Fibrin-based artificial ovary prototype: from animal models to human clinical application

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Summary

The artificial ovary is a promising experimental fertility restoration strategy for women who cannot undergo ovarian tissue transplantation due to the possible risk of reseeding malignant cells. This approach is based on isolation of ovarian follicles, followed by their encapsulation in a three-dimensional matrix to support follicle architecture during grafting. Since human ovarian tissue is not readily available for research purposes, different animal models can be used for the development of a bio-engineered ovary. However, findings with human and mouse follicles are often discrepant. The goal of this review is to describe the key steps involved in creation of an artificial ovary, discuss the different findings according to the model used, and contemplate, the latest achievements in humans with a view to prompt clinical application.

KEY WORDS: artificial ovary, fertility preservation, isolated ovarian follicles.

Introduction

Thanks to advances in cancer research, overall survival rates of young cancer patients are now as high as 80% (1). However, the toxicity of radio- and chemotherapy remains serious concern, especially its effect on a patient's fertility, which is why different fertility preservation strategies have been developed for young girls and women of reproductive age (2). Ovarian tissue cryopreservation (OTC), followed by orthotopic transplantation once in cancer remission, is the only available option for prepubertal girls and women who require urgent cancer treatment (2). Since the first live birth was achieved with this approach at Saint Luc's Hospital back in 2004, more than 130 live births have been reported worldwide (2), demonstrating ability of this technique to restore both endocrine and reproductive functions. Nevertheless, for certain malignancies, particularly blood-borne disease like leukemia, reimplantation of ovarian tissue is not advised due to the possible risk of reseeding cancer cells (3). For these patients, no clinical alternatives are currently available. However, OTC can still be proposed as a fertility preservation strategy while researchers continue to work on promising new fertility restoration techniques (4-6). Indeed, there are two main approaches now under investigation: (I) in vitro culture (IVC) of isolated follicles; and (II) in vivo growth of isolated follicles inside the so-called transplantable artificial ovary (TAO). Both are based on isolation of ovarian follicles, which are then encapsulated in a biomaterial to maintain their three-dimensiaonal (3D)

structure and provide an artificial environment for follicle survival and growth (7). Despite encouraging results with both of these experimental strategies (5, 8, 9), different challenges have emerged. The aim of this review is therefore to explore the latest achievements and main challenges encountered so far in creating the TAO, focusing especially on the disparate findings obtained with murine and human follicles. Efforts directed at ensuring prompt human clinical application are also discussed.

Artificial ovary strategy

Conceptually, an artificial ovary is a temporary substitute for natural ovary, in which isolated follicles, ovarian cells (stromal and endothelial) and a combination of growth factors can be assembled inside a biomaterial, with the essential role of replacing the native extracellular matrix (ECM) during the early days post-grafting (7) (Figure 1). First, a biopsy of ovarian tissue is retrieved prior to starting gonadotoxic therapy, and immediately cryopreserved or used to isolate preantral follicles that may also be frozen (10). Although the presence of a follicular basal membrane ensures the absence of contaminating blood cells or surrounding malignant cells, a follicle purging step involving three consecutive washes in saline solution is also required to guarantee the safety of the whole procedure (11, 12). Addition of ovarian cells to the population of isolated follicles is also fundamental. Indeed, as previously demonstrated, the co-presence of endothelial cells (13) and stromal cells (14) may promote revascularization and steroid hormone production after transplantation. In this regard, two different strategies can be applied: I) fresh ovarian cells may be retrieved from a second ovarian biopsy obtained after completion of cancer treatment; II) they can be isolated together with preantral follicles from a patient's frozen-thawed ovarian tissue, purged (15), and then combined inside a biocompatible and biodegradable scaffold to be transplanted back to the patient.

Ovarian follicle isolation

As previously mentioned, ovarian follicle isolation is the first essential step for creation of the TAO. Because of the scarcity of human ovarian tissue for research purposes, many studies on ovarian follicle isolation are based on various animal models (16-18).

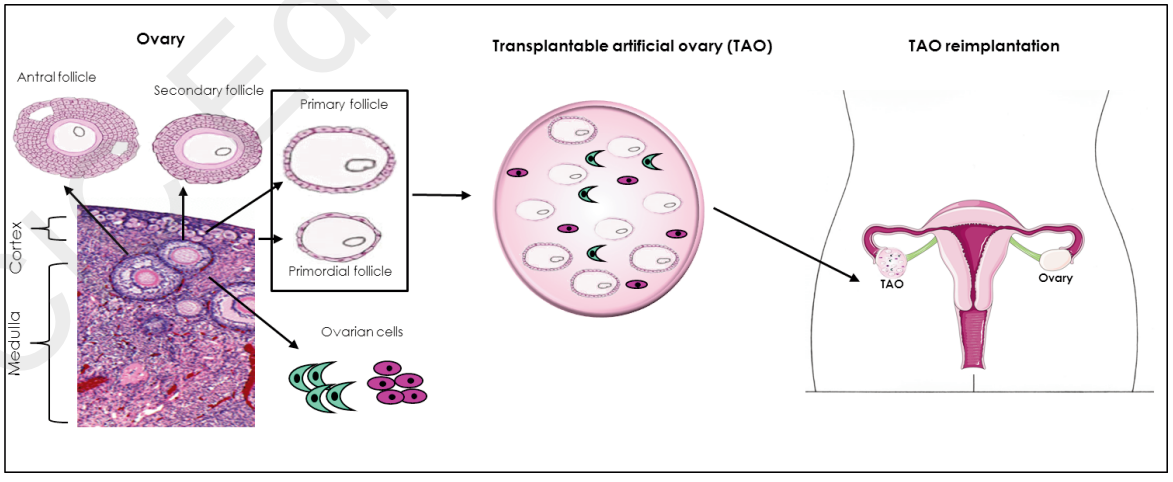

Figure 1 - Development of an artificial ovary prototype. On the left: histological section of an ovary showing the outer ovarian cortex containing mostly primordial and primary follicles, and the inner ovarian medulla with growing follicles (secondary and antral) and blood vessels. All follicle stages are interspersed throughout ovarian stroma. In the center: an artificial ovary prototype composed of a scaffold (pink) in which primordial-primary follicles and ovarian cells are encapsulated. On the right: reimplantation of the TAO in the peritoneal cavity once the patient is cured.

Animal models

The ideal animal model for an ovary should be as similar as possible to the human organ in terms of architecture, softness, follicle distribution, duration of folliculogenesis and number of ovulated oocytes per cycle. This makes non-human primates a good option for studying follicle isolation and subsequently in vivo growth (19, 20). However, besides all the ethical concerns around using these animals for research, the facilities they require and high costs involved render this model unsuitable for the majority of reproductive research centers. Among other mammals, rodents and domestic animals like cows, pigs, sheep and goats are currently the most widely used (21, 22). Ovarian follicle isolation from these animals is usually performed by mechanical methods, since it does not require use of any enzymes. There are two different approaches that can be applied. The first involves a calibrated tissue chopper, which enables repeated cutting of whole ovaries or ovarian tissue fragments that can then be further disaggregated in association with manual pipetting to increase follicle yield (17). The second utilizes a microdissector (manual or laser) under a dissecting microscope (23). While the former is definitely less time-consuming, both are usually effective for follicles larger than 120 μm, and when the surrounding theca layer needs to be kept intact (22, 23). However, the feasibility of the method is strictly dependent on the composition and softness of the ovarian tissue in question, which is quite variable between species (24).

Human model

In humans, this strategy does not yield comparable results. Since the majority of follicles in human ovarian tissue are primordial and primary, and located in a very collagen- rich ECM, solely mechanical means of isolation can only recover very small numbers. Moreover, residual stromal cells may also surround follicles, which is hazardous for safe follicle reimplantation (11). For this reason, optimized methodologies using combined mechanical and enzymatic tissue digestion have been developed (25-27). As recently confirmed by Dong et al. (28), human primordial follicles exhibit lower survival rates after IVC when they are isolated using a mechanical method rather than a combination of mechanical and enzymatic tissue digestion (28). It is exactly the opposite with mouse follicles, which are more susceptible to basal membrane dissociation when they are subjected to combined enzymatic and mechanical isolation compared to purely mechanical dissociation (22). These conflicting findings clearly demonstrate the limitations of translational outcomes and methodologies right from the outset of creating an artificial ovary, highlighting the importance of developing tailored protocols for human application. Another important aspect to be considered for human follicle isolation is the great variability in follicle number and ECM density between women. Hence, with a view to future clinical application, we developed a personalized human ovarian follicle isolation protocol, further optimizing our original procedure (26). In this new study (29), we hypothesized that when the first isolated follicles are continually subjected to enzymatic activity, their thin basal membrane may be compromised, leading to reduced follicle quality and even follicle loss. Therefore, unlike our previous method and all other protocols found in the literature, we divided our enzymatic digestion into three different time points, introducing a filtration step every 30 minutes to rescue the first isolated follicles. Our findings revealed that 35% of follicles were already fully isolated after the first cycle of enzymatic digestion. This allowed us not only to recover greater numbers of isolated primordial and primary follicles, but also more good-quality follicle, as confirmed by better follicle recovery rates after transplantation in a TAO than in our previous study (30). Even in the same species, there are differences in follicle distribution according to age. In human adult patients, the majority of the follicles are normally located in the rigid outer ovarian cortex. In very young girls, however, due to the small size of their ovaries and high ovarian reserve, a good many follicles can also be found in the softer interior ovarian medulla. Since leukemia most commonly affects children and adolescents, ideal candidates for the TAO, a follicle isolation protocol is

also needed to retrieve follicles from the ovarian medulla (31).

Choosing a matrix for the TAO

Thanks to the advent of tissue engineering and subsequent development of 3D systems, isolated follicles can be embedded inside different polymers with the main roles of (I) physically supporting follicles and ovarian cells in the initial period after transplantation, and (II) providing a dynamic physical environment for their growth (8). Different natural and synthethic scaffold materials are commercially available, but choosing the right matrix for the TAO remains one of the great challenges of research. Table 1 provides a summary of various natural (32-36) and synthetic (37) scaffolds already tested for the artificial ovary prototype. Ideally, the material chosen to encapsulate isolated follicles needs to be easy to handle, biocompatible, biodegradable, porous enough to allow exchange of nutrients and removal of gas and waste, and strike the right balance between rigidity and elasticity to maintain the spherical follicle architecture, but at the same time allow radial follicle growth. According to the literature, fibrin-based matrices have shown the most promise so far with both mouse and human follicles (38).

Fibrin-based artificial ovary

Basically, fibrin is a natural biopolymer, composed of two different proteins, namely fibrinogen (F; expressed in mg/ml) and thrombin (T; expressed in IU/ml), which in the presence of calcium ions activates a polymerization process to give rise to a mesh onto which different cells can be seeded. One of the major advantages of using fibrin is the ability to adjust its microstructure by simply varying F and T concentrations (38). This allows investigators to obtain different scaffolds in terms of porosity, rigidity and degradation rate according to their needs. Hence, the most important issue to address in this context is: how to determine the right combination of F and T for the artificial ovary matrix. The first study to investigate which fibrin formulation may be most appropriate for the TAO was conducted in our laboratory (39). In this IVC study, isolated human ovarian cells were encapsulated in nine different F/T combinations in order to compare cell seeding, density and distribution, and also cell survival and matrix degradation after 7 days of culture. Based on the results, just two fibrin combinations (F12.5/T1 and F25/T4) were selected for possible further transplantation (39). In the subsequent in vivo study (35), these matrices were used to encapsulate and graft murine preantral follicles and ovarian cells to Naval Medical Research Institute (NMRI) mice for 7 days. After one week of autografting, both combinations showed similar follicle recovery rates (31-32%) and growth patterns. These findings therefore demonstrated, for the first time, that a fibrin-based scaffold could make an effective artificial organ for grafting of mouse follicles,

Table 1. Different matrices for construction of an artificial ovary prototype.

Possible matrices for the artificial ovary prototype	
Synthetic	**Natural**
PEG (37)	Plasma clots (32)
	Collagen (33)
	Alginate (34)
	Fibrin (35)
	Decellularized ovarian ECM (36)

also yielding higher follicle recovery rates than a similar earlier study using alginate (34). In light of these promising results, the F12.5/T1 formulation was hypothesized to be a possible candidate for transplantation of isolated human ovarian follicles. Unfortunately, a subsequent xenografting study with F12.5/T1 and human follicles produced very discouraging results (40), prompting us to investigate which factors could be responsible for such a glaring discrepancy.

Impact of follicle stage on graft outcomes in a fibrin-based artificial ovary

Looking at follicle distribution inside the adult ovary, there is a clear difference between mice and humans. In humans, the majority of follicles are at the primordial and primary stage (Figure 2 A, B), while in mice, more than 50% of follicles are already at the secondary stage (35) (Figure 2 C, D). One of the possible explanations for the divergent outcomes obtained in our autografting and xenografting studies might thus be the presence of different follicle populations encapsulated in F12.5/T1**.** In order to elucidate whether follicle stage may play a role in our findings, we initiated a new study (42). Two distinct mouse follicle populations, primordial-primary and secondary respectively, were independently encapsulated in F12.5/T1 along with ovarian cells, and grafted to mice for 2 and 7 days. After both periods of grafting, follicle recovery rates, growth (Ki67) and follicle death (TUNEL) were investigated and compared. Our data showed that although both follicle populations were able to grow and survive, secondary follicles achieved better recovery rates (40% and 28%) than primordial-primary follicles (16% and 6%). However, it was unclear which factors in the procedure may have led to such discrepant results. We therefore repeated a similar in vivo study (43), but introducing a control group, in which murine primordial-primary and secondary follicles were immediately fixed in F12.5/T1 and analyzed by histology prior transplantation (day 0). Since small but comparable percentages of degenerated follicles were detected soon after isolation and encapsulation, we excluded any negative impact of the follicle isolation procedure. Scanning electron microscopy (SEM) analysis on day 0 revealed that both follicle developmental stages were well encapsulated in F12.5/T1, allowing us to rule out any lack of interaction between primordial-primary follicles and the matrix. After grafting, vascularization assessment (CD34) and transmission electron microscopy (TEM) analysis were carried out. On day 2 of transplantation, both follicle developmental stages were found to have a normal ultrastructure at TEM, comparable to follicles in mouse ovarian tissue, which also excluded any possible impairment by the grafting procedure itself. Interestingly, CD34 analysis showed a significantly greater network of new capillaries in the secondary follicle group than the primordial-primary group. This led us to speculate that the lower vascularization and hence higher hypoxia rates observed in primordial-primary follicles may be responsible for their inferior survival after grafting (43).

Impact of matrix rigidity on graft outcomes in a fibrin-based artificial ovary

Another possible hypothesis that was not previously investigated may be that human and mouse follicles have different physical requirements for survival and growth in a TAO. Indeed, other studies (44, 45) with isolated mouse primordial-primary follicles encapsulated and grafted in a different fibrin-based artificial ovary prototype (F20/T50) have shown that increased F/T concentrations may also play a role in follicle survival and development after grafting. As previously mentioned, adult mouse ovaries are generally softer and characterized by a largely heterogeneous follicle population in their cortex (Figure 2 C, D) (46). Conversely, adult human ovaries are stiffer, especially the outer collagen-rich ovarian cortex where the majority of primordial and primary follicles reside (Figure 2 A, B) (47). We therefore set about investigating whether the rigidity of the matrix could influence human follicle survival, a hypothesis that was recently confirmed in our laboratory (30). In this study, we demonstrated an increased follicle recovery rate (21%) after one week of xenografting when human primordial-primary follicles were encapsulated and grafted inside a more

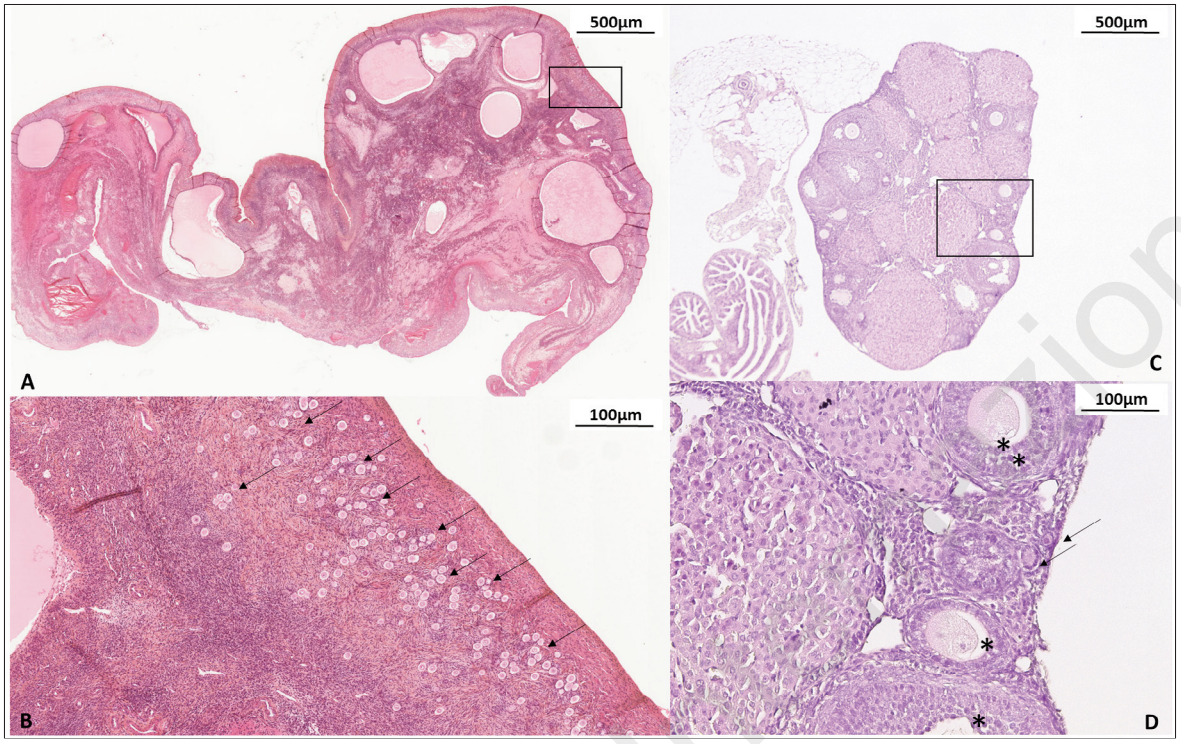

Figure 2 - Histological representation of human and mouse ovaries. A) Whole human ovary after hematoxylin-eosin-saffron staining. The black square indicates a portion of magnified ovary visible in figure B. B) Portion of magnified human ovarian cortex showing mostly primordial and primary follicles →. C) Whole mouse ovary after hematoxylin-eosin staining. The black square indicates a portion of magnified ovary visible in C. C) Portion of magnified mouse ovarian cortex showing primordial and primary follicles (black arrows) and secondary and antral follicles (asterisk). Human ovary pictures were obtained from the digital microscope platform of the University of Namur (www.histology.be/index.html) and kindly donated by Professor Poumay.

rigid fibrin-based TAO protoype (F50/T10). This work confirmed that the requiriments for encapsulation and grafting of mouse follicle are not the same as those for human follicles, nor is the follicle isolation procedure. Therefore, with a view to extrapolating our findings to potential clinical application, further studies with human ovarian tissue and larger animals that more closely resemble the physiology of the human ovary should be undertaken.

Fibrin-based artificial ovary prototype mimicking human ovarian tissue architecture

When developing a bio-inspired ovary for future clinical application, the human ovary is obviously the best model for investigation. Since many aspects of human ovarian tissue are still unexploited, a new study was initiated (48). Chiti et al. (48) evaluated human ovarian cortex from age-related women by SEM in order to analyze its microstructure and fiber thickness. This then served as control group against 4 experimental groups with increasing F/T concentrations (F12.5/T1, F30/T50, F50/T50 and F75/T75), where rigidity was also calculated. Follicle morphology and recovery rates were analyzed by histology. When F/T concentrations were augmented, fiber thickness was found to decrease, while fiber density and fibrin rigidity increased. Compared to fibers present in human ovarian tissue, F12.5/T1 exhibited significantly greater fiber thickness, which may explain the discouraging results previously obtained when human follicles were encapsulated and grafted in this F/T concentration. The F12.5/T1 combination was therefore excluded from further investigations. On the other hand, F50/T50 was shown to be similar to human ovarian cortex in terms of fiber thickness and rigidity. Since no differences were found in human follicle recov-

ery rates soon after encapsulation with any of the other F/T combinations, we concluded that F50/T50 may be the scaffold concentration that most closely resembles human ovarian tissue architecture.

Conclusions

Coniderable progress has been made in creating an artificial ovary over the last decade. However, when mouse ovary outcomes are extrapolated to humans, a less encouraging scenario is encountered, probably due to vast differences between mouse and human ovaries (24). Hence, despite mouse ovaries serving as an initially useful source of data for preliminary investigations into follicle survival and growth in the TAO, the results cannot be translated to a human context. For this reason, studies involving larger mammals and human tissue are essential before contemplating clinical application. A number of very recent papers on use of human ovarian tissue (12, 29, 48) have indeed reported that research in this field is edging ever closer to clinical practice. Although it is too soon to speculate on when it will be generally available, we can certainly say that two key aspects of the TAO are ready for clinical application. First, safe human ovarian follicle isolation protocols based on good manufacturing practice-endorsed products and specifically tailored to both adult and young patients have recently been formulated (12, 29, 31). Second, a fibrin-based scaffold specially designed to closely resemble the architecture of natural ovarian tissue (48) and hence allow human follicles to be seeded and grafted onto a biomaterial has already been approved by the US Food and Drug Administration. Our goal is firmly in sight.

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