REVIEW

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Fertility restoration by the cryopreservation of oocytes and ovarian tissue from the position of biomedical ethics: a review

Однією зі значних проблем хіміо/радіотерапії є втрата плідності, що може призводити до порушення психологічної рівноваги та якості життя жінок, які пережили рак. Останнім часом кріоконсервація тканин яєчника з їх наступною аутотрансплантацією відкрила нові можливості в галузі відновлення плідності. Поряд з психологічними та етичними питаннями, пов'язаними з процедурою, існує ризик ре-трансплантації пухлинних клітин і відновлення злоякісності. В цьому огляді ми приділяємо увагу новим досягненням у кріоконсервації яйцеклітин і тканини яєчників і намагаємося відповісти на питання про безпечність та ефективність відновлення плідності цим шляхом.

Introduction. Preservation of fertility in female patients diagnosed with cancer has recently been an area of intensive investigation. Women diagnosed with cancer prior to or during their reproductive period often have to deal not only with the uncertainty of long-term survival, but also with the partial or total loss of fertility function as a result of cancer treatment. Many treatments administered for cancer are gonadotoxic, mainly because of the utilisation of alkylating agents that damage both resting and actively dividing cells, and/or involve radiation therapy, leading to premature ovarian failure and infertility in the majority of these patients [28, 29, 50]. An ovarian radiation dose of more than 6 Gy usually culminates in permanent infertility. Cancer is not uncommon in younger women, and the treatment required for most of the common cancer types occurring in younger women implies either removal of the reproductive organs, or cytotoxic treatment that could partially or definitively affect reproductive function [48].

One method of preserving fertility in this circumstance is to cryopreserve embryos, oocytes, or ovarian tissue before initiating chemoradiotherapy. Cryopreservation of embryos has ethical and technical consequences. It is not applicable in cases of children with cancer or woman who do not desire to create an embryo for the future. Moreover, chemoradiotherapy should occur immediately after the diagnosis and there is not usually time for hyperstimulation.

Cryopreservation of oocytes. Till now about 150 babies in all over the world were born after the fertilisation of cryopreserved oocytes in standard IVF or ICSI protocols [53, 6]. Though survival rate of the oocytes after thawing varies between 24 % and 100 %, the implantation rate of the embryos created by fertilization of cryopreserved oocytes does not reach more than 3% [5].

The particular frailness of oocytes as compared to embryos with two pronucleus is due to morphological and functional conditions [16] . The most important of them are:

Volume-surface coefficient. The volume-surface coefficient of oocyte is very low, and this could cause deceleration of water displacement during freezing-thawing procedure and intracellular formation of crystals of ice. Nevertheless, the survival rate of embryos with two pronucleus is higher than survival rate of unfertilized oocytes, which suggests that not only volume-surface relationship influences enhanced frailness of oocytes [8].

Calcium influx. The destruction of cortical granules and influx of Ca^{2+} during the thawing could be responsible for the alteration of the fertilization process [55, 24, 61, 16].

Metabolic activation. The metabolic activity of the cytoplasm of the embryo is much higher compared with unfertilized oocyte, which could influence higher resistance to freezing-thawing damage. The increase of the intracellular calcium concentration, being a part of the activation processes, increases the resistance to the osmotic shock [5,13, 26].

Injury of the meiotic spindle. Microtubules are the cytoplasm structures which are most sensitive to freezing-thawing injury and destruction of meiotic spindle could cause dispersion of chromosomes, aneuploidies, fertilization failure and development arrest [40].

Moreover, recovery of mature oocytes requires hyper stimulation, which is often impossible within urgent chemoradiotherapy. Cryopreservation of ovarian tissue with subsequent autotransplantation remains a tenuous option in the effort to restore fertility after chemoradiotherapy. That is why cryopreservation of ovarian tissue, with subsequent autotransplantation remains a most perspective option in the purpose to restore fertility after successful chemoradiotherapy.

Cryopreservation of ovarian tissue. Human ovarian tissue transplantation was reported for the first time in 1996 [20, 21], and ovarian orthotopic transplantation was described in 2000 [34]. The success thus far in humans is very limited. Only two cases of live birth of child after autotransplantation of cryopreserved ovarian tissue have been reported: one after in vitro fertilisation [30], and other by natural conception [11].

In the context of artificial reproduction once the ovarian tissue is cryopreserved, future options include transplantation of the tissue back to the woman (autotransplantation), or to nude mice (xenotransplantation), or to culture the follicles *in vitro*. Two different surgical approaches have been used for autotransplantation in humans: orthotopic or heterotopic. Orthotopic transplantation places ovarian tissue at close proximity to the infundibulo-pelvic ligament with the hope that natural pregnancy may occur. Heterotopic transplantation is an alternative approach by which cryopreserved ovarian tissue is transplanted to a site outside of the pelvis [30, 21].

In comparison to mature oocytes primordial follicles are relatively small and resistant to cryoinjury, owing to a relatively inactive metabolism, lack of metaphase spindle, zona pellucida and cortical granules. By cryopreserving ovarian cortical tissue, thousands of immature oocytes can be stored without the need for ovarian stimulation [1]. Nevertheless, one of the problems is the low survival rate and low developmental potential of oocytes from frozen-thawed ovarian tissue. Segino and colleagues [47] describe the study of frozen-thawed ovarian tissue of three-week old female mice. High mortality in part of the granulosa cells of a follicle obtained from frozen/thawed ovarian tissue and delayed development of the preantral follicles isolated from frozen/thawed mouse ovarian tissues were demonstrated. Rimon et al. [43] represent the study group which included 6 women with cancer who underwent ovarian tissue cryopreservation (OTCP). The incidence of apoptosis and abnormal morphology of primordial and primary follicles in frozen-thawed samples

were two times higher than in control samples.

Among the injury agents which act during cryopreservation is the toxicity of cryoprotectants. In the study of Santos et al. [44] histological analysis showed that, compared to control fragments, all cryoprotectants (glycerol (GLY), ethylene glycol (EG), propanediol (PROH) or dimethyl sulfoxide (DMSO) significantly reduced the percentage of normal preantral follicles in ovarian fragments prior to or after cryopreservation.

Orthotopic transplantation. There has been a single case report of ovarian endocrine function but no ovulation following such transplantation [42]. Very recently Donnez et al. [12] reported return of ovarian endocrine function and a live birth following orthotopic transplantation of cryopreserved ovarian tissue in a woman treated for Hodgkin's lymphoma. Schmidt et al. [46] represent the study of ovarian function of women, who previously had cortical tissue from an entire ovary cryopreserved prior to chemotherapy because of malignant disease and then autotransplanted after the treatment. All three patients with autotransplanted ovarian tissue regained ovarian function as confirmed by return of menses, follicles on ultrasonography and normalised hormone levels. Authors conclude that autotransplantation of ovarian tissue led to return of ovarian function.

Meirow et al. [29] describe a live birth after in vitro fertilisation following the transplantation of thawed cryopreserved ovarian cortical tissue into the ovaries of a 28-year-old woman who had ovarian failure after high-dose chemotherapy for non-Hodgkin's lymphoma.

Heterotopic transplantation. Transplantation to a heterotopic site such as the forearm [38] or abdomen [39] is technically easier and less risky than orthotopic transplantation. It also allows easier monitoring of follicle growth. Evidently IVF-embryo transfer is necessary in order to achieve pregnancy. Regardless of the different attempts there are no pregnancies reported after heterotopic ovarian tissue transplantation in human. In 2001, Oktay et al. [37] , were the first to report return of ovarian endocrine function with the development of a dominant follicle and resumption of menstrual cycles in two women using this approach. In one case, after blocking the patient's pituitary function with gonadotropin releasing hormone antagonist, and stimulating her with human menopausal gonadotropin for 11 days, they performed percutaneous oocyte retrieval from the forearm, but the fertilisation could not be achieved with ICSI [36] . More recently, Oktay et al. [39], by transplanting the cryopreserved ovarian tissue beneath the abdominal skin, were able to restore ovarian function in a woman previously treated for breast cancer, but pregnancy did not occur.

Xenotransplantation. Mice with severe combined immunodeficiency (SCID) can accommodate tissues from foreign species without host-versus-graft response due to a deficiency in both T and B-cell mediated immunity [4]. Xenotransplantation of cryopreserved ovarian tissue into SCID mice has also shown success, with healthy follicles present in the graft when removed 22 weeks after the initial transplantation [34]. Indeed, following subcutaneous placement of human ovarian cortical tissue into mice, follicular growth in response to exogenous gonadotropin stimulation, follicle maturation, and corpus luteum formation have been observed [57]. Ishijima et al. [22] report the results of the cryopreservation of the ovarian tissue of dog by a vitrification method and the successful transplantation of this tissue into mice. Following some authors the use of this option gives the possibility to eliminate of cancer cell transmission since cancer cells do not penetrate zona pellucida. Another advantage could be the possible application in women in whom hormonal stimulation is contraindicated. Additional advantages of xenotransplantation include convenient monitoring of follicular development, and easy access to follicle aspiration [43] . However, this method is unlikely to be clinically available in

the near future. Recently, it has been demonstrated that oocytes of frozen-thawed human ovarian tissue transplanted into severe combined immunodeficient mice, and further matured in vitro, showed aberrant microtubule organization and chromatin patterns [25] . Moreover, possible transmission of zoonoses to humans is a serious concern.

Cryopreservation of whole ovaries. There are some very encouraging results showing return of fertility after the cryopreservation of whole ovaries of small animals [18, 9, 56] . An important limiting factor of restoration of fertility after the ovarian cryopreservation of big animals is the significant follicles' loss that occurs during the initial ischemia resulting from thrombosis after grafting [60] . The use of microsurgical techniques has led to improvements in graft survival [3] . Dissection of ovarian vessels during ovariectomy and perfusion of ovary with cryoprotectants through these vessels improved tissue survival and led to similar rates of follicular viability and apoptosis to ovarian cortical strips [23] .

A successful pregnancy was achieved following transplantation of frozen–thawed rat ovaries [56] . Most recently Arav et al. [2] reported successful restoration of endocrine activity, oocyte recovery and embryo development after cryopreservation and transplantation of whole sheep ovary. While these results are encouraging, the risk of metastatic disease remains an important concern. Moreover, in the fertility preservation approach in humans, removal of whole ovary is not advised because one can never completely exclude recovery of ovarian function after chemotherapy.

Risks. Samples of ovarian tissue usually are obtained from biopsies collected laparoscopically, and the procedure is accompanied with risks typical for laparoscopical surgery [19] . Nevertheless, the greatest concern with ovarian tissue cryopreservation and following transplantation is the possibility of reseeding a cancer, harboured within the ovary, with autografting frozen–thawed ovarian strips [10, 15].

Ideally, ovarian tissue cryopreservation for the purposes of future autotransplantation should be performed on patients with a low risk for cancer metastasis to the ovary. Fortunately, most of the malignant diseases encountered during the reproductive years do not metastasise to the ovaries. Exceptions include blood borne malignancies such as leukaemia, neuroblastoma, and Burkitt's lymphoma.

As summarised by Sonmezer and Oktay [50, 51] , cancers with a low risk of ovarian involvement include Wilms' tumour, Ewing's sarcoma, non-Hodgkin's and Hodgkin's lymphomas, non-genital rhabdomyosarcoma, osteogenic sarcoma, and squamous cell carcinoma of the cervix.

Cancers with moderate risk include adenocarcinoma of the colon, rectum, and appendix, upper gastrointestinal system malignancies, and cervical carcinoma with adeno/adenosquamous differentiation [31] .

High risk of ovarian involvement occurs with leukaemia, neuroblastoma, genital rhabdomyosarcoma, and Burkitt's lymphoma [7, 58, 49, 27, 59]. Histological evaluation of ovarian samples has been suggested in order to prevent cancer transmission, although it is not possible to completely eliminate the risk of transmission in haematological or disseminated malignancies.

There are proposals that patients with high risk cancers such as leukaemia and neuroblastoma should either not be given the option of ovarian autotransplantation, or ovarian tissue harvest should be performed after the first round of chemotherapy in order to ablate any neoplastic cells residing within the ovary.

However, in doing this, it is also wise to remember that the ovarian reserve may be compromised with each cycle of chemotherapy, which will diminish the longevity and survival of the grafts [42] .

Another risk of ovarian tissue transplantation is the malignant transformation. Germline mutations in the tumour suppressor genes BRCA1 and BRCA2 predispose women to breast and ovarian cancer. Female carriers

of BRCA1 or BRCA2 gene mutations have very high lifetime risks for breast and ovarian cancers [14] . In particular, patients with BRCA-1 and BRCA-2 mutations have a 60% and 10–20% lifetime risk of developing ovarian cancer, respectively [15]. Since no screening method has proven effective for ovarian cancer, prophylactic salphingo–oophorectomy should be discussed with all carriers of BRCA mutations, as soon as childbearing is completed or by age 35–40. Orthotopic transplantation should not be offered to these patients. Some authors propose a heterotopic transplantation as a possibility, but with the condition that transplanted tissue should be removed in its entirety as soon as fertility treatment is complete.

Mueller et al. [32] present the negative long-term effect of heterotopic transplantation of cryopreserved ovarian tissue. Ovarian tissue from rats was cryopreserved using a slow-freezing protocol. After thawing, the tissue pieces were transplanted under the splenic capsule in 14 rats of the same inbred strain and remained there for 210 or 300 days. Sex cord stromal tumours, consisting mainly of granulosa cells, were found in all of the rats. Although the hormonal situation in rats cannot be directly compared to that in humans, the development of sex cord stromal tumours in this animal model may be worth considering when cryopreserved ovarian tissue is transplanted heterotopically in fertility-preserving programs for cancer patients.

Poirot et al. [41] present the study in which from September 2000 to December 2004, 47 prepubertal girls were referred by oncologists for ovarian tissue cryopreservation. After informed consent, the ovarian tissue was collected and frozen by a slow cooling protocol until the temperature of liquid nitrogen. No surgical side effect occurred. No metastatic ovarian tumour was found.

Psychological problems. In an attempt to restore fertility after chemoradiotherapy, doctors and patients often make heroic sacrifices. It is unlikely that the risks and sacrifices of reaching maternity always help and not worse the psychological status of cancer survivors [51] .

The following quotation is from the review of Akar and Oktay, 2005 [1]: 'The patient had undergone bilateral mastectomy and lymph node dissection, followed by ovarian cryopreservation before chemotherapy with large doses of alkylating agents and stem cell transplantation at age 30. The patient had become and remained menopausal after chemotherapy. In this patient, ovarian cortical pieces were transplanted above the rectus abdominus fascia, because the forearm was not suitable as a result of previous extensive axillary lymph node dissection. After remaining in the menopause for six years, the patient's ovarian function returned within three months of ovarian transplantation. We then attempted percutaneous oocyte retrieval. After eight attempts, 20 oocytes were retrieved and eight were suitable for IVF. Only one oocyte fertilized normally and progressed to a four-cell stage embryo; no pregnancy occurred after transfer of the embryo.'

The case quoted above identifies risks of recurrence of malignancy as well as risks from hormonal stimulation and IVF technology. There is also the risk of misperceptions about elements of the natural image of maternity. In the process of fertility treatments the mother to be does not always enjoy good health. Some sense of corporal elements of reproduction are required in order to feel hopeful and positive about the situation. In the case described above by Akar and Oktay, eight unsuccessful attempts to overcome sterility from cancer treatment did not improve the personal comfort of the woman who had lost both breasts, the function of both ovaries, and damage to her arm. Perhaps, psychological counselling directed to encouraging women to accept their condition of infertility and help find another way to exercise the desire for maternity and to ameliorate a psychological imbalance [45] .

Conclusion. The effectiveness of oocytes and ovarian tissue cryopreservation still

remains in the phase of experiment.

The use of cryopreservation of mature oocytes for fertility restoration is limited by the particular frailness of the elements of their cytoplasm and by the fact that recovery of mature oocytes requires hyper stimulation, which is often impossible within urgent chemoradiotherapy. The implantation rate of embryos created from frozen- thawed oocytes is very low, and high risks of aneuploidies in the child are due to cryopreservation derived microtubule damage. The risks for mother, are coupled with hyperstimulation and laparoscopia procedures.

Sample collection of ovarian tissue does not need neither hyperstimulation, nor time for follicle growth and could be executed directly before or after the first round of chemoradiotherapy with the risks typical for laparoscopic surgery. On the contrary, the major concern with following transplantation of cryopreserved ovarian tissue is the possibility of tumour cells reseeding.

Besides the development of the technologies of cryopreservation, new cryoprotectants and surgery achievements there is still a problem of the low survival rate and low developmental potential of oocytes from frozen-thawed ovarian tissue.

While regardless of the different attempts there are no pregnancies reported after heterotopic ovarian tissue transplantation, the attempts of orthotopic transplantation has resulted in two cases of live birth. It seems that the orthotopic transplantation of ovarian tissue should be considered positive, when performed on patients with a low risk for cancer metastasis to the ovary and when the transplantation does not increase the risk of recurrence of malignancy.

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FERTILITY RESTORATION BY THE CRYOPRESERVATION OF OOCYTES AND OVARIAN TISSUE FROM THE POSITION OF BIOMEDICAL ETHICS: A REVIEW

The loss of fertility as a consequence of chemoradiotherapy is a considerable problem. It can affect the psychological equilibrium and quality of life for women cancer survivors. In recent years, the possibility of cryopresenrvation of ovarian tissue following auto transplantation, opens new promise in the attempt to restore fertility. In addition to psychological and ethical concerns of this procedure, there are risks of retransplantation of tumor cells and recurrence of malignancy. In this review we will focus on the most recent achievements in cryopreservation of oocytes and ovarian tissue and will attempt to answer questions about the safety and effectiveness of restoration of fertility by cryopreservation of oocytes or ovarian tissue.

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