern the follicles (Figure 1).

Visualization of follicles in human ovarian cortical tissue strips by LSCM
White spots resembling small follicles were detected in cortical tissue strips by a single and fast scanning at a low magnification (×4) using LSCM (Figure 2). Very bright round spots in a dark grey field of tissue were easy to distinguish and were counted, giving an estimation of the number of follicles in the tissue strips (Table I).

Figure 1. Fluorescent staining of human ovarian fragment with R123 (scale bar 1 mm).
Higher magnifications confirmed the classical three-dimensional structure of follicles. Nuclei were visualized as dark (absence of fluorescence), cytoplasm stained weakly and granulosa cells were stained bright (Figure 3).

**Mouse ovarian tissue transplantation as viability assay after exposure to R123 and confocal visualization**

Both grafted hemi-ovaries were recovered. Follicular development was observed in grafted tissue as seen on histomorphological evaluation of both grafts. There was no significant difference in follicular development in grafts that had been exposed to R123 compared to non-exposed controls (Table II, Figure 4).

**Table II. Mouse ovarian follicular development after exposure to R123, confocal visualization of follicles and allotransplantation**

<table>
<thead>
<tr>
<th></th>
<th>Primordial and primary (%)</th>
<th>Pre-antral, antral and ovulatory (%)</th>
<th>Total</th>
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<tr>
<td>B1</td>
<td>212 (85.1)</td>
<td>37 (14.9)</td>
<td>249</td>
</tr>
<tr>
<td>B2</td>
<td>298 (84.4)</td>
<td>55 (15.6)</td>
<td>353</td>
</tr>
<tr>
<td>Control</td>
<td>337 (88.2)</td>
<td>45 (11.8)</td>
<td>382</td>
</tr>
</tbody>
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B1, B2: Two mice with hemi-ovaries transplanted to the back muscle site. Control: mean percentage of six grafts (non-R123 and LSCM exposed). P-value = 0.2930.

**Discussion**

When preparing ovarian tissue for transplantation, it would be helpful to have a visualization technique for detecting small follicles that allow the subsequent use of the tissue. For this purpose, we studied both a laser confocal method and a simple stereomicroscopic method. Follicles in human ovarian cortical tissue strips were clearly detected by LSCM combined with exposure to the mitochondrial stain R123. We found that short-term exposure to R123 and LSCM was not toxic to follicular

**Table 1.** Estimation of follicle content of human cortical tissue strips with laser scanning confocal microscopy (LSCM) and follicle counting after haematoxylin–eosin (H&E) histological staining of the same tissue samples

<table>
<thead>
<tr>
<th>Strip number</th>
<th>Number of follicles</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>LSCM</td>
<td>H&amp;E</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
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<tr>
<td>3</td>
<td>6</td>
<td>7</td>
<td></td>
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<td></td>
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<tr>
<td>6</td>
<td>7</td>
<td>6</td>
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</table>

**Figure 3.** Laser scanning confocal microscopy (LSCM) of human primordial follicle with higher magnification. (A) Serial scanning of human primordial follicle, (B) LSCM of human primordial follicle: (a) granulosa cells, (b) cytoplasm, (c) nucleus (scale bar 10 μm), (C) the same follicle after histological examination with haematoxylin–eosin staining (scale bar 12 μm).

**Figure 5.** Visible human ovarian follicles in a thin piece of cortical tissue using glass-bottom dishes. (A) Low magnification under stereomicroscope (arrows) (scale bar 100 μm). (B) Higher magnification under microscope (scale bar 18 μm).
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development in mouse ovarian allografts. Stereomicroscopy using fine glass-bottom dishes to visualize primordial follicles in thin sections of human ovarian tissue was revealed as a simple alternative to LSCM for detection of follicles.

Cryopreservation and banking of ovarian cortical tissue in view of future preservation of female fertility is a well-known technique (Newton et al., 1996; Newton, 1998; Bedaiwy and Falcone, 2004; Oktay and Sonmez, 2004). Recent reports on successful ovarian tissue transplantation showed that ovarian activity and even fertility can be restored in patients at risk of losing their ovarian activity (Donnez et al., 2004; Meirrow et al., 2005; Oktay and Tilly, 2004; Smitz and Cortvrindt, 2004; Silber et al., 2005). Because an estimation of follicle density is mandatory to choose the right tissue strips with sufficient amount of primordial/early primary follicles, non-harmful visualization of follicles before ovarian tissue transplantation provides a useful tool. As the number and distribution of follicles in the cortical part of the adult women’s ovaries are very variable according to age and physiologic status, absence of any follicle in the ovarian cortical tissue strips selected for transplantation is a potential hazard (Lass et al., 1997).

Recording of follicle density can be equally important before cryopreservation or in vitro culture (Cortvrindt and Smitz, 2001).

We introduced two methods to inspect ovarian tissues for the presence of follicles before transplantation. R123, a vital mitochondrial stain that can be visualized in cells by means of LSCM, seems to be a good technique for this purpose. It is generally accepted that R123, as a permeant cationic fluorochrome, is taken up specifically by functional, active mitochondria. This means that only follicles surrounded with live granulosa cells will be visible, and it is not possible to detect dead follicles (Alonso-Pozos et al., 2003). As the aim was to have a rapid estimation of the presence of ovarian follicles in tissue strips, only a single scan of the tissue with a minimum energy source of laser beam and small magnification of the lens was done. This was enough to see white spots resembling follicles in a dark field of tissue, allowing us to estimate the number of follicles in the tissue. Higher magnifications and H&E staining confirmed the results. Grafting of mouse ovarian tissue after LSCM–R123 proved that follicles survive and are able to develop even up to the ovulatory stage. Thus, our animal model shows that the combination of LSCM and short-term R123 exposure is not cytotoxic for mouse ovarian tissue. This is supported by previous unpublished data showing that short-term exposure of mouse zygotes to R123 up to 90 min is not toxic for in vitro blastocyst development. However, if this method of evaluation is to become of practical value, viability of human xenograft after exposure to R123 and LSCM will have to be demonstrated. In addition to the confocal microscopy, we also introduced a very simple stereomicroscopic method to evaluate cryopreserved human ovarian cortical tissue strips after thawing. The basic success of this technique is the use of glass-bottom dishes and varying the light incidence.

Both methods presented here for follicle visualization in human ovarian cortical tissue have their own advantages and disadvantages. LSCM needs a well-equipped laboratory but gives a fair estimation of the number of follicles. It is clear that for lots of laboratories, it is not possible to use expensive techniques such as LSCM to evaluate the tissues. Also, the use of a fluorescent mitochondrial dye may not be acceptable for human tissue that has to be used subsequently for transplantation. Therefore, it was necessary to establish a simple technique allowing the tissue to be re-used with certainty. Using glass-bottom dishes under the stereomicroscope is a simple method, although this technique requires an experienced operator with sufficient knowledge of intact follicle morphology to detect the follicles by adjustment of the light and the lens. Using glass-bottom dishes prevents scattering of transmitted light during evaluation of the tissue, which is the case when using plastic dishes during evaluation of the tissue. The thickness and density of the tissue can be a limiting parameter. The best thickness seems to be 0.5 mm or less. The great advantage of this method is that no chemical dye has to be used and that the same tissue on which the visualization was done can be used for further experiments. However, the method is restricted to transparent tissues. In contrast, with LSCM whole tissues can be evaluated even if they are not transparent. Though previously described techniques such as Calcein AM and Picogreen (Cortvrindt and Smitz, 2001) are also very good methods that can be used to estimate follicular density in ovarian tissues, their cytotoxic nature prohibits their clinical use.

The two methods presented here for the detection of follicle presence in ovarian tissue may help clinicians and researchers to evaluate fresh or cryopreserved ovarian tissue before any experiments such as transplantation or long-term culture.

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References


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