Ovarian and fertility preservation prior to gonadotoxic treatment : efficacy and safety studies

Hoekman, E.J.

Chapter 7

General discussion and future perspectives

The evolution of assisted reproductive technologies (ART) has facilitated the development of methods and strategies to preserve fertility in patients with disease who need gonadotoxic treatment. However despite optimized techniques and accumulation of data, the evidence with regard to efficacy of most techniques is bounded by case series, which is especially the issue for ovarian tissue cryopreservation. The main purpose of this thesis was to evaluate several aspect of fertility preservation techniques. We studied and evaluated 3 different and clinically promising techniques which can be offered in young children and/or women to preserve fertility. With our studies we aimed to optimize treatment protocols and thereby guiding physicians and patients in choosing the most optimal treatment of fertility preservation.

Ovarian tissue cryopreservation

Ovarian tissue cryopreservation (OTC) is an upcoming and promising treatment option in several parts over the world: in some countries the techniques has already been integrated as an established and even standard technique instead of being experimental. Although OTC and followed by auto-transplantation is still considered an experimental technique in the Netherlands, we showed in **Chapter 2** that auto-transplantation of cryopreserved ovarian tissue is an effective method to restore ovarian activity in 86% of patients and results a live birth rate of 57% (4/7). However, the usage rate of cryopreserved ovarian tissue appears to be very low: only in 8.7% of women who underwent OTC, auto-transplantation is performed. This finding is in agreement with the literature: Rosendahl et al reported a usage rate of only 4.4% in the period 1999-2009.[1] Oktay and Oktem reported 59 cases of cryopreserved ovarian tissue and only 3 transplantations (5%).[2] Given the fact that auto-transplantation is not performed because ovarian function was restored after gonadotoxic treatment, an important question is: Do we perform ovarian tissue cryopreservation too often?

In **Chapter 2,** we showed that especially in breast cancer and perhaps in osteosarcoma patients, the risk of POI after gonadotoxic treatment was much lower than estimated. One can argue that fertility preservation is not necessary in these patients because of the low risk of POI. Additionally, in the meantime, new fertility preservation techniques have been developed like oocyte vitrification and in vitro maturation. Moreover the knowledge of the adverse risk of ovarian hyperstimulation on breast cancer patients has expanded.

The high number of patients that undergo OTC because of breast cancer in our study is probably due to the available options of fertility preservation at that particular moment (our cohort included 69 women from 2002 until 2015). Oocyte cryopreservation has been an option in the Netherlands from 2011 onwards. Additionally, because an high-estrogen environment level obtained by superovulation was not considered safe for breast

cancer patients at that time, thus superovulation was considered not to be an option in these patients. Therefore OTC was the only suitable suggestion in the early years of our cohort. However, more recently, multiple studies demonstrated the positive effect of the additional administration of Tamoxifen/ Letrozole to induce a lower peak estradiol level during superovulation for oocyte cryopreservation. Furthermore, it has been proven that superovulation, in combination with Tamoxifen/ Letrozole does not negatively affect survival in hormone receptor positive breast cancer patients which also provides a prolonging time schedule for fertility preservation. Therefore, cryopreservation of oocytes and/or embryo's has become a valid and safe option for fertility preservation in these women. This allows women to choose between the different techniques.[3-7]

The other patient group without reporting POI, are the patients who were treated for osteosarcoma during their childhood: 2 patients out of 5 already conceived naturally and delivered healthy babies. Overbeek et all, showed that hormonal markers of ovarian reserve (e.g. AMH and FSH), appear to be unaffected by cancer treatment in the majority of childhood cancer survivors (CCs). However, the proportion of CCs with abnormal markers increased significantly above the age of 35 years, especially those who were treated with pelvic radiation therapy (with or without additional alkylating chemotherapy). This implies that although ovarian function appears intact long after childhood cancer treatment, CCs remain at risk of a reduced reproductive life span.[8] Whether in childhood OTC and additionally auto-transplantation or oocyte cryopreservation, is the best solution in this reduced reproductive live span, is an important question that does not have a straight answer yet.

An often discussed disadvantage, with regard to reproductive life span is the issue that after unilateral oophorectomy for OTC the total amount of primordial follicles is reduced. However, it has been reported that after unilateral oophorectomy, menopause is induced only 1-1.8 years earlier.[9-11] Thus the argument of inducing POI by unilateral oophorectomy does not stand. One can argue unilateral oophorectomy solely because of OTC prior to cancer treatment, because of possible gonadotoxicity is unethical. All these considerations should be discussed in order to allow informed choice.
The other question about OTC, is the efficacy of ovarian transplants. Our study showed a duration time of restored ovarian function up to 57 months, whereas in the literature the ovarian function last up to 4-5years after auto-transplantation.[12-16] Therefore, multiple transplantations may and can be required to extend the duration of ovarian function.[14] In general, a survival rate op 50-80% of primordial follicles after freezing and thawing, has been demonstrated.[17, 18] The limited survival time of the graft may be attributed to the restricted amount of tissue that is transplanted, uneven follicular distribution as well as suboptimal cryopreservation/thawing protocols.[19] Also, chemotherapy before harvesting ovarian tissue compromises the longevity of the graft.[20] However, the major factor behind

the short life-span of the graft seems to be injuries during the extended time (several days) of warm ischemia after auto-transplantation.[21-26] Although, it has been showed that after xenotranplantation of human tissue, the vascularization seems to be completed within less than five days, and the local oxygen pressure in the tissue continues to increase for a period of 10 days.[27] From results after experiments in a rat model showed that physical manipulation of the transplantation site shortens the ischemic period of transplanted ovaries.[28] Also other methods to decrease the damage of the ovarian tissue due to ischemia-reperfusion have been suggested.[29-33] Incubating the graft or host with hyaluronic acid -rich biological glue combined with VEGF-A and Vitamin E gave better results.[34-36] More research is needed to optimize the survival of primordial follicles in frozen ovarian tissue and the longevity of the graft to increase the duration of ovarian function restoration after auto-transplantation. A major concern is the risk of reintroducing residual disease (undetected ovarian metastasis) into the patient. Ovarian metastases have been described for multiple malignancies: breast cancer, lung cancer, renal tumours, gynaecologic tumors, Ewing's sarcoma, Hodgkin's lymphoma, Non-Hodgkin's lymphoma, biliary duct cancer and other cancers of the gastrointestinal system.[37-43] In our cohort, at closure of follow up, none of the transplanted patients showed clinical evidence of ovarian metastasis in the ovary in which the cryopreserved ovarian slices were transplanted**.** This is without doubt due to the strict inclusion criteria: only women with low risk of ovarian metastasis are offered OTC. Despite strict inclusion criteria, the concern of reintroduction of cancer may limit the usability of OTC and auto-transplantation for cancer patients.

In the table below, the risk of ovarian involvement is displayed for different malignancies. In theory this risk resembles the risk for reintroduction of malignant cells after cryopreservation of ovarian tissue. Remarkably, in general the risk of metastases of breast cancer patients is negligible. At our institution OTC is offered only women suffering from a cancer in which the risk of ovarian metastases is < 0.2%.

Table 1. Risk ovarian metastases

< 0.2%	0.2-11%	>11%
Wilm's tumor	Coloncarcinoma	Leukemia
Non-Hodgkin	Breast cancer (lobular type), stage IV	Neuroblastoma
Squamous cell carcinoma of the uterine cervix	Lymphnode invasion/ adeno Cervical carcinoma	Burkitt's lymphoma
Nongenital Rhabdomyosarcoma		
Osteosarcoma		
Ewing's sarcoma		
Hodgkin's lymphoma		

Oktay K, Hum Repr. Update. 2001-4 (combined articles)

Furthermore, a question mark can be placed at the potential of Isolated Tumor Cells (ITC's) or micro-metastases to develop into a tumour after transplantation. The biological significance of ITC's and micro-metastases is a continuous discussion in the literature. For example in breast cancer patients, ITC's in the sentinel lymph node may have nonprognostic significance with respect to survival.[48] However, despite the reports of the non-prognostic significance of ITC's, studies have shown that undetected micrometastases in the ovaries of mouse models of Hodgkin's lymphoma and leukemia are able to develop into tumors.[7,44, 45] Therefore the finding of ITC's should not be ignored and tissue should not be used for transplantation. At last, it has been reported, that in case of organ transplants, transplantation of tumour cells has some potential to develop into tumours within the recipient.[46,47]

Given the concern about reintroduction of cancer we developed a tailor-made approach by identifying patient-tumour-specific markers. This resulted in the detection of malignant cells in the Fallopian tube in one case (1/47). However this ovarian spread had already been expected since this case was a women with peritoneal metastasis of esophageal carcinoma **(Chapter 3)**. In our perspective, in order to minimize the risk of transplanting ovarian metastases, it is recommend to analyse the remaining ovarian tissue after dissection and preparation of the cryopreserved ovarian slices by the pathologist.

More recently a new technique has been developed to bypass the risk of introducing ovarian micrometastasis: the use of *artificial* ovaries.[49-52] These ovaries consist of isolated pre-antra follicles assembled in a structure 3D matrix which allow follicles to grow and develop. Once transplanted to the patient, this *artificial* ovary would potentially restore fertility and endocrine function. Because of the absence of ovarian tissue, there is no risk of reintroducing undetected ovarian metastasis. Another introduced option is xenotransplantation of human ovarian slices, harvested at OTC, in immunodeficient mice. However this method requires animals, time, money and demanding infrastructure. Moreover it is unknown after how much time after xenotransplantation recurrent disease can be detected.

Given the absence of valid other options, the combination of strict indication with the tailor-made approach described in **Chapter 3** seems to be the most acceptable method to minimize the risk of transplantation of residual disease at this time. Therefore in our opinion the remaining ovary is best site to transplant ovarian tissue into, which offers the woman the possibility of a natural conception, follow up of the ovarian tissue by ultrasound is easy and removal for the transplanted chips after completion of family, if indicated, is easily performed by oophorectomy.

Ovarian transposition

We showed that ovarian tissue cryopreservation and consequent auto transplantation is safe, feasible and effective in selected women at risk of premature ovarian insufficiency because of scheduled administration of gonadotoxic treatment. However, in patients requiring pelvic or total body irradiation another technique to preserve ovarian function may be preferred: ovarian transposition.

Despite the fact that ovarian transposition (OT) has been performed for more than 50 years, there is only minimal data on the effectivity of OT. Our data in **Chapter 4,** showed that OT prior to pelvic radiation therapy (PRT) result in a significant increase in ovarian survival (OS) after pelvic radiation: 5 years OS 60.3% versus 0.0% respectively with and without OT (p = 0.00). Furthermore, additional brachytherapy and/ or low dose chemotherapy (as part of chemoradiation therapy) had no significant impact on ovarian survival. Previous studies have shown a correlation between age, the dosage of pelvic radiation and ovarian failure. Hamish et al. described ovarian failure at the age of 10 after exposure to 18.4 Gy but at the age of 30, ovarian failure occurred after exposure to 14.3Gy.[53] Although we found in our study that OT is effective especially until the age of 35, we should offer shared decision making in women aged until 40, especially since the age for attaining the first pregnancy is increasing.

Published data describe a wide range of ovarian survival after OT and RT. Therefore, we performed a systematic review in order to conclude on the efficacy of OT based on results of all reported studies **(Chapter 5)**. We found that ovarian survival that varies from 20-100% (38 studies, 765 patients). We also concluded that there was a significant and relevant heterogeneity between studies. Moreover most of the published data suffered from methodological flaws since most studies are case series and non-comparative. We found a most favourable outcome of ovarian survival in patients after OT and Vaginal Brachy Radiation Therapy (VBRT) with a range from 63.6-100%, thereafter OT prior to PRT, and at last OT prior to RT combined with chemotherapy. Thus, patients should be selected carefully regarding to treatment regimen, radiation field and transposition site to maximize benefits

versus side effects and complication ratio. Finally, it is important to bear in mind that recent improvements in image-guided and more tailor-made radiation therapy regimens may possibly improve ovarian survival rates, both after and without OT. Studies of cohorts of women that underwent OT prior to the current targeted radiation techniques are warranted, in order to be able to inform patients accordingly.

The most valid explanations concerning OT-failure, is the risk of ovarian migration after OT.[55] A second explanation of failure of OT is the diminished blood supply to the transposed ovaries due to the formation of scar tissue after radiation therapy, manipulation during surgery, or kinking of the infundibulopelvic ligament.[56] At last, it has been shown that, due to scattered radiation, a substantial loss of ovarian function might still occur despite adequate and persistent transposition.[57-59] Van Beurden et all showed that on the edge of the radiation field the dose amounted to 50% of the midline dose.[60] At 1 cm outside the lateral border the dose decreased to 14%, at 2cm to 9% at 3cm to 7% and at 4 cm to 5% of the midline dose. Unfortunately, we were not able to establish firm conclusions about the optimal location of the ovary prior to radiation therapy. This inherently means that the safest position of the ovary is as far cranio-lateral as possible, accompanied by a radio-opaque marker to identify the ovary prior to the radiation therapy. In case of pelvic (Non-) Hodgkin's lymphoma, the ovaries were often sutured at the uterus, with additionally a lead shield placed outside the abdomen due to a higher risk of receiving scatter radiation.

Besides ovarian failure, as known the uterus could be affected by radiotherapy and as a consequence radiation-induced damage, which is irreversible. Although there is no clear data indicating the dose of radiation to the uterus, it has been suggested to avoid attempting pregnancy after >45Gy irradiation therapy during adulthood and >25Gy in childhood, although possible uterine damage after 12Gy also has been described.[61-64] There have been some publications of spontaneous pregnancies after ovarian transposition and radiation therapy. However, these were only case series/ reports, and almost all patients were treated for M. Hodgkin and irradiated with an inverted Y field with the use of an lead shield minimizing the radiation dose at the uterus.[65-69] Pregnancies after other cancer types have been less successful: only one study reported successful pregnancies in women with pelvic / vaginal cancer.[70] Morice 1998 reported 12 pregnancies treated with surgery, OT and RT (and in some cases chemotherapy), in which the received radiation dose by the ovaries was expected to be 1.9Gy and the radiation those received by the uterus was not mentioned. Kurt and Cantor both reported a pregnancy after radiation therapy and ovarian transposition (because of rectal carcinoma and aneurysmal bone cyst respectively), of which the pregnancy resulted in immature delivery after 21 weeks of pregnancy.[71, 72] In case of desire to have children in patients with a radiated uterus, surrogacy can be an option. Only 4 case reports have been published, concerning surrogate pregnancy after ovarian transposition and pelvic radiation.[73-76] Despite a lack of data, the poor ovarian response to

ovarian stimulation and additional higher risk of pregnancy complications, surrogacy is the only option for both genetic mother and child in these women.[77] Thus ovarian transposition in means of fertility preservation offers the possibility genetically own children, however surrogacy will be needed.

With regard to complications after ovarian transposition, we found a small risk of complications **(Chapter 4, 5)**. The incidence of ovarian cysts was 10% (versus 2.5% life time risk of ovarian cysts in the normal population). This should be mentioned during counselling.[78]

Vitrification of oocytes

At start of our research, oocyte vitrification (ultra-rapid freezing of oocytes to minimize oocyte damage) was a fast developing technique in its early stage. This technique offers several advantages: a surgical procedure for ovarian transposition oophorectomy is not necessary, there is no need to create embryo's for cryopreservation, and above all, it does not carry the risk of reintroducing micro-metastases. Therefore cryopreservation of oocytes is an important option for women seeking possibilities to preserve fertility.

In general, technical skills are needed before implementing a new technique. We showed in **Chapter 6** how to optimize a learning-curve before the process of oocyte vitrification. Especially the process of thawing and warming needs to be trained and practised before augmentation and implementation of a vitrification program can be obtained. Based on our results in **Chapter 6,** we advise during training programs to start with non-human material such a mouse blastocysts. Compared to training models with non-biologic material, mouse blastocytes do have biological activity which can be accessed to evaluate the effectiveness of the process.

It has been estimated worldwide that more than thousand babies are born from cryopreserved oocytes.[74-81] However, it appears that the majority of the live births were from egg donor programs rather than from women treated with gonadotoxic treatment leading to POI.[82] Additionally, the effectiveness of oocyte vitrification prior to gonadotoxic treatment in cancer patients seems to be low: life birth rate is 6%.[79] Given the fast implementation of oocyte vitrification in women in whom gonadotoxic treatment is scheduled, the need for more data on the effectiveness of this technique is needed to draw firm conclusions. There has been much debate about the possible risks and side effects of this procedure, like ovarian hyperstimulation syndrome. Furthermore, the fear of major birth defects is not increased (1.3% vs 0.2%-2%).[80] Moreover the risks of the super-stimulation as well as complications as results of the follicle puncture are small (0.15%).[81]

Conclusions

In this thesis we showed that ovarian tissue cryopreservation can be performed in patients with various primary, malignant and non-malignant, diseases, however, low usage rates. This can possibly be improved by excluding breast cancer patients and young osteosarcoma patients as indication for preservation. Ovarian tissue (auto-)transplantation seems to be a safe and highly effective procedure to restore fertility, and a current preoperative tailor-made clinical risk assessment to reduce the risk of reintroducing malignant disease, seems to be a good alternative procedure in absence of other techniques.

The experimental status of ovarian tissue cryopreservation in the Netherlands, can be discussed and adapted as a standard treatment before the start of gonadotoxic therapy.

In women who need pelvic radiation therapy, ovarian transposition should be offered to preserve ovarian function to all fertile women below the age of 35 and has to be discussed in women age 35-40. Despite the low number of patients and the retrospective design of the study, we found >50% ovarian survival after 5 years. Furthermore, taking all considerations and pitfalls into account, ovarian transposition is a safe procedure, due to a small risk of complications. Moreover it can be considered in various cancer types, however taking the risk of ovarian metastasis into account. Furthermore, Intensity-Modulated Radiation Therapy (IMRT) and Volumetric Modulated Arc Therapy (VMAT) allows the radiotherapist to deliver radiation doses only to target tissues with reduced scatter radiation.[82-85] Thus, improving ovarian survival rates can update the data evaluating the effectivity of OT will be necessary.

A growing amount of hospitals all over the world, introduce the option of vitrifying oocytes. It is known that the vitrification procedure has been developed faster and most problems consequently have been identified during the process of thawing and warming of the oocytes. However, even using a well-tested commercially available protocol for vitrification and warming, such a protocol is still learning-sensitive. We showed that there is a learning-curve towards implementation of vitrification procedure, and therefore, we recommend skills-training before implementation, special attention has to be paid to the thawing and warming part of the process. Given the low success rates after oocyte vitrification, even after the recommended skills-training, new data should improve these rates in anticipation of new developments.
To date, only a few live births resulting from the cryopreservation of oocytes at the immature germinal vesicle stage or after In vitro maturation have been reported.[86-89] At last, developing techniques of in vitro maturation of primordial follicles in frozen ovarian tissue will overcome the risk of reintroducing metastasis and will provide numbers of oocytes to

become fertilitzed which will increase the chance of getting pregnant of genetically own children in women who were treated with gonadotoxic therapies.

Future perspectives

To improve efficacy and safety of ovarian and fertility preservation prior to gonadotoxic treatment, future perspectives regarding indication, quality assessment, safety, and the use of minimally invasive surgical techniques are obligatory. Research in this field enhances the following issues:

- OTC has been performed for various types of cancer and non-malignant diseases. However, future (additional) indications can be broaden to preserve ovarian tissue were ovaries are 'affected' by diseases such as endometrioses, benign ovarian (dermoid) tumours, BRCA1/2 mutations, or even malignant ovarian diseases, in which the total ovary needs to be removed.
- The development of a more precise risk assessment with regard to POI in combination with techniques to minimize reintroduction of malignancy will improve the efficacy and cost-effectiveness of ovarian transposition with consequent auto transplantation even more.
- Image-guided surgery using near infrared fluorescent probes (NIRF) is a new and promising technique that can be used to visualize structures/cells in real-time.[90] This technique will leave the tissue fragments unaffected and suitable for auto-transplantation purposes to restore fertility in cancer patients. Further development is needed and can be a perfect tool to rule out micro-metastases in tissue that will be auto-transplanted.
- Additionally, further development of full-field optical coherence tomography (FF-OCT) to improve depth (>100 μm) in tissue can be used to visualize normal ovarian structure and micro-metastases [91] and the distribution of oocytes prior to transplantation which is needed to improve effectiveness of ovarian tissue auto-transplantation.
- Prior and after auto-transplantation, studies concerning qualitative and quantitative-measurement of follicle survival and ovarian tissue vitality are needed, to optimize the success and survival time of the graft after ovarian tissue auto-transplantation.
- According to the malignant ovarian diseases and carriers of BRCA1/2 mutation, further development of in vitro maturation techniques of primordial follicles and/or the use of an "artificial" ovary, will bypasses the risk of reintroducing malignant cells.
- To improve the longevity of the grafts further in vivo research is needed to shorten the warm-ischemic period after auto-transplantation.
- Simplifying the technique of OTC. For example, the established technique of harvesting the ovarian cortex is to perform an oophorectomy. Perhaps, needle expiration of oocytes or ovarian cortex biopsy will be the future in OTC.

- Subsequently, the use of a medical device to auto-transplantant ovarian tissue by only punching the tissue into the remained ovary, will prevent unnecessary operations.
- Development and the use of IMRT/ VMAT will hopefully reduce the scatter radiation received by the transposed ovaries.
- Another cause of failure of ovarian transposition is the migration of the ovary to the pelvis. Whereas ovarian tissue only needs revascularization, why not perform ovarian tissue transplantation outside the radiation field?
- All this future research questions also includes a close cooperation with all the fields concerning fertility preservation, e.g. geneticist, embryologists, engineers and bioscientists.

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