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REVIEW ARTICLE

Innovations for Optimizing Fertility and Ovarian Functionality in Female Cancer Patients

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ABSTRACT

The survival rates and quality of life of patients undergoing cancer treatments have significantly increased in recent years. However, these advanced treatments are known to exert a potentially gonadotoxic effect, impairing the female population of germ cells and, consequently, leading to ovarian failure through hormonal dysfunction and premature menopause. Nowadays, it is strongly recommended that oncologists discuss the risks of infertility and alternatives with each patient individually, recommending a consultation with a reproductive specialist as early as possible. In this context, remarkable progress has been made in assisted reproductive techniques aimed at fertility preservation for female patients. The main currently applied methods to avoid total loss of ovarian activity as a result of chemo or radiotherapy include oocyte, embryo, or ovarian tissue cryopreservation. This topic review summarizes the recent advances in these techniques, which have increased the chances of fertility preservation and family planning, as well as providing hope for cancer survivors. Oocyte cryopreservation followed by *in vitro* fertilization and embryo transfer are currently the recommended first-line fertility preservation approaches. However, for prepubertal individuals, the technique of ovarian tissue cryopreservation is advised; this is, moreover, the best method for efficient maintenance of the follicular reserve and allows future tissue transplantation, which not only restores fertility but also resumes natural ovarian hormonal activity. Recently, the discussion about the reestablishment of ovarian function in a defective tissue has raised compelling arguments, based on the theory about the presence of germ stem cells in the ovary. Despite the relevance of the results and the psychological benefits, the number of female patients diagnosed with cancer who are offered any method of fertility preservation is low. Multidisciplinary teams working jointly with oncologists and fertility specialists are essential for better outcomes regarding the wellness of patients who recover from cancer. Although further studies are needed to improve some of the available approaches, for now, comprehensive availability of oocyte/embryo or ovarian tissue freezing services is necessary worldwide to meet patient needs.

1. INTRODUCTION

Despite the increasing number of diagnoses, the continuous advances in cancer therapies have significantly improved not only survival rates, but also patient quality of life. If currently, 1 in 51 women will be diagnosed with cancer by the age of 39 years,[1] for children and adolescents with hematological malignancies and solid tumors, an important decline in mortality in Western Europe and North America has been reported.[2] The use of new and more effective treatments has led to important rises in cure rates and an estimated 1 in 530 adults aged 20-39 years has survived cancer during childhood.[3]

Among these survivors, a considerable number are still of reproductive age and advanced treatments do not come without a negative side: they are known to exert a potential gonadotoxic effect, reducing the female population of germ cells and, consequently, leading to ovarian failure through hormonal dysfunction and premature menopause.[4] Because of the longer life expectancy, concern about the consequences of these treatments has gained attention. Many women who overcome cancer have not completed or even started their families – in the last decade the number of pregnancies among women over 30 years of age has considerably increased in comparison to adolescents and women in their 20s.[5] Fertility preservation is thus an important concern among cancer survivors and exerts a considerable psychological impact, as well as affecting the patient's and their parents' decisions about future family planning.[6] Loss of fertility is often associated with premature menopause, the side effect which also has great importance in the patient's wellbeing.[7] Early menopause symptoms are a result of endocrine changes that lead to irregular menstrual cycles, vasomotor symptoms with hot flushes and night sweats, mood changes, anxiety, vaginal dryness, and bladder symptoms, among others.[8]

Thus, medical teams should be familiar with the risks involved in a patient's specific treatment and provide advice about the best alternatives to avoid premature menopause and fertility losses. Guidelines strongly recommend that oncologists discuss the possibilities of infertility and alternatives with every patient, recommending a consultation with a reproductive specialist as early as possible.[9] In this context, assisted reproductive techniques have provided remarkable progress in oncofertility and fertility preservation treatments, using the established methods of oocyte, embryo, or ovarian tissue cryopreservation. This topic review summarizes the recent advances in these techniques, that have provided the chance of fertility preservation, family planning, and hope for cancer surviving patients.

2. IMPACTS OF CANCER TREATMENTS ON THE OVARIAN FOLLICULAR RESERVE

Any patient of reproductive age about to be subjected to potentially gonadotoxic treatments must be informed about this risk; in the case of children and adolescents, parents or a responsible adult should be advised.[10] Despite the fact that nowadays many oncologic therapies provide remission and cure, their side effects might include impairment of ovarian function. These iatrogenic effects occur in both acute and long-term manners, causing menopause symptoms in adult women, and delaying puberty and bone development in girls.[11]

A wide range of chemotherapeutic drugs are commonly applied for cancer treatments. These include alkylating agents, anthracyclines, taxanes, topoisomerase inhibitors, and vinca alkaloids, which are often used in combination. Among these, there is evidence that ovarian damage is mainly caused by alkylating agents,[4] but it is difficult to determine the impacts of individual drugs, since most treatments are based on drug associations.

Cyclophosphamide is known to reduce the primordial follicle reserve either due to toxicity of cells or to excessive follicle activation.[12,13] It is, moreover, applied to induce ovarian damage in rodent experiments.[14] Cyclophosphamide triggers apoptosis by rapid induction of DNA breaks and shifts in expression of pro- and anti-apoptotic genes.[15,16] In growing follicles, granulosa cells are exposed to the impacts of these alkylating agents, showing high rates of apoptosis, and inducing the follicles to atresia.[17] However, the reason for ovarian dysfunction is not only due to the direct impact in follicles, but also to an inflammatory process and vascular damage.[18] In addition, impacts on the embryo's follicle development and competence, when the mother is exposed to cyclophosphamide during early pregnancy or prior to conception, were described.[19]

For local pelvic malignancies, ionizing radiation is commonly adopted. According to a study, the dose necessary for depleting half the oocyte population is <2 Gy.[20] Usually, a dose of at least 6 Gy is applied and in some cases, it can be >80Gy, providing a high risk of ovarian failure independently of the patient's age.[21] Ionizing radiation causes direct or indirect damage to DNA, by breaking the DNA strand or inducing the

formation of free radicals that end up damaging the cell's genetic compounds.[21]

The severity of these impacts depends on the patient's age, type of therapy, pharmacological agent applied, dose, and type of cancer.[4] Approaches to alleviate these impacts in somatic cells and oocytes have also been tested.[22] However, solid and established alternatives for preserving ovarian cells and tissue before these treatments are performed have been widely applied and are commonly recommended to patients in these cases.

3. CURRENT FEASIBLE ALTERNATIVES FOR PRESERVING FEMALE FERTILITY

a. OOCYTE CRYOPRESERVATION

According to established guidelines from European countries, oocyte cryopreservation is the first-line method to be proposed for prevention of iatrogenic infertility in women of reproductive age.[10] In this technique, unfertilized mature oocytes are stored for future *in vitro* fertilization (IVF) or intracytoplasmic sperm injection. In general, patients of reproductive age are candidates for this technique, independently of the availability of a partner at the time of oocyte cryopreservation. When pregnancy is desired, the oocytes are thawed and fertilized *in vitro*, and the embryos are implanted. However, in comparison to cryopreservation of sperm and embryo, oocyte cryopreservation has been neglected for a long time due to the cell's morphological characteristics (large size with high water content, low cell surface to volume ratio, high content of cortical granules, and presence of spindle and zona pellucida), which lead to susceptibilities to chemical and physical stress, frequently related to low fertilization and pregnancy rates. Contrarily, ethical issues surrounding the storage of embryos have relighted the need for improvements in oocyte cryopreservation techniques.[23] The commonly established protocol for performing oocyte cryopreservation is shown in Figure 1.

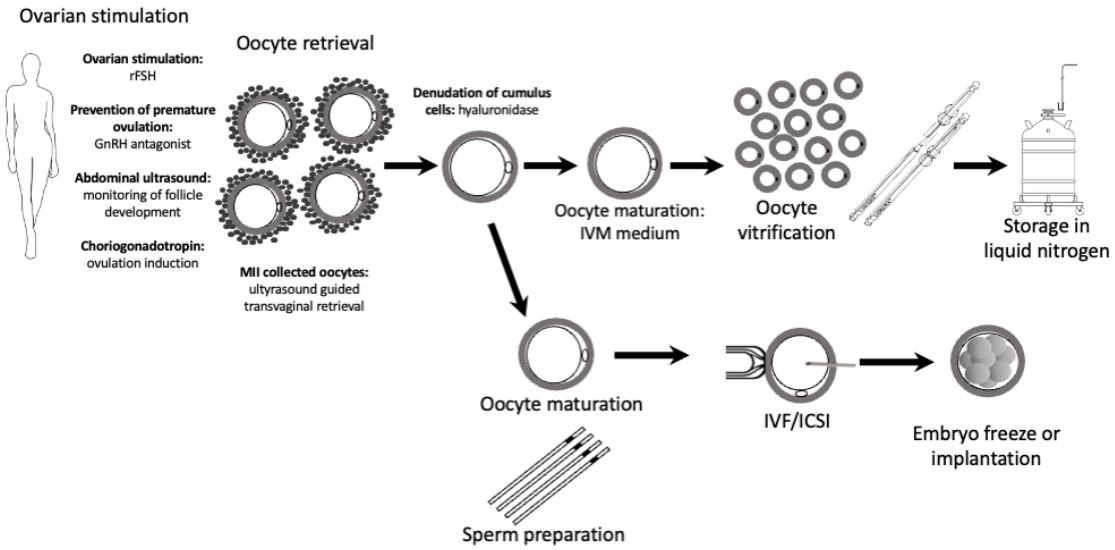

Figure 1. Commonly applied technique for oocyte cryopreservation. ICSI: intracytoplasmic sperm injection; IVF: *in vitro* fertilization; IVM: *in vitro* maturation; rFSH: recombinant follicle stimulating hormone.

The procedures for oocyte cryopreservation have improved in the last decade and vitrification is usually the chosen option.[24] In comparison to the slow-freezing method, vitrification enables better meiotic spindle recovery than slow freezing. Closed systems,[25] which avoid direct contact between oocytes and liquid nitrogen, have mitigated an old problem of vitrification: risk of cell contamination and infections. Thus, the use of these closed systems has been validated and widely applied in oocyte cryopreservation programs.

For collecting and storing oocytes, patients need to undergo ovarian stimulation, aiming to obtain a great number of oocytes for cryopreservation. The technique adopted for ovarian stimulation may vary according to patient's needs. For example, in patients with resistant ovarian syndrome or high levels of FSH no ovarian stimulation may be needed;[26] in contrast, patients with deficient oocyte maturation may be subjected to an association of rFSH and human menopausal gonadotropin.[27] The length of time that oocytes can be stored for after

adequate vitrification is unknown. According to the literature, storage for up to 13 years has led to the live birth of healthy babies.[28]

A negative aspect of oocyte cryopreservation is that this technique is not indicated for patients requiring an urgent start to their cancer treatment, since the ovarian stimulation procedure usually takes several days.[27] In these cases, there is no time to induce follicle development and subject the patient to oocyte retrieval, due to the urgent need for chemotherapy or radiotherapy. This technique is also not indicated for prepubertal patients; in these cases, since the hypothalamic-pituitary-gonad axis is not yet mature, it is not possible to perform ovarian stimulations through cycles of FSH treatment. The options for these patients will be discussed in Topics 3c and 3d.

In terms of effectiveness, growing evidence of successful outcomes has increased the reliability and applicability of oocyte cryopreservation in the field of human assisted reproduction. According to a recently published review,[29] the age at oocyte collection is the factor that most affects the prognosis, with the age of 35 years being the marking point, after which there are significantly reduced chances of success. Success rates of oocyte cryopreservation have been described in some studies conducted over the years. Usually, fertilization and pregnancy rates do not differ when comparing fresh and frozen-thawed retrieved oocytes.[30–32] Some quantitative aspects of fertilization and clinical pregnancy rates are shown in Table 1.

Table 1. Retrospective studies describing the success rates of oocyte cryopreservation procedures.

Author, year	Method	Fertilization rate (%)	Clinical pregnancy (%)
Talreja et al. (2020)[33]	Vitrification	86.2	63.6
	Fresh	83.4	60.5
Domingues et al. (2017)[34]	Vitrification	77.4	59
	Fresh	74.5	60.9
Cobo et al. (2010)[31]	Vitrification	74.2	55.4
	Fresh	73.3	55.6
Doyle et al. (2010)[35]	Vitrification	69.5	54.4
	Fresh	71.7	45.1

Recently, artificial intelligence has been applied to determine egg quality for women undergoing oocyte cryopreservation.[36] Violet technology can predict the probabilities of blastocyst development and live birth according to the quality of each egg, through application of a light microscope to capture images of the oocytes before they are frozen, and using a database comparison system. Automated closed-vitrification systems are also constantly being improved, aiming to avoid decreases in the efficacy of the vitrification protocol.[37]

b. EMBRYO CRYOPRESERVATION

The first pregnancy achieved after embryo cryopreservation was reported in 1983,[38] with the first baby born in 1985.[39] Since then, the technique has been improved and in 2022 the pregnancy rates range from 32%[40] to 35%[41] among different cohorts. The procedure is necessarily preceded by oocyte collection, applying part of the method previously described in Figure 1; however, the oocyte does not necessarily need to be cryopreserved before *in vitro* fertilization is performed. Clearly, embryo cryopreservation, similarly to oocyte cryopreservation, is not an option for prepubertal individuals and patients with an urgent need to start a gonadotoxic treatment. In addition, it is also not applicable for those patients who do not have a potential partner or do not wish to utilize sperm banks to have a baby.

Usually, embryos are cryostored after reaching at least the stage of 6 blastomeres. The summarized protocol is shown in Figure 2.[42–45] Regarding the cryopreservation procedure, in the last decade, vitrification has replaced slow freezing in embryos in different reproductive centers due to higher survival and implantation rates.[46] A systematic review performed in 2017 concluded that clinical pregnancy rates are higher when vitrification is performed in comparison to slow freezing.[47] Vitrification protocols have also been improved, and usually a mixture of ethylene glycol and dimethyl sulfoxide is applied as a cryoprotectant, in association with an extracellular cryoprotectant, such as sucrose.[48] Choosing the cryopreservation method is crucial because the ability of the embryo to survive reflects in the implantation ability.

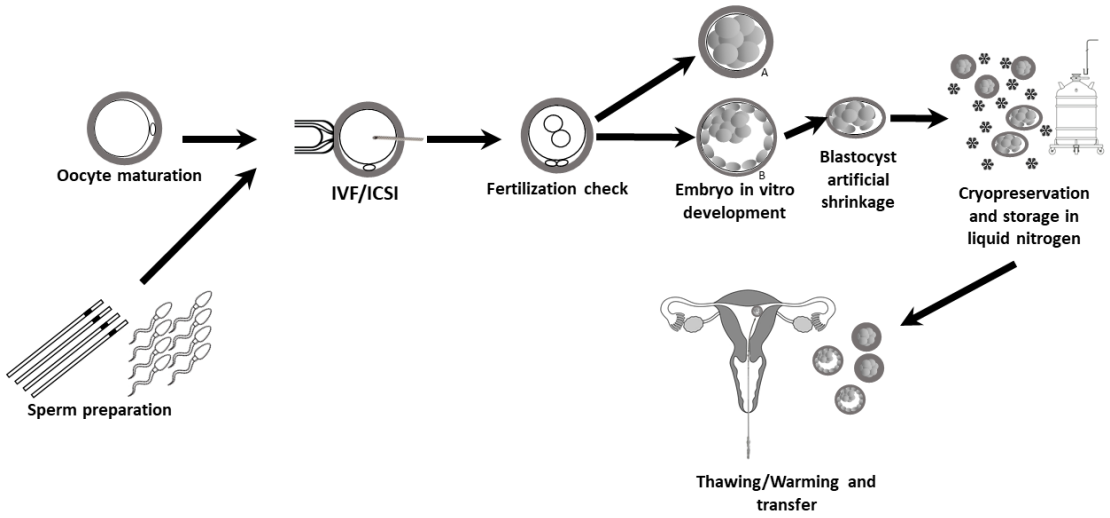

Figure 2. Commonly applied technique for embryo cryopreservation and transfer. The first steps of the technique are described in Figure 1 (ovarian stimulation and oocyte retrieval). After oocyte *in vitro* maturation, *in vitro* fertilization (IVF), or intracytoplasmic sperm injection (ICSI) are performed with prepared sperm. The fertilization procedure must be evaluated around 16-18h of IVF/ICSI to check for the presence of 2 nuclei and/or 2 polar bodies. Embryos must grow *in vitro* at least to the stage of 6 blastomeres to be cryopreserved as cleavage (A) or blastocyst (B) stages (in case of blastocyst cryopreservation, removal of blastocoel fluid is necessary).

Commonly, to perform the transfer after the cryopreserved embryo is thawed/warmed, patients showing a natural ovulatory cycle do not need further hormonal manipulation. However, hormonal stimulation may be performed to prepare the uterus for implantation. Some centers describe the use of luteal phase support with progesterone, and the use of hCG (as an ovulation trigger) and progesterone has been described.[49,50] In addition, patients with a thin endometrial wall, or other complications, such as prolonged menstruation and bleeding at ovulation are offered a treatment with ethinyl and/or progesterone.[48] For the transfer, embryos are deposited in the uterus through a catheter, in the lower to mid portion.[51] Analgesia or anesthesia is not mandatory for performing transcervical embryo transfer.[52] An alternative method to perform deposition of the embryos is by transmyometrial transfer, which is more invasive (a needle is inserted transmyometrially) but allows difficulties in transcervical embryo transfer to be overcome.[53]

Comparing the number of collected oocytes, fertilization rates, and live births, no differences were found between cancer or healthy patients who underwent the embryo cryopreservation procedure, demonstrating that this method is safe for application in oncologic patients.[54,55] In a cohort study, Kappy et al. (2021) reported that the majority of cancer patients who decided to preserve fertility, chose cryopreservation of embryos (36%) rather than oocyte or ovarian tissue cryopreservation.[56]

c. CRYOPRESERVATION OF OVARIAN TISSUE

Ovarian tissue cryopreservation is the only choice for preserving the fertility of prepubertal girls in need of a gonadotoxic treatment.[57] The first live birth using this strategy was announced in 2004;[58] and to date, more than 200 live births have been reported worldwide,[59] demonstrating the effectiveness of this method. However, the success rate is unclear because the total number of attempts performed up to now is unknown. This approach is also a tool for adult cancer patients needing to start chemotherapy within days of diagnosis, since it can be performed at any time during the menstrual cycle and there is no need for ovarian stimulation, or for the start of cancer treatment to be delayed.[60] Another important positive aspect of ovarian tissue cryopreservation is that it enables the storage of hundreds of primordial follicles, depending on the patient's age.

The cryopreservation of ovarian tissue safeguards oocytes associated with follicular cells in their natural extracellular environment. It is also a successful and irreplaceable alternative for natural hormone replacement therapy in cancer patients after gonadotoxic treatment is performed.[61] Recently, healthy individuals have also been

offered the benefits of this approach, with the aim of postponing physiological menopause and aging by autotransplantation of cryopreserved ovarian tissue, when the first symptoms of follicular depletion are present.[62,63] In a recently published review, authors conclude that in comparison to the commonly adopted hormonal replacement therapies, which usually include oral administration of synthetic hormones, ovarian tissue cryopreservation and transplantation has shown similar effectiveness in women undergoing physiological menopause.[64]

To perform cryopreservation, ovarian tissue collection must be performed before the patient is subjected to any chemo or radiotherapy. Usually, surgical collection can be made by laparoscopy, by which ovarian cortical biopsy samples are taken from either the right and/or left ovary.[58] The cortical strips should not be too thick, to allow ideal perfusion of cryoprotectants and uniform freezing, or too thin, to maintain an adequate number of follicles and vascular structure, which will be essential at transplantation. In some cases, removal and cryopreservation of the whole ovary is performed; in these cases, a more invasive procedure is necessary for retrieving the tissue (laparoscopic ovariectomy), by which the ovarian pedicle is clamped, and the gonad is immediately subjected to perfusion of cryoprotectants.[65] Perfusion of cryoprotectants in whole ovary cryopreservation may be optimized by application through the ovarian artery.[66]

Differently from cryopreservation of oocytes and embryos, freezing ovarian tissue presents a challenge due to its heterogeneous cell composition. Cells and extracellular components deserve extra attention, to guarantee their viability when the tissue is warmed/thawed and transplanted. Cryoprotectants such as ethylene glycol and dimethyl sulfoxide are applied for slow freezing.[58] The vitrification method has also been applied in some centers.[67] A recent experiment confirmed that when an adequate cryopreservation method is applied, refreezing-rethawing does not impair ovarian tissue viability and functionality.[68] The summary protocol for ovarian tissue cryopreservation is shown in Figure 3.

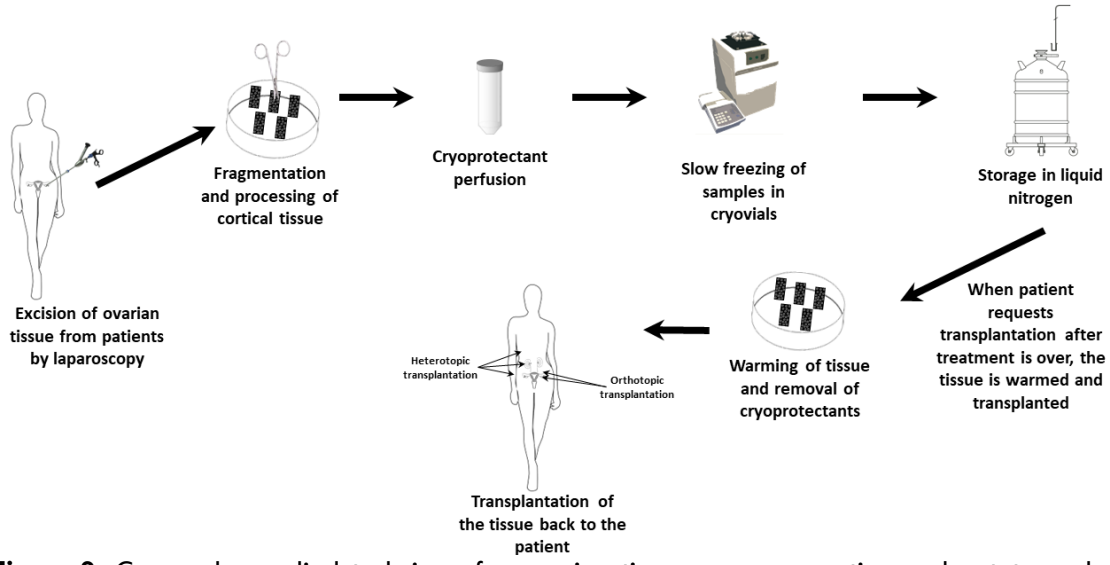

Figure 3. Commonly applied technique for ovarian tissue cryopreservation and autotransplantation. No hormonal treatment is necessary, and surgery may be performed at any phase of the menstrual cycle; the most commonly applied technique for harvesting the ovarian tissue is laparoscopy. Samples must be obtained before any chemo or radiotherapy occurs. After cryopreservation and storage, tissue may be warmed and retransplanted to the patient either orthotopically, when fertility restoration is desired (ovarian pedicle, ovarian medulla, peritoneal window), or heterotopically, when solely hormonal secretion restoration is intended (subcutaneous tissue, muscle, kidney capsule).

d. CRYOPRESERVATION OF ISOLATED FOLLICLES

The main limitation aspect of ovarian tissue cryopreservation is almost certainly the fact that, for some patients, there is a high risk of reintroducing malignant cells during transplantation, after tumor remission and recovery of healthy status.[69] This is because the cryopreserved ovarian tissue encloses the extracellular matrix, stroma, and vessels which may preserve remnant cancer cells

that presumably have not been subjected to the cytotoxic treatment (Figure 4). Malignant cells from leukemia,[70] borderline ovarian tumors,[71] and sarcomas[72] have been detected in thawed/warmed ovarian tissue, suggesting that the procedure needs to be avoided by patients in these conditions. Although the real magnitude of the malignancy reintroduction risk is not known, the cancer type, mass of malignant cells, and time of ovarian tissue harvesting influence this risk;[73] either way, alternatives to the use of ovarian tissue in such cases are seriously recommended.

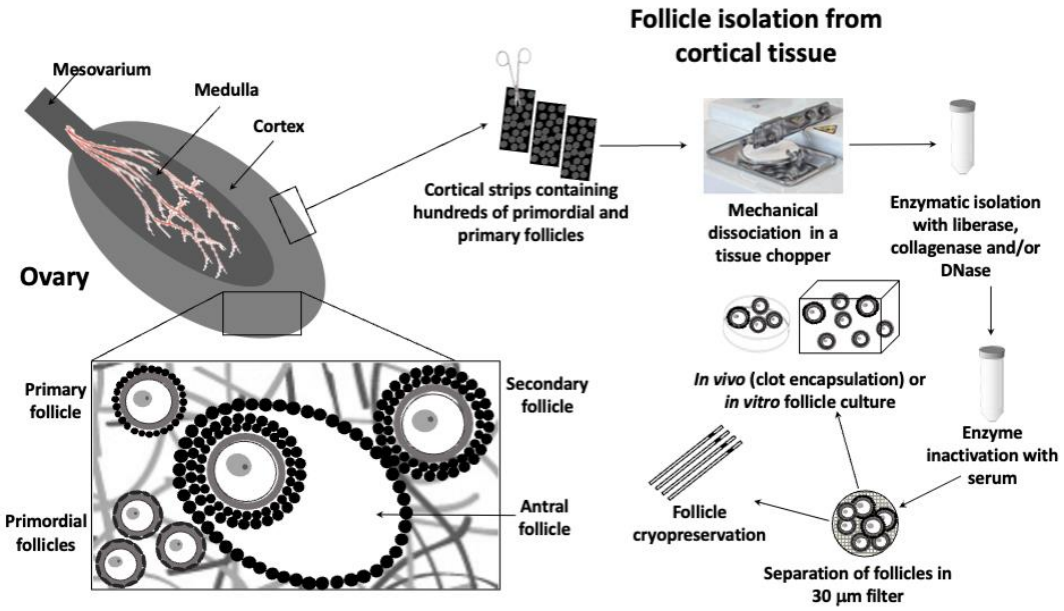

Figure 4. Ovarian cortex composition and isolation of preantral follicles aiming at cryopreservation or follicle growth. Most of the follicular population is made up of primordial and primary follicles; antral follicles are also present in the cortical region of the ovary but are excisioned prior to tissue cryopreservation.[74]

The benefits of isolating follicles before cryopreserving them include (1) the greater availability of follicles obtained in the ovary, in comparison to oocytes obtained from super ovulated patients, (2) favored cryoprotectant perfusion during cryopreservation, (3) avoidance of reintroducing malignant cells that may be present in the ovarian tissue, and (4) optimization of germ cell preservation, since the transplantation of ovarian tissue implies follicle loss due to ischemia.[75]

For cryopreservation of isolated follicles, their dissociation from the tissue can be performed by mechanical or enzymatic methods, which are commonly applied in association. Mechanical isolation is based on fragmentation of the ovarian cortex using scissors or a tissue chopper. In the enzymatic approach, liberase, collagenase, and/or DNase are used for digestion of the tissue and the release of intact follicles.[74,76,77] The choice of the enzyme is a crucial point and care must be taken to avoid or mitigate follicle damage during isolation. It also affects preantral follicles in different stages of development in a particular manner.[78] After isolation, the cryopreservation protocol is similar to that applied for oocytes, by submerging follicles in cryoprotectant solution and placing them in plastic straws for slow freezing or vitrification (Figure 4).

This methodology has been less frequently described in humans, despite the considerable advances in animals. The first successful case of a live birth after isolated follicle cryopreservation was described in mice, when offspring were obtained after follicle isolation, cryopreservation, *in vitro* culture, maturation, fertilization, and embryo transfer.[79] In these rodents, preantral follicles containing immature oocytes obtained from animals subjected to superovulation protocols showed a faster growth rate during *in vitro* culture.[80]

4. RESTORING FEMALE FERTILITY AFTER TREATMENT

a. OVARIAN TISSUE TRANSPLANTATION

Thawed ovarian tissue can restore ovarian activity and fertility after the patient's recovery by applying a surgical procedure with low risks for the patient.[81] This is the main technique used for restoring cryopreserved ovarian tissue and can be performed in a number of centers worldwide. Different transplantation approaches are available and reestablishment of ovarian function with

physiological secretion of steroid hormones, which is effective for replacing the standard approach of hormonal replacement therapy with synthetic hormones, is possible when ovarian tissue is reimplanted either heterotopically or orthotopically (Figure 3).

The choice of method and transplantation site is an essential factor that directly affects graft viability. By definition, orthotopic transplantation of ovarian tissue means its reimplantation in the pelvic cavity;[82] the two established techniques for orthotopic transplantation are (1) by fixing the cortical strips in the remaining decorticated ovarian medulla when present,[83,84] and (2) by creating a peritoneal window.[58] This method allows natural conception to occur, since the cavity contains the appropriate environment to allow follicular development, in terms of pressure, oxygenation, and the presence of peritoneal fluid, as well as oocyte catching by the fallopian tube.[85]

In the heterotopic transplantation approach, other anatomical locations, such as subcutaneous tissue (forearm, abdominal wall) and muscles (abdominal wall, intramuscular or under the fascia), are chosen. Heterotopic transplantation may circumvent invasive surgical approaches when performed in an easily accessed region; however, this method does not allow natural conception, so is adopted when solely reestablishment of ovarian function is desired by the patient, without fertility restoration. However, one case of biochemical pregnancy has been reported by heterotopic ovarian tissue transplantation to the abdominal wall,[86] after oocytes were aspirated from the functional grafts and subjected to IVF and embryo transfer.

Fixation of the tissue is made by stitches or applying surgical glue and for both techniques no vascular anastomosis is performed.[58] Thus, after transplantation, survival of the graft is dependent on the local revascularization process and ischemic injury is inevitable, causing massive follicular loss.[87] Although cryopreserving and transplanting grafts of reduced thickness is favorable with respect to freezing efficiency and reduced post-transplantation ischemia, it is known to accelerate depletion of the follicular pool.[87] Treatment of the graft with antioxidants added to the freezing solution[88] as well as administration of growth and angiogenic factors to the patient after transplantation[89] have been investigated, aiming to promote faster revascularization and prevent excessive follicular loss.

According to a report from one of the main current reproductive centers performing ovarian tissue transplantation, the mean time of ovarian functionality remains at least 5 years after transplantation; this timescale may be higher if tissue was recovered before to age of 22.[85] Thus, if necessary, future transplantation of further strips may be performed when signs of ovarian insufficiency are persistent.

After transplantation, ovarian functionality was restored in 95% of patients subjected to the procedure (assuring that primordial follicles are present in the ovarian fragment).[90] Results published by three clinical centers showed success rates of 50% in pregnancy and 41% in live births.[91] By the year 2020, more than 200 live births were assumed to have occurred after ovarian tissue cryopreservation and transplantation.[59]

The negative aspect of this technique is, as previously stressed, the risk of reimplanting malignant cells in patients in recovery from leukemia and other types of cancer. A recently published literature review discussed how photodynamic therapy can mitigate this risk by *ex vivo* purging of malignant cells after cryopreservation and before transplantation: by applying selective phototoxicity directed to malignant cells it would be possible to preserve the follicular structure and viability as well as to avoid damage to stromal and vascular cells.[92] Other alternatives to mitigate this risk are discussed in Topics 4b and 4c.

b. *IN VITRO* CULTURE OF FOLLICLES

Transplantation is not the only possible way to recover immature follicle activity after ovarian tissue has been cryopreserved. Furthermore, when cryopreservation is performed in isolated follicles, two options are available: *in vitro* culture, aimed at full development of these follicles until oocytes reach the MII stage, or embedding them in a matrix and proceeding to transplantation (this alternative is further discussed in Topic 4c).

Most isolated follicles obtained either before or after cryopreservation from the ovarian tissue are in the primordial or primary stage. Although the full mechanism by which primordial quiescent follicles are activated remains unclear, their activation and initial growth are regulated by specific endocrine and local factors. Oocyte signaling pathways are involved in this process,[93] with the pathways PTEN, suppressing, and mTOR, promoting this activation.[94,95]

In rodents, primordial follicles retrieved by enzymatic isolation from ovarian tissue were cultured *in vitro* until oocytes reached the MII stage, then, mature oocytes were fertilized, embryos were transferred, and living offspring were

obtained.[96,97] For humans, the whole multi-step *in vitro* culture system for primordial follicles was also described.[98] The summarized protocol for *in vitro* activation and development of human primordial follicles is described below.[98]

1. Primordial follicle activation and development to the secondary stage (6-10 days). *In vitro* culture of small tissue fragments in medium supplemented with insulin, transferrin, selenium, FSH, and ascorbic acid.
2. Mechanical isolation and culture of secondary follicles (8 days). Medium supplemented with activin A and FSH.
3. Retrieval of cumulus oocyte complexes from follicles presenting antral cavity. Culture (4-6 days) of complexes in medium supplemented with Activin A and FSH until oocytes reach 100 µm in diameter.
4. Maturation of oocytes in SAGE IVM medium supplemented with FSH and LH until detection of polar body and cumulus cell expansion.

As described, the major step of this protocol occurs with follicles included in the ovarian tissue, which contribute to the success of the procedure, since in this case the physical environment and contact with neighbor cells and the extracellular matrix are maintained. It is believed that manipulation of the tissue disrupts the signaling pathways that regulate follicle activation.[93] On the other hand, secondary follicles do not show good survival rates within ovarian tissue after this stage when *in vitro* culture is performed; thus, they need to be released from the tissue and cultured individually to prevent follicle interactions.[94,98]

The most significant applicability of *in vitro* follicle development is for fertility preservation, due to the widespread ovarian tissue cryopreservation. Unfortunately, this is not yet applicable in the clinical routine, as it is under development and refinement, aiming to provide an ideal protocol reproducible for human primordial/primary follicles to reach the antral stage and oocytes to reach the mature stage, allowing IVF. In any case, the findings described so far provide considerable advances in the knowledge of the oogenesis process, greatly contributing to improvements in fertility preservation.

c. THE TRANSPLANTABLE BIOENGINEERED OVARY

The search for a way to avoid the reintroduction of malignant cells to the patient has led to advances in tissue engineering approaches, aiming to provide an adequate environment for the development of germ cells and restoration of reproductive function. The bioengineered transplantable ovary was conceived to safely allow transplantation of primordial and primary isolated follicles, supporting follicle survival and development after transplantation. This approach not only allows fertility but also adequate secretion of hormones in a tailored manner, using the patient's own ovarian tissue to obtain cells and an artificial matrix source.[99]

An adequate matrix must be adapted to maintain primordial and primary follicles, since these structures represent more than 90% of the ovarian follicular population and are the most resistant to cryoinjury.[100] Furthermore, not only a matrix is necessary: for adequate follicle activation and growth, viable and numerous stromal cells are mandatory to develop the theca cell layer, and endothelial cells are necessary to constitute vessels. Thus, the crosstalk between follicles and the other components of the system demands the re-creation of an environment that ideally mimics the human ovary.

To compose the engineered artificial ovary, addition of isolated somatic ovarian cells is provided by processing the patient's tissue. Fibroblasts and endothelial cells may be isolated from the tissue by enzymatic digestion.[101] They are then sorted and added to the artificial ovary matrix to establish a natural communication between the follicles and surrounding stroma cells and enhance the revascularization process.[102,103] These somatic cells may be obtained either from the cryopreserved tissue before gonadotoxic treatment is performed, or even after it, by using a fresh ovarian biopsy at the time of transplantation, avoiding any chance of contamination with malignant cells.[104]

The choice of the matrix to assemble stromal cells and follicles is challenging. Synthetic[105] and natural[106] options of polymer sources have been tested. Synthetic matrices may be specifically tailored according to the requirements for clinical application and can be largely and uniformly manufactured;[107] however, they might lack extracellular molecules that are essential for cell adhesion.[108] One example of a polymer that adequately constitutes the ovarian artificial ovary is poly(ethylene glycol).[108] Natural polymers, such as collagen, allow better cell interaction, but might lack adequate mechanical strength and impair composition modifications due to their complex structure.[109] Fibrin and alginate are natural alternatives for developing a matrix.[110,111]

The engineered transplantable ovary is a promising alternative that could enormously benefit cancer

patients. It also allows reestablishment of natural hormone secretion in a physiological manner, after ovarian function is lost due to anticancer treatments. However, despite showing promise, this technique is still under development to be tested as a suitable physiological environment to allow human gametogenesis.

5. GERM CELL RENEWAL: DOES POSTNATAL OOGENESIS HAPPEN IN THE HUMAN OVARY?

It is supposed to be a consensus that performing heterotopic ovarian tissue transplantation does not allow a naturally conceived pregnancy. However, cases have been described where patients with confirmed premature ovarian failure became pregnant after performing heterotopic ovarian tissue transplantation.[112]

This is probably explained by the theory that reestablishment of physiological serum estradiol levels could restore the defective remaining ovarian tissue. Treatment with ethinyl estradiol in patients with premature ovarian failure led to an ovulation rate of 32% against 0% observed in the placebo group,[113] suggesting that reestablishment of serum estrogen can restore function of a previously "menopausal" ovary. Contrarily, other authors are skeptical regarding this hypothesis.[114] In fact, in 2006, Oktay and colleagues did not believe that hormone secretion by the transplanted ovarian tissue would be able to initiate follicular activity by the menopausal in situ ovary,[115] taking into consideration that chemotherapy injured oocytes are committed to fragmentation and die within 4 hours.[116] The most suitable hypothesis then was that the ovarian reserve could be recovered by another mechanism, which is contrary to what has been stated for a long time: the inability of female germ cell reserve to renew after birth.

After confirmed premature ovarian failure, hematologic stem cell transplantation in mice led to oocyte regeneration[117] and recovery of ovarian function in human patients.[118] Recently, autologous stem cell transplantation directly to the ovary increased the development of antral follicles, improving the success rates of IVF procedures in poor responders[119] and allowing the birth of a healthy baby from a 45-year-old woman with confirmed premature ovarian failure.[120] Another work, however, refuted the possibility of blood or bone marrow cells to become oocytes.[121] According to the authors, although fluorescent-labeled cells from the donor were found in the host ovary associated with developing oocytes, they did not show any differentiated germ cell characteristics. Indeed, Veitia et al. confirmed the genetic similarities between the newborns and the mothers, excluding any chances of a relationship between the baby and the donor.[122] This, however, does not exclude the possibility that blood cells support the development of new follicles suitable for ovulation. Adult stem cells release paracrine soluble factors such as cytokines, growth factors, and chemokines, this being the potential reason for the association of bone marrow stem cells with the reestablishment of ovarian function.[123,124]

The question about where potential germline stem cells come from, however, remains. Taking into consideration the theory that paracrine/endocrine factors released by the transplanted ovary may induce differentiation of germline stem cells in the in-situ ovary, as proposed by Oktay et al. in 2011, it is possible that the new oocytes arise from a cell population already present in the ovary: ovarian stem cells.[125] A likely population of very small ovarian stem cells lying on the ovarian surface epithelium was described according to its expression of SSEA-4, a pluripotency-related marker.[126,127] However, successful experiments were performed after use of cortical tissue or whole ovary, leaving in doubt the real localization of these cells in the ovary. After ovarian cortex digestion and cell sorting, mouse and human germline stem cells were cultured *in vitro*, spontaneously generating 35-50 μm oocytes; injection of these cells into human menopause ovarian cortex led to the formation of follicles, after xenografting.[128] In addition, transplantation of these cells into ovaries of infertile mice produced offspring.[129] However, in the case where the in-situ ovary has ovarian stem cells waiting for an appropriate niche to develop, why does it not restore follicle activity before the heterotopic transplantation? There is a chance that potential germ cells may have come from the grafted tissue, entered the bloodstream and reached the in-situ ovary, as an appropriate niche[112,130], since little is known about their availability in the menopause ovary.

If in rodents the presence of stem cells with the potential to develop into new oocytes in the adult ovary has been confirmed[131], in humans it is still controversial. A study performed by Wagner et al. described that six cell types expressing DDX4, a cytoplasmic RNA helicase commonly used as a marker for germ cells in adult ovaries, are present in the cortex, according to single-cell transcriptome and cell surface antigen profiles.[132] These cells comprise oocytes, granulosa cells, endothelial cells, perivascular cells, and stromal cells. However, recently a workflow optimization developed by Alberico et al. concluded that mistaking cell types

during magnetic-assisted or fluorescence activated cell sorting is avoided when gene expression profiles are evaluated by single-cell RNA sequence analysis.[133] The authors declare that optimization of the previously applied technique allows identification of cell types expressing genes that closely align with primitive germ cells, and even markers of meiosis-I commitment and progression, such as *SYCP3, STAG3, SMC3,* and *SMC1a*, are present.[133]

These works lead us to assume that there is a close relation between the presence of stem cells in the inactive ovary and the reestablishment of fertility in both humans and mice. However, although low, one should always consider the possibility of spontaneous fertility restoration after hematologic stem cell transplantation in patients with confirmed premature ovarian failure. Even though no conclusive works have been published so far, the possible restoration of follicle population in the inactive ovary must be considered. Thus, the discussion remains on the availability of cells that have the potential to renew the female follicular population in the ovary. Confirmation of their existence could potentially open new opportunities for creating alternatives to restore ovarian function and, consequently, fertility after undergoing a gonadotoxic treatment.

6. CONCLUSION

Despite the effectiveness of some methods and the psychological benefits to patients, the number of women diagnosed with cancer that are offered a method of fertility preservation is low. Multidisciplinary teams working jointly with oncologists and fertility specialists are necessary, as well as widespread availability of oocyte/embryo or ovarian tissue freezing services.

Oocyte cryopreservation followed by IVF and embryo transfer remains the most commonly applied technique for preserving fertility in adult cancer patients, this being the first-line fertility preservation technique to be recommended. However, it cannot be assured this is in fact the most appropriate alternative, due to lack of knowledge and experience in other techniques, such as ovarian tissue cryopreservation, in most regions of the world. For prepubertal patients, ovarian tissue cryopreservation is the only available technique for preserving ovarian function; ideally, this option should be discussed as a first-line approach in cases of treatment with a high gonadotoxic risk, where chemotherapy must be immediately started, and in pediatric patients.

CONFLICTS OF INTEREST STATEMENT

The author has no conflict of interest to disclose.

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