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Review

OVARIAN TISSUE CRYOSTORAGE AND GRAFTING: An Option to Preserve Fertility in Pediatric Patients with Malignancies

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□ *Fertility preservation in childhood cancer has become an important area of investigation due to increasing survival rates after cancer therapy. For these patients with an increased risk of infertility and premature ovarian failure, cryopreservation of ovarian tissue is a promising tool to preserve at least part of the reproductive potential. In recent years significant improvements have been achieved in this area, and 2 live births after autografting of frozen–thawed ovarian tissue have been reported. However, further research is needed to assess the clinical effectiveness of ovarian cryopreservation, to optimize the technique, and to limit the risk of reintroducing cancer cells in the patient with the graft.*

Keywords fertility, ovarian tissue cryopreservation, ovarian tissue grafting, pediatric cancer treatment, precocious ovarian failure

In childhood patients with cancer, the 5-year survival rate has relevantly increased in the last years, and for some kinds of malignancy it currently reaches 70–80% [1]. It has been estimated that by the year 2010 about 1 in every 570 persons aged 20–40 years will be a long-term survivor of childhood cancer [2]. Female children who survive to cancer are candidates to suffer

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from cancer treatment-related problems involving their reproductive potential, such as infertility and/or premature ovarian failure (POF) [3].

The ovaries are quite sensitive to cytotoxic treatments, especially to alkylating agents and ionizing radiations, whose effect often causes the premature exhaustion of the follicle pool with substantial consequences on quality of life [4, 5]. Awareness of the effects of oncostatic treatments on fertility results in an increasing number of patients seeking help to preserve their possibility to childbearing [6].

In adults several options are available, including hormonal "shields" (GnRH analogues, oral contraceptives, etc.), surgical procedures (ovariopexis, etc.), and cryostorage of embryos and/or unfertilized oocytes, but they are not suitable before puberty. In pediatric patients, the harvesting and storage of ovarian tissue is at present the most promising procedure, although its results in terms of restoration of fertility are still very preliminary.

EPIDEMIOLOGY OF CANCER IN CHILDHOOD

Cancer among children (<14 years old) is relatively uncommon, representing 1–2% of all malignancies. Even though childhood cancer remains the leading cause of disease-related mortality among children 1–14 years of age, a remarkable improvement in the survival rates has been achieved in the last three decades, with an overall increase of life expectancy [7]. The most common cancers in childhood are acute leukemias and brain tumors, followed by neuroblastoma, Wilm's tumors, and non-Hodgkin lymphomas. Less frequent cancers are Hodgkin disease, rhabdomyosarcoma, soft tissue sarcoma, germ cell tumor, retinoblastoma, and osteosarcoma [8].

EFFECTS OF CHEMOTHERAPY AND RADIOTHERAPY ON THE YOUNG OVARY

The ovaries reach the maximum content of primordial follicles and oogonia during the 16th–20th week of gestation, when follicles number is approximately 6–7 million. Their number progressively decreases in the last months of pregnancy, it is reduced to half at birth, and decreases throughout life until menopause.

Cytotoxic treatments increase the rate of follicle loss, with ovarian damage varying according to the patient's age, the extent of ovarian follicular reserve, and the type of treatment (mono/polychemotherapy, radiotherapy with or without direct pelvic irradiation, combined treatments, etc.) [9]. Since the ovaries accomplish both endocrine and reproductive functions, the damage may finally result in several clinical problems, including amenorrhea, menstrual irregularity, failure to develop secondary sex characteristics, infertility, and POF [9].

Some patients are still fertile after anticancer therapy, but since radiations and some commonly used chemotherapeutic agents are mutagenic, these long-term survivors are concerned about the potential effect of the treatment on the health of their offspring. Adverse effects on oocyte DNA may theoretically lead to an increase in the rate of miscarriage, stillbirth, congenital malformations, aneuploidy, and single gene-related diseases, or to an increase in the risk of cancer in the offspring [reviewed in 10].

Recently, however, a report of the Childhood Cancer Survivor Study did not identify excess adverse outcomes for chemotherapeutic agents to this respect. In 4029 pregnancies, no significant associations were observed between the previous cancer treatment and pregnancy outcomes, although the risk of miscarriage was higher among women whose ovaries were directly irradiated (relative risk [RR]: 1.86, $p = .14$) or near the radiation field (RR: 1.64, $p = .03$) [11]. Even a large, retrospective study revealed that among patients that conceived after autologous transplant of allogenic stem cells, pregnancies were likely to have a successful outcome [12].

Chemotherapy

In general, all chemotherapeutic drugs are cytotoxic for the ovary to some extent [13], but those more often associated with a high risk of gonadal dysfunction are alkylating agents (cyclophosphamide, busulfan, melphalan, chlorambucil, procarbazine, dacarbazine, isofosfamide, nitrogen mustards, and thiotepa) [14–16]. There is also a moderate risk of gonadal injury with cisplatin, carboplatin, and doxorubicin [17]. Other agents, such as bleomycin, actinomycin-D, mercaptopurine, vincristine, 5-fluorouracil, and methotrexate, are associated with low risk of ovarian toxicity [6].

Cyclophosphamide is the more harmful agent for oocytes and granulosa cells: it acts in a dose-dependent manner and is highly effective in causing oocyte death and follicle depletion [16]. Overall, intensive, high-dose chemotherapy (such as the one required before bone marrow transplantation) brings the highest risk of developing POF and leads to subsequent ovarian failure in almost all cases [18, 19]. A large retrospective study concerning pregnancy outcome in patients submitted to hematopoietic stem cell transplantation showed that only 0.6% of 37,362 patients conceived in the years following the therapy [12].

Ovarian injury increases in parallel with the dose of the administered chemotherapy: for example, myelo-ablative doses of alkylating agents induce POF at all ages [20]. The extent of follicular destruction depend on the type of treatment and on the administered dose, but even the age of patients deeply affects the final loss of endocrine and reproductive functions. Younger patients have more primordial follicles than adults and the gonadal damage is on average less severe than in adult women. The high number of follicles in the ovarian cortex allows the young patient to retain her reproductive

potential after chemotherapy better than adult women [20]. However, these young girls have a consistently increased risk to develop a POF in their future life [21]. Serological markers of ovarian reserve (anti-Müllerian hormone, inhibin B) and sonographic measure of ovarian size suggest that subclinical abnormalities of ovarian function may occur in these patients despite normal menses and gonadotropin levels [22].

Combining various chemotherapeutic agents further increases gonadal damage: the MOPP/ABV chemotherapy (chlorethamine, vincristine, procarbazine, prednisone, doxorubicin, bleomycin, vinblastine) was reported to cause amenorrhea in 89% of patients older than 25 and in 20% of those below 25 years of age [23].

Radiotherapy

The degree of gonadal damage induced by ionizing radiations depends on the dose, width of irradiation field, administration modalities, and patient's age at the moment of exposure [24]. Radiotherapy may induce a dose-dependent depletion of primordial follicles with consequent retardation of puberty or POF; the LDL-50 (lethal dose needed to disrupt half of the follicles) in humans is approximately 2 Gy [24].

Girls treated with whole abdominal and/or pelvic irradiation for Hodgkin disease, Wilm's tumor, or other solid tumors (e.g., rhabdomyosarcoma, neuroblastoma) are at high risk of POF [24]. When ovarian transposition is performed prior to radiotherapy, however, ovarian function is retained in the majority of adolescent females [25]. Ovarian transposition is not a completely risk-free procedure: increased risk of adhesions formation (even with intestinal obstruction), functional ovarian cysts formation, and fallopian tube damage impairing future fertility has been reported [26].

Spinal irradiation for the treatment of acute lymphoblastic leukemia and brain tumors may result in significant ovarian damage in young women [27].

Patients who receive a stem cell transplant with total body irradiation (TBI) are at greater risk of developing POF: virtually all patients submitted to TBI after age 10 will develop a POF, whereas approximately 50% of TBI-treated girls under 10 years of age will suffer acute loss of ovarian function [14, 28]. Even though the remaining 50% appear not to suffer from ovarian failure in the short term, they still have an increased risk of diminished reproductive potential linked to exposition to cytotoxic therapy [26].

The depletion of oocytes after irradiation is proportional to their initial number (ovarian reserve); consequently, radiation therapy is more harmful for older people than for children, whose ovaries retain a huge pool of follicles even after irradiation. It has been demonstrated that the radiation dose at which POF is likely to develop is inversely proportional to age at the time of treatment [29]. Thus, while doses of 6 Gy may be sufficient to produce irreversible ovarian damage in women over 40 years, 10–20 Gy are

needed to induce POF in the majority of females treated during childhood [30].

OVARIAN RESERVE (OR) AND ONCOSTATIC TREATMENT

Treatment protocols are continually evolving to improve survival and reduce adverse effects, including anti-reproductive effects. At present the impact of childhood cancer treatment on OR is poorly known, as large-scale studies assessing how OR is affected by cancer treatment are lacking.

Moreover, gonadal damage is not detectable before puberty, as the hypothalamic–pituitary–ovarian axis is quiescent. After adolescence, the assessment of ovarian function includes the monitoring of puberty development, with assay of plasma sex steroids and measurement of ovarian volume by ultrasound [31]. However, the only presence of an endocrine ovarian activity and of ovulation are not indicative of the extent of OR. Several studies show an unequal distribution of primordial follicles in ovarian cortex and consequently even an ovarian biopsy is not an accurate tool to estimate the OR [32]. Moreover, it might raise ethical concerns to take a biopsy to determine ovarian reserve in a young patient. All the available biochemical markers of ovarian activity (FSH, estradiol, inhibin B, anti-Müllerian hormone) and the dynamic tests to assess OR (e.g., clomiphene citrate challenge test) are suitable only after puberty and cannot predict in a fully reliable way the life expectancy of an ovary damaged by cytotoxic therapy.

A classification of the infertility risk estimated on the basis of the type of cancer and the associated treatment considers high-risk, medium-risk, and low-risk therapies according to the proportion of survived patients that show impaired fertility [29]. The highest risk (more than 80%) of infertility is associated with TBI, pelvic radiotherapy, chemotherapy for bone-marrow transplantation and treatment with alkylating drugs [29].

PRESERVING FERTILITY IN PEDIATRIC PATIENTS: OVARIAN TISSUE CRYOSTORAGE/AUTOGRAFTING

History and Rationale of Ovarian Cortex Cryostorage

The idea of autologous transplantation of human ovarian tissue is not novel: in 1906 a surgeon reported on this procedure with fresh tissue, but only in the 1950s, when cryoprotectants were discovered, did the aim to freeze ovaries and preserve their function become realistic [33, 34]. However, no progress was made in this field until the 1990s, when recognition of the potential clinical application in reproductive medicine renewed interest in ovarian cryostorage and grafting.

The potential of saving at least part of the follicles by means of repeated biopsies of the ovarian cortex, their cryostorage, and subsequent

post-thaw grafting in the same subject renders this technique useful even for pediatric patients. In fact, no hormonal ovarian stimulation (which would not be possible in a prepubertal girl) is required before ovarian biopsy. This implies also that biopsies may be performed as soon as cancer has been diagnosed, which makes the technique a good option even in case of very aggressive cancers that require to rapidly begin treatment.

It is reasonable to offer ovarian tissue cryopreservation to pediatric patients only if they have a realistic chance of long-term survival, if the scheduled treatment is associated with a risk of infertility and/or POF exceeding 50%, and if the therapy will not significantly impair the function of the uterus [29].

Sampling and Freezing Ovarian Tissue

Ovarian cortex in childhood is rich of hundreds in immature, primordial, and primary follicles, which survive cryopreservation better than growing and mature follicles. Various groups have studied in animal species the freezing technique, confirming the good feasibility of cryopreservation for the ovary and obtaining successful pregnancies after thawing and autografting [35, 36].

The ovarian cortical tissue is removed via laparoscopy and cut into strips of 1–3 mm in thickness (up to 1 cm^2 total area) to ensure an optimal penetration of cryoprotectants. As general anesthesia is required, the operation is frequently scheduled at the same time of bone marrow aspiration or placement of a central catheter for chemotherapy administration. Ovarian fragments are immediately observed by a pathologist to confirm the presence of follicles and contemporaneously assess the absence of a massive cancer infiltration.

It is possible either to remove the entire ovary or to take multiple ovarian samples [37]. There is little difference for an experienced surgeon in terms of either complications or duration of the operation for these two procedures, and a very high rate of primordial follicle survival after freeze–thawing of the whole human ovary was reported [38]. However, the removal of the whole ovary is difficult to accept in case of children whose future fertility is not certainly compromised after chemo/radiotherapy. Indeed, the occurrence of POF depends on several factors and does not invariably happen in all cases.

The standard method for human ovarian tissue cryopreservation is the so-called "slow equilibrium freezing," during which an albumin-containing medium is used and propanediol (PROH), dimethyl sulfoxide (DMSO), or ethylene glycol (EG) are used as cryoprotectants in combination with sucrose [39]. A programmed, authomatic, slow-rate cooling is applied, and tissue

fragments are finally put in cryovials and merged in liquid nitrogen where they may be kept for years.

Post-thawing Options: Autografting

The thawing procedure of ovarian fragments is accomplished by rapid warming at room temperature, using solutions containing a progressively decreasing sucrose concentration. A histological evaluation of follicle survival is immediately accomplished, and then the biopsies are available for subsequent use.

The main problem of ovarian banking is currently how to restore ovarian function and fertility using frozen/thawed ovarian tissue, with high effectiveness and acceptable risks. The ideal option would be autografting the tissue at the orthotopic site, in the ovarian natural site. The possibility of a natural pregnancy after spontaneous ovulation of the ovarian tissue grafted at the orthotopic ovarian site has been shown in animals such as the sheep, whose ovaries are histologically similar to humans [35]. Functional studies on the endocrine activity, follicular development, and reproductive lifespan have been performed in primates after ovarian tissue autografting [40]. In humans, a satisfactory resumption of ovarian endocrine function and follicular growth in grafted ovarian biopsies was repeatedly documented [41–44], although the endocrine activity of the grafted tissue was found to be limited to a few months [43].

The first human live birth after orthotopic transplantation of frozen–thawed ovarian tissue was recently reported [45]. Conception occurred after spontaneous ovulation from a grafted ovarian fragment in a woman previously treated with chemotherapy and irradiation for Hodgkin lymphoma, whose ovaries were left in the pelvis, but underwent POF (FSH 91.1 U/L) after the oncostatic treatment [45]. Several thawed ovarian strips were laparoscopically placed inside the residual ovary after some months from the end of the therapies: the resumption of ovulation was documented by ultrasound and was observed to occur in the area of the graft. After 3 ovulatory cycles, a spontaneous pregnancy occurred that ended with the birth of a healthy baby [45]. This study was criticized because since the patient did not undergo bilateral oophorectomy, ovulation could have resulted from the residual ovarian tissue and not from the graft [46]. However, ovulation is likely to have arisen from the grafted tissue as Donnez and Dolmans were able to directly visualize a growing follicle in the grafted tissue and not in the remaining native ovaries during a laparoscopy performed some months after transplantation [47].

A second case of a live birth after ovarian autografting was reported by Meirow et al. [48]. A young woman diagnosed to suffer from a Hodgkin lymphoma was submitted to ovarian fragment recovery and cryostorage. After high-dose poli-chemotherapy a POF took place, documented by the abrupt

rise of circulating FSH levels and by undetectable blood levels of inhibin B and anti-Müllerian hormone [48]. After cancer treatment, autografting of ovarian cortical fragments under the capsule of the ovaries that had been left in the pelvis was accomplished. Some weeks later, spontaneous growth of a follicle was documented, the oocyte was retrieved by ultrasound-guided puncture and in vitro fertilization (IVF) was performed, after which the patient conceived and delivered a healthy newborn [48]. Even in this case the ovaries had been left, and although they seemed to be totally nonfunctional after chemotherapy, the spontaneous resumption of ovulation cannot be ruled out with absolute certainty.

It must be remarked that to conclusively prove the origin of a pregnancy from a cryopreserved and autografted ovarian fragment, bilateral ooforectomy should be done: this procedure is not considered ethical in humans because not all women undergo POF after treatment and also because the remaining ovaries, although atrophic, can be used as a site of grafting as in the forementioned cases. Experiments in oophorectomized animals are needed to definitely prove that ovulation occurs from a thawed, grafted ovarian slice.

An alternative approach is ovarian tissue heterotopic (outside the pelvis) transplantation. The grafting at a heterotopic site, such as the forearm [49, 50] or the abdominal wall [43, 51, 52], has been successfully accomplished and it is easier to perform than pelvic transplantation as it does not require general anesthesia or abdominal surgery. This technique allows the graft to be closely monitored by ultrasound, but oocytes have to be collected by transcutaneous puncture, and an IVF procedure has to be performed.

The first live birth in a primate after heterotopic grafting was recently reported following oocyte retrieval from a fresh ovarian fragment grafted under the abdominal wall, its fertilization by IVF and the replacement of the resulting embryo in a surrogate mother [53]. In humans, ovarian function was shown to be restored in 2 patients for several months after transplanting ovarian tissue fragments to a tissutal pocket in the forearm [54]. In one patient who received ovarian autografting under the abdominal skin, the recovery of some oocytes was demonstrated, but most of them were immature or morphologically abnormal, and only one was fertilized by IVF, giving rise to a good-looking, 4-cell embryo that was replaced in utero, but did not implant [51].

It seems possible that the site of grafting could influence the competence of the grafted tissue to respond to gonadotropins and promote the development of mature, viable oocytes. Maybe local factors, such as vascularization, oxygen tension, external pressure, temperature, and substrate availability, affect the possibility of sustaining the growth and development of normal follicles and oocytes. By now the pelvic site and, in particular, the ovarian tissue (when left during cancer treatment) seems to be the best site to graft frozen–thawed ovarian tissue.

Thawed ovarian fragments do not need vascular anastomosis when transplanted in a well-vascolarized tissue. Ovarian tissue is endowed with abundant genes for angiogenesis factors; however, hypoxic tissue damage occurs while waiting for neovascularization, which starts after more than 48 h. The survival of primordial follicles (that is around 90% after thawing) ranges between 5 and 50% after graft [55, 56], the most crucial factor being the degree of ischaemic injury after transplantation. It would be useful to promote angiogenesis [57] and minimize hypoxia after transplantation [52], but administration of angiogenesis-promoting factors (e.g., VEGF) to the recipient does not bring improvements because it stimulates only the growth of a superficial capillary network [40].

To better prevent ischaemic follicular loss it has been proposed to take the intact ovary preserving blood vessels [38]. This technique implies the vascular micro-anastomosis to a vascular pedicle (e.g., the forearm vessels) to preserve perfusion of ovarian tissue. After the end of oncostatic therapy, the whole ovaries may be transposed again with a new reanastomosis to the pelvic vessels. Although promising, this technique is suitable only for patients treated by exclusive pelvic radiotherapy; this is not the usual case for children.

Freezing the whole ovary is also an option: this promising research is still at the beginning and requires some technical adaptations, such as vascular perfusion with a heparinated solution to avoid vascular thrombosis in the ovarian pedicle just before freezing and identification of the optimum cryoprotectant to be used with the whole organ [38].

Risks of Ovarian Tissue Autografting

A major concern associated with autografting of ovarian tissue is the risk of transmitting metastatic cancer cells in a subject that has been successfully treated for the same cancer. Theoretically, cryopreservation of ovarian tissue and autografting is a suitable procedure only in case of malignancies with a very low risk for ovarian micrometastasis. In fact, the risk of transferring cancer cells back to the patient depends on the type of cancer, its likelihood to metastasize in the ovary, and even on the overall mass of transferred malignant cells.

Animal studies showed that the risk of reimplanting cancer cells with cryopreserved–thawed ovarian tissue is concrete: the ovarian cortex derived from a mouse with a lymphoma expressing the donor-specific gene Zfy-1 (a type of lymphoma that is very rare in humans) was able to transmit the specific disease to a healthy female mouse after grafting [58].

Among childhood cancers, a low incidence (<0.2%) of ovarian micrometastasis has been detected in Wilm's tumor, Ewing sarcoma, osteogenic sarcoma, nongenital rhabdomyosarcoma, and lymphomas [59, 60]. On the contrary, both leukemia and neuroblastoma expose the recipient to a high risk (>11%) of cancer cell transmission [59, 61], contraindicating

autografting. Ovarian biopsies from patients with Hodgkin lymphoma were found to be free from cancer cells when examined with the conventional histological procedures [62], and the occurrence of ovarian metastasis is extremely rare in Hodgkin disease according to the clinical practice. The risk of transmitting cancer seems to be extremely low even when grafting ovarian tissue belonging to non-Hodgkin lymphoma patients to SCID mice [63].

At present, leukemia is the most controversial indication for cryopreservation and transplantation of ovarian tissue. It is self-evident that leukemia cells are inside the ovarian vessels at the time of the biopsy and cryostorage maintains their malignant potential, which may be later expressed after thawing and grafting. The collection of ovarian tissue after one or more chemotherapy cycles could lower the risk of disease transmission, but would also result in a loss of primordial follicles and in a lower effectiveness of cryopreservation.

In all cases, but particularly for leukemia patients, it is essential to detect residual micrometastatic disease in the frozen ovarian tissue. A standard histological evaluation is not sufficient to rule out microscopic malignant cell nests [64]. Using polymerase chain reaction (PCR), a single neoplastic cell among $>10^5$ may be detected [65]. PCR, immunohistochemistry, and Northern blot analysis represent the best options to analyze ovarian tissue to identify metastatic cells expressing cancer-specific chromosomal translocations and/or other tumor markers [66].

An alternative approach is the purging of ovarian tissue from tumor cells by means of the insertion of follicle suspension in plasma clots [67, 68]. Ovarian tissue purging from MCF-7 breast cancer cells has been obtained in vitro after incubation of a suspension of MCF-7-contaminated ovarian tissue with cytotoxic T cells retargeting through the bispecific antibody BIS-1 [69]. In this study, a highly efficient cancer cell aggression by T lymphocytes in the presence of BIS-1 was shown, and the fluorescent detection system demonstrated tumor cells depletion after the purging procedure. In the meanwhile, ovarian follicles remained morphologically intact and viable.

Recently, the survival of human follicles isolated from ovarian tissue and individually cryostored has been described [70]. This interesting technique is somewhat in the middle between ovarian tissue cryopreservation and oocyte cryopreservation. Autografting of isolated follicles via injection of a follicle-containing solution under the capsule of the residual ovary brings a risk of malignant cell transmission that is definitely lower than the one occurring with ovarian tissue autografting.

Indeed, a thorough evaluation prior to ovarian tissue autografting is required to confirm the safety of the procedure on an individual basis [71]. In case of a high risk of disease transmission or when metastases are found in ovarian biopsies, alternative options should be considered.

Alternatives to Ovarian Tissue Autografting: Xenografting, in Vitro Follicle Maturation (IVM), Oocyte Reconstruction by Nuclear Transfer

Alternative strategies to ovarian tissue autografting when the risk of transmitting cancer is high are object of active investigation, but still are largely hypothetical. *Xenografting* is the graft of human frozen–thawed ovarian tissue into a host animal to obtain the maturation of human gametes within the animal organism. Several immunodeficient animal lines (e.g., SCID mice) could be used as xenograft recipients. The animal serves as an incubator for human follicle maturation and the occurrence of cancer cell transmission to the host is not a problem [72]. The possibility of transmitting cancer cells through the oocyte to the woman receiving it during in vitro fertilization is practically absent as cancer cells do not penetrate the zona pellucida [73].

Development of human ovarian follicles in host animals receiving xenografted ovarian tissue has reached the antral stage [74–77], and the occurrence of ovulation and corpus luteum formation has been reported [78]. No data are currently available either on the final maturation stages of follicles or on the developmental potential of oocytes derived from these follicles. Even considering nonhuman experiments, no live births have been produced from oocytes derived from xenografted ovarian tissue.

A major concern with xenografting is the transmission of animal-derived zoonoses to the recipient woman through the oocyte. Moreover, the ethical acceptability of obtaining human gametes in a host animal is very low. As a consequence, the clinical application of ovarian tissue xenografting appears to be quite unlikely, at least in the near future.

The technique of *growing in vitro human ovarian follicles (IVM)* would represent a good alternative to autografting, being potentially able to provide mature oocytes without any risk of cancer transmission. Unfortunately, this technique is still in its infancy and a few experimental data have been provided so far.

In the murine model, less than 2% of the primordial follicles may mature in vitro, originating an oocyte able to be fertilized, develop, and reach the blastocyst stage; only one live birth was reported to occur from IVM, and was affected by relevant metabolic disorders, including severe obesity [79].

In humans, the growth of primordial follicles cultivated in vitro was obtained up to the secondary stage, but no more [80]. Major problems in IVM are the length of in vitro culture required to grow primordial follicles to the preovulatory stage (about 80 days), in turn leading to a very high risk of bacterial or fungi contamination, and the poor knowledge about the intraovarian factors that initiate follicle growth before the gonadotropin-responsive phase. Follicle IVM is not risk-free, as epigenetic embryo abnormalities secondary to incomplete methylation of the DNA of immature oocytes have been reported in the mouse [81, 82].

Oocyte reconstruction is an application of cloning technology and is performed via membrane electro-fusion of a germinal vesicle (GV) nucleus (karyoplast) with an enucleated egg cytoplasm (cytoplast). In the mouse, GV stage oocytes reconstructed by nuclear transfer have been shown to undergo meiosis and allow fertilization and embryo development up to the blastocyst stage [83].

In the mouse, cryopreserved immature oocytes have been used as a source of GV nuclei to perform GV transfer with fresh cytoplasts deriving from enucleated, fresh GV oocytes; these reconstructed GV oocytes are genetically normal and able to complete meiosis [84]. It has been hypothesized that GV nuclei from cryopreserved ovarian follicles could be used to reconstruct oocytes with fresh cytoplasts taken from the GV oocytes of routine IVF patients. This possibility is, at present, merely speculative.

Ethical Concerns of Ovarian Cryopreservation

It must be underlined that ovarian tissue harvesting and transplantation in pediatric patients is still an experimental technique. The following issues are major priorities that must be considered when proposing this option to the patients and to their parents [85]:

1. The procedure must not harm the patient by delaying cancer treatment.
2. No remnant cancer cells must be reintroduced with autografting.
3. Damaged cryopreserved oocytes must not be fertilized and implanted.
4. The informed assent from adolescent patients and the informed consent from parents is mandatory; all the potential risks, including the risk of surgery to obtain ovarian tissue and the future fate of the stored tissue, must be thoroughly discussed before going forward; similarly, the chance of obtaining a successful fertility preservation must be clearly prospected on the basis of the available published data.
5. Policies to protect the patient's future rights to use her gametes and addressing the disposition of gametes if the patient dies must be developed.

A recently published normative analysis on ethical issues concerning ovarian tissue cryopreservation for adolescent cancer patients has concluded that more advanced research should be done before young patients can be ethically enrolled in ovarian cryopreservation programs [85]. Indeed, the present technology of ovarian cryopreservation does not guarantee to preserve fertility in these patients, but it gives a reasonable hope to reach this goal and is definitely worth being proposed in highly specialized institutions. According to other authors [86, 87] we think that ovarian cortex banking can be ethically offered to adolescent patients before any oncostatic treatments that bears a high risk of compromising their future fertility.

CONCLUSIONS

Fertility preservation in children and adolescents diagnosed with cancer has become an important area of investigation due to increasing pediatric cancer survival rates. While for sexually mature women there are several options to preserve fertility, for adolescents and childhood patients the most promising way at present is cryostorage of ovarian cortical tissue coupled with autografting after thawing. In the last years, there have been significant improvements in this research area and 2 live births have been reported in women submitted to orthotopic autografting of frozen–thawed ovarian cortical strips [45, 48]. However, too few cases have been performed to allow a correct assessment of the clinical effectiveness of the technique as well as of the risk of reintroducing cancer cells in the patient with the graft. Future studies will determine in a larger number of patients whether an acceptable ovarian tissue functional longevity can be achieved after cryopreservation and grafting, and whether fertility can be restored with acceptable risks of cancer recurrence.

In conclusion, ovarian tissue cryopreservation should be considered still experimental and should be performed only in a few specialized institutions. Moreover, further research in this area is definitely necessary.

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