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Light element distribution in fresh and frozen–thawed human ovarian tissues: a preliminary study

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KEY MESSAGE

This study describes novel synchrotron-based X-ray fluorescence analyses in human ovarian tissues and reports on the utility of monitoring light element distribution in these samples to reveal the tissue integrity and oocyte quality. For the first time, the light element composition of ooplasmic vacuoles is identified.

ABSTRACT

Research question: Does synchrotron X-ray fluorescence (XRF) provide novel chemical information for the evaluation of human ovarian tissue cryopreservation protocols?

Design: Tissues from five patients undergoing laparoscopic surgery for benign gynaecological conditions were fixed for microscopic analysis either immediately or after cryopreservation. After fixation, fresh and slowly frozen samples were selected by light microscopy and transmission electron microscopy, and subsequently analysed with synchrotron XRF microscopy at different incident energies.

Results: The distributions of elements detected at 7.3 keV (S, P, K, Cl, Fe, and Os) and 1.5 keV (Na and Mg) were related to the changes revealed by light microscopy and transmission electron microscopy analyses. The light elements showed highly informative findings. The S distribution was found to be an indicator of extracellular component changes in the stromal tissues of the freeze–stored samples, further revealed by the transmission electron microscopy analyses. Low-quality follicles, frequent in the freeze–thawed tissues, showed a high Na level in the ooplasm. On the contrary, good-quality follicles were detected by a homogeneous Cl distribution. The occurrence of vacuolated follicles increased after cryopreservation, and the XRF analyses showed that the vacuolar structures contained mainly Cl and Na.

Conclusions: The study demonstrates that elemental imaging techniques, particularly revealing the distribution light elements, could be useful in establishing new cryopreservation protocols.

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Introduction

Chemotherapy, radiation therapy, or both, in women with cancer may compromise their fertility because its gonadotoxicity and side effects may lead to premature ovarian failure or loss of both endocrine and reproductive function (Mahajan, 2015). Such an event is maximally sensitive when pre-pubertal girls and young women are affected. In women of child-bearing or pre-pubescent age, the international guidelines recommend clinicians to discuss the possible risk of infertility caused by diseases or drug treatment and help them select the best method to preserve their fertility (Lambertini et al., 2016). Only a few actual clinical options, however, are present for preserving female fertility: cryopreservation of the oocyte, embryo, or ovarian tissue (Lambertini et al., 2016). Embryo cryopreservation is the most frequently used method but is feasible only in post-pubertal women and requires 2–4 weeks preparation together with a partner or a donor. Although it requires time for ovarian stimulation, oocyte cryopreservation can also be an option, which is clearly more practicable than embryo banking. Some side-effects are related to the fact that mature oocytes are fragile owing to their large dimensions, high water content and chromosomal arrangement (Rodriguez-Wallberg and Oktay, 2012). Nevertheless, recent promising data have been reported using properly modified vitrification protocols, resulting in improved oocyte survival and higher fertilization and pregnancy rates (Cobo and Diaz, 2011); these methods have already been used in clinical practice (Practice Committees of American Society for Reproductive Medicine, Society for Assisted Reproductive Technology, 2013). Considering the increasing number of reports and clinical experiences, ovarian tissue cryopreservation and banking start to appear as a promising clinical option and are the only viable method for pre-pubertal girls and for women who need immediate chemotherapy (Oktay et al., 1998). The use of immature oocytes and ovarian tissue, however, is still experimental.

During laparotomy or laparoscopy (or after oophorectomy), small fragments of the cortical ovarian tissue containing a large amount of quiescent primordial and primary follicles can be removed, cut into thin slices and cryopreserved (Fabbri et al., 2004). When needed after remission, the cortical stripes are thawed and regrafted into the patient, either in the ovaries (orthotopic graft) or in a remote area of the body (etherotopic graft), such as the forearm (Gamzatova et al., 2014). A number of reports have shown that human follicles in tissue slices can survive cryopreservation after cooling to −196°C in liquid nitrogen (Oktay et al., 2001), and the technique allows restoration of gametogenic, steroidogenic function, or both (Gamzatova et al., 2014). The intrinsic plasticity of the ovarian tissue sustains its aptness for freezing; however, compared with freeze–storage of isolated cells, cryopreservation of the ovarian tissue yields some problems related to the complexity of the tissue architecture and heterogeneous cell composition (oocytes, granulose and stromal cells) (Fabbri et al., 2010). The cryopreservation protocols must consider a balance for the optimal conditions for each cell type, and the cryoprotectants used must have an adequate penetration to all the cells to avoid ice crystal formation (Fabbri, 2006).

As in many other laboratories, slow–freeze techniques are currently used in our laboratory, whereas new vitrification protocols involving rapid cooling of ovarian tissue fragments are starting to be explored (Silber et al., 2010). To date, more than 30 live births have been reported using the slow–freeze technique in the ovarian tissue (Mahajan, 2015) and only two live births after vitrification (Suzuki et al., 2015). The damage extent and the quality of the follicles after rewarming of freeze–stored tissues are usually evaluated morphologically using light microscopy (Fabbri et al., 2014) and transmission electron microscopy (TEM) (Camboni et al., 2008). Obviously, it would be important to access further molecular information on the quality of the ovarian tissues and the changes that occur during freeze–storage or after rewarming. A similar knowledge would further advance state-of-the-art protocols. To improve the evaluation of the damage and the underlying biochemical mechanism at this level, we aimed to test the applicability of some emerging synchrotron microscopy approaches. In particular, as a possible integration with conventional techniques, we experimented with the applicability of synchrotron radiation-based X-ray fluorescence (XRF) microscopy at different incident energies. X-ray fluorescence analysis is a multi-elemental, highly sensitive technique based on the detection of X-rays emitted from sample atoms excited with X-ray photons. This technique can provide semi-quantitative or quantitative information, as the intensity of fluorescence is related to the level of the element within the sample. Over the past decade, the investigation of biological samples using XRF has been favoured by the development of high-flux and highly focused X-ray beams at different synchrotron facilities (Ortega et al., 2007; Pascolo et al., 2013).

In the present study, we explored the capabilities of two XRF set-ups at different spatial resolutions and at different energies on both fresh and cryopreserved human ovarian tissues to reveal the possible elemental changes related to treatments and conservation. The samples used were pre-selected using light microscopy and TEM to compare the elemental distributions with conventional morphological evaluations.

Materials and methods

Patients and sample preparation

The ovarian tissues were obtained from five patients who underwent laparoscopic surgery of total or partial oophorectomy for benign gynaecological conditions (four patients had ovarian cysts; one had an endometrial polyp (**Supplementary Table S1**) at the Gynaecological and Obstetrics Department of the Institute of Maternal and Child Health IRCCS Burlo Garofolo, Trieste, Italy. The bioptic samples were collected after obtaining informed consent from the patients. The research was approved by the Institutional Review Board of IRCCS Burlo Garofolo on 13 September 2016 (authorization number 10/2016). For each patient, the ovarian tissue samples were analysed immediately after surgical excision (t0- control tissue) and after freezing–thawing protocols (t1) by the three microscopy techniques described below. The biopsy specimen was immediately immersed in 20 ml of a flushing medium with heparin (SynVitro® Flush, Origio, Berlin, Germany) at 37°C and then transferred into a culture dish (Falcon, Becton Dickinson, Bedford MA, USA) containing a flushing medium. The cortical part was mechanically separated (using a scalpel) from the medullar tissue and cut into small fragments (1–2-mm thickness and 5-mm width) (Isachenko et al., 2012; Keros et al., 2009). The control tissue (t0) slices (two to three for each patient) were immediately fixed for evaluation using light microscopy and TEM. The other fragments (four to six slices for each patient) were slowly frozen and stored in liquid nitrogen for 3–6 months before defrosting. Thereafter, they were fixed (t1). The slow–freezing and thawing protocols are described in **Supplementary Appendix**.

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