Isolation of pre-antral follicles from human ovarian medulla tissue

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BACKGROUND: Cryopreservation of ovarian tissue for fertility preservation is based on the ovarian cortex that contains the vast majority of the follicular reserve, while the remaining tissue, the medulla is discarded. The present study describes the development of a gentle method for isolating pre-antral follicles from human ovarian medulla and evaluating its follicular content.

METHODS: Medulla was collected from 40 girls/women aged 3–35 years undergoing cryopreservation of the ovarian cortex. Follicle density was assessed for all patients and pre-antral follicles were isolated from 22 patients. On the basis of the neutral red (NR) staining of follicles and enzymatic digestion with a mixture of Collagenase IV and Liberase Thermolysin Medium, viable pre-antral follicles were isolated.

RESULTS: NR accumulated in follicles resulting in a distinct red staining within the medulla. Follicle density of the medulla varied from 0 to 9824 follicles/gram of medulla and was significantly higher ($P<0.001$) in the 3–9-year age group when compared with older groups (10–35 years). Enzymatic digestion combined with follicle identification by NR yielded a high output of isolated and viable pre-antral follicles from medulla, of which, 3607 follicles were collected and classified. The percentage of primordial and growing follicles decreased and increased, respectively, with age ($P<0.0001$ and $P<0.0007$).

CONCLUSIONS: Discarded medulla contained a considerable pool of pre-antral follicles, especially in young girls. Our new method allowed the isolation of viable pre-antral follicles from human ovarian medulla and provides a unique opportunity for basic scientific studies and for culture and grafting purposes.

Key words: ovarian medulla / human follicles / isolation / vital staining / fertility preservation

Introduction

Young women and girls with a malignant disease, such as cancer, face the risk of losing ovarian function and becoming sterile as a side effect of the gonadotoxic treatment they receive. By low-temperature cryostorage of the ovarian tissue prior to initiation of a potential sterilizing gonadotoxic treatment, it is now possible to restore fertility by transplanting the frozen/thawed tissue once the patient has recovered (Schmidt et al., 2003a; Donnez et al., 2004). Considerable success has been achieved in terms of restoration of hormonal cyclicity in patients (Oktay and Karlikaya, 2000; Radford et al., 2001; Andersen et al., 2008; Silber et al., 2008; Kim et al., 2009) and, currently, 12 healthy children have been born worldwide as a result of transplanting frozen/thawed ovarian tissue (Donnez et al., 2010).

A woman’s fertility potential is linked to the follicular reserve stored in her ovaries. Owing to the anatomical architecture of the ovary >90% of the total follicular reserve, mainly the resting primordial follicles, is at any moment in time located within the thin poorly vascularized layer of the outermost part of the ovary (i.e. the cortex). In contrast, growing follicles are almost exclusively located in the cortico-medullary border and in the medulla proper, which is well vascularized (van Wezel and Rodgers, 1996). Therefore, as part of the ovarian cryopreservation procedure, the cortex is isolated to a thickness of 1–2 mm (von Wolff et al., 2009): this helps to ensure preservation of the majority of primordial follicles, which are also more resistant to cryo-injury than growing follicles because metabolism is relatively low, the oocyte is surrounded by only a few granulosa cells (GCs) and primordial follicles are small in size (Smits and Cortvrindt, 2002; Hovatta, 2005; von Wolff et al., 2009).

There is a gradual decline of follicular density from the cortex to the medulla region. In connection with the current practise of isolating the outermost part of the cortical tissue and preparing 1–2 mm thick slices for fertility preservation, it is unknown how many follicles are actually being lost by discarding the medulla. Based on a protocol for isolating
human pre-antral follicles from the cortex published by Dolmans and et al. (2006), we have developed a new method for isolation of follicles in the medulla and used it to evaluate the follicular density, the number of follicles and their developmental stage in the medulla.

Materials and Methods

Patients and collection of ovarian medulla

Ovarian medulla was obtained from women who were having one ovary laparoscopically removed for fertility preservation, because of a disease in which adequate treatment (e.g. chemotherapy and/or radiation therapy) posed a high risk of destroying all ovarian follicles and rendering the woman sterile. Only the cortex was used for fertility preservation as previously described (Andersen et al., 2008), whereas the medulla was discarded.

The residual ovarian tissue which remained after isolating the cortex to a thickness of 1–2 mm was defined as the medulla, although it cannot be excluded that small amounts of residual ovarian cortex might be present within this tissue. Medulla was obtained from 40 girls and women, aged 3–35 years (median, 24 years), and collected from April 2009 to May 2010. A few small pieces of human ovarian tissue, obtained during trimming of the tissues prior to cryopreservation, were on a small number of occasions also included. The diagnoses, which indicated ovarian cryopreservation was required, included Hodgkin’s disease (n = 12), mammary cancer (n = 10), leukaemia (n = 3) and various others (n = 15), which in no patient was related to an endocrinological (e.g. polycystic ovary syndrome) and/or ovarian disease. In adult women the ovary was collected at various times during the menstrual cycle.

The follicular density in the medulla tissue was assessed in all 40 patients, whereas follicle isolation was only performed in 22 patients, for different reasons as stated in the ‘Results’ section.

Fertility preservation by cryopreserving ovarian tissue has been approved by the Minister of Health in Denmark and by the ethical committee of the municipalities of Copenhagen and Frederiksborg (J. no. J/KF/01/170/99).

Histological evaluation of the ovarian tissue

A small piece of fresh cortex from a few patients and medulla tissue from each patient was weighed and processed for histology. The tissue was fixed in Bouin’s solution immediately after preparation, dehydrated in ethanol and embedded in paraffin. The entire tissue fragment was cut into serial sections (30 μm thick), mounted on glass slides and stained with Mayer’s haematoxylin and periodic acid Schiff’s reagent.

Counting follicles visualized with neutral red in the human ovarian medulla

Between one and three pieces of fresh medulla from each patient were rinsed in phosphate-buffered saline (PBS) and the weight recorded. The medulla was homogenized in a tissue sectioner (Mcllwain Tissue Chopper; Mickle Laboratory, Guildford, UK), and adjusted to 0.3 mm. The cutting procedure was repeated several times until pieces of ~0.3–0.5 mm3 were obtained. Tissue pieces were transferred to a 35 mm culture dish (Nunc™ Δ Dish, Nunc A/S, Roskilde, Denmark) containing 3 ml of 37 °C warm McCoy’s 5a culture medium containing sodium bicarbonate supplemented with 25 mM HEPES (Invitrogen, GibCO). 0.1% human serum albumin (CSL Behring 20%, Marburg, Germany), 2 mM glutamax (Invitrogen, GibCO), 0.05 mg/ml penicillin/streptomycin (Invitrogen, GibCO™), 5.5 mg/ml transferrin, 6 mg/ml selenium, 10 mg/ml insulin (Invitrogen Corporation, GibCO™) and 50 mg/ml ascorbic acid (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The pieces of medulla were incubated in culture medium supplemented with 50 μg/ml of neutral red (NR) solution (2-amino-3 methyl-7-dimethyl-aminophenazonchloride; cat# N2889, Sigma-Aldrich Logistik GmbH, Schnelldorf, Germany) for 4–6 h at 37 °C. NR is a weak cationic supravital dye, soluble in water, which at slightly acid pH yields a deep red colour. NR readily diffuses through the plasma membrane and concentrates in the lysosomes of viable cells (Allison and Young, 1964). It is thus possible to distinguish between viable, damaged or dead cells (Borengen and Puermer, 1985), and NR has been used to assess the viability of porcine and ovine oocytes, granulosa and theca cells (Campbell et al., 1996; Shores et al., 2000; Brankin et al., 2003). NR is therefore especially concentrated in follicles making it possible to distinguish follicles in larger pieces of tissue. Before counting follicles in the tissue under a stereomicroscope (Leica MZ12, Microsystems Ltd), 5 ml of Dulbecco’s PBS (Biochrom AG, Berlin, Germany) was added to dilute the NR, and the number of red coloured follicles in each piece of medulla was recorded. The NR dye will only stain viable follicles and therefore dead follicles were not included in the density analysis. Thus, NR staining provides an estimate of viable follicles within the ovarian tissue and not an absolute quantification of follicle numbers.

Isolation and classification of follicles from medulla

Ovarian medulla pieces, prepared in the tissue sectioner as described above, were transferred to a 90 mm culture dish (Nunc™ Δ Dish, Nunc A/S) containing 12 ml preheated McCoy’s medium as described above. For enzymatic digestion of the medulla pieces, the culture medium was supplemented with 50 μg/ml of NR solution and a mixture of Liberase TM (Thermolysin Medium; 0.04 mg/ml, Roche Diagnostics GmbH, Mannheim, Germany) and Collagenase IV (0.2 mg/ml, Sigma-Aldrich), followed by incubation at 37 °C for 70–80 min with gentle agitation. The enzymatic digestion was terminated by addition of an equal volume of 4°C PBS supplemented with 10% fetal bovine serum (FBS, Invitrogen, GibCO). The cell suspension was aspirated up and down in a 1-ml pipette to further mechanically disrupt the digested tissue. Follicles with red colouration were immediately identified under a stereomicroscope (Leica MZ12, Microsystems Ltd), 5 ml of Dulbecco’s PBS (Biochrom AG, Berlin, Germany) was added to dilute the NR, and the number of red coloured follicles in each piece of medulla was recorded. The NR dye will only stain viable follicles and therefore dead follicles were not included in the density analysis. Thus, NR staining provides an estimate of viable follicles within the ovarian tissue and not an absolute quantification of follicle numbers.

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of GCs surround a growing oocyte) and late secondary follicle (three or more layers of GCs surround a growing oocyte).

**Viability assessment with fluorescent dye**

To assess NR as a vital staining, small fragments of ovarian tissue (total number exceeding 200) were divided into two groups; one dish with ovarian fragments serving as a negative control in which cell death was induced by 60 min incubation in 70% ethanol and freezing (i.e. −20°C) prior to NR staining, while the other dish with ovarian fragments was incubated with NR as described above. Following 4 h of incubation with NR both groups were assessed for follicle number. Pieces of medulla included as a negative control were without staining for NR, but the presence of follicles was confirmed by histological evaluation.

Fragments containing NR-stained follicles in the non-treated tissue were isolated by enzymatic digestion and the fluorescent vital dye carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, Vybrant CFDA SE Cell Tracer Kit, Invitrogen, Oregon, USA) was used to confirm the viability of the isolated NR-stained follicles. CFDA-SE, which is non-fluorescent, passively diffuses into the cytoplasm of cells. Once inside the cell, intracellular esterases remove acetate groups and convert the molecule to the fluorescent ester, carboxyfluorescein succinimidyl ester (CFSE), which is retained within cells and covalently couples, via its succinimidyl group, to intracellular molecules (Parnell, 1999). If the cell membrane is compromised, which is characteristic for a dead or dying cell, the CFSE leaks from the cell and staining will not be observed.

Isolated NR-stained follicles (n = 64) of different stages (varying from 40 to 160 μm in diameter) were incubated in 5.57 μg/ml CFDA-SE for 20 min at 37°C. Subsequently, the medium was replaced with fresh pre-warmed medium and incubated for another 20 min at 37°C to ensure complete modification of the probe. Follicles were washed in PBS and transferred onto glass microscope slides in 15 μl drops of PBS. A PAP-pen (Invitrogen, Camarillo, CA, USA) was used to draw a hydrophobic barrier around the PBS drop on the slide, followed by mounting with a cover slip. The approximate excitation and emission peaks of CFSE were 492 and 517 nm, respectively. Follicles labelled with CFSE were visualized by fluorescence microscopy with a Zeiss Axioskop microscope (Broch and Michelsen Instrument AS, Zeiss, West Germany) using a standard fluorescent filter set and captured with Leica DFC420C software (Leica Microsystems Ltd., Heerbrugg, Germany).

**Statistical analysis**

Statistical analysis was performed using GraphPad InStat. Data were analyzed using parametric analysis complying with requirements for normal distribution, individuality and homogeneity of variances. Linear regression analysis was performed (i) after log transformation of the follicular density/follicular number and (ii) on the percentile distribution of pre-antral follicles in relation to the age of the patient. One-way analysis of variance (ANOVA) test was performed to evaluate (i) number of follicles per gram of medulla tissue and (ii) total number of follicles in the medulla, in relation to the age of the patient. Tukey–Kramer multiple comparisons test was used to determine the significant differences between the four age groups.

ANOVA test was also performed on log-transformed data to evaluate the distribution of pre-antral follicles within the four age groups. Statistical significance was set at P < 0.05.

**Results**

**Visualization of follicles with NR**

NR accumulated in ovarian follicles within 1–4 h of incubation, depending on the thickness of the pieces, giving the viable follicles an orange-red colouring. Figure 1A depicts how the red colouration was concentrated in circular structures inside slightly stained stromal tissue, which made the follicles easy to visualize. By staining a thin slice of human cortex with NR, it was possible to visualize the characteristic gradient of follicles within the ovarian tissue (Fig. 1C), showing the cortex as being the thin, poorly vascularized, outer part of the ovary packed with the majority of the primordial follicles. NR also identified follicles within the medulla and stained in addition the nearby blood vessel (Fig. 1B), showing the medulla as being richly vascularized, and closest to the centre of the ovary but also containing groups of small pre-antral follicles.

The presence of small pre-antral follicles in cortex and medulla was also assessed by histological evaluation. The cortex contained a high density of pre-antral follicles, and density was closely related to age (Fig. 1D), but the medulla also contained both pre-antral and more advanced stages of folliculogenesis (Fig. 1E) confirming the picture observed with NR.

**Follicle density in the medulla**

Medulla was collected from 40 young women and girls followed by staining with NR and assessment of follicle density. The follicular density in the medulla of each of the 40 patients is shown in Fig. 2. The follicular density of the medulla varied from 0 to 9824 follicles per gram of medulla and showed a significant inverse linear correlation with age (r = −0.57, P < 0.0001; Fig. 2).

Total number of follicles in the medulla for 40 girls and women is depicted as a line graph in Fig. 3A, varying from the highest number of 82 258 follicles observed in a 7-year-old girl to absence of follicles in three different women aged 27, 32 and 34 years. The 40 female patients were grouped according to age into four intervals; 3–9, 10–19, 20–29 and 30–35 years, and average follicle density was calculated for each group as shown in Table I. Total number of follicles in medulla was highest in the group of 3–9 years, and declined drastically in the group of 10–19-year-old girls, followed by a low plateau level of follicles in the medulla in the two last groups (Fig. 3A). Follicles ranged in average number from 51 020 follicles in the medulla of 3–9-year-old girls to 2738 follicles in the medulla of the 30–35-year-old women (Table I). Statistical analysis showed that the total number of follicles and follicle density per gram of medulla was significantly higher in the 3–9-year age group when compared with the other three groups (10–35 years) (Table I), although the follicular density showed a huge variability within each age group. Within pieces of medulla from one patient a pronounced variability in follicular density was also observed. In the most extreme case, the follicular density varied from 166 follicles per gram of tissue in one fragment to 1060 follicles in another fragment.

The weight of the medulla in an adult woman’s ovary (20–39 years of age), as obtained in connection with cryopreservation of the cortex for fertility preservation, was around 6-fold higher than that of girls (3–9 years of age) (Table I). However, the mean number of follicles per gram medulla from young girls (3–9 years of age) was 17-fold higher than the follicle density in medulla from girls aged 10–19 years, and 29–55-fold higher than the follicle density in medulla from a woman (20–35 years of age) (Table I).

Representative light microscopic images of NR-stained medulla from four girls and women, one from each age group, visualizes the gradual decline of follicle density in relation to age (Fig. 3B–E).
Isolation of follicles by enzymatic digestion and NR staining

To confirm that the red structures detected by NR were actually follicles and to allow a detailed assessment of follicle morphology, enzymatic digestion was used to isolate follicles from the medulla. Enzymatic digestion with Liberase TM resulted in incomplete tissue digestion, irrespective of the enzyme concentration and the period of incubation. The tissue remained poorly dissolved, was stretchy and with follicles still tightly embedded in the tissue (data not shown). Enzymatic digestion with Collagenase IV alone resulted in a total disruption of the tissue, and the basal membrane of the isolated follicles became severely damaged (data not shown). A suitable mixture of Liberase TM and Collagenase IV gave a complete and homogeneous digestion of medulla with the least damage to the follicular basal membrane. By mechanical disruption of the tissue, the NR-stained follicles were released from the medulla and could easily be collected (Fig. 4A and B). Isolated NR-stained follicles of different pre-antral developmental stages, from primordial to secondary pre-antral follicles (ranging from 40 to 212 μm in diameter), were collected continuously throughout the enzymatic digestion of the medulla (Fig. 4A–D). The isolation procedure took 3–5 h depending on the follicular density.

Viability assessment of NR-stained follicles

Red-stained follicles were observed in most (>80%) of the ovarian fragments under normal NR staining conditions of viable tissue (Fig. 4E), while not a single red-stained follicle was observed in ovarian fragments that served as negative control after induced cell death (Fig. 4F). In each of the medulla fragments used for negative control, the presence of follicles was confirmed by histology (Fig. 4G). To verify the viability of the isolated NR-stained follicles fluorescent vital dye CFDA-SE was used to stain the follicles after isolation. Viability assessment with CFDA-SE showed that all except one of the 64 isolated NR-stained follicles (98.5%; Fig. 4H) were also fluorescent (Fig. 4I), which supported the assumption of NR as a vital stain.
Pre-antral follicles were isolated from 22 of the 40 patients. In the remaining 18 patients follicles from the medulla were not isolated because of unsuccessful enzymatic treatment \((n = 3)\), the absence of follicles within the tissue \((n = 4)\), the isolated follicles did not meet the requirements of the morphology and viability assessment \((n = 5)\) or because the isolation procedure was too time consuming to be performed on the day of cryopreservation \((n = 6)\).

Thousands of follicles were isolated by enzymatic digestion, combined with NR, from the collected medulla of the 22 girls and women aged 3–34 years and assessed for viability and morphology. By microscopic analysis the morphology of each isolated follicle was assessed, and only follicles with a well-preserved morphology and NR staining were collected for classification. Isolated follicles with a visibly damaged basal membrane or oocyte extrusion were discarded. A total of 3607 isolated follicles passed the viability and morphology assessment, of which 2079 follicles were isolated from the 3–9-year-old girls \((n = 4)\), 771 follicles from the 10–19-year-old girls \((n = 3)\), 401 follicles from the 20–29-year-old women \((n = 9)\) and 356 follicles were isolated from the 30–34-year-old women \((n = 6)\). The 3607 isolated follicles were assigned to one of five...
groups according to their diameter as given in Fig. 4J–N; <60 μm (n = 2934, Fig. 4J), >60–75 μm (n = 413, Fig. 4K), >75–100 μm (n = 199, Fig. 4L), >100–150 μm (n = 39, Fig. 4M) and >150 μm (n = 22, Fig. 4N). Following histological preparation, pictures of representative follicles from each size group are shown in Fig. 4O–S.

### Table I Mean follicle density in the human ovary medulla in relation to age.

<table>
<thead>
<tr>
<th>Density parameters</th>
<th>Age (in years)</th>
<th>*P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3–9</td>
<td>10–19</td>
</tr>
<tr>
<td>No. of patients/medulla pieces</td>
<td>4/7</td>
<td>6/12</td>
</tr>
<tr>
<td>Weight of medulla piece (μg)</td>
<td>52.3 ± 13.6</td>
<td>99.4 ± 4.6</td>
</tr>
<tr>
<td>Follicle count per piece</td>
<td>396 ± 186</td>
<td>50 ± 13</td>
</tr>
<tr>
<td>No. of follicles per gram tissue</td>
<td>5616 ± 2235^a</td>
<td>334 ± 138^b</td>
</tr>
<tr>
<td>Total weight of medulla (g)</td>
<td>0.56 ± 0.1</td>
<td>2.21 ± 0.5</td>
</tr>
<tr>
<td>Total no. of follicles in medulla</td>
<td>51020 ± 16363^a</td>
<td>6302 ± 2806^b</td>
</tr>
</tbody>
</table>

The different follicle density parameters are listed in relation to four age groups (a total of 40 female patients aged 3–35 years of age). On the basis of these parameters, an estimate of mean follicular densities/follicular numbers were calculated. Across each row values with different letters are significantly different (*P < 0.001). Data are mean ± SEM.

^a Analysis of variance.

^b Values with different letters differ significantly (*P < 0.001).

**Discussion**

The present study is, to our knowledge, the first to isolate human pre-antral follicles from discarded ovarian medulla obtained in connection with cryopreservation of cortex for fertility preservation. The number and the density of pre-antral follicles in medulla were much higher than we expected especially from younger women and children. Further, the study demonstrated that a new method involving NR in combination with a new blend of enzymes is suitable in order to isolate viable pre-antral follicles. Collectively, medulla that is normally discarded during cryopreservation of ovarian tissue is an unexpected valuable source of human pre-antral follicles that can be utilized in a number of different ways. First, this opens a new avenue for the development of additional methods for fertility preservation in cancer patients. In rodents, it has been shown that isolated frozen/thawed follicles transplanted back to a sterile ovary can restore fertility (Carroll and Gosden, 1993), and it may be envisioned that human isolated pre-antral follicles may also survive cryopreservation and may, if needed, be transplanted to the women from where they originate and thereby restore fertility. If the NR proves to be non-toxic with no long-term negative effects on the follicles, this new method may therefore be developed into a technique that, in combination with cryopreservation of the cortex, enhances the potential of fertility preservation. Alongside development of such therapeutic applications, the availability of human pre-antral follicles provides an unparalleled opportunity to study human follicular development and perform in vitro studies.

In the present study, the medulla was defined as the residual ovarian tissue that remained after isolation of the cortex. The residual ovarian tissue was in most cases contaminated with small fragments of cortex that resulted from trimming the ovarian tissue for fertility preservation. However, we do not consider this contamination to exert any significant effects on the results obtained and in the distribution of the follicle classes observed.
Isolation of human pre-antral follicles

Figure 4. Top panel: Isolation of pre-antral follicles from human medulla tissue by enzymatic digestion and NR staining. (A) Fresh ovarian medulla tissue from a 6-year-old girl digested with Liberase and Collagenase IV, combined with NR staining. NR-coloured primordial follicles are collected. White arrows: isolated NR-coloured follicles. Dark orange: NR-coloured follicles still embedded in medulla tissue. (B) Primary and secondary follicles released from the digested medulla tissue. Follicles isolated from medulla tissue: (C) 160 μm diameter (D) 212 μm diameter. Middle panel: Viability assessment of follicles stained with NR. (E) More than 200 fragments of ovarian tissue were incubated with NR: red-stained follicles were observed in >80% of fragments. (F) Negative control; cell death induced by 60 min incubation in 70% ethanol at −20°C prior to NR staining: no red-stained follicles observed in >200 small fragments of ovarian tissue. (G) Histological evaluation of negative control tissue showing the presence of follicles despite negative NR staining. (H) A 160 μm diameter follicle that was NR positive in situ, following isolation from fresh ovarian tissue. (I) Positive CFDA-SE staining of the 160 μm diameter NR positive follicle. Bottom panel: Classification of pre-antral follicles. A total of 3607 follicles from 22 girls and women aged 3–34 years were isolated and pooled according to diameter. (J–N) Microscopic analysis of follicle Groups 1–5; (J) ≤60 μm, (K) >60–75 μm, (L) >75–100 μm, (M) >100–150 μm and (N) >150 μm. Scale bars: 100 μm. (O–S) Histological analysis revealed developmental stage, and representatives of each group are depicted. O, primordial/early primary follicle; P, late primary follicle; Q, early/mid secondary follicle; R, mid/late secondary follicle; S, late secondary follicle. Scale bars: 50 μm.
In order to develop a new method for follicle isolation, we modified previous experiences with enzymatic digestion of human cortex tissue and found the combined use of NR very effective. A mixture of Liberase TM and Collagenase IV was found to be optimal for disruption of the less dense medulla. Different types of collagenase (Ia, II, IX and XI) have been used for the enzymatic isolation of ovarian follicles (Hovatta et al., 1999; Oktay and Karlikaya, 2000; Martinez-Madrid et al., 2004), but in the light of studies indicating a risk of some collagenase preparations to interfere with follicle quality (Eppig, 1994; Hovatta et al., 1999), Liberase has emerged as a new powerful tool to help maintain the quality of isolated follicles (Dolmans et al., 2006). However, our attempts to disrupt the medulla with Liberase TM alone were disappointing, since the tissue was only partly digested and most follicles were still surrounded by stroma. The incomplete disruption of the tissue by Liberase TM treatment was previously observed as a disadvantage, because the consequence was a lower number of fully isolated follicles compared with collagenase treatment (Dolmans et al., 2006). In contrast to Liberase TM treatment, Collagenase IV treatment alone dissolved the medulla and the quality of the isolated follicles was poor, with disruption of the basal lamina and premature oocyte extrusion as reported by Eppig (1994) and Hovatta et al. (1999). A mixture of the two enzymes was tested in order to combine the softening actions of the Liberase TM with the disruptive actions of Collagenase IV, which proved effective in dissociating medulla, while the simultaneous presence of NR allows a fast identification, and therefore isolation of viable follicles.

Quality assessment of the isolated follicles was based on the general follicle morphology and on follicle viability analysed by NR and the vital fluorescent dye CFSE-DA. This study demonstrates that NR can be used to detect viable follicles within ovarian tissue and furthermore be used as a valuable visualization tool during the isolation procedure. Based on positive CFDA-SE staining of isolated NR-coloured follicles, we verified NR as a vital stain of healthy follicles. In connection with the present method of isolating human pre-antral follicles, the use of NR will ensure that predominantly viable follicles are used for research and/or possible fertility preservation. The staining patterns observed following incubation of tissue in NR are consistent with patterns observed using other vital stains/dyes, such as rhodamine 123 and Calcein AM staining of human cortical tissue, which demonstrated that follicles could be visualized as bright areas within slightly stained stromal tissue (Cortvrindt and Smitz, 2001; Smitz and Cortvrindt, 2002; Soleimani et al., 2006). However, in contrast to rhodamine 123 and Calcein AM, staining with NR does not apparently compromise follicle survival, as it may avoid some of the disadvantages, such as photo-oxidative and solvent toxicity damage, which have been reported for rhodamine 123 and Calcein AM. Nemes et al. (1979) showed that the uptake of NR had no deleterious effects on enzymatic activity within cellular organelles, and if this non-toxic effect of NR can...
be confirmed on follicles in future studies, it may be a valuable tool for follicle isolation in connection with therapeutic applications.

In this study, 3607 fully isolated follicles were acquired from discarded medulla collected from 22 girls and women aged 3–34 years. In the remaining 18 women, the isolation procedure was unsuccessful because the correct mixture of enzymes was not used and/or other practical arrangements proved unsuitable. Compared with previously published methods using cortex that contains a high density of follicles, the present yield of follicles was surprisingly high. Based on follicle diameter measurements of isolated follicles, we showed a very significant decline in the number of follicles with a diameter \(< 60 \mu m\) in relation to age, and a significant increase in the number of follicles exceeding 60 \(\mu m\) in diameter in relation to age. Overall, we found that the primordial follicle was the most predominant type not only in the cortex but also in the medulla. Schmidt et al. (2003b) showed that in the cortex of the adult human ovary 94% of the follicles were primordial, 5.3% primary and 0.7% secondary. Our studies revealed that the predominant follicle type in medulla of the adult ovary (20–34 years of age) is also the primordial/early primary follicle (55–56% primordial/early primary, 39–40% late primary/early secondary and 5% secondary follicles). However, there is a relatively lower abundance of primordial/early primary follicles in the medulla when compared with the cortex, suggesting that the potential contamination with cortex is low and does not appear to exert a major effect on the results obtained. The distinctive lower percentage of primordial/early primary follicles and an increasing number of growing follicles in the medulla of aging women when compared with cortex is in accordance with the early work of Block (1952). Moreover, our results showed that the primordial/early primary follicles represent the vast majority (81–88%) of follicles also in the medulla of girls under the age of 10 years and resembles the distribution found in the cortex of prepubertal girls (98–99% primordial follicles) (Schmidt et al., 2003b). In addition, the number of isolated primordial/primary follicles from the medulla is likely to be an underestimation of their actual presence because their small size made them more difficult to recognize than the larger follicles within the digested tissue. In light of these results it is suggested that discarding the medulla from girls under the age of 10 years during cryopreservation of ovarian tissue should be reviewed and a possible freezing protocol for the medulla, and/or follicles isolated from the medulla, should be considered. It is of great importance for these young girls to preserve the majority of their follicular reserve in order to prolong their period of fertility, as it is not yet clear how long transplanted tissue actually functions within the ovary.

The fact that the vital dye NR accumulates in viable follicles made it possible to visualize red stained follicular structures within the slightly stained stromal tissue and to estimate the follicular density of viable follicles within the tissue. Dead follicles, which might have been compromised by the preparation procedure, were therefore not included in the total follicle counts, and the follicle densities found in the present study are likely to slightly underestimate the actual total number of follicles in medulla. Our results showed that the follicular density in medulla from 40 women showed a significant inverse linear correlation with age, although a large variation in follicular density existed. This extends earlier observations from cortical biopsies showing an almost linear decay curve of primordial follicles in relation to age (Faddy, 2000; Qu et al., 2000). Furthermore, we showed that the most drastic decline in medulla follicle density was apparent in girls over the age of 10 years and throughout the reproductive years.

The abundance of follicles within the medulla of girls under the age of 10 years was especially pronounced when follicle density was expressed as mean number of follicles per gram of medulla, showing a 29–55-fold higher density of follicles in the medulla of 3–9-year-old girls compared with adult women over the age of 20 years. The presence of such a high number of follicles within the medulla of 3–9-year-old girls, of which 88% of isolated follicles were \(< 60 \mu m\) in diameter (primordial/early primary follicles), suggests that during childhood the follicular reserve is not completely localized to the cortical region of the ovary.

In addition, our results showed a huge variability of follicular density within each age group and within the same patient. However, these results resemble other studies obtained with cortex (Lass et al., 1997; Schmidt et al., 2003b). Lass et al. (1997) studied follicle density in ovarian cortical biopsies obtained from 60 women and found a range of 0–160 follicles/mm\(^3\). Schmidt et al. (2003b) also found a huge difference in follicular density from patient-to-patient and between different cortex pieces of the same ovary. Therefore, it is most likely that the varying follicle densities observed within the medulla follow the varying distribution pattern of follicles in the cortex.

At the present time, availability of human follicles is often the limiting factor in the development of new reproductive techniques. Mechanisms that regulate follicle development and atresia are only partially understood and more information on these fundamental processes is required. This could be achieved by exploiting the available pre-antral follicles in medulla (following removal of the cortex for cryopreservation) in order to develop new methods of follicle culture and study the effects of various growth factors and conditions in culture.

The current practise of collecting cortex tissue in 1–2 mm thick slices secures a large amount of the primordial follicles for fertility preservation. Simultaneously, the thin slices allow a fast penetration of cryoprotectants, which probably is important in limiting the potential toxic effect on the tissue and helping to ensure follicle survival. Although the actual thicknesses of the cortical slices used for cryopreservation are likely to vary from one laboratory to the other, the present study showed that a large number of follicles are likely to be discarded via the medulla in most cases. It is probably not feasible to cryopreserve the medulla tissue itself, since thin slices of around 1–2 mm thick are likely to contain only few follicles, at least in adult women. The best option for developing new fertility preserving measures will probably be to develop cryopreservation methods for isolated follicles or, alternatively, develop in vitro maturation methods to obtain mature oocytes, especially in cases where one entire ovary is removed for fertility preservation. However, both approaches require a substantial research effort to reach the therapeutic level.

In conclusion, a considerable number of pre-antral follicles is lost when discarding the medulla during the current practise of isolating the cortical tissue for cryopreservation, especially in young prepubertal girls. Moreover, a new method for isolating pre-antral follicles from the available human medulla has been established, which provides a unique opportunity to isolate small human follicles for basic scientific studies and may in the future be developed into an additional method of fertility preservation.

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