Isolated ovarian follicle culture: a promising strategy for fertility preservation

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Summary

Ovarian tissue cryopreservation represents one among the most preferred strategies for fertility preservation currently. However, concerns regarding the transmission of malignant cells during the transplantion of stored ovarian tissues, is a major restraint in recommending the procedure to patients diagnosed with all kinds of malignant disorders. On the contrary, use of isolated follicles for restoration of fertility in such patients could completely evade the possibility of cancer re-introduction after treatment. Follicles housed in the ovarian environment *in vivo* prevail under the mechanical and the chemical/nutritional support of the ovary. Although not complete, recent knowledge about the dynamics of follicular progression has led to improvements in the culture system adopted. This review aims at summarising the culture of isolated follicles *in vitro*, particularly emphasising the efforts made to mechanically and nutritionally support the follicle. Advances in follicular culture systems could prove useful to highly improve the efficiency of current fertility restoration strategies and evade the concerns associated with the same.

KEY WORDS: fertility preservation, follicle culture, three dimensional support, isolated follicles.

Introduction

Cancer prevalence among women is till date, a major medical concern. Studies suggest that, 1 out of 51 women would have had an invasive cancer diagnosed by 39 years of age (1). Recent reports supported by the national cancer institute, have shown a 0.8% increase in cancer affected children during the past decade (2). However, recent advances in chemotherapy, radiotherapy, and bone marrow transplantation can cure as high as 90% of women and children affected by cancer and other disorders requiring such treatment. On the other side, ionizing radiation and aggressive chemotherapy can result in some degree of premature ovarian failure in almost 100% of patients requiring such therapy (3). Whole body irradiation coupled with intensive chemotherapy associated with bone marrow transplantation, poses one of the greatest threats to treated patients. The ovarian reserve is completely abolished after treatment regimes including alkylating agents such as busulphan (4). Moreover, several studies have shown that a radiation dose as low as 5- 20 Gy, is sufficient to cause gonadal function impairment (5-7).

Concerns in routine ovarian tissue cryopreservation

Cryopreservation of the ovarian tissue is one of the mainstays in fertility preservation strategies adopted today among cancer affected women. The fact that ~25 live births have been reported till date using this procedure (8), could easily ar-

gue against considering this procedure under experimental phase any longer. Moreover, ovarian tissue cryopreservation is the only option available today for preservation of fertility in pre-pubertal girls and patients, requiring immediate initiation of potentially gonadotoxic anti-cancer treatment (9). Unfortunately, the most notable concern for ovarian tissue cryopreservation and transplant after the course of treatment, is the probability of re-introducing malignant cells back into the cured individual with very high chances of propagating the cancer again (10).
Ovarian metastases have been reported for most malignancies including breast cancer, lung cancer, renal tumors, neoblastomas, Ewing's sarcoma, Hodgkins's lymphoma, Non-Hodgkin's lymphoma, biliary duct cancer and other gastrointestinal cancers (11-16). Studies evaluating the incidence of ovarian metastasis in different cancers show that ovarian involvement is highest in gastric cancer (55.8%), colon cancer (26.6%), breast cancer (24.2%), pulmonary carcinoma (23.4%), lymphoma (13.3%) uterine cancer (13.1%) and leukaemia (8.4%) (17).
Routine histological examination of the ovarian tissue fragments have proven to be inefficient in predicting the prevalence of malignant cells in the transplanted tissue (18). In this context, transplantation of stored ovarian tissue is particularly warranted in leukaemia, being a systemic disease with very high chances of metastasizing to the ovaries. In a study involving 18 leukaemia patients, routine histology and immunohistochemical analysis showed no presence of malignant cells in the biopsied ovarian tissue. However, highly sensitive reverse transcriptase PCR (RT- PCR) revealed the presence of molecular leukemic markers in the tissues of 9 out of 16 of these patients, previously thought to be safe from ovarian metastasis (18). Although, there is no conclusive report demonstrating the re-introduction of malignant cells through ovarian tissue transplanted after storage, studies in animal models have shown growth of intraperitoneal masses after transplantation of ovarian tissue from leukemic patients (18). Hence, transplantation of stored ovarian tissue in general can only be offered with extreme caution for re-storation of fertility in cancer treated women. Considering these issues recent research in fertility preservation is focused on alternative approaches to circumvent this problem. Cryopreservation, culture and methods to re-implant isolated ovarian follicles rather than whole tissue, is one among the most focussed topics in this context. Depending on age, the ovarian cortex houses thousands of dormant primordial follicles that can be isolated (19). These immature follicles represent the largest population of ovarian follicles and are more resistant to cryopreservation than advanced stage follicles (20). Furthermore, early stage follicles have shown to maintain normal morphology and ultrastructure following freezing, making them excellent candidates for long term preservation. The most important advantage of isolated follicle culture and transplant arises from the fact that the malignancy cannot cross the basal lamina of the follicle and the oocyte is protected from cancer cell invasion (21). Moreover, improvements in follicular development *in vitro* can also help these patients to obtain a larger number of oocytes for *in vitro* fertilization techniques, hence overcoming the concerns of re-transplantation all together. Henceforth, improvements in the isolation, culture and re-introduction of ovarian follicles can greatly improve current fertility preservation strategies and can provide a risk free method for the restoration of fertility in women affected by chemo/radiotherapy induced premature ovarian failure.

Strategies for isolaton of ovarian follicles

Several methods have been tried over the years for the successful isolation of ovarian follicles. Mechanical isolation of follicles have been in practice since the last 2 decades employing several instruments. The most commonly used techniques involve the use of tissue choppers (22), homogenizers (23), cell dissociation sieves (24) as reviewed by Valdevane et al. (25). Larger pre antral follicles have been isolated by microdissection using insulin needles (26). Mechanical isolation using fine needles has the advantage of preserving the basal lamina and the thecal layers intact maintaining the integrity of the follicle (27). A recent study successfully attained isolation of bovine primary follicles using mechanical disruption of ovarian cortex using a pasteur pipette. Furthermore, these follicles were demonstrated to survive *in vitro* culture for 21 days and form visible antral cavities (28). Enzymatic digestion has been the preferred method

of choice for the isolation of primordial/ primary follicles since these small follicles ranging (30-50um) are manually very hard to isolate. Although, enzymatic isolation can improve follicle yields compared to mechanical methods (29, 30), these aggressive enzymes can compromise follicular survival (27, 31, 32). A recent study observed spontaneous degeneration of isolated human follicles in culture, after enzymatic isolation. In spite of using a purified enzyme blend in place of traditional crude mixtures of collagenase, human follicular isolation could not be efficiently established using this enzymatic method (33). Furthermore, enzymatically isolated follicles particularly tend to lose their basal lamina causing granulosa cells to migrate away from the oocyte (34). Since isolation of follicles for culture is a crucial step in individual follicle culture, fine tuning the method and optimising the stage of follicle to be isolated is necessary for further development of this technique.

Towards a 3 dimensional culture of follicles *in vitro*

The most promising results in follicular development *in vitro* was attained by Eppig and O'Brien et al. in mouse model, producing live offspring from primordial follicles (35, 36). In spite of a decade of research following this initial success, similar results are yet to be obtained in larger mammals and human. Conventional 2 dimensional cultures fail to mimic the *in vivo* follicular environment. Culture of follicles directly on treated membranes or tissue culture surfaces destroys the spatial arrangement of follicles. Granulosa cells are seen to attach on the culture surface and cause follicular flattening (35). Growth of the oocyte and meiotic maturation relies on the signals exchange through gap junctions between the oocyte and surrounding granulosa cells (37). Loss of these gap junctions causes premature ovulation and degeneration of the oocyte (38). These signals are crucial for sharing paracrine factors that in turn promote growth of both cell types (39). This is supported by the fact that, oocyte is not able to transport amino acids and carry out glucose and cholesterol biosynthesis independently, in the absence of granulosa cell secreted factors (40).

Mimicing the *in vivo* follicular environment

Recent efforts on improving the *in vitro* maintenance and growth of follicles can roughly said to be aimed at enhancing two most crucial factors that determine fate of the follicle *in vitro.*

1) Physical/mechanical support to the growing follicle

Research conducted in the murine model has demonstrated the importance of biomechanical environment in determining follicular growth and considers this physical support to be as crucial as the hormonal millieu of the follicle (41). To this end an array of different strategies has been adopted till date to spatially support the follicle and provide the physical stimuli that it needs to survive *in vitro* reviewed by (27). Use of V shaped microwell plates has seen to be useful to some extent in maintaining three dimensional architecture of bovine (42) and human follicles (43). Collagen owing to its natural presence in the extra cellular matrix surrounding the follicle has been used to support *in vitro* follicular growth since the emergence of 3 dimensional culture systems. Embedded culture of follicles in collagen (44) was found to be superior to collagen membrane inserts (45) in supporting follicular growth and architecture *in vitro*. Combelles et al. (46) demonstrated that follicles embedded in collagen matrix maintained their three dimensional architecture and demonstrated neuronal like extensions arising from the granulosa cells towards the oocyte. Recent success with the use of collagen in supporting antral cavity formation of early bovine primary follicles (28) indicates that collagen gels still have scope for research, to be considered an efficient compound to mechanically support *in vitro* follicle growth. However, shrinkage of the gel and decreased microscopic visibility over time are the most common problems faced with the use of collagen (29). Furthermore, the need for enzymatic treatment to dissolve the gel at the end of culture is another matter of concern (47). The most widely used three dimensional follicle encapsulation system till date is alginate (48, 49). Alginate encapsulation coupled with the use

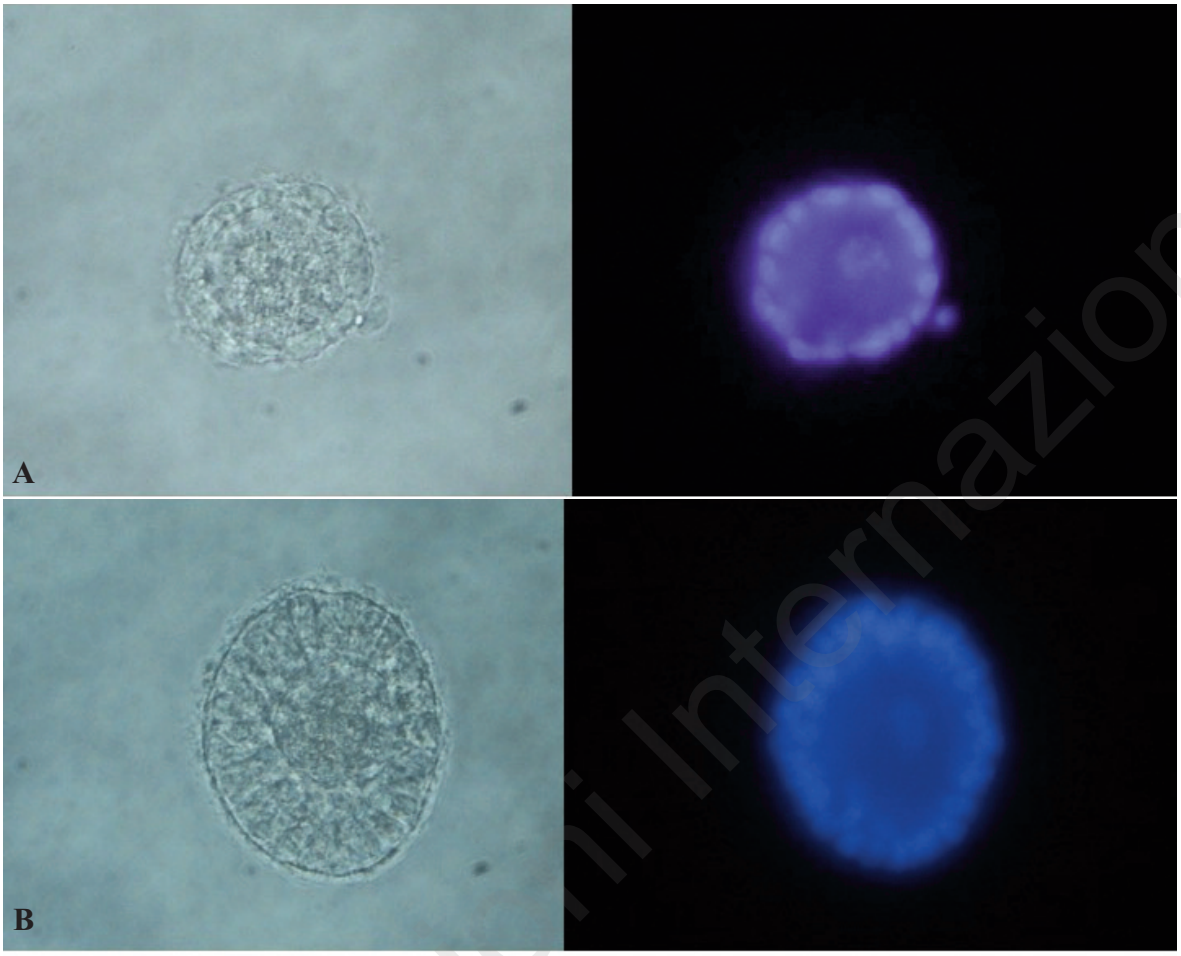

Figure 1 - A) Isolated primordial follicles stained with Hoechst 33324 (nuclear stain): oocyte nucleus clearly visualized. B) Isolated secondary follicles stained with Hoechst 33324 (nuclear stain): oocyte nucleus clearly visualized.

of V shaped microwell plates have been tried to support human and (49) primate (50, 51) follicles. Encapsulation with alginate has the advantage of promoting nutrient, oxygen, hormone and growth factor exchange between the follicle and the culture medium. Although, the comparatively rigid nature of alginate has seen to inhibit follicular growth in mouse follicles (52), human (53) and primate follicles (54) seem to prefer the rigid support provided by the alginate matrix. Primate follicles have been successfully cultured over long periods in alginate gels (55). This could be directly accounted to the highly fibrous nature of the ovarian cortex seen in human, bovine and primate ovaries compared to mice. Incorporation of extracellular matrix components to alginate have been tried to develop synthetic matrices that improve the performance of alginate supported cultures (56). Studies on the functions of extracellular matrix have revealed that it plays a crucial role in coordinating cell behaviour, cellular differentiation and secretion which are inevitable for follicular advancement (57). Our group demonstrated that alginate incorporated with collagen IV, a major extracellular component in ovary enhances the growth of isolated human follicles in culture. Moreover, ultrastructural analysis of these follicles revealed that culture of follicles embedded in alginate + collagen IV better preserved their three dimensional follicular architecture (58).

Matrigel is a commercially available extracellular matrix composed of collagen IV, laminin, fibronetin, entactin, heparin sulphate and proteoglycans along with an array of growth factors (EGF, FGF, IGF-1, PDGF and TGF-β) that has been tried for the growth of follicles (53, 59, 60). The incorporation of bio-engineering has

led to the development of novel dynamic matrices in follicle culture. These compounds differ from traditional materials by their ability to more efficiently accommodate the drastically expanding follicle. Fibrin containing matrices are widely being tested recently, particularly after the recent success of developing a mature oocyte from a macaque primary follicle for the first time (61). Biodegradable matrices composed of Interpenetrating Networks of fibrin (IPN) (62) that intercalate with matrix proteins could better ensure sustained delivery of growth factors to the follicle (33). VEGF containing IPN matrix has recently been shown to support *in vitro* follicle growth, oocyte maturation and the subsequent development of a live offspring. Novel hydrogels comprising intercalating peptides that lyse in response to follicular proteases could dynamically allow expansion of the growing follicle. Hence, the future of designing matrices for the *in vitro* growth of follicles lies in incorporating concepts of bio-engineering and tissue culture to develop dynamic culture systems that adjusts to the changing dimensions of the follicle.

2) Chemical/nutritional demands of the follicle

A recent research has revealed that in order to extrapolate the success obtained in culturing rodent follicles into larger species a multi-step dynamic culture system is required to cater to the various transitional stages of mammalian follicular development (43, 63, 64). To this end, follicular development will have to address 3 major events: 1) Primordial follicle activation and initiation of growth, 2) Pre-antral to antral follicle transition, 3) Development of fertilizable oocytes from tertiary follicles (27).

Hence the primary step to be considered is the *in vitro* activation of primordial follicles. Unfortunately, there are no conclusive reports on the factors that control early follicle recruitment and growth. However, it seems safe to assume that the process should involve a complex interplay of inhibitory, stimulatory and maintenance factors (65). Recent research suggests a role of Phosphatidylinositol- 3-Kinase (PI3K) – Akt signalling pathway of the oocyte in kick starting the follicular growth (66). Fine tuning of the hormonal and chemical milieu of the follicle is crucial to attain developmentally competent oocytes that complete cytoplasmic and nuclear maturation at a desired pace. Consequently several hormones and signalling molecules have been proposed to obtain optimal follicle growth *in vitro.* A few bio active components trialled have been discussed below.

Follicle Stimulating Hormone (FSH) is one among the most repeatedly trialled hormone to this end. FSH and estradiol are shown to have positive effects on antrum formation in granulosa cells in rodents (67) and pigs (68) for a long time. FSH along with LH activates cAMP system and activates the enzymes responsible for steroidogenesis in granulosa cells (69). FSH has also been shown to have positive effects on long term culture of bovine ovarian cortex in addition with GDF-9 and bFGF (70). Other studies have also reported the role of FSH in ensuring pre antral follicle survival and growth of primate (50) and human (53) follicles in 3 dimensional culture.

A bi-phasic requirement of FSH was revealed in a recent study that attained for the first time a 2 cell embryo from primate follicles cultured *in vitro* (51). Here they exposed follicles to high FSH levels before antrum formation and marginal levels after appearance of the antrum (51). In fact exposure to elevated levels of FSH for prolonged periods could disrupt the control of the oocyte over granulosa cell proliferation and differentiation (51) and lead to the pre-mature loss of trans zonal projections between oocytes and granulosa cells (71).

Epidermal Growth Factor (EGF) is yet another factor promoting pre antral follicle growth (72) as it induces granulosa cell proliferation and folliculogenesis (73) and progesterone synthesis by activating FSH receptors in granulosa cells. Positive effect of EGF has also been demonstrated in various animal models like pig (74), cow (75) and hamster (76).

Presence of Basic Fibroblast Growth Factor (bFGF) bioactivity is demonstrated in granulosa cells (77) and early growing follicles (78, 79). A combination of FSH, EGF and bFGF have recently gained acceptance as optimal media components that have given interesting results in the bovine model (28).

Activin protein has been localized in granulosa cells of human follicles. Hence, possible roles of activin as a follicular growth have been investigated (43, 71) based on the stimulatory effect of

activin on ovine pre-antral (80) and caprine follicles (81). This group demonstrated that activin not only does stimulate the growth of follicles but may also have functions in aiding the directional proliferation of granulosa cells by promoting the polarized expression of connexin proteins. Since loss of cellular polarization is a major threat *in vitro,* causing granulosa cells to proliferate undirectionally, activin could serve as an important modulator of cellular progression in the follicles. Activin was also seen to improve granulosa zona focal adhesions, the loss of which is one among the major concerns of *in vitro* follicular culture.

Fetuin, a glycoprotein component in serum and follicular fluid (82, 83) has been used to substitute the use of serum avoiding the concerns of using contaminated animal derivatives in culture. Schroeder et al. showed that fetuin increased zona pellucida solubility during oocyte maturation *in vitro* and supported a serum free culture environment (84). A protease inhibitory effect of fetuin was put forward for this observation. Fetuin has been suggested to improve cellular differentiation, growth and attachment *in vitro* (85, 86). Furthermore, fetuin was seen to maintain the integrity of alginate gels in long term culture (51).

Apart from the culture components oxygen tension is a key factor in determining the behaviour of any culture environment. Follicle culture *in vitro* has been regularly conducted at atmospheric oxygen tension (20% v/v /140 mm Hg) (50). Theoretically, follicles should be maintained at around 5% oxygen tension owing to the low partial pressure of oxygen in the peritoneal cavity i.e. 40mmHg (87). Low oxygen culture has been beneficial for the culture of rat pre antral follicles improving oocyte, viability maturation, parthenogentic activation and fertilization rates *in vitro* (88). Caprine pre natural follicles exhibited higher percentage of antrum formation at 5% oxygen as compared to 20% (89). Higher levels of reactive oxygen species are frequently associated with high partial pressure of oxygen and this oxidative stress induces cytotoxicity (90). Subsequently, culturing under low oxygen tension has seen to reduce cumulus cell apoptosis in canine oocyte cumulus complexes in culture (91). Lately, a higher number of healthy oocytes were also derived under low oxygen conditions, during *in vitro* culture of follicles (51).

Conclusions

In spite of several years of research, many key factors determining the complex process of follicular maturation still remains a mystery. Further knowledge of these fundamental mechanisms are necessary to extrapolate these factors *in vitro* in turn making *in vitro* follicle maturation possible. Analysis on studies conducted till date reveals a thin balance of several signalling molecules and factors that fine tune the optimal growth of the follicular unit. Hence, studies aimed to optimize each progressive step in follicular maturation, to more closely mimic what occurs *in vivo*, would be needed to attain a successful *in vitro* follicular growth. Improvements made in culture conditions of follicles *in vitro* could eventually avoid the need to transplant whole ovarian tissues to patients opting for fertility preservation. Furthermore, ability to transplant individual follicles grown *in vitro* could eliminate concerns like ischemic damage, graft death and accelerated proliferation of follicles following transplant that exhausts the whole tissue in a single attempt. Hence, single follicle culture and transplant could aid in attaining numerous competent oocytes minimising the wastage of follicles, ensuring long term results for the patient.

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