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Cryopreservation of ovarian cortex in girls and young women diagnosed with cancer

Kriokonserwacja kory jajnika u dziewcząt i młodych kobiet z rozpoznaniem nowotworu

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Summary

In the last years, thanks to an incredible development of medicine and using more aggressive forms of cancer treatment, the number of cancer survivors increases. Currently in Poland, 5% of people diagnosed with cancer are under 35 years old, which means in procreative age. It is particularly important to pay attention to the standard of their lives. It is correlated with many aspects, also the will to have children. In cases of girls and young women, an effective cancer treatment should be preceded by an offer of a proper method enabling fertility preservation. One of such methods is cryopreservation of ovarian cortex.

Cryopreservation of ovarian cortex is an experimental, but very promising method of preserving fertility especially for young women, also before puberty. In contrast to other methods, requires no hormonal stimulation prior to collection the tissue so it doesn't delay cancer therapy. After at least 2 years from the end of cancer treatment and exclusion of recurrent disease, ovarian tissue can be transplanted ortho- or heterotopic.

The aim of the study is to emphasize the need to inform women about the fertility preservation methods even before the cancer treatment. The issue of the fertility preservation is becoming increasingly essential due to the growing number of the cancer survivors.

Streszczenie

W ostatnich czasach, dzięki niezwykłemu postępowi medycyny i stosowaniu coraz agresywniejszych form leczenia przeciwnowotworowego, rośnie liczba ozdrowieńców. Obecnie w Polsce 5% osób z nowo rozpoznaną chorobą nowotworową to osoby poniżej 35 roku życia, a więc w wieku prokreacyjnym. Należy zwrócić szczególną uwagę na jakość ich życia. Z jakością życia wiąże się wiele różnych aspektów, m. in. chęć posiadania potomstwa. W przypadku dziewczynek i młodych kobiet skuteczne leczenie przeciwnowotworowe powinno być zatem poprzedzone propozycją odpowiedniej metody umożliwiającej zachowanie płodności. Jedną z takich metod jest kriokonserwacja kory jajnika.

Kriokonserwacja kory jajnika jest eksperymentalną, ale bardzo obiecującą metodą zachowania płodności, skierowaną przede wszystkim do młodych kobiet, również przed okresem pokwitania. W przeciwieństwie do innych metod, nie wymaga stymulacji hormonalnej przed pobraniem tkanki, dzięki czemu nie opóźnia leczenia przeciwnowotworowego. Po upływie przynajmniej 2 lat od zakończenia leczenia i wykluczenia nawrotu choroby można przeszczepić tkankę jajnika w sposób orto- lub heterotopowy.

Celem pracy jest uświadomienie lekarzom konieczności informowania kobiet jeszcze przed rozpoczęciem leczenia przeciwnowotworowego o metodach zachowania płodności.

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INTRODUCTION

It is estimated that ca. 700 thousand to 1 million couples in Poland suffer from infertility (transient or constant), induced by male or female factor. There are many reasons of infertility, e.g. immunological disorders, semen anomalies, endometriosis, sexually transmitted diseases (STD) or cancer treatment. The cancer itself may diminish fertility by malnutrition syndrome, depression,

elevated temperature, opportunistic infections. During the next decade the number of people with infertility caused by cancer treatment will probably increase (1-3).

Fertility among oncological female patients may be diminished by the decrease of the number of ovarian follicles, hormonal disorders or malfunction of reproductive system. Infertility may occur already during treatment as period loss or a few years after the completion of treatment as premature ovarian failure (POF). Even after the reoccurrence of period after the completion of treatment, fertility may be highly disordered. The transient loss of period is caused by damage to mature follicles, while lasting loss – by damage to both mature and primary follicles (4).

The risk of infertility is closely correlated with treatment method, duration of treatment, doses, application method, cancer type, as well as age of a patient and her fertility before the treatment (often difficult to estimate) (tab. 1) (5).

Based on the data of National Register of Cancer in years 1999 and 2009, a significant increase of cancer survivors can be seen, due to major medical progress and even more aggressive forms of treatment. Currently 5% (ca. 7000 a year) of people diagnosed with cancer are below 35 years of age. It is very important to pay attention to the standard of their lives.

Among the majority of young women diagnosed with cancer, the desire to become a mother in the future becomes stronger than before diagnosis, although some of them fear parenthood because of the possibility of disease recurrence. During the study it was stated that s much as 81% of patients and 93% of their parents was interested in the possibility of fertility preservation (7).

Taking into consideration the information above, doctors should talk to the patient and explain possible consequences of cancer treatment, as well as propose a method of fertility preservation (fig. 1) (8-11).

CRYOPRESERVATION OF OVARIAN CORTEX

Cryopreservation of embryos and *in vitro* fertilization (IVF) is a widely accepted method that requires certain age of a patient (after puberty) and ovaries stimulation (in case of hormone-dependent cancer the ovaries are stimulated with tamoxifen and aromatase inhibitors). Mainly, it is a method for patients who have a partner or are willing to use the service of semen bank. A sure advantage of IVF is a big percentage of pregnancies ended with birth (effectiveness of 40%).

Cryopreservation of ovarian cortex is still an experimental, but very promising method that is possible also for women before puberty. This method still needs a lot of research concerning its safety and effectiveness and the use of this method requires approval of bioethical board.

On the contrary to other methods of fertility preservation, it does not require hormonal stimulation prior to collection of tissue, which does not delay the treatment. However, the method is not recommended to women after 40 years of age due to low ovarian reserve. Ovarian reserve amounts to 300 thousand egg cells at the moment of birth and decrease with age. Currently, it is estimated that among 95% of women above 30 years of age the amount of egg cells is ca. 12% of primary ovarian reserve, and among women above 40 years of age – only 3%.

Table 1. The risk of amenorrhea depending on the treatment (6).

Level of risk	Treatment protocol	Application
High risk Among > 80% of women suffer from amenorrhea	1. Irradiation of abdomen or pelvis with doses > 6 Gy among adult women, > 15 Gy among women before puberty and > 10 Gy after puberty 2. TBI 3. CMF, CEF, CAF w 6 in among women > 40 y.o. 4. Cyclophosphamide 5 g/m^2 among women > 40 y.o. and 7.5 g/m^2 among women < 20 y.o. 5. Alkylating agents prior to BMT/SCT 6. Alkylating agents together with TBI or pelvis irradiation 7. Protocols containing procarbazine 8. Irradiation of skull/brain > 40 Gy	1. Various cancer types 2. BMT/SCT 3. Breast cancer 4. Various cancer types 5. BMT/SCT 6. BMT/SCT, HL, neuroblastoma, ovary cancer 7. HL 8. Brain cancer
Medium risk 30-70%	1. CMF, CEF, CAF w 6 in among women 30-39 y.o. 2. AC among women > 40 y.o. 3. Irradiation of abdomen or pelvis in 10-15 Gy before puberty 4. Irradiation of abdomen or pelvis in 5-10 Gy after puberty 5. Irradiation of spine with doses > 25 Gy	1. Breast cancer 2. Breast cancer 3. Wilms' tumor 4. Wilms' tumor, neuroblastoma 5. Spine, brain cancer, return of ALL, NHL
Low risk < 20%	1. CMF, CEF, CAF w 6 in among women < 30 y.o. 2. AC among women 30-39 y.o. 3. Protocol ABVD, CHOP, COP 4. AC 5. Multidrug therapy	1. Breast cancer 2. Breast cancer 3. HL, NHL 4. AML 5. ALL
Very low/no risk	1. Methotrexate, 5-fluorouracil 2. Vincristine in politherapy 3. Radioactive iodine	1. Breast cancer 2. Leukemia, HL, NHL, neuroblastoma, Wilms' tumor 3. Thyroid cancer

Gy – Grey; HL – Hodgkin's lymphoma; TBI – Total Body Irradiation; BMT – Bone Marrow Transplant; SCT – Stem Cell Transplant; NHL – Non-Hodgkin lymphoma; AML – acute marrow leukemia; ALL – acute lymphoblastic leukemia; AC, CMF, CEF, CAF, ABVD, CHOP, COP – treatment schemes

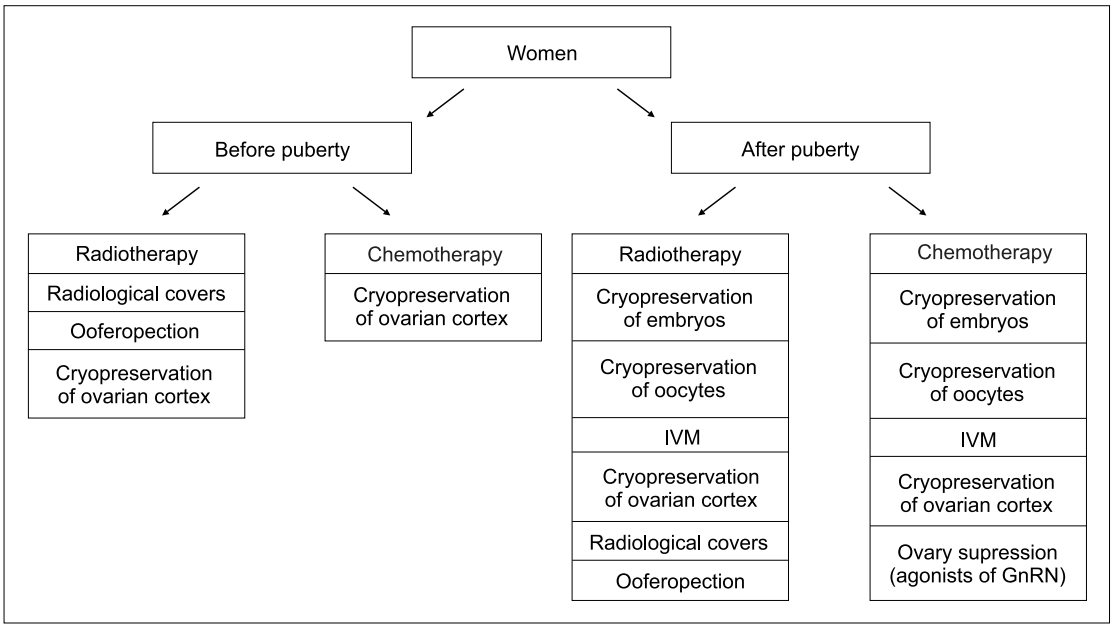

Fig 1. Female fertility preservation options.
IVF – *in vitro* fertilization; IVM – maturation of cells *in vitro*, GnRH – gonadoliberyna

Proposed criteria including the patient to the group of collection and cryopreservation of ovarian cortex are mainly: age under 40, risk of POF at the level of min. 30%, will to get pregnant, impossibility of IVF prior to oncological treatment, no contraindications to surgery, signed consent to treatment, exclusion of any possibility of transplanting cancer cells (12).

Until now, in spite of many attempts of retransplant, there were 14 children born thanks to this method (13). Pregnancy was achieved both naturally and by IVF procedure.

Ovarian cortex contains in majority the immature, primordial follicles arrested in the diplothene stage of prophase I of meiosis. The follicles have diameter of 30-60 μm and contain oocyte of 9-20 μm. They are surrounded by one layer of granular cells being in constant connection with the oocyte through intercellular connections. The antral follicle, also called a Graafian follicle, have a diameter of more than 200 μm, and is surrounded by zona pellucida, separating it from the granulose cells. By the end of the follicular, or proliferative phase oocyte takes peripheral place and is surrounded by two to three layers of granulosa cells, so-called cumulus oophorus (14). The cumulus oophorus layer of the preovulatory follicle will develop an opening and excrete the oocyte with a complement of cumulus cells in a process called ovulation. The oocyte is now called the ovum and is competent to undergo fertilization.

Primordial follicles are more resilient to freezing and defreezing because of smaller size (easier penetration of cryoprotectant), big ratio between the area and volume, low metabolism, as well as lack of spindle apparatus, zona pellucida and mature granular cells. However, after defreezing the loss of follicles is more likely than in case of freezing embryos.

Cryopreservation of ovarian cortex can be divided into three stages.

Stage 1. Collection and freezing of ovarian tissue (Silber)

The tissue of ovary can be collected in two ways: through collection of fragments of ovarian cortex or isolated follicles. Currently, the tissue is collected by fragment of ovarian cortex. Other options are yet impossible due to technical problems (15).

The tissue of ovary is collected by laparoscopic method in general anesthesia (the procedure takes ca. 45 minutes) or during surgery aiming in removing tumor, which decreases the amount of surgeries needed. Depending on the amount of collected tissue, several fragments of ovarian cortex is prepared (10-50). The material has to be instantly moved to the laboratory of aided reproduction on Leibovitz 15 base being covered with ice. In the laboratory the cortex is divided into thin slips (1-2 mm thick). The numer of slips depends on the amount of the material, estimated loss of egg cells and age of the patient. It is important that all the fragments are the same size in order to ensure good cryoprotectant (substance preventing cell damage) penetration. The choice of cryoprotectant is essential. It is known that while using glycerol 90% of follicles is lost, with 55% while using propylene glycol and 25% – ethylene glycol.

Every fragment is sank in special flasks for cryovial sample storage containing 1 ml of cryoprotectant (usually 1.5 mol/l DMSO and 0.1 mol/l sacharosis from the base and 10% of patient serum) for 30 min in temperature of 4°C. Then the flasks are transported to special freezers and the temperature is gradually decreased. At first from 4°C to -9°C with speed of 2°C/min, later the temperature is decreased to -40°C with speed of 0.3°C/min. In the end, before transporting to liquid nitrogen, in which the flasks are stored, the temperature is decrease to -140°C with speed of 10°C/min (16, 17).

Stage 2. Exclusion of possible metastasis

The possibility of transferring cancer cells along with the transplant has been the subject of many studies. Meirow et al. in their research showed that ovarian cortex transplant was not correlated with transferring Hodgkin's lymphoma cells (18). Kim et al. showed that collection of ovary tissue prior to high dose chemotherapy for HD or NHL creates no risk of disease transmission during self-transplant, although it cannot be completely excluded (19). Gosden, Rutherford and Norfolk reckon that recurrence of disease after ovary tissue transplant among patients with HL or Wilms' tumor is very unlikely to happen (20).

In order to exclude metastasis some amount of tissue is directed to histopathological, immunohistochesmical or PCR research. It is an important problem which requires assessment of risk correlated with cancer type, the frequency of finding cancer cells in ovarian tissue fragments while using techniques based on molecular biology. One of experimental methods may also be xenotransplant which is based on transplanting the ovarian tissue to a mouse with immune deficiency. During research antral follicles development was observed. Other observations were made concerning formation of corpus luteum and concentration of progesterone among animals. There is little data concerning maturing of human follicles in animals. Immunocytochemical analyses show irregular microtubules and DNA, however, it is not known if the reason was the xenotransplant itself or the process of freezing and defreezing. It has not been stated yet if human oocytes maturing in animals are functionally and ultrastructurally correct. Another problem is ethical aspect concerning safety of maturing follicles, as well as mixing human and animals' DNA (21). Xenotransplant is not a therapeutic method used in humans.

In some cases, several cycles of chemiotherapy is implemented before the collection of ovarian cortex to destroy possible cancer cells in gonads. An alternative options is also IVM and IVF (9, 12, 22).

Stage 3. Retransplant of ovrian cortex

Surely, one of the most difficult stages is the retransplant of ovary tissue, during which more follicles are destroyed than during freezing and defreezing. The percentage of functioning follicles varies between 5 and 50%. The transplant of ovary fragment without vascular pedicle is correlated with ischemia and hypoxia (ovarian cortex tolerates ischemia for 3 hours, but in temperature of 4°C), while the process of revascularization may last up to 48 hours. An additional factor decreasing the effectiveness of this method is high sensitivity of ovarian stromal cells to low temperature (the level of apoptosis is higher in frozen and defrozen tissues than in fresh ones, despite of the time of incubation).

The flasks with ovarian cortex fragments are defrozen in room temperature for 2 minutes and later put in water bath of 37°C for the next 2 minutes. After the time needed the fragments of ovarian cortex are transported from the flask to plates with tissue culture. The material has to be washed several times in order to get rid of the rest of cryoprotectant.

The ovary tissue may be transplanted ortho- and heterotopic way. In orthotopic transplantation the ovary tissue is put in pelvis in the place where the tissue was collected from or around this place. The slips of ovarian cortex may be implanted in course of laparoscopic or mini-laparotomic surgery. They are placed in specially prepared pockets of ovarian peritoneum, close to vessels and oviduct. The pockets may be prepared a few days earlier. After the cut, the edges should be electrocoagulated, in order to stimulate angiogenesis and neovascularisation, which can prevent the effect of ischemia and loss of many follicles. It is also said that therapy with antioxidants or substitution of factors stimulating vessel growth may prevent to some extent ischemia. The cut may be done lengthwise or crosswise and the fragments of ovary are placed next to each other. Then the pocket is closed. Apart from that, in case of removal of both ovaries, there is a possibility of sewing the ovary tissue to peritoneum (to triangular pockets of assimilable cellulosic membrane). Natural fertilization is possible only after orthotopic transplant.

Heterotopic transplantation is based on placing the ovary tissue in hypodermic tissue of forearm or stomach, e.g. in the pocket in the rectus abdominis muscle or breast muscle. The method of transplantation is analogical to orthotopic one – tissue is placed in specially prepared pockets. Experiments on animals show that transplant within stomach area is much more effective than in forearm, due to possible injuries, lower temperature than required to maturing of follicles (higher level of survival and development of cells). Heterotopic transplantation is technically easier and is characterized by better accessibility. Currently, one pregnancy was achieved after heterotopic transplantation, although it resulted in miscarriage in I trimester.

It was stated that after ovary tissue transplantation hormonal and reproduction function return many years after the procedure. Menstrual cycle return usually after 4-5 months (8 weeks to 8 months), due to differences in ovarian reserves and time of transplant, which is consistent with the time of folliculogenesis.

Retransplantation of ovarian cortex in most cases should be done around 2 years after the completion of cancer treatment, always after consulting with oncologist, when the risk of metastasis is much lower (9, 23, 24).

DISADVANTAGES OF CRYOPRESERVATION OF OVARIAN CORTEX

Cryopreservation of ovarian cortex is nowadays a promising method, although characterized by low effectiveness. An important issue is also the necessity of surgery and correlated with it general anesthesia. Another important problem is the probability of transplanting ovarian cortex with cancer cells. The risk of cancer increases among the patients with hematological cancer and this causing

metastasis to ovary. Until now, it was not observed to implant cancer among women with breast cancer.

Depending on method used, loss of follicles may occur. The choice of cryoprotectant as well as way of tissue preparation, retransplant and ischemia play an essential role in this matter.

Cryopreservation of ovarian cortex is also a very expensive method. The price contains surgery, freezing and storage of tissue as well as retransplant.

CONCLUSIONS

Cryopreservation of ovarian cortex is a relatively new, but very promising method. It enables to preserve fertility even among young girls, regardless of the method of cancer treatment. It is essential to remember that it is not only the life of a patient that counts. The aim is to provide as high as possible standard of life through the attempt to assist in realization of dream of motherhood (24).

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