REVIEW

Preservation of Fertility in Women Undergoing Chemotherapy: Current Approach and Future Prospects

RONIT ABIR,^{1,2} BENJAMIN FISCH,¹ AHUD RAZ,¹ SHMUEL NITKE,¹ and ZION BEN-RAFAEL¹

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Purpose: Anticancer treatment causes ovarian failure.

Methods: Some hormones may have a protective effect on the ovary. Cryopreservation (freezing) of oocytes has had very limited success, and therefore, currently its use before chemotherapy is not a feasible option. However, cryopreservation of embryos is possible. Another solution is oocyte donation followed by in vitro fertilization (IVF).

Results: Ovarian cortical slices containing primordial follicles have been cryopreserved successfully. To restore fertility, cryopreserved-thawed tissue taken from cancer patients before therapy could be replanted after recovery. The possible risk of malignancy restoration could be eliminated by obtaining unilaminar follicles from cryopreserved-thawed tissue and growing them in vitro, followed by routine IVF.

Conclusions: Although women who undergo chemotherapy face limited options for fertility preservation, intensive studies in cryopreservation and in vitro maturation of follicles harbor hope for brighter prospects in the future.

KEY WORDS: primordial follicles; chemotherapy; "protective" hormonal treatment; cryopreservation; in vitro maturation.

THE PROBLEM

As cancer treatment improves, more young women of reproductive age survive. However, the radiation and chemotherapy used to cure the primary illness also

cause ovarian failure, premature menopause, and amenorrhea (1,2). These have been associated mostly to cases of women with malignancies that affect young patients, such as breast cancer, lymphoma (Hodgkin's and non-Hodgkin's), and leukemias and related blood dyscrasias, but there are also some reports of ovarian failure in cases of choriocarcinoma, thyroid cancer, and other cancers (1). The mechanism of follicular damage induced by chemotherapy is not yet understood, since most of the follicles in the human ovary are inactive primordial follicles and do not undergo mitotic division (3), whereas chemotherapeutic drugs are supposed to affect only dividing cells (4). One possible explanation is that these drugs cause damage only in growing follicles, and through the hypophysial feedback mechanisms, more primordial follicles are recruited to the growing pool and so on. In vitro studies of rat granulosa cells have shown that these cells are the primary targets for cyclophosphamide-induced ovarian damage (5–7). However, cyclophosphamide was also shown to cause damage to oocytes in vitro and in vivo (5,6). It is possible that this is due to the fact the granulosa cells serve as nurturing cells to the oocytes through gap junctions (5).

A number of factors determine the level of ovarian damage caused by anticancer treatment (1,2).

The Agent Used. The main chemotherapeutic agents that induce ovarian damage are alkylating agents such as cyclophosphamide, melphalan, and chlorambucil; other drugs such as *cis*-platinum and vinca alkaloids have also been implicated. Combination chemotherapy is associated with an increase in ovarian failure. Radiotherapy damage also depends on the position of the

¹ Department of Obstetrics and Gynecology, Rabin Medical Center, Beilinson Campus, Petah Tikva 49100, Israel.

² To whom correspondence should be addressed at IVF Research Laboratory, Department of Obstetrics and Gynecology, Rabin Medical Center, Beilinson Campus, Petah Tikva 49100, Israel.

ovaries within the abdominal radiotherapy field. Another factor that increases ovarian damage is combined chemo- and radiotherapy.

The Treatment Protocol. The dose of the chemotherapeutic agent, the duration of administration, and the total dosage received by the patient are all important factors that affect the level of ovarian damage. Patients who receive several cycles of chemotherapy are more likely to have severe ovarian dysfunction.

Patient Age. The younger the patient, the lower the likelihood of severe premature ovarian failure, and vice versa (1,2,8,9).

MEDICAL AND SURGICAL MEANS OF OVARIAN PROTECTION

To limit radiation damage to the ovaries, surgical transposition can be used to place at least one ovary as far out of the main radiation field as possible (1). However, because of scattered radiation, a substantial loss of ovarian function might still occur (1,10–12).

Several studies have shown lesser ovarian damage after chemotherapy in young, prepubertal girls (8,9). This may be attributed to the age of the patients and, also, perhaps to their premenarchial hormonal status. If the latter is true, mimicking the prepubertal state may protect the ovary. Therefore, various studies have suggested the possible use of hormones during or before chemotherapy as follows.

Contraceptive Pills. Comparison of ovarian biopsy findings in patients with Hodgkin's lymphoma who received chemotherapy with versus without contraceptive pills showed a higher number of primordial follicles in those who received the contraceptives (13,14).

Gonadotropin-Releasing Hormone (GnRH) Analogue. This hormone prevents follicles from growing and undergoing mitotic divisions by blocking gonadotropin induction. The mechanism of action of GnRH analogue may involve direct suppression of gonadotropin receptors in the ovary, leading to atresia of the already developed follicles as well as inhibition of recruitment of small follicles into the proliferating pool (4,5). Currently, GnRH analogue treatment appears to be the most promising approach for prevention of cytotoxicity associated ovarian failure (15). It appears that these analogues act on an earlier stage of folliculogenesis than cyclophosphamide, thus preventing the follicles from reaching the chemotherapy-sensitive stage (5,6). Ovarian biopsy samples from rats given a GnRH analogue in combination with cyclophosphamide yielded more follicles than those from rats treated only

with cyclophosphamide (4,16), and the rats' fertility was maintained (16). Similar results were achieved with rhesus monkeys (17). In a recent study, GnRH analogue was given to 18 of 36 lymphoma patients treated with combination chemotherapy or combined radio- and chemotherapy (15,18). Fifteen of the 16 surviving patients who received the analogue began menstruating regularly within a few months of termination of therapy, compared with only 7 of 18 women who were not given GnRH analogue.

Progesterone or One of Its Active Analogues. Another study showed that the administration of combined progesterone and cyclophosphamide to rats maintained their fertility better than GnRH analogue administration (16), and only with progesterone were the normal fecundity rates in the rats maintained. Medroxyprogesterone acetate (MPA) was given to 12 Hodgkin's lymphoma patients who received combination chemotherapy (19). At the end of treatment, ovarian biopsy study by light microscopy showed that, although there was a significant decrease in the amount of primordial follicles in the women given MPA, their number was still significantly higher than in the women who did not receive MPA. However, electron microscopy study revealed that, despite the higher numbers, the quality of the primordial follicles from the MPA group was low and there were signs of intracellular damage (thick basal membranes, various nuclear changes, changes in the shape of the mitochondria etc.). This raised the question of the actual ovarian "protective" effect of progesterone. Therefore, other solutions for fertility preservation should be sought for candidates of anticancer treatment.

CRYOPRESERVATION OF OOCYTES AND EMBRYOS AND OVUM DONATION

Cryopreservation (freezing) of oocytes from various mammalian species, including humans, has had very limited success (20,21). It has been linked with spindle damage and thus with chromosomal abnormalities (21), parthogenesis of oocytes (22), zona hardening, and low oocyte survival rates after thawing (21). There have been very limited reports of human pregnancies and births from a frozen-thawed oocyte after routine in vitro fertilization (IVF) (23–25). In a recent study, 1- or 2-day-old unfertilized frozen-thawed oocytes from an IVF program survived better than thawed-fresh oocytes cryopreserved immediately after collection. Nevertheless, embryonic development after intracytoplasmic sperm injection (ICSI) was better from frozen-thawed freshly collected oocytes compared with aged unfertil-

ized oocytes (26). Pregnancies were achieved only from freshly collected thawed oocytes but none of these pregnancies went successfully to term. Then, in 1997, Porcu *et al.* (20) reported a normal birth from a frozen-thawed oocyte after ICSI. The oocytes were frozen with 1,2-propanediol (PROH) and sucrose in a solution containing 30% plasmanate. Thus, the use of cryopreservation with ICSI could perhaps lead to higher fertilization rates of cryopreserved-thawed oocytes (23–28). Immature germinal vesicle-stage mouse (29) and human (30–32) oocytes have also been frozen successfully, but some studies showed difficulties in both in vitro maturation of these oocytes (29) and further embryonic development (31). Since oocyte cryopreservation is still very experimental, it is not yet an option for female patients before chemo- and radiotherapy.

Cryopreservation of embryos is, however, a routine procedure at IVF units (33). Therefore, before chemotherapy women can undergo IVF treatment and have their embryos cryopreserved (2,34). However, this is far from an optimal solution. Prior to cryopreservation of oocytes and embryos, superovulation is required, and this might have unpleasant side effects. Furthermore, in many cases the patients are women who do not yet have partners or young girls, such that donor sperm must be used, which could be controversial.

Another option is oocyte donation followed by routine IVF (35–37). This, too, has several drawbacks: there is a shortage of donated oocytes, and most women would prefer to use their own oocytes before turning to this alternative. The use of oocytes from ovaries of aborted fetuses could serve as a putative source of donated ova, although there are ethical debates about this issue (38). It is unknown whether oocytes from fetuses have the same developmental capacity as those from adults, and few studies to date have investigated the growth of fetal follicles. In one work, fresh and frozen-thawed fetal ovaries from mice were transplanted under the kidney capsule or to the bursal cavity of oophorectomized mice (39). The fetal allografts restored the cyclic activity and the follicular development in the hosts, and in 33% of the cases of transplantation in the bursal cavity, the hosts became pregnant from the donor fetal oocytes. Fetal primordial follicles from bovine (40) and baboon (41) were shown to initiate in vitro growth when cultured in ovarian slices. In another study (42) fresh and cryopreserved-thawed human fetal ovaries after rapid freezing were placed in organ culture. Although a polar body was extruded in some of the oocytes, the oocytes did not reach the size associated with mature adult oocytes; also, the extrusion of the polar body may have indicated atre-

sia and not nuclear maturation (3). Further research is needed in this area to clarify the feasibility of this option.

CRYOPRESERVATION AND TRANSPLANTATION OF OVARIAN CORTICAL SLICES

Contrary to the difficulties with oocytes, primordial follicles that are found in the ovarian cortex (43) are less sensitive to cryopreservation damage (44,45). It is, therefore, possible to freeze and store ovarian tissue from cancer patients prior to therapy to restore fertility and replant the cryopreserved-thawed tissue once the patients are cured. It is noteworthy that retrieval of ovarian tissue involves only a simple laparoscopic operation, without the side effects of hormonal stimulation for IVF.

Transplantation of primordial follicles into ovaries of sterile mice induced growth of these follicles to preovulatory sizes (45–47). Transplantation of frozen-thawed primordial follicles frozen with dimethylsulfoxide (DMSO) to ovaries of sterile mice resulted in live births from the transplants (47,48). In another study, transplantation of mouse ovarian slices frozen with DMSO resulted in live births (49). Frozen-thawed and fresh ovarian autographs frozen with DMSO restored fertility in oophorectomized sheep (44), and two lambs were born: one from a frozen-thawed biopsy and another from a fresh biopsy.

The kidney capsule of immunodeficient mice serves as a site for transplantation of ovarian slices from various species and as a model for the development of follicles, since the transplants are not rejected and receive a rich blood supply (39,45). Ovarian cortical slices from sheep and cats were transplanted in this way and developed to late antral stages (50). In a very recent study, human ovarian cortical slices were transplanted to SCID/*hpg* mice and grew to antral stages after administration of follicle stimulating hormone (FSH) stimulation to the hosts during a graft period of at least 17 weeks (51). Similar results were achieved with transplanted frozen-thawed marmoset ovarian slices frozen with DMSO (52). Human ovarian tissue has been successfully frozen with various cryoprotectants: DMSO, PROH, glycerol, and ethylene glycol (53–55) (Fig. 1). The follicles appeared histologically normal after cryopreservation (52) and survived under the kidney capsule of immunodeficient mice for up to 18 days (53). A recent study suggests that the best method of preparing tissue for freezing is exposure for 30 min to 1.5 M solutions of ethylene

Fig. 1. Photographs of sections in human ovaries. (A) A section showing a group of primordial follicles in human ovarian tissue. Paraffin section. (B) A section showing a primordial follicle in a human ovarian tissue after cryopreservation with propandiol and sucrose. Paraffin section. H&E; original magnifications, $\times 400$.

glycol or DMSO at 4°C and subsequent freezing in these solutions (55). However, Shaw *et al.* (56) reported the transmission of lymphoma via grafts of both fresh- and frozen-thawed ovarian tissue from diseased donor mice to healthy recipients. This study highlights the risks of clinical transplantation of ovarian biopsy samples to women who recovered from cancer, especially a bloodborne cancer (54).

IN VITRO MATURATION OF FOLLICLES FROM OVARIAN BIOPSIES

The risks of ovarian replantation could be eliminated by obtaining unilaminar follicles from frozen-thawed tissue and growing the follicles in vitro, followed by routine IVF (58,59). Secondary preantral follicles from various mammalian species have been grown in culture

to antral stages with FSH induction (60,61). Secondary mouse follicles have been matured in vitro and live pups born from the fertilized oocytes (60–63). FSH has also been shown to stimulate growth of secondary hamster follicles (64). Large secondary pig follicles were cultured in collagen gels to antral stages (65), and secondary human follicles isolated enzymatically for 37 hr at 4°C were cultured for 4 days to antral stages (66). Mechanically isolated human secondary and early antral follicles were cultured for a few weeks (67) (Fig. 2), and their viability and growth were better than those of follicles cultured after enzymatic isolation (66). However, not only is the number of secondary follicles low in human ovaries (53,68), but there is a very high natural atretic oocyte loss in early antral human follicles (67). Therefore, maturing the more abundant unilaminar follicles (53,68) presents a more favorable option. Unfortunately, the signals that induce their growth into secondary preantral follicles are unknown, although it is presumed that they are transmitted through interactions with the surrounding stroma cells (3).

There are two possible approaches to the culture of primary and primordial follicles. The first is to culture whole slices of ovarian tissue (organ culture), such that the structural integrity of the ovarian tissue is maintained and hence the interactions between the surrounding stroma cells and the follicles are retained. Mouse primary follicles have been cultured in slices, and the follicles reached preovulatory stages (69). Eppig and O'Brien (70) cultured mouse primordial follicles in organ culture to preantral stages. These secondary follicles were further matured after enzymatic isolation, and in one case, a live pup was born after IVF. In another study slices from fresh- and frozen-thawed adult human ovarian tissue were placed in organ cultures, and unilaminar follicles survived for up to 3 weeks (71). In some cases secondary follicles were observed in the histologic sections, but because direct monitoring of these cultures was not possible, it was not clear whether they originated from unilaminar follicles. It is noteworthy that the studies describing cultures of fetal follicles used this culture system (40–42).

The second approach is to culture isolated unilaminar follicles. This culture system enables direct monitoring of the follicles during the culture period, which is of special importance considering the poorly populated human ovarian tissue in adults (53,68). Enzymatically isolated mouse (72) and mechanically isolated bovine (73) unilaminar follicles have been cultured to multilaminar stages in collagen gels.

Enzymatically isolated rat follicles cultured with stroma cells on poly-L-lysine-coated plates grew to preovulatory stages (74). Enzymatic isolation of primordial (75) and primary (76) follicles has also been successful in the pig, and granulosa cells from primary follicles proliferated in culture (76). Recently Oktay *et al.* (77) described morphologically normal human follicles that were isolated by a combined enzymatic and mechanical method and studied by electron microscopy and fluorescent viability markers. Primordial human follicles from fresh- and frozen-thawed ovarian tissue cultured by Oktay *et al.* (77) survived in culture for up to 5 days, but no indication of actual growth was observed. In a pilot study recently performed in our department (78), human unilaminar follicles were cultured in collagen gels and an increase in the number of granulosa cell layers and in the sizes of the oocytes was observed in 40% of the follicles from both fresh- and frozen-thawed ovarian tissue during a 24-hr culture period.

SUMMARY

Women who are about to receive anticancer treatment face limited options for fertility preservation, but intensive studies, currently under way, hold better promise. Oocyte cryopreservation has had very limited success (20–32) and is currently not a feasible option for women before cancer treatment. Although in some cases embryo cryopreservation may be considered before chemotherapy, this is far from the optimal solution because many of the patients are either very young or unmarried. Oocyte donations are a possibility (2, 35–37), but apart from the shortage in donated oocytes, most women prefer to use their own germ cells before turning to this alternative. Human ovarian cortical slices containing primordial follicles have been cryopreserved successfully (53–55). Therefore, in various hospitals worldwide (79–82), including our own, ovarian biopsy samples from cancer patients before therapy are frozen, with the hope for a breakthrough in this field. Cryopreservation of human ovaries would be a good alternative in cases of irreversibly damaged ovaries due to anticancer treatment, despite the use of “protective” treatment with chemo- and radiotherapy. Although some promising results with replantation of ovarian tissue have been noted in animal studies, there is a risk that its clinical application would restore not only the fertility but also the malignancy, especially leukemias, which are very common in younger patients (56,57). It is possible that in the near-future cryopre-

Fig. 2. Photographs of cultured human follicles. (A) A section in a human preantral follicle after 8 days in culture. Note the thick granulosa layer, the visibly normal oocyte, and the theca layer at an initial stage. Plastic section. Toluidine blue; original magnification, $\times 400$. (B) A section of an early antral human follicle that reached antral stages ($850\ \mu\text{m}$) from a preantral stage. Note the granulosa layer, the visibly normal oocyte, the theca layer, and the antrum. Paraffin section. H&E; original magnification, $\times 200$. Reprinted with permission of the American Society for Reproductive Medicine (Ref. 62).

served-thawed ovarian tissue will be transplanted to women after pathologic markers confirm the biopsy samples contains no malignant cells (58–81). Once the technology of in vitro maturation of human unilaminar follicles is established, it will be possible to switch to a revolutionary method of IVF using oocytes that were

matured and fertilized in vitro, thus avoiding the dangers of ovarian replantation. The availability of such treatment will probably lead to its demand not only from patients with cancer but also from healthy women who choose to postpone childbearing until later in life and therefore wish to retain their fertility.

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