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FERTILITY PRESERVATION

A novel fibrin-based artificial ovary prototype resembling human ovarian tissue in terms of architecture and rigidity

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Abstract

Purpose The aim of this study is to optimize fibrin matrix composition in order to mimic human ovarian tissue architecture for human ovarian follicle encapsulation and grafting.

Methods Ultrastructure of fresh human ovarian cortex in age-related women ($n = 3$) and different fibrin formulations (F12.5/T1, F30/T50, F50/T50, F75/T75), rheology of fibrin matrices and histology of isolated and encapsulated human ovarian follicles in these matrices.

Results Fresh human ovarian cortex showed a highly fibrous and structurally inhomogeneous architecture in three age-related patients, but the mean ± SD of fiber thickness (61.3 to 72.4 nm) was comparable between patients. When the fiber thickness of four different fibrin formulations was compared with human ovarian cortex, F50/T50 and F75/T75 showed similar fiber diameters to native tissue, while F12.5/T1 was significantly different (p value < 0.01). In addition, increased concentrations of fibrin exhibited enhanced storage modulus with F50/T50, resembling physiological ovarian rigidity. Excluding F12.5/T1 from further analysis, only three remaining fibrin matrices (F30/T50, F50/T50, F75/T75) were histologically investigated. For this, frozen-thawed fragments of human ovarian tissue collected from 22 patients were used to isolate ovarian follicles and encapsulate them in the three fibrin formulations. All three yielded similar follicle recovery and loss rates soon after encapsulation. Therefore, based on fiber thickness, porosity, and rigidity, we selected F50/T50 as the fibrin formulation that best mimics native tissue.

Conclusions Of all the different fibrin matrix concentrations tested, F50/T50 emerged as the combination of choice in terms of ultrastructure and rigidity, most closely resembling human ovarian cortex.

Keywords Human ovarian tissue microstructure · Scanning electron microscopy · Fibrin matrix · Porosity · Isolated follicles · Artificial ovary

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Introduction

Ovarian tissue cryopreservation and transplantation after cancer remission is a promising fertility restoration strategy that has already led to more than 100 live births worldwide [1–3]. Nevertheless, cancer patients at high risk of ovarian involvement cannot undergo ovarian tissue transplantation after being cured because of reasonable concerns about disease recurrence [4–7]. With a view to offering these women future alternatives allowing them to conceive, in recent years, we have been developing a transplantable artificial ovary. Conceptually, the artificial ovary requires cryopreservation of ovarian tissue before initiation of cancer treatment, followed by follicle isolation and encapsulation inside an artificial matrix, before grafting to the patient with the goal of restoring her endocrine and reproductive functions [8]. Despite encouraging results showing that a safe and efficient follicle isolation procedure is now feasible for human application [9–12], creating the appropriate scaffold to encapsulate and temporarily support human follicles after grafting remains a challenge [13].

Many different matrices have already been tested [14–21], with a fibrin-based scaffold emerging as the elective choice for construction of an artificial ovary prototype [22]. Since the majority of studies in this field have been conducted with mouse follicles, it is vital to investigate the right combination of fibrinogen and thrombin (F/T) to use in humans. These two components are the principal constituents of fibrin and key players in controlling the porosity and rigidity of the scaffold, and hence the ability to encapsulate and graft human follicles. Previous studies [23, 24] have suggested that while a fibrin matrix with low concentrations of fibrinogen and thrombin (F12.5/T1) is suitable for encapsulation and grafting of mouse secondary follicles, it may lack adequate stiffness to support primordial-primary follicles. This hypothesis was subsequently confirmed by Paulini et al. [21] using a scaffold with higher concentrations of fibrinogen and thrombin (F50/T10) to encapsulate and graft human primordial-primary follicles [21]. However, in order to design an artificial matrix that mimics the physiological ovarian environment, it is first necessary to gather more knowledge on the physical structure of the native human ovary.

Our study therefore aims to investigate human ovarian tissue microstructure in order to identify the fibrin formulation that best resembles the natural milieu of the human ovary in terms of architecture, porosity, and rigidity.

Materials and methods

Experimental design

Fresh human ovarian cortex samples ($n = 3$) were obtained from three age-related women (28–34 years of age) after laparoscopic surgery for benign gynecological disease after gaining informed consent. These biopsies and four different fibrin clot formulations (F12.5/T1, F30/T50, F50/T50, F75/T75) were analyzed by scanning electron microscopy (SEM) to assess and compare their ultrastructure. Fibrin matrices were also investigated for storage or elastic modulus (G') in order to evaluate their stiffness. Based on the significant difference in fiber thickness and porosity between F12.5/T1 and human ovarian cortex, as well as our previous unpublished findings on human follicles [25], we excluded F12.5/T1 from subsequent histological analysis. Frozen cryovials of ovarian cortex from different patients ($n = 22$) were then collected from the ovarian tissue bank, thawed and processed for follicle isolation, as previously described in one of our recently submitted papers [26]. Once isolated, preantral follicles (between 14 and 50) were embedded in the three remaining fibrin clot formulations (F30/T50, F50/T50, F75/T75) in order to compare follicle recovery and loss rates after fibrin encapsulation.

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). After obtaining informed consent, fragments of ovarian tissue were collected from patients affected by mesothelial cysts, rectovaginal nodules without associated ovarian endometriomas, and pelvic inflammatory disease.

Fibrin clot reconstitution

Reconstitution and dilution of the two components (fibrinogen and thrombin) of the fibrin sealant kit (Tissucol, Baxter, Lessines, Belgium) and fibrin clot formation were previously described by Paulini et al. [21]. Fibrinogen (100 mg/mL) was diluted in saline solution (0.9% NaCl) to obtain four concentrations, 12.5, 30, 50, and 75 mg/mL, and thrombin (500 IU/mL) in 40 mmol/L $CaCl_2$ to achieve concentrations of 1, 50, and 75 IU/mL. For fibrin polymerization, 100 μL of the appropriate fibrinogen concentration was mixed once with 100 μL of the corresponding thrombin concentration and incubated at 37 °C for 15 or 45 min depending on the F/T combination. The samples were then fixed in Karnovsky solution (2.5% glutaraldehyde [Agar Scientific, Brussels, Belgium] and 4% paraformaldehyde in 0.1 M phosphate-buffered saline [PBS]) for 24 h or immediately processed for rheological investigations.

Morphological analysis of human ovarian cortex and different fibrin matrices

Ovarian biopsies were immediately transported on ice to the laboratory in minimal essential medium plus Glutamax™

(MEM; Gibco, Invitrogen, Merelbeeke, Belgium). The medulla was removed with surgical scissors and the cortex fixed in Karnovsky solution for 24 h. After fixation, the ovarian fragments and four different fibrin matrices were immersed overnight in a solution of 30% glycerol in 0.1 M PBS (4 °C), plunged into liquid nitrogen, and cryofractured. The samples were washed in fresh 0.1 M PBS three times for 5 min each, before post-fixation with a solution of 2% osmium tetroxide (OsO_4) in 0.1 M PBS in the dark. After 1 h, the washing step was repeated three times, as previously described. The samples were then dehydrated with a graded series of ethanol (30–100%), dried to the critical point, coated with gold film (Cressington sputter coater 208HR), and analyzed by SEM (JSM-7600F, JEOL) set at 15-keV electron beam energy.

Rheological analysis of the mechanical properties of different fibrin formulations

In order to measure the rigidity of the four different fibrin matrices, their linear viscoelastic properties were investigated by small-amplitude oscillatory shear rheology using an MCR 301 (Anton Paar, Germany) rheometer. Temperature was controlled by a convection oven operating under air. Measurements were obtained at 37 °C using 8-mm parallel-plate geometry. Sandpaper was fixed to the lower plate to avoid slipping and ensure good adhesion of the samples to the steel surface. Each fibrin formulation was analyzed and compared for G'.

Thawing of frozen ovarian cortex and follicle isolation

Cryovials of frozen ovarian cortex from different patients (n = 22) were thawed and processed for follicle isolation, as recently described [26]. Briefly, after mechanical tissue dissociation, the ovarian tissue was incubated in 10 mL of PBS with Ca^{2+} and Mg^{2+} (Gibco, Thermo Fisher Scientific, Ghent, Belgium) in the presence of 0.28 Wünsch units/mL Liberase DH (Roche Diagnostics) and 8 Kunitz units/mL DNase I (Roche Diagnostics, Brussels, Belgium) in a water bath (37 °C) for up to 90 min with gentle agitation and pipetted every 15 min. The enzymatic digestion step was halted by adding a similar volume of PBS + 10% heat-inactivated fetal bovine serum. Fully isolated follicles were retrieved with the help of a stereomicroscope and 130-μm micropipette by two operators.

Follicle encapsulation and histological analysis

Because of the considerable difference in fiber thickness and porosity between human ovarian cortex and the F12.5/T1 combination encountered after SEM analysis, this fibrin formulation was not further investigated for follicle encapsulation. Only the three remaining F/T combinations were used to embed human follicles for histological analysis. For follicle encapsulation, a droplet of 12 μL fibrinogen (30, 50, and 75 mg/mL) was deposited on a glass petri dish, to which between 14 and 50 isolated preantral follicles were added (3 μL). The droplet was then mixed with 15 μL thrombin (50 and 75 IU/mL) and incubated at 37 °C for 15 min. After polymerization, the fibrin clot was gently detached, covered with 2% melted agarose (UltraPure, Thermo Fisher Scientific, Ghent, Belgium) and immediately fixed in 4% formalin [27]. After 24 h of fixation, the clots were embedded in paraffin for histology. Each fibrin clot was cut into 5-μm serial sections and every second section was stained with hematoxylin and eosin to count and compare the encapsulated follicles and follicular structures. Follicles were identified by a surrounding basal lamina and GCs with a central oocyte. Follicular structures were defined as groups of cells organized in round formations with no visible oocyte.

Statistical analysis

The Kruskal-Wallis test was applied to compare fiber thickness between ovarian tissue samples and fibrin matrices, and follicle recovery and loss rates after fibrin encapsulation. A p value < 0.05 was considered statistically significant. All statistical analyses were performed with GraphPad Prism 7 software (GraphPad, La Jolla, USA). Dunn's multiple comparison test was also conducted post hoc.

Results

Microstructure of human ovarian cortex and fibrin matrices

The overall architecture of ovarian tissue showed no significant difference in fiber density or fiber organization between patients (Fig. 1). Indeed, all ovarian cortex fragments were found to be highly fibrous and structurally inhomogeneous. A visible cluster of primordial-primary follicles is shown in Fig. 1c. There was also no significant difference in mean ± SD fiber thickness (61.3 to 72.4 nm) between patients.

As illustrated in Fig. 2, all fibrin formulations exhibited a fibrillary structure and more homogeneous organization of their filaments than did human ovarian tissue. Moreover, when F/T concentrations were raised, we observed an increase in the number of fibers, and hence fibrin consistency. On the other hand, the porosity of fibrin networks as well as fiber diameter decreased at higher F/T concentrations (Table 1). When mean ± SD fiber thickness was compared with human ovarian tissue, a significant difference was observed only with F12.5/T1 ($p < 0.01$), while F50/T50 and F75/T75 showed very similar fiber thickness values to ovarian cortex (Fig. 2).

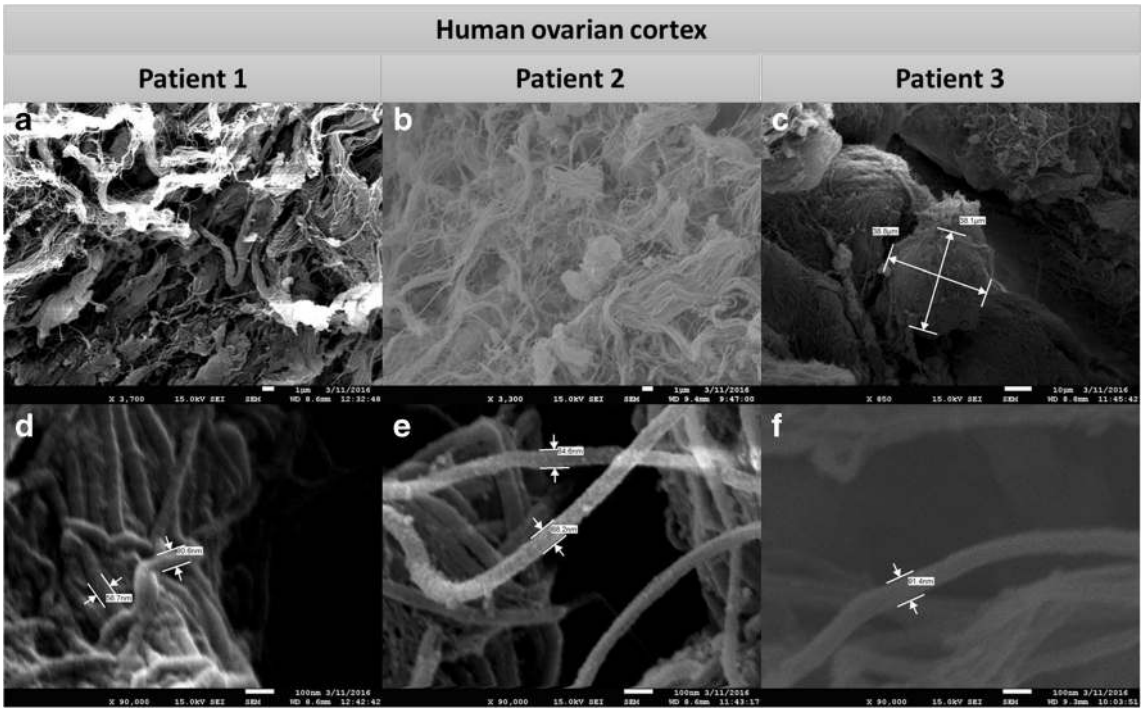

Fig. 1 SEM analysis of fresh human ovarian cortex. No significant difference was found between ovarian tissue fragments from the three patients. Patients 1 (**a**) and 2 (**b**) show heterogeneous distribution of collagen fibers. In patient 3, we can observe a cluster of primordial-primary follicles (one of them marked with crossed white arrows) of 38 μm (**c**). Fiber thickness (white arrows and bars) was measured in ovarian samples from all patients (**d**–**f**)

Viscoelastic properties of different fibrin formulations

As previously mentioned, four different fibrin matrices (F12.5/T1, F30/T50, F50/T50, F75/T75) were investigated for G'. Dynamic oscillatory shear measurements were taken at 37 °C in order to characterize the viscoelastic response of the samples. First, strain sweep tests were conducted at a frequency of 1 rad/s to determine the limits of the linear regime of deformation. Based on these results, the amplitude of deformation used in frequency sweep tests was fixed at 0.4% for all samples to ensure measurements within the linear regime. In all samples, experimental storage moduli showed fully elastic behavior (namely with G' almost independent of the frequency of oscillatory shear flow). Corresponding values of the storage moduli (140, 1100, 3800, and 9300 Pa) are presented in Fig. 3 for all four concentrations. Higher F/T concentrations gave rise to increased G', resulting from more cross-linking points within the systems, yielding a more rigid fibrin matrix.

Follicle encapsulation and histological analysis

After follicle isolation, a total of 696 follicles were retrieved and encapsulated in the three selected fibrin formulations: 216, 228, and 252 follicles were placed in F30/T50 ($n = 7$ clots), F50/T50 ($n = 7$ clots), and F75/T75 ($n = 7$ clots) fibrin clots, respectively, and processed for hematoxylin and eosin staining. Then, to evaluate whether fibrin matrix concentrations would influence follicle recovery and loss, we counted and compared the number of follicles detected in the three different fibrin formulations. As shown in Table 2, mean follicle recovery and loss rates ranged between 44 and 47% and 29 and 39%, respectively. However, when mean ± SD values of follicles found and lost were compared, no statistical difference was noted between the three fibrin formulations shortly after encapsulation (Table 2). Figure 4 shows isolated follicles encapsulated in the different fibrin scaffold concentrations.

Discussion

With a view to creating a transplantable artificial ovary, our aim was to optimize fibrin scaffold composition [16, 21, 23, 24] for human follicle encapsulation and grafting.

Human ovarian cortex and fibrin matrix microstructure

One of the key aspects in the design of the artificial ovary is a supporting matrix [8]. Among all different matrices used in in vivo studies [14–21, 28], a fibrin-based scaffold appears to be the best choice [22]. However, our previous studies have demonstrated that while a fibrin matrix with low concentrations of fibrinogen and thrombin (F12.5/T1) may well be

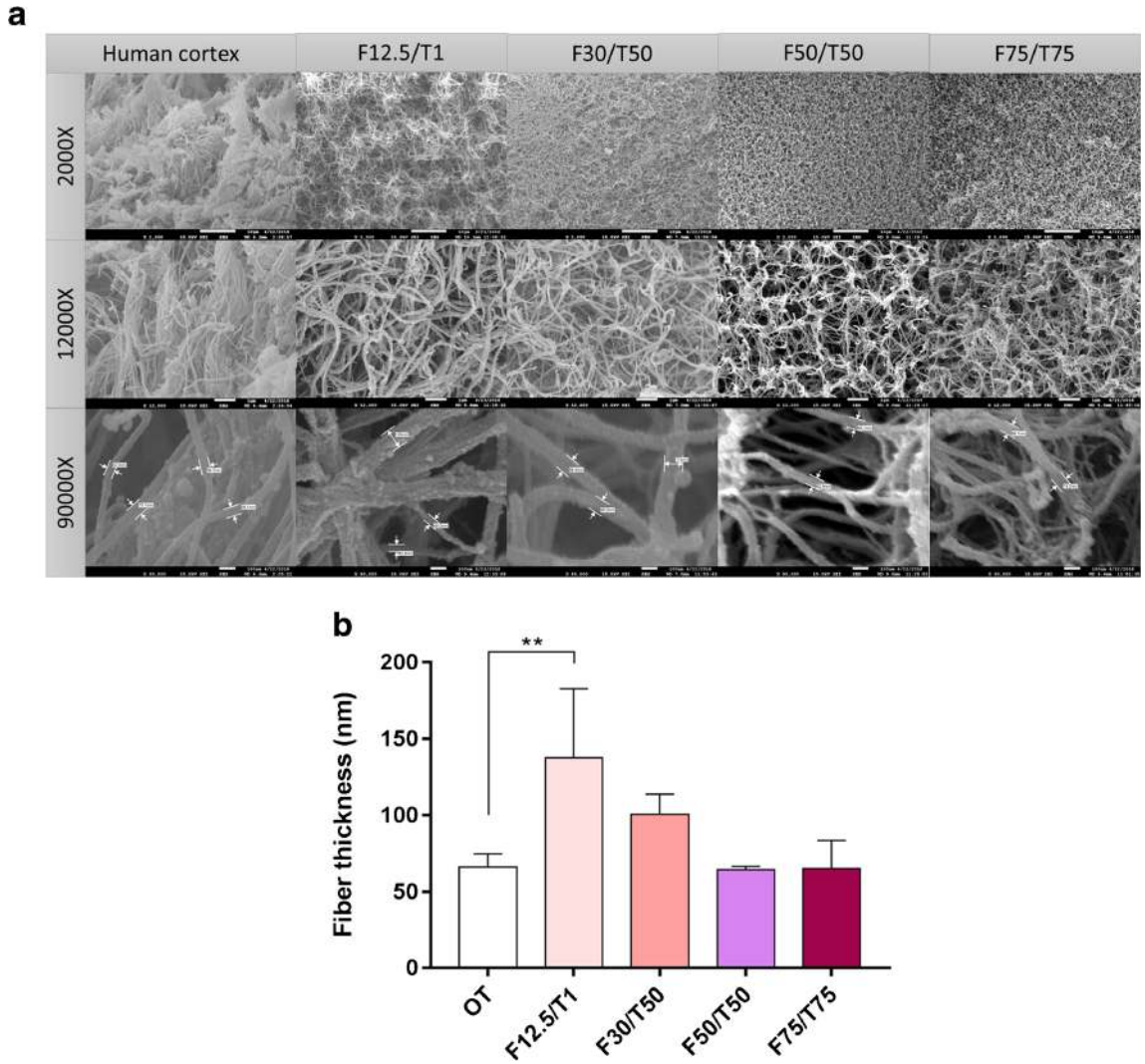

Fig. 2 Ultrastructure of human ovarian cortex and different fibrin formulations. SEM image of human ovarian cortex and fibrin matrix formulations at different magnifications (× 2000; × 12,000; × 90,000) (**a**). Graphic representation of fiber thickness in human ovarian tissue (OT) and four fibrin formulations (**b**)

suitable for encapsulation and grafting of mouse secondary follicles, it could lack the required stiffness to support human primordial-primary follicles [16, 23, 24]. This hypothesis was reinforced when we observed that human preantral follicles showed better survival rates with higher F/T concentrations (F50/T10) [21]. We therefore decided to conduct a more in-depth analysis of the ultrastructure of human ovarian cortex and compare our findings with different fibrin formulations based on in vivo studies from the existing literature [22].

Table 1 Fibrin fiber thickness according to different fibrin formulations. Increased F/T concentrations exhibited decreased fiber thickness

	Fiber thickness (mean ± SD)
F12.5/T1	137.5 ± 45.5 nm
F30/T50	100.8 ± 13.2 nm
F50/T50	64.5 ± 2.3 nm
F75/T75	65.4 ± 18.4 nm

Indeed, our goal was to identify the fibrin formulation that most closely mimics physiological ovarian cortex architecture.

In the present study, we demonstrated that fragments of ovarian cortex collected from women of similar age have a generally similar architecture, with a very dense, fibrous, and inhomogeneous structure characterized by interlaced collagen fibers of comparable thickness. It has been proved that collagen fibrils, which constitute one of the most abundant components of the ovarian extracellular matrix [29, 30], tend to increase in number [31] and diameter with age [32]. We showed that conducting a comparative analysis between human ovarian cortex microstructure and fibrin formulations is an efficient way of identifying a supporting scaffold that best resembles the architecture of the human ovary, achieving optimal porosity to encapsulate and transplant follicles. Considering the vast physiological differences that exist between rodent

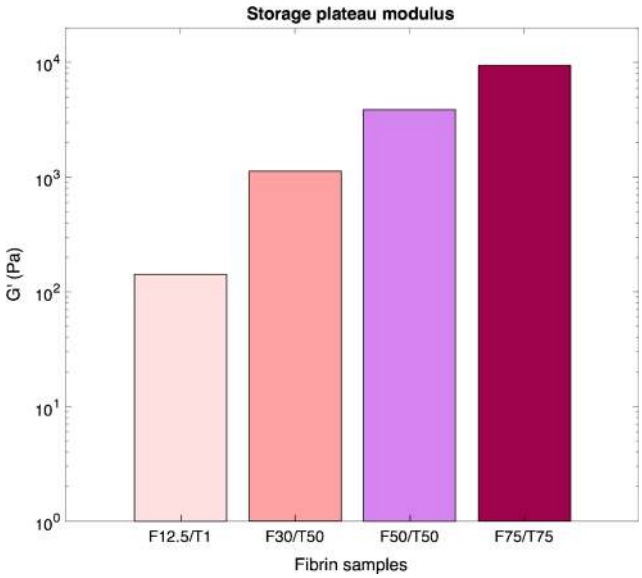

Fig. 3 Graphic representation of fibrin rigidity (storage modulus G') according to different fibrin formulations. The stiffness (G') of fibrin matrices was positively correlated with increased concentrations of fibrinogen and thrombin

and primate ovaries [33], use of mouse follicles for the design of an artificial ovary prototype, as suggested by Laronda et al. [19], may not necessarily be translatable to humans, so caution should be exercised. Hence, in our study, we considered human ovarian tissue architecture as the reference standard for creation of the artificial ovary prototype. Importantly, the great difference found between F12.5/T1 and ovarian tissue ultrastructure may also explain the low human follicle recovery rates encountered in our previous study, when preantral follicles were encapsulated in F12.5/T1 and grafted to mice [25]. Indeed, some fibrin matrix combinations exhibited a less dense and compact structure, with more homogeneous and organized distribution of fibrin fibers than human ovarian cortex. In addition, an inverse correlation was found between F/T concentrations, fiber thickness, and pore size, as documented in other reports [34–36]. Interestingly, when F/T concentrations were increased to F50/T50 and F75/T75, the formulations exhibited fiber thickness comparable to human ovarian cortex. This led us to hypothesize that these combinations may mimic the physiological environment of human follicles more closely, making them good candidates for the artificial ovary prototype. However, another important feature to consider in the development of a transplantable artificial ovary is the rigidity of the matrix. It has been previously demonstrated that ovary stiffness [37], hence also that of the artificial ovary prototype [21, 38], may influence follicle behavior. We therefore decided to investigate the storage modulus of the four selected fibrin matrices and compare it with that of human ovarian tissue [39]. As expected, increased concentrations of fibrinogen and thrombin yielded higher G' levels, and consequently greater rigidity, in line with earlier reports from the literature [36, 40]. Nevertheless, only F50/T50 showed similar rigidity to human ovarian tissue [39].

Table 2 Follicle recovery rates, follicular structures, and follicle loss after fibrin matrix encapsulation. Follicle recovery rates, follicular structures, and follicle loss with different fibrin formulations were compared shortly after encapsulation

Fibrin formulation	Follicle recovery	Follicular structures	Follicle loss
F30/T50 ($n = 7$)	46 ± 21%a	26 ± 17%b	38 ± 21%c
F50/T50 ($n = 7$)	44 ± 24% a	18 ± 11%b	39 ± 20%c
F75/T75 ($n = 7$)	47 ± 22%a	23 ± 14%b	29 ± 30%c

Different letters in each column (a, b, c) indicate significant differences ($p < 0.05$)

Follicle encapsulation

Based on the great difference encountered between human ovarian cortex and F12.5/T1 ultrastructure, as well as previously discouraging findings with human follicles encapsulated in F12.5/T1 and subsequently grafted [25], we excluded this fibrin formulation from further histological investigations. From the other three fibrin combinations, around 50% of follicles were recovered soon after fibrin polymerization, which is consistent with earlier studies using human follicles [21]. However, a non-negligible percentage of follicle loss was found with all fibrin formulations, as previously stated for mouse follicles [24]. Taking into account that most follicles are normally located at the border of the fibrin clot, this loss may be partially explained by retraction of the fibrin matrices after fixation and dehydration, which typically occurs during histological processing. In the context of future clinical application, it should be borne in mind that the fibrin clot needs to be grafted to the patient as soon as it is created.

General conclusion

In conclusion, among all the different fibrin matrix concentrations tested, F50/T50 appears to be the combination of choice in terms of ultrastructure and rigidity, most closely resembling human ovarian cortex. Such findings are essential to help us standardize fibrin matrix architecture.

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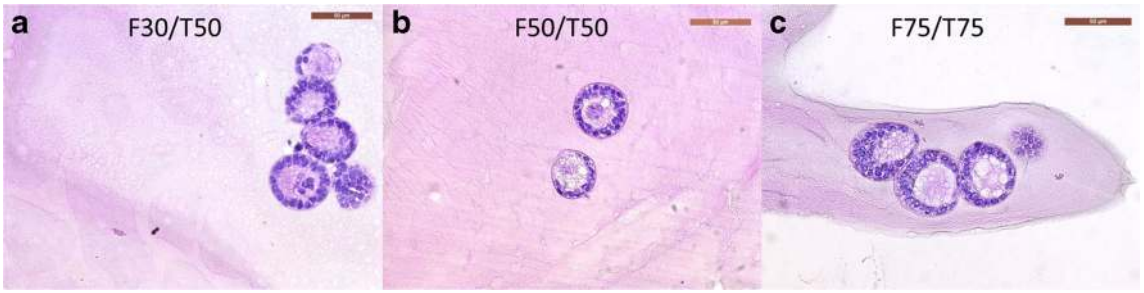

Fig. 4 Histology of isolated human follicles in different fibrin formulations. Follicles encapsulated in F30/T50 (**a**), F50/T50 (**b**), and F75/T75 (**c**). Scale bar 50 μm

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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