In situ identification of follicles in ovarian cortex as a tool for quantifying follicle density, viability and developmental potential in strategies to preserve female fertility

Sir,

We read with interest the article by Chambers et al. (2010). The authors emphasized the benefit of using neutral red (NR) staining for quantifying follicle density, viability and developmental potential in ovarian cortical tissues. They compared the advantages of their preferred method over our previously published research (Soleimani et al., 2006). There are some misinterpretations that we would like to discuss further.

(i) The authors mentioned the toxicity of methanol as a solvent for rhodamine 123 (R123) used in our article. The final concentration of methanol in the medium used for our experiment was 0.1% (v/v), which is lower than the minimum concentration that is considered toxic in any reference. Moreover, we determined our final concentration of R123 (1 ng/μl) to be non-toxic compared with a control group both in medium and in a Laser Scanning Confocal Microscopy (LSCM) visualization setting via mouse zygote assay (unpublished data) and mouse ovarian tissue (OT) transplantation (Soleimani et al., 2006). On the other hand, the invasive procedure used by Chambers et al. (2010) for rinsing the ovine ovaries utilized 70% (v/v) ethanol, which is much more toxic for handling OT and is not discussed in their manuscript.

(ii) In the same article, we also introduced a novel, simple and cost-effective method to evaluate follicles in thin human OT cortical strips which Chambers et al. (2010) did not mention. This method has important advantages over any chemicals used for staining such as NR. OT strips with a thickness of ~<0.5 mm are easily visualizable using stereo-microscopy, and we therefore question the advantage of vibratome1000 assisted slicing of OTs to a thickness of 80–100 μm. To our knowledge there will be no need for any staining to visualize follicles if using a thickness of <0.5 mm. OTs thicker than 0.5 mm can be visualized accurately by R123 and LSCM, and NR is not capable of giving the estimation of the follicles in that thickness at all.

(iii) We believe that the method proposed by Chambers et al. (2010) for OT transplantation is not well adapted to a human clinical setting. They propose using OTs of a thickness of 100 μm, which are very fragile and easily damaged while holding for transplantation.

(iv) The authors did not mention whether or not they used adhesive mounting during the cutting of OTs. On the basis of the operating instructions of the Vibratome1000 and our experience, direct mounting will damage the tissue due to the pressure of the holder. To prevent damage, a cyanoacrylate base specimen adhesive must be used. This adhesive is environmentally toxic and may influence the experiment (United States Patent 4200549).

(v) In Figure 1d, a follicle with a diameter of 225 μm is shown. If OTs were sectioned into 80–100 μm thickness slices as mentioned in methods and materials section, this follicle must be cut into three slices and is not likely to be alive, contrary to the body text and figure legend.

(vi) Human OTs in clinical trials must be prepared and transplanted as soon as possible using minimally invasive techniques. NR, as described by Chambers et al. (2010) delays visualization for ~4 h due to long incubation time while the incubation time for R123 is <60 min that can be combined with thawing procedure. On the other hand, stereomicroscopy offers the greatest advantages in time efficiency.

(vii) While the authors mentioned some references demonstrating the non-toxicity of NR, some of the same publications state that some ultrastructural changes can occur after uptake of NR (Nemes et al., 1979). A toxicity screening should be done before clearing NR for use in any reproductive research trials.

(viii) Due to potential false results, TUNEL staining has to be performed in conjunction with other methods such as immunohistochemistry. Because R123 only stains healthy follicles with active mitochondria (Johnson et al., 1981; Chen et al., 1982; Davis et al., 1985; Emaus et al., 1986; Chen, 1988), there was no need for evaluation of the follicles for specific apoptotic markers in our study.

(ix) There are two main advantages to staining with R123 over NR. Most importantly, R123 allows the visualization of healthy, active mitochondria, thereby giving a more direct indication of cellular health. NR staining of the lysosomes is a less direct indicator of cell health. In addition, R123 can be washed from cells completely, while NR remains permanently localized within the lysosomal compartments (Winckler et al., 1974; Nemes et al., 1979).

(x) Unlike the disadvantages for NR mentioned by Chambers et al. (2010), R123 staining can help visualize the cell structure of the follicle, accurately demonstrating developmental stages (Soleimani et al., 2006).

(xi) The accuracy of the detection rate of the follicles in human OTs using R123 in our study was 96% (24/25, cryopreserved OTs post thawing) which was incorrectly reported by Chambers et al. (2010) as 50%. While using NR, they reported a...
maximum accuracy of 40.3% in human and 69.3 and 71.2% in fresh ovine and frozen ovine OTs, respectively. This emphasizes the differences between ovine and human OTs and it confirms the advantage of stereo-microscopy and R123 over the NR method.

(xii) The efficacy of stereo-microscopic evaluation of human OTs has been proved by assessing follicular density before xenografting into the back muscle of SCID mice, resulting in a significantly high number of MII oocytes obtained (Soleimani et al., 2010).

In conclusion, we believe that Chambers et al. (2010) misrepresented our findings and failed to prove the superiority of using NR over R123 and stereo-microscopy.

References


Reply: In situ identification of follicles in ovarian cortex as a tool for quantifying follicle density, viability and developmental potential in strategies to preserve female fertility

Sir,

If we have overlooked aspects of the work of Dr Reza Soleimani and Prof. Dr Petra De Sutter we sincerely apologize, as this was not our intention. We are very concerned by the comments detailed in their Letter to the Editor and are dismayed that highly respected colleagues feel we may have misinterpreted their work. We have therefore looked in detail at each of the points raised.

(1) We acknowledge the authors’ comments regarding solvent toxicity, and recognize that the concentration of methanol used in the study by Soleimani et al. (2006) was low. However, the comments made in our Introduction (p. 2560) relate to concerns surrounding the use of fluorescent dyes for follicle visualization in general, not specifically R123. We also detailed the use of Calcein AM as reported by other workers (Cortvrindt and Smitz, 2001), which also often requires reconstitution in a low concentration of solvent prior to use. There is no dispute that these fluorescent compounds are not water-soluble, but instead require the use of a non-aqueous solvent for reconstitution. We have also commented on the potential damaging effects of fluorescent light on tissues, the use of which is essential for the visualization of both R123 and Calcein AM. We feel that the key point here is the concern regarding the use of any fluorescent dye for follicle visualization; indeed this fact was alluded to by the authors themselves in their own publication (Soleimani et al., 2006).

We recognize that the dose of R123 used by Soleimani et al. (2006) was shown to be non-toxic via mouse zygote assay and mouse ovarian tissue (OT) transplantation. Indeed, we specifically included comments on this evidence in the Introduction of our original submitted manuscript, but this section was subsequently amended in response to the recommendations made by the reviewers during the peer-review process.

With regard to the use of 70% (v/v) ethanol unfortunately it appears that Dr Soleimani and Prof. De Sutter may have confused two of our methodologies, namely: (i) the protocols associated with ovine cortical tissue culture and (ii) the methods used for neutral red staining. These methods are quite discrete. The brief washes in 70% (v/v) ethanol relate only to the aseptic preparation of abattoir-derived ovine ovaries that are to be used for long-term culture, as we have detailed previously (Newton et al., 1999). A 70% (v/v) ethanol wash is not a component of the neutral red staining method. In support of this we would like to draw attention to the fact that ethanol washes were not used during the processing of our human tissue samples, which were collected under sterile surgical conditions and used for neutral red assessment only.

(2) The authors comment on their method of evaluating follicles in thin human OT cortical strips. We are aware that stereo-microscopic