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ARTICLE

Survival and growth of human preantral follicles after cryopreservation of ovarian tissue, follicle isolation and short-term xenografting

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Professor Christiani Amorim received her PhD from the Federal University of Santa Maria. She then worked at the Florence University, in Italy, and, subsequently, served as Associate Professor at the Brasília University, in Brazil. She is currently Professor at the Catholic University of Louvain, in Belgium. In recent years, Professor Amorim has focused her attention on the development of a transplantable artificial ovary to restore fertility in cancer patients. Her pioneering studies have served as the basis for establishing the field of ovarian tissue engineering, and she has been actively organizing the first group on reproductive tissue engineering.

Abstract In women, chemotherapy and radiotherapy can be harmful to the ovaries, causing loss of endocrine and reproductive functions. When gonadotoxic treatment cannot be delayed, ovarian tissue cryobanking is the only way of preserving fertility. This technique, however, is not advisable for patients with certain types of cancer, because of the risk of reintroducing malignant cells present in the cryopreserved tissue. Our objective is therefore to develop a transplantable artificial ovary. To this end, cryopreserved human preantral follicles were isolated and embedded in fibrin formulations prepared with 50 mg/ml fibrinogen and 10 IU/ml thrombin supplemented or not with 3% hyaluronic acid, and respectively xenografted to specially created right and left peritoneal pockets in eight nude mice. On days 0 and 7, the animals were killed and the matrices retrieved. On day 7, no difference was observed in the recovery rate of follicles embedded in fibrin alone (23.4%) or fibrin-hyaluronic acid (20.5%). Ki67 staining confirmed growth of the grafted follicles and terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling assay revealed 100% of the follicles to be viable in both groups on day 7. In conclusion, fibrin seems to be a promising material for creation of an artificial ovary, supporting follicle survival and development.

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Introduction

Transplantation is currently the only option able to re-establish ovarian function from cryopreserved ovarian tissue in cancer survivors (Donnez and Dolmans, 2013). This technique has resulted in successful ovarian function restoration and more than 60 pregnancies to date (Donnez and Dolmans, 2015). There is legitimate concern, however, about the possible presence of malignant cells in cryopreserved fragments, which could lead to recurrence of the primary disease after reimplantation (Greve et al., 2012; Meirow et al., 2008; Rosendahl et al., 2010). Dolmans et al. (2013) and Sonmezer and Oktay (2004) classified malignant diseases into three categories according to the risk of ovarian involvement. Leukaemia, neuroblastoma and Burkitt lymphoma were found to run the highest risk of metastasizing to the ovaries, so transplantation of ovarian tissue after disease remission is not advisable for these patients.

To avoid the risk of reintroducing malignant cells after cancer treatment, the development of an artificial ovary could offer a solution. Indeed, our previous studies have shown that isolated mouse preantral follicles can survive and grow after transplantation (Chiti et al., 2016; Luyckx et al., 2014; Vanacker et al., 2014). This occurred mainly with the use of a fibrin formulation with low fibrinogen and thrombin concentrations (Chiti et al., 2016; Luyckx et al., 2014), which yielded a higher recovery rate of isolated murine preantral follicles. When this fibrin matrix was used to xenograft isolated human follicles, however, the results were significantly inferior, showing a recovery rate of only around 2% (Amorim CA, unpublished results). Such low concentrations of fibrinogen and thrombin may negatively affect these human follicles, as they need a more rigid environment to maintain their three-dimensional structure, vital to their survival and development (Xu et al., 2006). Moreover, despite high concentrations of proteins in the extracellular matrix, ovarian tissue is also made up of glycosaminoglicans, which play an important role in tissue morphogenesis (Dairkee and Glaser, 1982). Hyaluronic acid is one of the glycosaminoglicans present in human ovarian tissue (Haslene-Hox et al., 2015) and has already been successfully used for in-vitro culture of mouse preantral follicles (Desai et al., 2012). Apart from its numerous advantages in tissue engineering, such as being recognized by cellular receptors and interaction with several extracellular matrix proteins (Donegan et al., 2010), it was reported that varying hyaluronic acid concentrations could also affect the stiffness of the matrix (Desai et al., 2012).

The goal of our study was therefore to evaluate whether a fibrin formulation with higher concentrations of fibrinogen and thrombin would constitute a suitable matrix to graft isolated human preantral follicles, as we know that the human ovary is more rigid than the mouse ovary, so human follicles may well require a stiffer matrix for their survival. We also wanted to investigate whether addition of hyaluronic acid to the fibrin matrix to make it more rigid would improve follicle survival and growth during xenotransplantation.

Materials and methods

Experimental design

Preantral follicles and ovarian stromal cells were isolated from human frozen–thawed biopsies. Between 20 and 50 preantral follicles were embedded in fibrin clots without hyaluronic acid (fibrin group) or with hyaluronic acid (fibrin-hyaluronic acid group), together with 50,000 ovarian stromal cells in each group. The clots were respectively xenografted to right and left pockets specially created in the inner wall of the peritoneum of eight cycling adult female nude mice (two clots per recipient: fibrin and fibrin-hyaluronic acid) for 10 min (day 0) or 7 days (day 7). Thereafter, the animals were killed and the clots were removed, fixed and processed for histology and immunohistochemistry.

Ethics

Use of human ovarian tissue was approved by the Institutional Review Board of the Université Catholique de Louvain on 2 June 2014 (IRB reference 2012/23MAR/125, registration number B403201213872). Use of proliferative endometrium, obtained from our university biolibrary, was approved by the Institutional Review Board of the Université Catholique de Louvain on 28 July 2008, EudraCT number 2008/001805-40. Guidelines for animal welfare were approved by the Committee on Animal Research of the Université Catholique de Louvain on 19 June 2014 (reference 2014/UCL/MD/007).

Collection of ovarian tissue

Ovarian biopsies were taken from 10 women (between 21 and 37 years of age) after obtaining informed consent. All patients underwent laparoscopic surgery for benign gynaecological disease. Biopsies were immediately transported on ice to the laboratory in minimal essential medium plus Glutamax™ (MEM; Gibco, Invitrogen, Merelbeeke, Belgium). Once in the laboratory, the medullary part of the biopsy was removed with surgical scissors and the cortex was cut into strips for cryopreservation.

Ovarian tissue freezing and thawing

Freezing and thawing of the ovarian tissue strips were carried out according to the method previously reported by Amorim et al. (2009), using a cryoprotective solution containing 10% DMSO (Sigma, St Louis, MO, USA) and 2% human serum albumin (Flexbumin, Baxter, Lessen, Belgium) in minimal essential medium.

Ovarian sample distribution

One ovarian strip from each patient was used for follicle isolation. Three to four follicles from each patient were then