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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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KEYWORDS: artificial ovary, cancer, fertility preservation, fibrin matrix, hyaluronic acid, mouse

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

used for viability analysis and the remaining follicles were divided into two groups (fibrin and fibrin-hyaluronic acid) for further xenografting to one mouse. In two cases (both day 0) where the ovarian strip was too small (~2 × 2 mm), samples from two patients were pooled together to have enough follicles to carry out the experiment.

Enzymatic digestion of ovarian tissue, follicle recovery and ovarian stromal cell isolation

The protocol previously implemented by Vanacker et al. (2011) was used to isolate human follicles. Preantral follicles were picked up over the course of 45 min (Dolmans et al., 2008). Two to five follicles from each patient were subsequently processed for live/dead assays to evaluate follicle viability after isolation. After follicle isolation, the solution was used to isolate ovarian stromal cells (Luyckx et al., 2014).

Fibrin clot formation

Reconstitution and dilution of the two components (fibrinogen and thrombin) of the fibrin sealant (Tissucol, Baxter) and fibrin clot formation were achieved according to the method described by Luyckx et al. (2014) with some modifications. Fibrinogen (100 mg/ml) was reconstituted in a solution containing 3000 KIU/mL bovine aprotinin, a fibrinolysis inhibitor, at 37°C. Thrombin (500 IU/ml) was reconstituted in 40 mmol/ml calcium chloride ($CaCl_2$) solution. The reconstituted thrombin was diluted in 40 mmol/l $CaCl_2$ solution to obtain a final concentration of 10 IU/ml.

Simultaneously, a solution of hyaluronic acid (6% w/v; Sigma) was prepared by dissolving the hyaluronic acid powder in sterile distilled water at 37°C. To form the two groups, 100 mg/ml fibrinogen was diluted (1:1) in saline (0.9% sodium chloride) or 6% hyaluronic acid to obtain a solution of 50 mg/ml fibrinogen (fibrin group) or 50 mg/ml fibrinogen plus 3% hyaluronic acid (fibrin-hyaluronic acid group). The hyaluronic acid concentration used in this study was based on the highest concentration that could be achieved to be homogenously mixed with fibrin.

A droplet of 7.5 μl fibrinogen (50 mg/ml) with or without hyaluronic acid was deposited on a glass petri dish, to which 30–50 isolated preantral follicles and 50,000 human ovarian stromal cells (1 μl) were added. This droplet, containing isolated human ovarian follicles and cells, was then mixed with a 7.5 μl-volume of thrombin on a plastic petri dish. For fibrin polymerization, the droplet was incubated at 37°C for 30 min, after which the fibrin clot was gently detached and grafted to the mice.

Follicle viability

Follicles were incubated in 100 μl Dulbecco's phosphate-buffered saline (PBS; Gibco, Invitrogen, Merelbeeke, Belgium) containing 2 μmol/l calcein-AM and 5 μmol/l ethidium homodimer-I (Molecular Probes, Leyden, the Netherlands) for 45 min at 37°C in the dark (Cortvrindt and Smitz, 2001). After exposure to fluorescent dyes, follicles were observed under an inverted fluorescence microscope (Leica DMIL, Van Hopplynus Instruments, Brussels, Belgium) and classified into four categories depending on the percentage of dead follicular cells, according to a classification established by our group: V1, follicles with the oocyte and all granulosa cells viable; V2, minimally damaged follicles with less than 10% dead follicular cells; V3, moderately damaged follicles with 10–50% dead follicular cells; V4, dead follicles with both the oocyte and all follicular cells dead (Martinez-Madrid et al., 2004).

Grafting fibrin clots to nude mice

Nude female mice aged 8–10 weeks (Swiss nu/nu, Charles River Laboratories, France) were used for this study. Their housing conditions, anaesthesia and analgesia have been described elsewhere (Vanacker et al., 2014). The surgical protocol and peritoneal pocket preparation have also been previously reported (Luyckx et al., 2014). Two fibrin clots were transplanted to each mouse: on the right side, a pure fibrin clot and on the left, a fibrin-hyaluronic acid clot. The abdominal wall and skin were then closed. Four mice were grafted and the clots were immediately removed and fixed in 4% paraformaldehyde for histological and immunohistochemical analyses (day 0). After 1 week, the remaining animals ($n = 4$) were killed by CO_2 asphyxiation and the grafts were recovered and fixed in 4% paraformaldehyde.

Histological analysis

After fixation, clots and grafts recovered on days 0 and 7 were dehydrated, embedded in paraffin and serially sectioned (5-mm thick sections). Every fourth slide was stained with haematoxylin–eosin (Merck, Darmstadt, Germany) for histological evaluation, namely localization of the grafted matrix and observation of graft degradation and vascularization. The remaining slides (Superfrost Plus, Menzel-Glaser, Germany) were kept for further investigations. Sections were examined with a Zeiss Axioskop microscope (Zeiss, Wetzlar, Germany).

Follicle recovery

After evaluating histological sections from both groups on day 0, we found that some of the structures considered to be isolated preantral follicles under the stereomicroscope (Leica MZ8) were actually spherical groups of cells or follicular structures (granulosa cells without an oocyte) (**Figure 1**). These structures were therefore subtracted from the number of follicles embedded in the clots and took into account only the number of follicles found in histological sections on day 0, calculating day 7 recovery rates based on this. To this end, the follicle recovery rate was adjusted on day 7 using a correction factor to recalculate the number of follicles embedded in clots before grafting:

$$Number\ of\ \ follicles\ embedded\ in\ the\ clot\ (D7) = \frac{F_F \times F_{RD0}}{100}$$

Where:

F_F = number of follicles added to the fibrinogen droplet

F_{RD0} = recovery rate of follicles on day 0

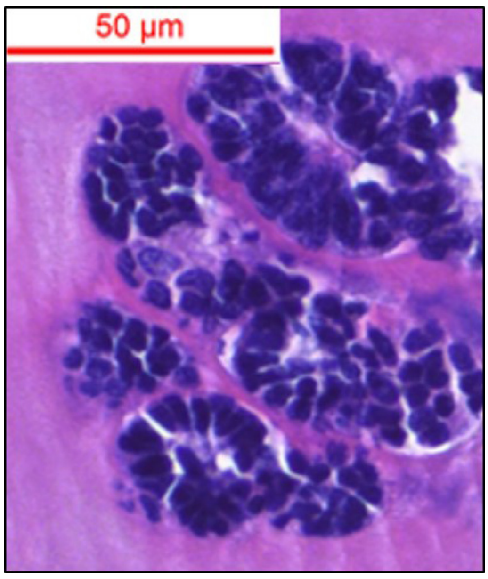

Figure 1 Micrograph of a haematoxylin-eosin stained section of a day 0 clot showing random groups of cells and follicular structures in a fibrin clot.

Follicle analyses

Two slides (eight sections), representative of each graft at histology, were used for analysis, with adjacent slides used for the different evaluations as described below. For immunostaining, the slides were first deparaffinized with Histosafe and rehydrated in 2-propanol.

Follicle apoptosis

Apoptosis was analysed by TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) to detect strand breaks of DNA occurring during the apoptotic process using the In Situ Cell Death Detection Kit, TMR Red (Roche Applied Science, Mannheim, Germany), according to the protocol previously described by Martinez-Madrid et al. (2007). TUNEL-positive and -negative granulosa cells were counted for each follicle using ImageJ, a freely available image-processing and analysis programme developed at the National Institutes of Health (http://rsb.info.nih.gov/ij/).

They were then classified into live or dead categories following criteria defined in the follicle viability section.

Granulosa cell proliferation

Proliferation of grafted follicles and cells in clots was analysed using mouse anti-human Ki67 as the primary antibody (dilution 1:100, clone MIB1, ref M7240, Dako, Glostrup, Denmark) and Envision anti-mouse (ref K4001, Dako) as the secondary antibody. Proliferative endometrium, obtained from our university biolibrary was used as a positive control. Negative controls consisted of the dilution solution without any primary antibody. Follicles with at least one Ki67-marked granulosa cells were considered as growing.

Granulosa cell activity after xenografting

Inhibin-alpha was used to assess granulosa cell activity. Inhibin-alpha is secreted by granulosa cells from human follicles to inhibit follicle-stimulating hormone secretion by the pituitary gland. Slides were incubated with the primary antibody, mouse anti-human inhibin-α, clone R1, mAb (dilution 1:100, ref. MCA 951 S, Serotec, Oxford Bio-Innovation, Oxford, UK) overnight at 4°C, before being incubated for a further 60 min at room temperature with the secondary antibody, Envision anti-mouse (DAKO). Fresh human ovarian tissue was used as a positive control. Negative controls consisted of the dilution solution without any primary antibody. Follicles with at least one inhibin-alpha-marked granulosa cell were considered healthy.

Table 1 Follicle diameter (µm) after isolation.

Patient	*Follicle diameter (mean ± SD)*
1	43.8 ± 4.8
2	38.8 ± 6.3
3	48.8 ± 2.5
4	38.8 ± 11.8
5	42.5 ± 2.9
6	45.0 ± 10
7	41.3 ± 2.5
8	43.8 ± 11.1
9	46.3 ± 4.8
10	40.0 ± 11.5

Statistical analysis

One-way analysis of variance was used to compare the follicle diameter in the different patients and Turkey's multiple comparison test was used as a post-hoc test. Statistical comparison between the two groups (fibrin versus fibrin-hyaluronic acid) was carried out using a two-tailed paired t-test. The chi-squared test of independence was used to compare the follicle classes between the two time periods (day 0 and day 7), follicle viability after grafting (TUNEL staining) and granulosa cell activity (inhibin-alpha staining). $P < 0.05$ was considered statistically significant.

Results

Follicle population before xenografting

After freezing, thawing and isolation, a total of 36 follicles were measured and tested for viability. As measurement of all isolated follicles before grafting is time consuming and could therefore negatively affect their viability, only follicles used for viability testing were evaluated to estimate the size of follicles isolated for xenotransplantion. No statistically significant difference in follicle diameter was observed between patients (**Table 1**). With follicle viability after cryopreservation and isolation, of the 36 follicles, 95% (34/36; 95% CI 0.81 to 0.99) were live follicles (V1) (**Figure 2**) and 5% (2/36) minimally damaged (V2).

Follicle analyses after xenografting

On day 7, all eight fibrin clots were easily identified by the non-absorbable sutures. Histological analysis confirmed that

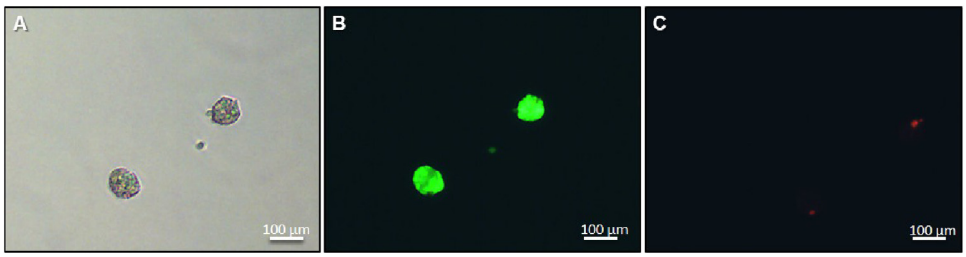

Figure 2 (A) Viability testing of human ovarian follicles soon after isolation. Light microscopy pictures of isolated follicles at 20× magnification; (B and C) follicle viability assessment by calcein-AM and ethidium homodimer-I.

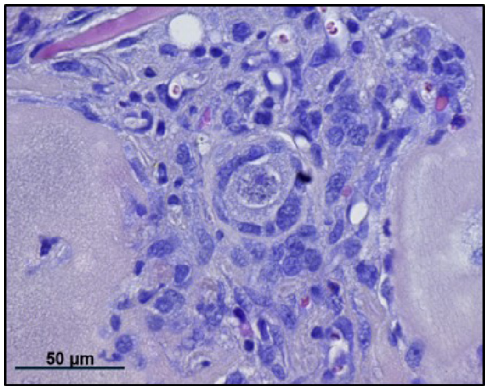

Figure 3 Primary follicle and ovarian cells in a fibrin clot on day 7 stained with haematoxylin-eosin.

all retrieved grafts contained ovarian follicles with normal oocytes and granulosa cells, a linear basement membrane, and even the presence of a homogeneous zona pellucida in larger follicles (**Figure 3**). The number of follicles found in both groups after the different periods of grafting is shown in **Table 2**. After applying the previously described formula, the corrected recovery rate for day 7 was 23% (27/115), compared with 20% in the fibrin-hyaluronic acid group (26/127), showing no statistical difference in recovery rates between the two groups after 7 days of xenotransplantation.

Follicle stage proportions varied between groups and days of xenografting. In the fibrin group, a significant decrease was observed in the number of primary follicles, and a significant increase in the number of secondary follicles after 7 days of xenotransplantation (both $P < 0.05$). Surprisingly, significantly higher population of primordial follicles ($P < 0.05$) was observed. On the other hand, no difference was found between day 0 and day 7 in the fibrin-hyaluronic acid group. On day 0, the proportion of primordial follicles was significantly higher in the fibrin-hyaluronic acid group than the fibrin group ($P < 0.05$). On day 7, although the proportion of primary follicles was statistically lower in the fibrin group ($P < 0.05$), the proportion of secondary follicles was statistically higher compared with the fibrin-hyaluronic acid group ($P < 0.05$) (**Figure 4**). Follicle growth in both groups was confirmed by Ki67 staining after 7 days of xenografting (**Figure 5A**).

A total of 45 follicles were analysed for apoptosis using TUNEL assays. On day 0, all follicles from the fibrin group were stained and classified as V1 (13/13) (**Figure 5B**), whereas 12 out of 13 follicles analysed from the fibrin-hyaluronic acid group were V1 (92%) and 1 (8%) was V2. After 7 days of xenografting, 12 out of 13 follicles analysed from fibrin group were V1 (92%) and 1 (8%) was V2. Five out of six follicles analysed in fibrin-hyaluronic acid group were V1 (83%) and 1 (17%) was V2. No statistical difference was observed in the viability of follicles from either group after the different periods of xenografting.

Granulosa cell activity in isolated follicles was confirmed by inhibin-alpha immunostaining (**Figure 5C**). On day 0, 93% (13/14) and 100% (9/9) of follicles showed inhibin-alpha-positive granulosa cells in the fibrin and fibrin-hyaluronic acid groups, respectively. After 7 days of xenografting, the proportion of follicles with inhibin-α-positive granulosa cells did not differ from day 0, yielding figures of 88% (14/16) and 100% (11/11) for fibrin and fibrin-hyaluronic acid clots, respectively.

Discussion

The concept of a transplantable artificial ovary hinges upon the isolation, encapsulation and grafting of preantral follicles inside a suitable matrix to avoid reseeding malignant cells back to the patient after complete cancer remission (Amorim, 2011; Luyckx et al., 2014; Vanacker et al., 2014). In our previous studies (Luyckx et al., 2013, 2014), different concentrations of fibrinogen and thrombin were tested to graft isolated murine preantral follicles, aiming to construct a standardized artificial ovary (Luyckx et al., 2014). Our promising results with isolated mouse follicles (Chiti et al., 2016; Luyckx et al., 2014) using a fibrin formulation with lower concentrations of fibrinogen and thrombin could not, however, be reproduced with isolated human follicles (Amorim CA, unpublished results). We believe that this was due to the lack of rigidity of this fibrin formulation, which was not able to maintain the three-dimensional structure of human follicles. For this reason, we decided to investigate if a stiffer fibrin matrix containing higher concentrations of fibrinogen and thrombin could have a positive effect on isolated human follicles. To this end, a fibrin formulation was tested, incorporating 50 mg/ml fibrinogen and 10 IU/ml thrombin, a combination successfully used by Germain et al. (2015) to create a matrix for the apical papilla of human teeth, supporting angiogenesis and cellular differentiation. This fibrin clot was easy to manipulate and did not immediately polymerize, which gave us time to properly mix the two fibrin components. Moreover, its handling allowed swift transplantation. To improve follicle survival, we also tested fibrin clot supplementation with hyaluronic acid, a polysaccharide found in most connective tissues and successfully used for cell encapsulation

Table 2 Recovery rate of preantral follicles after xenotransplantation.

Group	*Time interval*		*Follicle number (mean ± SD)*	*Follicle recovery (mean ± SD)*
Fibrin	D0	Follicles embedded in the clot	36.3 ± 15.9	57.5 ± 27.5%
		Follicles found after grafting	8.0 ± 7.0	
	D7	Follicles embedded in the clot	50.0 ± 0.0	13.5 ± 10.8%
		Follicles found after grafting	6.8 ± 5.4	
	D7 (after applying correction factor)	Follicles embedded in the clot	28.8 ± 0.0	23.4 ± 18.7%
		Follicles found after grafting	6.8 ± 5.5	
Fibrin-hyaluronic acid	D0	Follicles embedded in the clot	36.8 ± 13.6	64.8 ± 11.2%
		Follicles found after grafting	23.8 ± 9.8	
	D7	Follicles embedded in the clot	49.0 ± 0.8	13.3 ± 4.4%
		Follicles found after grafting	6.5 ± 2.1	
	D7 (after applying correction factor[a])	Follicles embedded in the clot	31.8 ± 0.5	20.5 ± 6.8%
		Follicles found after grafting	6.5 ± 2.1	

$n = 4$ for D0 and D7 of fibrin and fibrin-hyaluronic acid; D0, day 0; D7, day 7.
No statistically significant differences were found for D0 (fibrin versus fibrin-hyaluronic acid), D7 (fibrin versus fibrin-hyaluronic acid) and D7 (after applying correction factor) (fibrin versus fibrin-hyaluronic acid).
[a]See Materials and methods for correction factor calculation.

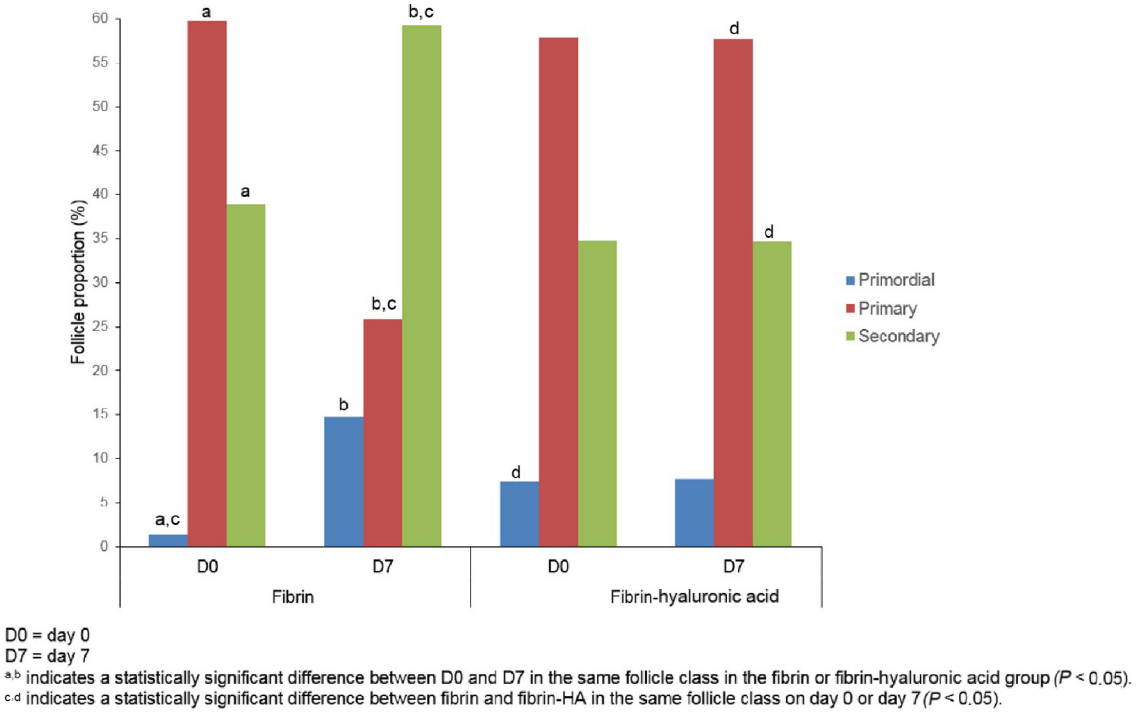

Figure 4 Proportions of primordial, primary and secondary follicles on days 0 and 7 (error bars represent 95% confidence intervals).

in tissue engineering (Gasperini et al., 2014). Our results, however, revealed that hyaluronic acid supplementation did not increase proportions of recovered follicles.

Although the follicle recovery rate was better using a fibrin formulation with higher concentrations of fibrinogen and thrombin, it was inferior to outcomes reported for isolated murine follicles (Luyckx et al., 2014) with our previous fibrin formulation. This is probably due to the stage of development of isolated follicles; in humans primordial and primary follicles are mainly isolated (Amorim et al., 2009; Dolmans et al., 2006; Vanacker et al., 2011); however, in mice, most are already at the secondary stage. Indeed, when Chiti et al. (2016) separated mouse primordial-primary and secondary follicles before allografting, they observed a significantly lower recovery rate in the group of smaller follicles compared with larger ones (6% versus 27%). According to these investigators, one of the possible reasons for such a low recovery rate of primordial and primary follicles may be the insufficient stiffness of the matrix. Our results seem to corroborate this hypothesis, as we were able to improve follicle recovery rates by increasing fibrin rigidity using elevated fibrinogen and thrombin concentrations. We cannot state, however, with any

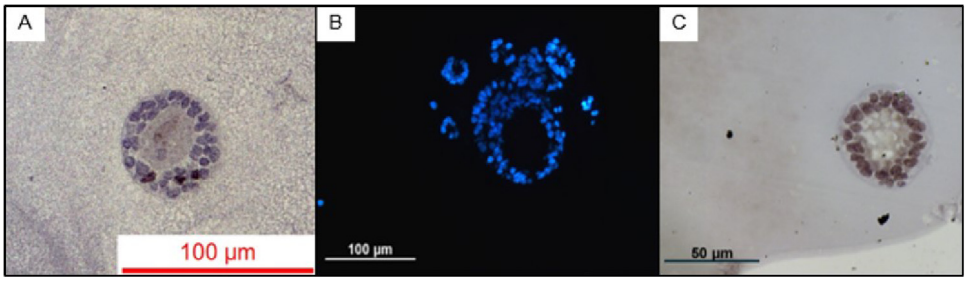

Figure 5 mmunohistochemical analyses. (A) Small secondary follicle with Ki67-positive granulosa cells in brown (fibrin group, day 7); (B) TUNEL assay showing 100% of granulosa cells to be viable after 7 days of grafting (fibrin group); (C) inhibin-alpha activity identified by brown staining in a preantral follicle (fibrin-hyaluronic acid group, day 7).

certainty that this is the only reason for our findings. Indeed, we can also hypothesize that such follicle loss could at least be partially due to the normal fate of the most of the follicles commencing growth, namely atresia (Gougeon, 1996). It is also important to bear in mind that, although a recovery rate of around 22% may seem low, it is similar to results reported by Nisolle et al. (2000) after 1 week of xenografting of human ovarian tissue.

Follicle growth in our fibrin matrices was confirmed by Ki67 immunostaining, which proves that the remaining population of isolated follicles can resume their development after transplantation. When compared with the results obtained by Dolmans et al. (2007) on xenografting of isolated human preantral follicles in plasma clots, our proportions of primordial and primary follicles in the fibrin and fibrin-hyaluronic acid groups, respectively, were similar. A much higher proportion of secondary follicles, however, was observed. As studies on ovarian hyaluronic acid have focused on its involvement in the development of antral follicles and ovulation (Irving-Rodgers and Rodgers, 2005), or mice preantral follicles in three-dimensional in-vitro culture (Desai et al., 2012), further investigations into hyaluronic acid involvement in the growth of preantral follicles are required to interpret our findings.

Follicle health status was evaluated by TUNEL assay and inhibin-alpha immunostaining. Again, no difference was observed between the groups; both showed high follicle viability after 7 days of xenografting and granulosa cells from most follicles expressed inhibin-alpha. Such encouraging results demonstrate that despite high follicle loss after xenotransplantation, the remaining follicles are viable and show signs of granulosa cell proliferation.

It is important to stress that, in this study, we used stromal cells isolated from frozen-thawed biopsies, whereas in a clinical setting, these cells would be isolated from a fresh ovarian biopsy retrieved from the patient after cancer treatment to avoid the potential risk of inadvertently adding malignant cells to the artificial ovary (Amorim, 2016). Indeed, we conducted a study (Soares et al., unpublished data) with fresh ovarian tissue retrieved from three patients at the time of ovarian tissue transplantation. All three had cancer and had undergone chemotherapy cycles. Stromal cells isolated from their tissue showed cell yield, viability and in-vitro proliferation to be similar to fresh controls from healthy patients (without cancer treatment), which suggests that chemotherapy does not negatively affect stromal cells (Soares et al., 2015a). To ensure that any malignant cells possibly present in cryopreserved tissue from which preantral follicles would be isolated would not find their way into the artificial ovary, we improved our isolation protocol by adding three steps of washing of follicle suspensions (Soares et al., 2015b).

In conclusion, isolated human follicles were able to survive after encapsulation in fibrin clots and short-term xenotransplantation. Fibrin looks to be a promising material for creation of an artificial ovary matrix, supporting isolated human follicles after grafting. More studies, however, are needed to confirm these findings.

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