Combination of fertility preservation strategies in young women with recently diagnosed cancer

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Summary

Background/Aims: The study describes clinical management and outcomes of currently available fertility preservation techniques in a set of 154 young female cancer patients. *Methods:* Patients in reproductive age with newly diagnosed cancer were offered embryo or oocyte cryopreservation, ovarian tissue cryopreservation and the administration of GnRH analogues during chemotherapy. Particular attention was given to the technical aspects and clinical application of these fertility preservation techniques. *Results:* During the study period (2004-2009), 154 young female cancer patients were offered fertility preservation counseling. Patient's average age was 29.4 years and average parity was 0.7 children. Administration of GnRH analogues (n = 123, 79.9%) and ovarian tissue cryopreservation (n = 15, 9.7%) were the most commonly used fertility preservation strategies. In 20 cases (16.1%), the combination of several fertility preservation techniques was offered to individually selected patients. *Conclusions:* Combination of fertility preservation techniques gives young cancer patients the best chance for future fertility and should be concentrated in specialized centers.

Key words: Cancer; infertility; Fertility preservation; Oocyte cryopreservation; Ovarian tissue cryopreservation.

Introduction

Incidence of cancer in reproductive age and childhood is on the rise. However, today more and more cancer patients can be completely cured due to earlier diagnosis and the availability of very effective anti-cancer therapies. One of the most common long-term consequences of cancer treatment is infertility due to the destruction of gonadal cells. Of the different modalities of oncology treatment, chemotherapy represents the greatest threat to reproductive function. The most gonadotoxic chemotherapeutics are alkylating agents, platina derivates and taxans. Some of these drugs, especially cyclofosphamide, are also widely used for immunotherapy in rheumatology (systemic lupus erythematodes, rheumatoid arthritis, etc.) [1].

The destruction of ovarian follicles by chemotherapy leads to a disruption of ovarian function. The degree of ovarian function disruption depends on the actual number of *primordial follicles (PMF)* in the ovary before the onset of chemotherapy. The number of ovarian follicles slowly decreases in life due to follicle maturation in reproductive age and also as a result of their ongoing apoptosis [2]. The actual number of follicles in the ovary is mainly determined by the patient's age at the time of cancer diagnosis. A complete loss of ovarian follicles leads to *acute ovarian failure (AOF)*. AOF is characterized by amenorrhea and acute menopausal syndrome (hot flushes and night sweating), which occur due to absence of the ovarian steroids (mainly estrogens and progesterone) normally produced by the follicles. The impact of chemotherapy sometimes only causes a significant decrease of PMF (not complete loss). Such cases are asymptomatic with regular menstrual cycle and the normal production of ovarian steroids, but a patient's ovarian reserve of PMF is considerably reduced and she is at a high risk of the onset of *premature ovarian failure (POF)* in the years following treatment. That is why all cured cancer patients are strongly advised not to postpone motherhood for too long. POF is defined as the onset of menopause before the age of 40, with amenorrhea and acute menopausal symptoms. In cases where AOF or POF occur, the patient becomes completely infertile due to the absence of PMF containing oocytes, and the only chance of becoming pregnant is to use an oocyte donor.

In the last ten years, a lot of research and clinical effort have been devoted to developing fertility preservation strategies which would preserve the reproductive potential of successfully cured cancer patients. The importance of the future fertility of cancer patients has prompted the creation of a new subspecialty of reproductive medicine recently referred to as *oncofertility*. This term was suggested by Woodruff in 2004 mainly because this problem involves primarily oncologists and specialists in reproductive medicine. The main goal of this new 'inter' discipline is research and development into methods of fertility preservation for patients in connection to cancer diagnosis, treatment and survivorship [3]. Oncofertility amalgamates biomedical and translation research, clinical medicine and the social sciences. Preserving fertility in the face of other medical (non-cancer) conditions is also becoming more prevalent, and so is the postponement of future fertility for social reasons.

Several fertility preservation strategies are currently available for young cancer patients before embarking on their oncology therapy. The optimal approach is chosen on a strictly individual basis and depends on the type of cancer, the type of treatment (e.g., radiation and/or chemotherapy), time available till the onset of treatment,

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the patient's age, and whether the patient has a partner [4]. To date, the most effective technique, and also the oldest, is *embryo cryopreservation* (EC). The human embryo is very resistant to damage caused by cryopreservation and delivery rates per embryo transfer using cryopreserved embryos are promising (18-20%) [5]. *Oocyte cryopreservation* (OC) is an alternative to embryo storage. Freezing mature oocytes has been a technical challenge because mature human oocytes are extremely sensitive to temperature changes. An alternative cryobiology approach exists called oocyte vitrification, which consists of solidifying liquid by ultra-rapid cooling. This renascent and promising technique is becoming an effective alternative to conventional slow freezing protocols [6]. However, both approaches (EC and OC) require in vitro fertilization (IVF) with the need for ovarian stimulation (usually for 2 to 4 weeks), which delays cancer treatment. The EC method additionally requires the participation of the male partner of the cancer patient. When these criteria cannot be met, more experimental preservation methods must be considered. A young female patient with newly diagnosed cancer should be informed about the possibility of *ovarian tissue cryopreservation* (OTC). After the patient has been cured, thawed ovarian tissue may be later auto-transplanted back to the pelvis to attempt spontaneous conception or carry out ovarian stimulation and subsequent IVF. Other emerging treatment options for fertility preservation include medication to prevent chemotherapy-induced oocyte damage. Clinically the most successful is the *administration of gonadotropin-releasing hormone analogues* (GnRH-a) during systemic cancer treatment (gonadotoxic chemotherapy or total body irradiation). Several human studies demonstrate that the GnRH-a are well tolerated and may protect long-term ovarian function [7-9]. Although the results of these studies are limited by sample size and lack of any randomized control group, randomized controlled trials are currently underway internationally to evaluate this strategy in young women with cancer. The *oocyte donor* (OD) program remains an established clinical option with a very high success rate for those who accept the use of non-parental genes or choose not to use experimental techniques or who fail to conceive with the above-mentioned methods.

The aim of this study is to describe the management and outcomes of currently available fertility preservation techniques in a set of 154 female cancer patients in reproductive age. The data presented here show that the most effective clinical results regarding the future fertility of young female cancer patients can be achieved using a combination of currently available fertility preservation techniques.

Materials and Methods

Female patients with newly diagnosed cancer in reproductive age (15-35 years) were referred by their oncologist for counseling at the Brno University Hospital Fertility Preservation Center (FPC) from January 2004 until November 2009. During their appointment, patients were informed about the risk of fertility impairment or fertility loss due to planned cancer treatment and about the possibilities represented by the available fertility preservation techniques. After considering several parameters, such as the type of cancer, proposed treatment protocol (e.g., radiation and/or chemotherapy), time available till onset of treatment, patient's age and whether the patient had a partner, the individual risk of threat to their fertility was assessed. An individual optimal fertility preservation strategy was advised from one of the following options:

– ovarian stimulation with embryo or oocyte cryopreservation (EC,OC);

– ovarian tissue cryopreservation (OTC);

– administration of GnRH-a during cytotoxic chemotherapy.

In many cases, a combination of GnRH-a co-treatment and one of the cryopreservation techniques was recommended. Every patient signed an informed consent form, containing detailed information about proposed fertility preservation techniques approved by the institutional ethics committee. All patients also had an option to refuse any of the proposed fertility preservation techniques. All administrative and statistical data monitored in the study were processed and statistically evaluated by standard statistical software (SPSS). We shall describe below in greater detail all the techniques mentioned above.

Embryo cryopreservation

Before EC all patients were stimulated with recombinant FSH follitropin alfa (Gonal-F, Merck Serono) together with the gonadotrophin releasing hormone antagonist cetrorelix (Cetrotide 3 mg, Merck Serono) according to a standardized protocol. Ovarian stimulation started at day 2 of the patient's menstrual cycle with a daily dose of 75-225 IU of FSH according to basal FSH levels and vaginal ultrasound (US) results. On day 7, cetrorelix in a dose of 3 mg was added to FSH to prevent any premature LH surge and oocyte retrieval was planned for day 10-14 of the patient's menstrual cycle. Oocytes were collected by US-guided vaginal needle aspiration under short general anesthesia. Collected oocytes were assessed by an embryologist and mature oocytes were fertilized with spermatozoa from the patient's partner using the intracytoplasmatic sperm injection (ICSI) technique. The partner of the patient gave written informed consent for the use of his spermatozoa which was collected before fertilization. All embryos created by fertilization were grown in vitro in an embryo culture (Vitrolife) for 2-4 days according to their growth potential, which was assessed daily by an embryologist. Usually at day 3 of culture, embryos were cyropreserved using the slow freezing technique by using a controlled rate freezer (Planer Kryo 10). The commercial freezing set Freeze-Kit 1TM (Vitrolife) was used for embryo preparation before the cryopreservation procedure. After successful cancer treatment, cryopreserved embryos can be used to achieve pregnancy by frozen-thawed embryo transfer (FET). To produce good pregnancy rates, the endometrium of recipient woman has to be prepared by administering estrogen and progesterone. We used estradiol valerate and micronized progesterone oral tablets for endometrium preparation according to standardized protocol [10]. FET was performed if endometrial thickness was at least 7 mm (measured by vaginal US). Embryos were always thawed one day prior embryotransfer by using the commercial embryo thawing kit Thaw-Kit 1TM (Vitrolife). After the thawing procedure, embryos were cultivated for 24 hours in a standard IVF medium and only those embryos that showed further development (50% or more of surviving blastomeres) were used for embryo transfer.

Oocyte cryopreservation

This second possible technique is suitable mainly for women without a regular male partner or adolescents. Ovarian stimulation and the oocyte collection process have been described in the previous paragraph. Collected mature oocytes containing both polar bodies (MII oocytes, metaphasis of second meiotic division) can be cryopreserved by the conventional slow freezing technique or by vitrification (ultra-rapid cooling). The conventional slow freezing technique used in our study is described in detail by Fabri *et al*. [11]. Ooocytes were prepared for freezing with the ready-to-use commercial media system Oocyte-Freeze (Medicult) containing 1,2-propanediol and sucrose as cryoprotectants and later cryopreserved in straws using a controlled rate freezer (Planer Kryo 10). The vitrification technique used by our lab is described by Kuwayama *et al*. [12]. For vitrification we also used the commercial media system Vitrification Cooling (Medicult) containing ethylene glycol, propandiol and sucrose as cryoprotectants and eight vitrification straws (High Security Vitrification Straw) for oocyte storage. According to strict European regulations (European Tissue Directive, 2004/23/EC), we used the so-called "closed" vitrification system, which avoids direct contact between the cryopreserved material and liquid nitrogen. The oocyte thawing process is analogous to the embryo thawing technique. Oocytes cryopreserved using the slow freezing technique were processed with the OocyteThaw (Medicult) thawing kit according to the manufacturer's instructions. Previously vitrified oocytes were thawed using a Vitrification Warming set (Medicult). Thawed oocytes were fertilized using standard ICSI and cultivated in vitro for 2-4 days according to their growth potential, which was assessed daily by an embryologist. Written informed consent of a patient's male partner allowing embryo transfer was required. Embryo transfer was performed by the standardized technique described in the previous paragraph.

Ovarian tissue cryopreservation

Ovarian tissue cryopreservation, the third technique available, was developed according to the previously published studies of several authors [13-16]. Ovarian tissue was obtained using the laparoscopic approach under general anesthesia. In a majority of cases, a sample of ovarian cortex sized 10 x 20 x 1-2 mm was obtained from both ovaries. In some patients with a severe risk of premature ovarian failure due to planned highly gonadotoxic chemotherapy, one entire ovary was removed. In all cases, a further 2-3 small (1 x 1 x 1 mm) samples of ovarian cortex were removed from both ovaries at random from the ovarian surface. Theses samples were assessed by a pathologist to exclude any metastatic involvement of the ovaries by primary diagnosed malignancy. Ovarian tissue intended for cryopreservation was transported to the embryology lab in a cryopreservation medium (G-Fert, Vitrolife) at human body temperature. The tissue was inspected under the microscope in a laminar box of the embryology lab. If 2-5 mm in diameter ovarian follicles were found to be growing in the sample, they were aspirated and oocytes were sought in the follicular fluid. Providing the patient had previously agreed, retrieved oocytes found in the follicular fluid could be matured in vitro and later cryopreserved as described in the previous paragraph. The acquired ovarian cortex tissue was cut lengthwise into smaller pieces, approximately 5 x 1 x 1 mm, with the help of scalpel. In cases of whole ovary removal, the ovarian cortex was separated from the ovarian medulla by digital dissection or with the help of scissors. Ovarian medulla is not suitable for cryopreservation because it does not contain PMF. Small strips of ovarian cortex were incubated in the cryopreservation medium EFS2 Freezing Kit (Vitrolife) for 90 min and loaded into cryotubes (4-6 strips to each cryotube). After careful identification, cryotubes with ovarian tissue were inserted into a controlled rate freezer (Planer Cryo 10) and cryopreserved according to the protocol used for routine embryo freezing. Whenever the vitrification technique was applied, the following commercially made vitrification kits were used: RapidVit Cleave (Vitrolife) or Vitrification Cooling (MediCult). Cryoprotectant exposure was performed according to the manufacturer's instructions and cryotubes loaded with processed ovarian strips were plunged directly into liquid nitrogen.

Administration of GnRH-a during chemotherapy.

We advise the administration of GnRH-a to our young female cancer patients during chemotherapy in order to prevent or decrease the rate of ovarian damage. The administration of GnRH-a during chemotherapy to prevent ovarian damage is sufficiently effective, especially in cases where less gonadotoxic chemotherapy regimens are being administered. This was confirmed by our previous observational study on female Hodgkin lymphoma (HL) patients [8]. This technique is used separately or in combination with the other fertility preservation methods described above. If less gonadotoxic chemotherapy is planned to cure a patient's malignancy, then the administration of GnRH-a is advised as a stand-alone technique to prevent ovarian damage. If highly gonadotoxic chemotherapy protocols must be used to achieve a patient's complete cure (e.g., alkylating agents, high cumulative doses, myeloablative chemotherapy before bone marrow transplantation), the patient is advised to combine GnRH-a administration during chemotherapy with one of the cryopreservation fertility saving techniques. The GnRH-a triptorelin (Diphereline SR 3 mg, Ibsen) is administered to female cancer patients during the whole time of chemotherapy in the form of intramuscular injection once a month (usually together with the pulse of chemotherapy).

Results

Over a period of five years (2004-2009), a cohort of 154 patients scheduled for gonadotoxic chemotherapy or immunotherapy was referred for consultation to prevent fertility impairment. The basic characteristics of the patient set are described in Table 1. The average age of patients included in the study was 29.4 ± 6.3 years. All women were Caucasian. The average body mass index (BMI) in the set of patients was 23.8 ± 3.1 kg/m^2. The majority of women were nuliparas or primiparas. The mean parity in this set of patients was 0.7 ± 0.3 children per patient. The length of systemic chemotherapy or immunotherapy was 5.2 ± 1.8 months on average. The mean time from cancer diagnosis to the start of systemic cancer treatment (needed to perform the planned methods of ovarian protection) did not exceed 14 days.

The spectrum of oncology or rheumatology diagnoses of patients referred for fertility preservation counseling is described in Table 2. The majority of treated patients suffered from newly diagnosed malignancy of the blood or lymphatic system. The most common diagnosis was Hodgkin lymphoma. This malignancy has very good

Table 1. — *Basic characteristics of the set of patients referred for consultation to prevent fertility impairment (Assisted Reproduction Center, Department of Obstetrics and Gynecology of Brno University Hospital, years 2004-2009).*

Follow-up parameter	Mean value
Age	29.4 ± 6.3
Body mass index (BMI)	23.8 ± 3.1 kg/m^2
Parity	0.7 ± 0.3
Race	Caucasian (100%)
Length of systemic chemotherapy	5.2 ± 1.8 months
Time to treatment start	13.5 ± 2.6 days

Table 2. — *The spectrum of oncology or rheumatology diagnoses of patients referred for fertility preservation counseling (Assisted Reproduction Center, Department of Obstetrics and Gynecology of Brno University Hospital, years 2004-2009).*

Diagnosis	ICD 10 code	No. of patients	%
Hodgkin lymphoma (HL)	C81	101	65.6
Non-Hodgkin lymphoma (NHL)	C82-85	15	9.7
Leukemia	C91-97	12	7.8
Systemic lupus erytematodes (SLE)	M32	11	7.1
Breast malignancy	C50	5	3.2
Gynecology malignancy (vagina, uterus, ovary)	C51-56	4	2.6
Gastrointestinal malignancy	C18-20	1	0.6
Bone malignancy	C40-41	2	1.3
Malignacy of head and neck	C00-13	3	1.9
Total		154	100

ICD - International Classification of Diseases.

Table 3. — *Representation of fertility preservation techniques chosen by the patient and theirs counseling physician (Assisted Reproduction Center, Department of Obstetrics and Gynecology of Brno University Hospital, years 2004-2009).*

Fertility preservation technique	No. of patients	% from total
Embryo cryopreservation (EC)	4	2.6
Oocyte cryopreservation (OC)	3	1.9
Ovarian tissue cryopreservation (OTC)	16	10.4
GnRH analogues (GnRH-a)	123	79.9
No fertility protection	8	5.2
Total	154	100
Combination of fertility preservation techniques (cryopreservation+ GnRH analogues)	20	16.1 (20 from 124)

prognosis quod vitam – five-year survival is 85-98% [17]. The other commonly occurring diagnoses referred to FPC were systemic lupus erythematodes with visceral involvement, breast malignancies, gynecological malignancies and head or neck malignancies.

All referred patients were individually consulted regarding their risk of fertility impairment or loss due to planned gonadotoxic chemotherapy. All patients were offered one or more fertility preservation techniques described in the methodology section. Table 3 describes the fertility preservation techniques chosen by the patients and their counseling physician. The most commonly used technique of fertility preservation was GnRH-a administration during chemotherapy (79.9%). Ovarian tissue cryopreservation was the second most preferred fertility preservation technique (10.4%). In 20 cases (16.1%), a combination of more than one fertility preservation technique was offered to individually selected patients. In these cases, administration of GnRH-a during chemotherapy or immunotherapy was combined with one of the cryopreservation strategies (EC, OC or OTC). Where a combination of fertility preservation techniques was applied, the case was categorized for statistical evidence into one of the cryopreservation sub-groups. A small number (5.2%) of referred patients decided not to use any of the offered fertility preservation supportive strategies before or during their oncology therapy. Detailed results of the fertility preservation techniques are summarized in Table 3 and analyzed in more detail in the following paragraphs.

Embryo cryopreservation

Embryo cryopreservation was the first technique implemented into our Fertility Preservation Program. This routinely available method in reproductive medicine was utilized by our first oncology patient in May 2004. The results of embryo cryopreservation and its usage by our women cancer patients are summarized in Table 4. Since 2004, we have used this technique in four patients and gained 18 oocytes suitable for fertilization. We have successfully frozen 12 embryos in total, with a mean number of 3.3 embryos per patient. The embryos of three patients, who successfully come through their oncology treatment, are still cryopreserved and ready for FET. In one case, cryopreserved embryos have been used for treating infertility caused by the oncologic therapy. This 21-year-old young woman was diagnosed with advanced stage Hodgkin lymphoma. Her chemotherapy was postponed for 16 days while her oocytes were acquired. Four embryos were cryopreserved for later use. After almost four years following successful cancer treatment the FET of three embryos was accomplished. These embryos were successfully thawed and transferred, but unfortunately the patient did not become pregnant. The FET was possible to carry out because the woman still had the same male partner, who agreed with the procedure. This patient is now dependent on hormonal replacement therapy and her infertility is being treated by oocyte donation.

Oocyte cryopreservation

During the study period, oocyte cryopreservation techniques were used by three cancer patients. Details of these cases are presented in Table 5. A total number of 15 oocytes were cryopreserved (5.0 oocytes per patient). Four of the 19 retrieved oocytes (21.0%) were not suitable for cryopreservation due to oocyte immaturity (diploid germinal vesicle oocytes) or their degeneration during the preparation procedure. None of these three cancer patients have yet asked to use their oocytes to become pregnant. The disadvantage of both this tech-

Table 4. — *Results of embryo cryopreservation and their usage by women cancer patients. (Assisted Reproduction Center, Department of Obstetrics and Gynecology of Brno University Hospital, years 2004-2009).*

Monitored parameters - embryo cryopreservation	Value
Number of patients (stimulated IVF cycles)	4 cycles
Total number of retrieved oocytes	18 oocytes
Mean number of oocytes per cycle	4.5 oocytes
Total number of created embryos	15 embryos
Fertilization rate	83%
Total number of cryopreserved embryos	12 embryos
Percentage of embryos not suitable for cryopreservation	20%
Mean number of cryopreserved embryos per cycle	3.3 embros
Monitored parameters - embryo usage	**Value**
Number of patients decided for cryo-embryotransfer	1
Total number of thawed embryos	4
Mean number of thawed embryos per cycle	4
Number of vital embryos after thawing	3 (75%)
Number of vital embryos after thawing and 24h co-cultivation	3 (100%)
Number of performed cryo-embryotransfers	1
Mean number of embryos transferred to uterus	3
Number of pregnancies after cryo-embryotransfer	0
Pregnancy rate	0%

Table 5. — *Results of oocyte cryopreservation in women cancer patients. (Assisted Reproduction Center, Department of Obstetrics and Gynecology of Brno University Hospital, years 2004-2009).*

Oocyte cryopreservation - monitored parameters	Value
Number of patients (stimulated IVF cycles)	3 cycles
Oncology diagnoses	2x HL, 1x NHL*
Total number of retrieved oocytes	19 oocytes
Mean number of oocytes per cycle	6.3 oocytes
Total number of cryopreserved oocytes	15 oocytes
Mean number of cryopreserved oocytes per cycle	5.0 oocytes
Percentage of oocytes not suitable for cryopreservation (diploid GV oocytes, fragmentized oocytes)	21%
Oocyte cryopreservation technique	slow freeze (Planer) 2x vitrification (MediCult) 1x
Mean time of chemotherapy postponement	18.6 days

* HL – Hodgkin lymphoma, NHL – non-Hodgkin lymphoma.

nique and EC is the need for ovarian stimulation and the postponement of planned cancer treatment. In this sub-set of patients, chemotherapy had to be postponed for an average of 18.6 days.

Ovarian tissue cryopreservation

The technique of ovarian tissue cryopreservation has been developed in our laboratory since 2005. Initially, we used a Planer controlled rate freezer; in 2007 we started to freeze ovarian tissue by the vitrification technique. During the period of this study the technique was applied in 16 cases of young female patients prior to highly gonadotoxic chemotherapy. In the majority of cases, patients suffered from blood or lymph node systemic malignancy (81%) - Hodgkin lymphoma (9x), non-Hodgkin lymphoma (3x) and acute myeloid leukemia (1x). A summary of ovarian tissue cryopreservation cases is presented in Table 6. The average age of women taking advantage of this technique was 26 years. The youngest patient was 13 years old and she decided for the procedure after taking advice from her parents. The patient set consisted of mostly nulliparous women (88%). All women were Caucasian. Average BMI in the sample was 23.7 kg/m^2. The length of systemic chemotherapy or immunotherapy averaged 7.1 months. Average time from fertility preservation counseling to the beginning of chemotherapy was very short, not exceeding one week. Ovarian tissue harvesting was conducted for all cases in this subgroup by laparoscopic surgery. The length of surgery did not exceed 60 min and no surgical complications were observed. The patient length of stay in hospital was two days at most. In the majority of cases (88%), only an ovarian cortex sample (approximately sized 10 x 20 x 1-2 mm) was removed from both ovaries. In the last two patients, where there was a very high risk of permanent ovarian failure, one entire ovary was removed. All of the ovarian tissue cryopreserved samples have yet to be thawed for the auto-transplantation and restoration of ovarian function. The digested characteristics of the patient set and the results of ovarian tissue cryopreservation are reported in Table 7.

GnRH analogues

The administration of GnRH analogues during chemotherapy to protect ovarian function was introduced into clinical practice in our center in 2003. During the follow-up period (2004-2009) of this study, we administered this medication to 123 patients during chemotherapy. This has been the most widely used method (79.9%) from the portfolio of fertility preservation techniques offered. Quite a large proportion of the women (n = 20, 16.1%) receiving GnRH analogues during chemotherapy have chosen to combine this approach with one of the cryopreservation techniques (EC, OC or OTC) (Table 3). The protective effect on ovarian function of administering GnRH analogue during chemotherapy has been described in detail in our previously published prospective case-control study on patients (n = 72) with newly diagnosed Hodgkin lymphoma. We documented a statistically smaller number of premature ovarian failure cases in the group of patients receiving GnRH analogues compared to the control patient group with follow-up periods of six and 12 months. After analyzing the study group according to the gonadotoxicy of chemotherapeutical regimens used, it was clear that the protective effects of GnRH analogue co-treatment were statistically significant only in the patient subgroups receiving less gonadotoxic chemotherapeutics [8]. These observations thus show the unsatisfactory protective effect of administering GnRH analogues to protect ovarian function in advanced cancer cases, where high dosage combined chemotherapy has to be administered.

Table 6. — *Ovarian tissue cryopreservation in cancer patients – cases overview (Assisted Reproduction Center, Department of Obstetrics and Gynecology of Brno University Hospital, years 2004-2009).*

Pac. no.	Age at cryo	Pac. parity	Oncol. diag.	Time to chemo	Chemo length	Body mass	Cryo date	Cryo tubes	Cryo technique	Cryo media
	Years			*Days*	*Months*	*kg/m²*	*dd.mm.yy*	*No.*		
1	23	0	HL	5	8	24	16.12.04	1	Planer	Vitrolife
2	22	0	HL	7	8	25	08.04.05	6	Planer	Vitrolife
3	28	0	NHL	4	6	22	23.03.06	3	Planer	Vitrolife
4	33	1	NHL	8	8	26	28.03.06	4	Planer	Vitrolife
5	19	0	HL	12	8	28	27.06.06	3	Planer	Vitrolife
6	20	0	HL	5	8	24	23.08.06	4	Planer	Vitrolife
7	19	0	HL	6	8	23	29.01.07	4	Planer	Vitrolife
8	33	0	SLE	6	6	22	21.05.07	6	Planer	Vitrolife
9	29	0	AML	8	7	21	24.05.07	4	Planer	Vitrolife
10	24	0	HL	5	8	28	16.10.07	3	Vitrifikace	MediCult
11	13	0	NHL	9	6	20	16.05.08	4	Vitrifikace	MediCult
12	31	0	HL	11	8	21	06.02.09	4	Vitrifikace	Vitrolife
13	30	0	HL	10	8	23	02.04.09	4	Vitrifikace	Vitrolife
14	28	0	HL relaps	6	6	25	15.04.09	4	Planer	Vitrolife
15	34	1	ovarian ca	8	6	26	19.05.09	9*	Vitrifikace	MediCult
16	31	0	tongue ca	7	5	22	07.07.09	7*	Vitrifikace	Vitrolife

HL: Hodgkin lymphoma; NHL: non-Hodgkin lymphoma; SLE: systemic lupus erythematodes; AML: acute myeloid leukemia.
* complete removal of one ovary (unilateral ovarectomy).

Table 7. — *Ovarian tissue cryopreservation – patient's set characteristics and results. (Assisted Reproduction Center, Department of Obstetrics and Gynecology of Brno University Hospital, years 2004-2009).*

Patient's set characteristics	Value (unit)
Average women's age in the time of cryopreservation	26.0 ± 6.2 years
Women's average parity	0.1 ± 0.3
Average body mass index (BMI)	23.7 ± 2.4 kg/m²
Race	caucasian (100%)
Average length of chemotherapy	7.1 ± 1.0 months
Average time left to beginning of chemotherapy	7.3 ± 2.3 days
Ovarian tissue cryopreservation results	**Value**
Ovarian tissue harvestin technique	laparoscopy (100%), length of surgery - 60 min. max. no surgical complications
Amount of tissue collected	14x - ovarian cortex strips 2x - unilateral ovarectomy
Average no. of frozen cryotubes	4.3 ± 1.8
Cryopreservation technique used	10x Planner slow freezing 6x vitrification
Vitrification media used	3x Vitrolife 3x MediCult
Cases of ovarian tissue auto-transplantation	0

Discussion

Rapid advances in the field of oncofertility have been made in last decade [18]. Our study presents the clinical outcomes of quite a large cohort of female patients requiring fertility preservation counseling. The complete portfolio of fertility preservation techniques offered to patients is another important strength of our study. One of the limitations of results presented here is the difficulty in following up the future fertility of the patients in the long term. The introduction of an international registry of patients who have used certain fertility preservation techniques and the later follow-up of their children could improve our knowledge about clinical efficacy.

Embryo cryopreservation has historically been the first available technique for preserving fertility in young female cancer patients. This technique became the gold standard of care with the best overall results for conception after successful cancer treatment. The pregnancy rate per embryo transfer varies between 10-30 % [19]. In our cohort, we reported embryo transfer of frozen-thawed embryos after successful cancer treatment, but the patient, unfortunately, did not conceive. The main disadvantage of this technique is the need for ovarian hyperstimulation with FSH and postponing the planned cancer treatment by an average of 2-4 weeks. Nevertheless, usage of "soft" hyperstimulation protocols (clomifene, tamoxifene, letrozole) is possible in cases of estrogen sensitive tumors [20]. A further drawback for this technique is its unavailability to young single women patients without any regular male partner, who would provide sperm for oocyte fertilization (analogically it is also unavailable for adolescents and children). This method is less preferred at our center because of the aforementioned inconveniencies of embryo cryopreservation and the availability of new fertility preservation techniques. This technique has been especially relegated in favor of OTC, which does not require ovarian stimulation or postponing the cancer treatment.

The *cryopreservation of oocytes* could be the favored option for women without a male partner. On the other hand, ovarian stimulation and postponing the start of chemotherapy is still necessary. Conventional slow freezing with ice crystal formation inside the cell could seriously damage the oocyte (cytoskeleton breaks, zona pellucida hardening) and reduce its future fertility compe-

tence. The renaissance of the vitrification freezing technique and development of new potent cryoprotectants (e.g., sucrose, propandiol) in the last decade have returned this method once again to clinical practice [21]. Before 2008, more than 35,000 oocytes were cryopreserved and more than 600 children were born, mostly using the conventional slow-cooling method. The overall live-birth rate per cryopreserved oocyte is approximately 2-10%, which is much lower than that of in vitro fertilization (IVF) using fresh oocytes [6]. The pregnancy outcomes reported in the literature are limited in terms of sample size and length of follow-up. They are also mostly meta-analyses of published cases rather than the comprehensive follow-up we present here. Oocytes could also be frozen in the diploid stage of folliculogenesis (germinal vesicle oocytes, metaphase I oocytes) and after thawing matured in vitro to their haploid metaphase II stage. With these advances in cryobiology, current oncofertility techniques could be pooled together. In our study, we have found that combining the cryopreservation of ovarian tissue together with immature diploid oocytes is very promising. This technique, which was first suggested by Revel [22] and has also been applied in our laboratory, could rapidly increase women's chances of becoming pregnant after a complete cure for their cancer.

The first reports of *ovarian tissue cryopreservation* date back to 1950. Their results were unsatisfactory, mostly due to the suboptimal laboratory procedures used. The technique was later significantly improved on animal models [23]. The main goal of this strategy is to save intact primordial follicles for later use, i.e., after the patient has been cured of cancer [13, 16, 24]. In comparison with EC or OC techniques, our patients who decided for OTC did not have to lose valuable time before starting chemotherapy and had no exposure to estrogens needed for ovarian hyperstimulation. Neither was it necessary to meet the requirement for having a regular male partner. If the tissue is harvested using a laparoscopic technique, it is possible to start systemic oncology treatment 2-3 days after surgery. The cryopreservation protocols used in our laboratory include slow freezing with previous exposure to cryoprotectants (1.5 M propandiol, 0.1 M sucrose) or the vitrification of ovarian cortex strips loaded into cryotubes. The vitrification technique is now preferred, because it is easier and faster to complete and the overall costs are comparable to conventional slow freezing. Despite the aforementioned advantages of this approach, OTC still remains an experimental method. In our center, clinical usage is approved by the ethics committee and the patient's written consent is required [25]. The risk of cancer recurrence can not be completely avoided following orthotopic auto-transplantation of ovarian cortex strips. Moreover, the period during which hormones of the transplanted ovarian tissue continue to function is limited. Auto-transplantation should be carefully scheduled to the time when childbearing is desired by the treated couple, and the oncology disease is in long-term complete remission. Ovarian hyperstimulation with a conventional IVF/ICSI cycle is usually required to achieve pregnancy after auto-transplantation. The thawed ovarian tissue can be used for the auto-transplantation, xeno-transplantation or in vitro culture of primordial follicles. In human medicine, the best results were reported with the auto-transplantation of tissue avascular cortical fragments. Before 2008, 12 clinical pregnancies and the birth of five healthy children were reported by applying this novel technique on cancer patients [26]. In the patient set reported in our paper, we have not yet performed the auto-transplantation procedure. Those of our patients who wished to store their ovarian tissue are still involved in cancer treatment or post treatment follow-up, waiting for the recommendation from their oncologist regarding the optimal childbearing time. Notable achievements have also been recorded with the xeno-transplantation of human ovarian tissue into immunodeficient mice. But the question is, however, who would agree with the embryo transfer of an embryo grown in vitro into the renal capsule of mice? In 2009, Xu *et al*. from Woodruff lab in Chicago, reported the first successful in vitro human primordial follicle culture. The isolated follicles were grown in a three-dimensional alginate matrix and after 30 days of culture the follicles reached the antral stage of folliculogenesis and contained haploid oocytes ready for fertilization [27]. In our opinion, this approach has great potential for future clinical usage and our lab has recently started to cooperate with colleagues at Northwestern University (Chicago, IL, USA) to implement the follicle culture technique into practice.

The use of GnRH analogues to protect ovarian function during chemotherapy is controversial and not generally accepted by all oncofertility specialists. The problem of the first human studies exploring this hypothesis was their retrospective design and the heterogeneity of patients and chemotherapy protocols used [28-30]. The results of better designed and prospective studies investigating the benefits of GnRH analogues in oncofertility are more promising. These studies have proved the protective effect of administering GnRH analogues during chemotherapy to save ovarian reserve [7, 9]. Our previously published results indicate the insufficient protective effect of GnRH analogues in preventing primordial follicle destruction by highly gonadotoxic myeloablative chemotherapeutic regimens before bone marrow transplantation [8]. Hopefully, we will be able to evaluate exactly the protective effect of GnRH analogues by double blind, multicenter, prospective, randomized trials, which are currently ongoing. The main advantage of this fertility preservation technique is its simplicity and non-invasiveness. In addition, the amenorrhea induced by administering GnRH analogues helps to protect pancytopenic and immuno-compromised patients during chemotherapy against heavy menstrual bleeding and any need for the extensive usage of expensive hematopoietic factors and blood derivates. According to our clinical experience with fertility preservation counseling, we recommend offering GnRH-analogue co-treatment during chemotherapy to the majority of our young female cancer patients. This approach is strongly recommended in can-

cer patients with early-stage disease and patients where low-dose chemotherapy with an absence of alkylating agents is recommended by an oncologist. In advanced cancer stages, where highly gonadotoxic chemotherapy has to be applied (such as hematological malignancies with bone marrow transplantation planned, recurrent disease, early relapse after primary treatment, etc.), the patient is advised that administering GnRH analogue itself does not sufficiently protect ovarian reserve. In these cases, we recommend combining the GnRH analogue administration with one of the cryopreservation techniques (EC, OC or OTC) in order to increase the patient's fertility chances in future (see also Table 3). A combination of ovarian tissue cryopreservation before the start of chemotherapy, together with GnRH analogue administration during cancer treatment, has become especially popular with our cancer patients more recently [31].

Fertility preservation counseling requires *multidisciplinary cooperation* among various medical specialties. In our experience, primary roles should be taken by a reproductive medicine specialist, an oncologist and an embryologist. We recommend setting up interdisciplinary *fertility preservation centers*. These centers could easily connect various medical specialists, apply currently available fertility preservation techniques, gather and disseminate the latest knowledge in oncofertility and coordinate research and development in this field (www.oncofertility.northwestern.edu, www.fertilitypreservationcare.com, www.ivfbrno.cz/cor). This interdisciplinary cooperation between an oncologist and reproductive medicine clinician, however, should not be limited to the time of cancer diagnosis, but it should continue right throughout the patient's entire reproductive age.

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