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Ovarian tissue cryopreservation - new opportunity to preserve fertility in female cancer patients

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Summary

Introduction: Malignant disease and the therapy are major factors that may result in complete loss of fertility. There are several strategies for fertility preservation in fertile women faced with cancer. A modern and potentially effective method of reproductive function protection is ovarian tissue cryopreservation.

Materials and Methods: This paper summarizes the medical and scientific knowledge in this interesting multidisciplinary medical field. Furthermore, the authors' own experience with this novel and interesting method of ovarian tissue protection is presented. Ovarian tissue was obtained during laparoscopic surgery in five nuliparous women (aged 19-33) with a diagnosis of lymphoma before chemotherapy from 2004 to 2006. After laboratory preparation, tissue was frozen by a slow cooling technique and stored in liquid nitrogen.

Results: In total 75 women with malignant lymphoma before chemotherapy were referred to our center for consultation - 68 chose ovarian inactivation by GnRH analogues during chemotherapy, two IVF cycles with embryo or oocyte cryopreservation and five ovarian tissue cryopreservation. In these five women one to two slices of ovarian cortex from both ovaries were recovered. Totally 20 cryotubes with three pieces of tissue in each were cryopreserved. In no case was metastasis of cancer cells found by histological evaluation.

Conclusions: Cryopreservation of ovarian tissue represents an effective alternative or addition to the cryopreservation of embryos or oocytes for women at risk of premature ovarian failure due to chemotherapy. Reproductive function protection requires close cooperation between oncology departments and assisted reproduction centers.

Key words: Cancer, Infertility, Chemotherapy, Ovary, Cryopreservation.

Introduction

After cardiovascular diseases, tumor diseases are the second major cause of death in both men and women of reproductive age despite significant developments in cancer diagnostics and therapy. According to data published by the American Cancer Society, 600,000 women are newly diagnosed with cancer in the USA every year, of whom 60,000 (10%) are of reproductive age [1]. The situation in the Czech Republic is similar. According to data collected by the Czech National Oncology Registry (NOR), 58,000 women of reproductive age were diagnosed with cancer in 2003 [2]. Newly developed diagnostic methods are increasingly effective at detecting cancer growth at earlier stages and due to advanced methods of chemotherapy and radiotherapy the number of patients who have been cured successfully or who are in long-term remission of the disease is on the rise.

The American National Cancer Institute (NCI) has estimated that in 2010 one in 250 adults will have a history of successful treatment of a malignant tumor [3]. Chemotherapy as one of the basic modalities of cancer therapy often leaves permanent side-effects in patients including infertility as a result of irreversible damage to the gonads [4]. The current methods of assisted reproduction can give cured cancer patients a chance of having their own children. In addition, intensive efforts over the past few years have also been made to develop procedures to prevent infertility during the course of cancer therapy.

Materials and Methods

Chemotherapy and infertility

The mechanism of the action of chemotherapeutics on ovaries has not yet been fully explained. Most theories assume the toxic effect on membrana granulosa cells or the oocyte leading to the terminal atresia of the follicle. Cytostatic agents largely interfere with the cell cycle of fast growing cells, including membrana granulosa cells, the division of which takes place under the control of pituitary gonadotropins. There is some indirect evidence that gonadotropic stimulation in particular is a prerequisite for the effect

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of a chemotherapeutic agent on the ovary. The administration of alkylating substances results in the fast destruction of follicles with the subsequent maturation of immature follicles and accelerated depletion of primordial follicles [5].

Cytostatics differ in their gonadotoxic effects. Whereas alkylating cytostatics are some of the substances with the most aggressive effects, methotrexate and 5-fluoruracil show minimal adverse effects on the follicular apparatus of ovaries or spermatogenesis. Clinical consequences of ovarian damage in women of reproductive age are manifested as secondary amenorrhea or oligomenorrhea, which may be corrected spontaneously after the completion of therapy. Some women, particularly those over 30 years of age, may show permanent amenorrhea and may develop premature ovarian failure with a hormone profile indicating hypergonadotropic hypogonadism [6].

The risks of premature ovarian failure in women who have undergone chemotherapy depend on the patient's age, gonadotoxic agent used, therapeutic regimen, and the total cumulative dose. An overview of gonadotoxic chemotherapeutics is provided in Table 1 and the cumulative gonadotoxic dosage of selected chemotherapeutic agents is presented in Table 2.

Table 1. — *Overview of gonadotoxic cytostatics.*

Alkylating substances	Cyclophosphamide Chlorambucil Melphalan Busulphan Carmustine (BCNU) Lomustine (CCNU) Methoxamine
Anti-metabolites - platinum derivatives, Vinca alkaloids, Taxanes	Cisplatine, carboplatine Vinblastine, Vincristine Paclitaxel Docetaxel Cytosine arabinoside Procarbazine

Table 2. — *Cumulative dose of cytostatics that induced permanent azoospermia in more than 50% of patients.* Adapted according to Schrader M. *et al.*: "Oncology", 2001, *24,* 326 [32].

Cyclophosphamide	7.5 g/m^2
Procarbazine	2.5 g/m^2
Chlorambucil	1.4 g/m^2
Vincristine	1.0 g/m^2
Paclitaxel	1.3 g/m^2
Cisplatine	4.0 g/m^2
Carmustine	2.5 g/m^2

Gamete damage prevention methods

Sperm cryopreservation prior to cancer therapy has been a routinely used method for protecting male fertility at our department for a number of years [7]. This procedure is frequently employed by physicians in the majority of clinical fields, which shows that both the professional and non-professional communities are well aware of it. Oocyte cryopreservation in female patients appears to be an analogous method to the above-mentioned sperm cryopreservation in male patients. This method has undergone intensive development particularly over the last ten years. However, its routine use in practice is prevented due to a relatively low success rate in achieving fertility from cryopreserved oocytes [8]. Technological procedures of cryopreservation and thawing, which would be capable of preventing zona pelucida hardening and damage to the assembly of microtubules (meiotic spindle, which an essential factor for completing meiosis in the mature oocyte) [9], are currently being investigated very intensively.

Another clinically used procedure to protect fertility prior to cancer therapy is the performance of an IVF cycle with fertilization followed by the cryopreservation of embryos after they have been cultured in vitro [10]. This method has been well elaborated from a technological point of view and has been introduced in practice. However, its use is limited by several serious conditions. One of the most limiting factors is that cancer in a patient must be detected at a relatively early stage and the patient should be in good health allowing postponement of the initiation of cancer therapy by approximately three to four weeks in order to undergo ovarian stimulation followed by oocyte retrieval. Another limitation that is of importance from a legislative and ethical perspective is the existence of the patient's partner who should give his written consent for the performance of an IVF cycle and provide his sperm to be used to fertilize the patient's retrieved oocytes.

Ovarian tissue cryopreservation (OTC) is an analogous method to the cryopreservation of sperm, oocytes, and embryos. This method has been investigated intensively over the last few years. One of the major advantages of this method is that it can be performed during clinical staging of the tumor disease without the need to postpone the start of anticancer treatment. Another huge advantage of this method is that the patient does not need to undergo hormone treatment, which could affect the biological behavior of some types of malignant cells.

All of the above-mentioned methods are based on the developed AR methods and knowledge obtained in the cryopreservation of human gametes and embryos. With their significant germinant potential, gametes are predetermined to become the first victims of aggressive chemotherapy. The process of folliculogenesis and the maturation of an oocyte are controlled particularly by hormones. Gonadoliberin analogues (GnRH-a) belong to a group of clinically used drugs capable of affecting these processes. Pilot studies show that by using these drugs the development of oocytes can be stopped at the stage of primordial follicles (as seen in the prepubertal development of a female). The resulting oocytes inhibited in this way will then show a significantly lower sensitivity to chemotherapy [11]. The protective effect of GnRH analogues on ovarian tissue is now being verified in a prospective cohort study in patients with Hodgkin's lymphoma (being conducted at our department).

In 2004, the birth of the first healthy child after orthotopic autotransplantation of ovarian tissue was reported [12], showing the clinical utilization of the procedure and giving young women diagnosed with malignant tumors a chance to have their own children. The following text contains an overview of the knowledge and successful treatments achieved in this interesting multidisciplinary area.

Indication for ovarian tissue cryopreservation

Ovarian tissue cryopreservation is an alternative to the prevention of ovarian damage or failure in patients who have recently been diagnosed with a tumor disease and who should undergo chemotherapy as soon as possible [13, 14]. The main goal of the whole

process is to offer a patient who has completed anticancer treatment and shows no signs of remission of the basic disease the possibility of reimplanting the cryopreserved ovarian tissue either into the small pelvis or into another part of her body (heterotopic autotransplantation).

In addition, this method can also be employed for a number of benign systemic diseases that require cytotoxic chemotherapy (e.g., systemic lupus and some other diseases, particularly diseases of the immune system). Furthermore, this method can be used in some ovarian diseases such as recurrent ovarian cysts and extensive pelvic endometriosis [15, 16].

Ovarian tissue cryopreservation procedure (OTC)

This topic has been addressed in a number of experimental studies over the past ten years. In their 1996 pilot study, Hovatt and Newton tested cryopreservation procedures using different cryoprotective agents and confirmed that ovarian tissue resists deep freezing [17, 18]. Later, Newton and particularly Gook found that it is primordial follicles that show the greatest resistance to the overall freezing and thawing process, allowing mature MII oocytes to develop from originally frozen and subsequently thawed ovarian tissue implanted in immunodeficient mice [19, 20].

Until now, successful transplantation of ovarian tissue that underwent a freezing-thawing cycle has been reported not only in mice, but also in sheep, rabbits, and marmoset monkeys [21-23]. These studies show a clear correlation between tissue damage and the duration of ischemia during tissue retrieval and processing. The decrease in the number of primordial follicles due to hypoxia was estimated to be 50-65%. Some of the studies indicate that the cells of ovarian tissue are able to survive ischemia for a period of up to three hours.

The potential use of substances such as antioxidants (ascorbic acid) and vascular endothelial growth factors (VEGFs), which would allow a decrease in the number of cells damaged due to cryopreservation [24], is currently under investigation.

These positive results have prompted the development of cryopreservation procedures and protocols in human medicine. Two basic types of cryopreservation protocols differing by cryoprotectant used are currently being utilized. Their advantages and limitations are summarized in Table 3. Evaluation of the success of cryopreservation procedures (the survival of primordial follicles and the rate of fibrotization of the graft after transplantation) is based on methods of histological examination of tissues.

Table 3. — *Cryopreservation protocols - ovarian tissue cryopreservation.* Adapted according to Gook DA *et al.*: *"European Journal of Obstetrics, Gynaecology and Reproductive Biology"*, 2004, *113S*, 41 [33].

Protocol with dimethyl-sulfoxide (DMSO)	Protocol with 1,2-propanediol (PROH)
Developed in 1994	Developed in 1996
Ovarian cortical strips with a thickness of 1 mm	Ovarian cortical strips (2 mm x 4 mm x 1 mm)
Dehydration: solution 1.5M DMSO + 10% Leibovitz L-15 medium, 15 minutes	Dehydration: solution 1.5M PROH + 0.1M sucrose-phosphate + 10 mg/ml human serum albumin, 90 minutes
Slow freezing: 2°C/min to –7°C, 0.3°C/min to –40°C, 10°C/min to –140°C, stored in liquid nitrogen	Slow freezing: 2°C/min to –7°C, 0.3°C/min to –30°C, 50°C/min to –150°C, stored in liquid nitrogen
Fast thawing: 2 min in air 24°C, 3 x 2 min in water bath 22°C	Fast thawing: 2-3 min in water bath 37°C

Table 4. — *Patients who underwent ovarian tissue cryopreservation at the Clinic of Gynaecology and Obstetrics in Brno.*

Patient no.	Age	Parity	Cancer diagnosis	Subsequent canter therapy	Dispenz.
1	24	0	NHL	CHT(R-CHOP) + RT 30 Gy	Relapse
2	19	0	HL	CHT (BEACOPP) + RT 30 Gy	Remission
3	31	0	HL	CHT (ABVD) + RT 35 Gy	Remission
4	28	0	Ca cerv. uteri	Radical trachelectomy + RT	?
5	22	0	HL	CHT (BEACOPP) + RT	?

HL: Hodgkin's lymphoma; NHL: Non-Hodgkin's lymphoma; CHT (x): chemotherapy (regimen); RT: radiotherapy.

In 2004, the first euploid embryo generated after autotransplantation of a frozen sample of ovarian tissue followed by the birth of a child after orthotopic transplantation in a patient treated for lymphoma [12, 25] was reported. In the latter paper [25], the ovarian tissue was transplanted orthotopically, i.e., in the area next to the original ovary destroyed by chemotherapy. The patient showed sporadic ovulation. It is therefore possible from a theoretical point of view that the pregnancy might have resulted from the original ovary left in situ rather than from the transplanted ovarian tissue. In their paper published in 2005, Israeli authors reported the pregnancy and birth of a healthy child after orthotopic autotransplantation of ovarian tissue followed by an IVF cycle in a 28-year-old woman three years after she had undergone chemotherapy for non-Hodgkin's lymphoma [26].

The whole OTC procedure is more complex than the cryopreservation of an embryo or oocyte. Unlike routine freezing of gametes, it resembles procedures of the cryopreservation of organs and other tissues (e.g., kidneys, liver, etc.) used in transplantology, as it requires the preservation of more types of cells. More than 90% of ovarian cortical cells consist of primordial follicles surrounded by a layer of granulous cells and fibrous tissue. Most follicles are located up to 1 mm under the cortical epithelium of the ovary. To use this tissue for transplantation and preserve the growth potential of follicles it is assumed that the integrity of the adjacent structures of ovarian tissue should also be preserved.

Tissue retrieval and processing

Laparoscopy in combination with short-term general anesthesia is the best way to perform biopsy. Due to the localization of primordial follicles in the ovary, several slices of the ovarian cortex with a thickness of 1-3 mm have to be collected. The total volume of the tissue collected and the extent of resection has to be decided. The literature describes several procedures ranging from the removal of the whole ovary or its parts to the collection of small samples of tissue using bioptic forceps. The optimum method with

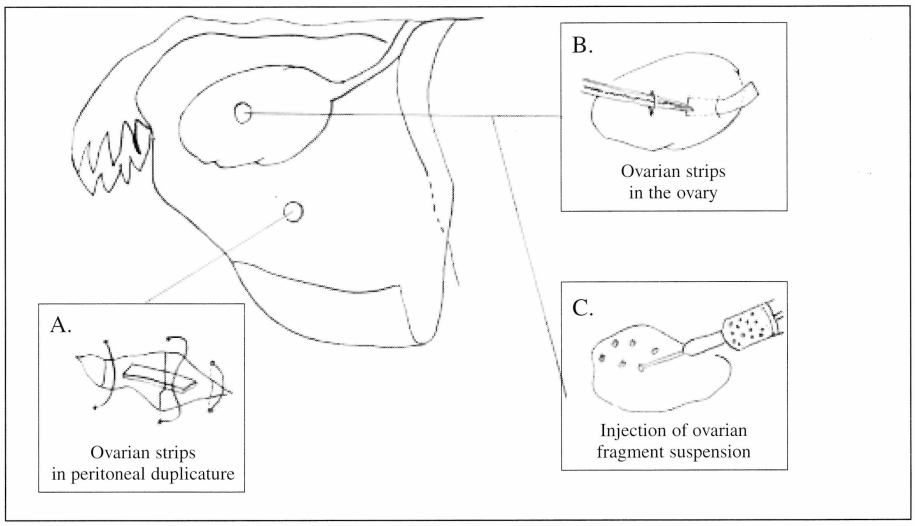

Figure 1. — Orthotopic ovarian tissue transplantation techniques (own material).
A) Ovarian strip placement in peritoneal duplicature; B) Placement of ovarian strips in the ovarian cortex; C) Injection of ovarian fragment suspension into ovarian cortex.

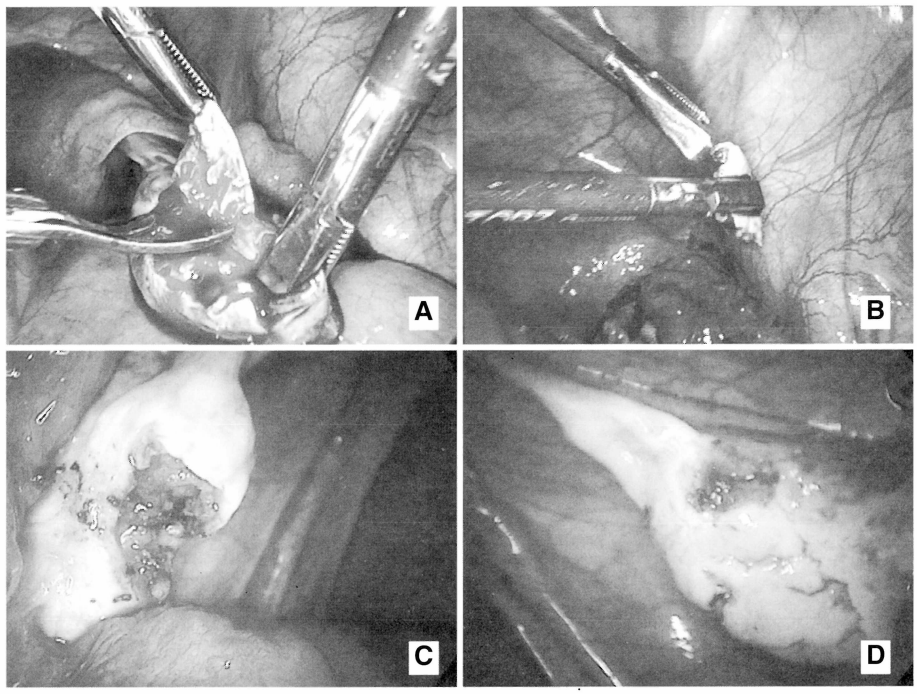

Figure 2. — Laparoscopic biopsy of ovarian tissue (own materials).
A) Cortical ovarian biopsy; B) The sample collected; C) The ovary after biopsy and coagulation of the edges of the wound; D) Random ovarian biopsy after treatment by coagulation.

regard to further processing appears to be the retrieval of two to three strips of tissue from both ovaries followed by processing to smaller pieces sized approximately 10 x 3 x 2 mm ("cortical strips") prior to cryopreservation.

The duration of ischemia is the principal limiting factor for the survival of follicles in the bioptic sample. To minimize ischemia-induced damage in experiments with animal models, the perfusion of the whole ovary was performed using cryoprotectants followed by freezing of the sample as a whole [27]. However, observations made by the authors showed that survival rates of follicles after freezing of the whole ovary did not differ much from those obtained using the method of freezing cortical strips.

Ovarian tissue autotransplantation

Methods of ovarian tissue autotransplantation in humans can be divided into orthotopic and heterotopic. Orthotopic transplantation means that the graft is placed in the small pelvis in the ovarian fossa or directly in the hormone-inactive ovary. Samples can be

placed either in the peritoneal duplicature and fixed by a suture or directly in the ovary using different techniques [12] (Figure 1). The most promising appears to be placement of the ovarian strips directly to the ovarian cortex and fixation through a suture (Figure 1B) [26].

Some authors have also demonstrated that compared to other regions (subcutis, muscle tissue) peritoneal tissue is better suited to transplantation since the loss of primordial follicles is smaller [28]. One disadvantage of orthotopic transplantation is that the surgery is more invasive and sometimes requires laparotomy. However, use of the laparoscopic approach prevails, particularly due to the advanced nature of this surgical method.

Heterotopic transplantation of the graft into hypodermis is another option. With this option the surgery is a far less invasive procedure, without the need of general anesthesia in most cases. Transplantation techniques into the subcutis of the forearm and hypogastrium have been described. Studies with monkeys have also confirmed the ability of the ovarian graft to form mature oocytes in a foreign environment of subcutis outside the small pelvis. Human medicine has succeeded in developing a diploid embryo in the autotransplant but pregnancy after embryo transfer has not yet been achieved [29].

Monitoring and further use of the autotransplant

Restoration of the menstrual cycle in a patient with iatrogenic amenorrhea after chemotherapy (hypergonadotropic hypogonadism) provides clinical evidence that the transplant is functioning. According to papers published so far, menstruation started spontaneously in an interval of six to 12 months after transplantation.

The simplest way of monitoring the function of the transplanted ovarian graft is to examine the levels of ovarian steroids and gonadotropins. This laboratory method is readily available and inexpensive. Another approach which is quite advanced and offers higher sensitivity is to monitor new markers and parameters of ovarian function and pool - inhibins (INH-A, INH-B) and antimüllerian hormone (AMH). Their levels in the blood start to rise several weeks before significantly decreased levels of gonatropin are detected.

Another way of monitoring the function of the autotransplant is bioptic verification combined with subsequent histopathological analysis or some molecular biology methods (FISH or PCR probe on the structures of ovarian tissue). The principal disadvantage of this approach is that it is invasive and there is a risk that the functional pool of the transplant sample will decrease.

In the case of long-time remission of cancer, the use of the autotransplant can be considered to obtain oocytes, followed by fertilization and the development of an embryo. In the case of orthotopic transplantation, spontaneous conception is theoretically possible although the chances of success are low. In the case of heterotopic transplantation it seems necessary as well as effective to use stimulation using gonadotropins followed by the application of oocyte retrieval techniques (OR) and in vitro fertilization techniques (IVF).

Safety of ovarian tissue cryopreservation

The retrieval of ovarian tissue is performed laparoscopically under general anesthesia and the risks associated with this intervention usually do not lead to major complications that would necessitate the postponement of cancer therapy. However, these risks should not be underestimated and the patient has to be properly informed by the surgeon and sign an informed consent.

The risk of relapse of tumor disease after autotransplantation because of the transfer of tumor cells present in ovarian tissue has to be taken into account. The majority of solid tumors in the ovary do not form metastases. Some tumor diseases have been described as forming metastases in the ovary (e.g., lobular breast carcinoma, some intestinal tumors – Kruckenberger's tumor). Such risk in systemic hematopoiesis-associated tumor diseases as leukemia is not accurately known, and other options of protecting ovarian function such as those described in the introduction of this paper should be considered. Prior to freezing and particularly prior to autotransplantation, it is highly recommended to perform histological or immunohistochemical analysis of a part of the sample for the possible presence of malignant cells related to the respective type of the basic cancer disease. A contraindication for this method will certainly be in patients with a genetic predisposition for the development of breast or ovarian carcinoma, i.e., gene-positive for breast cancer antigen (BRCA1, 2).

Recommendations for practice

Ovarian tissue cryopreservation is a method that has been intensively studied over the last few years. In some countries a number of specialized laboratories and centers have been established offering this method to female patients of reproductive age. The scientific debate on the advantages and potential risks led to the foundation of the expert working group and the approach recommended by the American Society for Reproductive Medicine (ASRM) [30]. Although the guideline was issued in 2004, the following is some of what is included:

- Until now the method has only been experimental;
- Written consent from the patient and approval of the Ethics Committee are required;
- Interdisciplinary cooperation is essential - gynecologist, pathologist, oncologist, embryologist, geneticist, psychologist;
- The method should not be offered commercially as an insurance against reproductive ageing.

EU Directive No. 2004/23/EC setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells came into force in Europe in April 2004. This directive defines the minimum standards and conditions for centres that deal with these procedures. The application of these standards may significantly reduce the availability of these medical procedures and services; on the other hand it ensures maximum safety and quality.

Our experience with ovarian tissue cryopreservation techniques

In cooperation with the Internal Clinic of Hematooncology at the Faculty Hospital, Brno, five cancer patients have undergone the biopsy and freezing of ovarian tissue at our department since January 2005. Table 4 provides an overview of indications of the intervention and detailed characteristics of patients. In all cases, the laparoscopic method was used to obtain 1-2 ovarian cortical strips with a maximum size of 5 x 10 mm and a thickness of up to 2 mm from each ovary (Figure 2). Furthermore, random biopsies from two different locations in both ovaries were performed. The samples sized up to 1 x 1 mm were examined histologically to determine the number of primordial follicles as the ovarian pool indicator and to exclude the presence of malignant cells.

The tissue retrieved was processed and frozen according to the cryopreservation protocol using 1,2-propanediol (Table 3). The retrieved ovarian tissue was placed in G Fert medium (Vitrolife) and transported to the laboratory within three minutes in a heat-insulated container. The tissue was cut in the same medium into pieces 2 x 4 x 1 mm. Pieces were frozen in EFS2 medium (Freezing Kit -Vitrolife) by a slow cooling technique using Planer freezer. The cryotubes were finally plunged into liquid nitrogen and stored. Totally 20 cryotubes with three pieces of tissue in each were cryopreserved. No cases of metastastic cancer cells were found by histological evaluation. The mean number of primordial follicles was 15.5 per 2 mm.

All patients included in the study were informed about the method and signed the informed consent. The project was approved by the Ethics Committee of the Faculty Hospital of Brno. None of the frozen samples have been used in transplantation so far. Currently, the running of this financially and organizationally demanding project is possible due to financial support from the Czech Republic Ministry of Health.

Discussion

Ovarian tissue cryopreservation as compared to cryopreservation of oocytes and embryos after fertilization has one major advantage: it does not require ovarian stimulation using gonadotropins and poses no typical risks such as ovarian hyperstimulation syndrome, or possible inflammatory or thromboembolic complications. Furthermore, ovarian stimulation is relatively contraindicated in hormone-sensitive tumors (breast carcinoma is discussed in particular) where this therapy might lead to the faster progression or dissemination of a primary tumor. Another important factor is that the start of cancer therapy does not have to be postponed for two to three weeks because of ovarian stimulation.

The minimization of ischemic damage to tissue during retrieval and processing is a very important factor for the functioning of the graft. Primordial follicles show lower sensitivity to ischemic damage while more extensive damage due to ischemia can be observed in adjacent granulous cells. The survival rate was shown to depend on the level of cell differentiation and the number and type of cellular organelles. In spite of this, the results obtained with ovarian tissue are far more encouraging as compared to oocyte cryopreservation. Further improvement is possible with the use of new cryobiological techniques such as vitrification (very fast freezing after covering the sample with liquid nitrogen). The improvement of freezing techniques to freeze the whole ovary is also a promising topic to be investigated in the future. Recently, Martinez-Madrid *et al.* have reported very good survival rates of primordial follicles after freezing and subsequent thawing of a whole human ovary [31]. In his work performed with bovine ovaries, Bedaiwy and Falcony also achieved excellent results in autotransplantation of the whole ovary using microvascular anastomosis techniques [27]. This approach is associated with one particular difficulty - finding a suitable method to preserve the whole ovary including its blood supply structures.

The size of the functional ovarian pool after transplantation of ovarian tissue is the basic prerequisite of long-term functional grafts. Histological studies have shown very uneven distribution of primordial follicles in the ovarian cortex, which are a morphological correlate of its function. As yet it has not been possible to predict how long a graft of ovarian tissue or an ovary damaged by chemotherapy or any other treatment will function after autotransplantation.

Prior to the use of frozen ovarian tissue the question will necessarily arise as to how soon after the successful completion of anticancer treatment it is suitable to perform the transplantation of ovarian tissue in a female patient. The decision depends particularly on the patient's state of health and test results during follow-up at the particular oncological clinic. Generally, it is recommended that transplantation should only be performed if the remission of cancer has not occurred for a period of two years.

Conclusion

Although many new studies describe the restoration of the function of ovarian tissue after autotransplantation and despite the birth of a child being repeatedly described after the use of these methods, it should be understood that there are a number of questions concerning this procedure that have to be answered. Before indicating this procedure, the general status of physical and mental health of a patient should be considered and proceeding should be strictly on an individual basis. Despite major progress in cryobiology, ovarian tissue cryopreservation should still be regarded as an experimental method. The practical performance of this procedure certainly requires early and close cooperation of the clinical oncologist, gynecologist-specialist in reproductive medicine, and embryologist.

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