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ORIGINAL ARTICLE

Restoration of Reproductive Potential after Autotransplantation of Frozen Ovaries in Mice Pretreated with Cyclophosphamide

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Purpose: Infertility due to ovarian failure that is caused by antineoplastic chemotherapeutic agents is one of the primary problems of female cancer patients who are in their reproductive years. It has become important to preserve the reproductive potential of female cancer patients. This study was conducted to determine whether autotransplantation of frozen ovaries can restore reproductive potential.

Methods: This study included 30 female mice that had normal reproductive potential. The mice were divided into 4 groups: the positive control, the negative control, the comparison group, and the experimental group. The positive control group received right total oophorectomy, and the negative control group received bilateral total oophorectomy. Greater than or equal to 90% of the left ovary was removed in the mice of the comparison group, and then cyclophosphamide was administered. In the experimental group, the right ovary taken out by right total oophorectomy, and this was crypreserved using the vitrification method. And then cyclophosphamide was administered. The cryopreserved ovary was autotransplanted to the left gonadal fat pad after greater than or equal to 90% of the left ovary was removed. The reproductive performance in each group was analyzed according to the pregnancy rate after mating.

Results: In the positive control group, all five mice became pregnant, and the number of fetuses was 4 to 5 (mean=4.60±0.55). In the comparison group, the pregnancy rate was 50%, and the mean number of fetuses was 1.40±0.55. In the experimental group, 7 of 10 (70%) mice became pregnant, and the mean number of fetuses was 4.71±2.56. There was no significant difference in the number of fetuses between the positive control and the experimental group (p=0.093), but there was a significant difference in the number of fetuses between the comparison group and the experimental group (p=0.019).

Conclusion: The results of this study suggest that autotransplantation of frozen ovaries using the vitrification method may restore the impaired ovarian function induced by antineoplastic chemotherapeutic agents.

Key Words : *Antineoplastic chemotherapeutic agent, Ovarian failure, Cryopreserved ovary, Vitrification method, Autotransplantation*

중심단어 : 항암 화학요법제, 난소부전, 냉동난소, 유리화법, 자가 이식

INTRODUCTION

With advancements in combined antineoplastic chemotherapy and radiotherapy, female cancer patients in their reproductive age can survive for a long time, but cases in which reproductive potential was lost due to ovarian failure after cancer treatment have also increased. Since autotransplantation of cryopreserved ovaries for the restoration of reproductive potential was introduced in 1994, many studies on this procedure have been performed.(1) Based on these studies, Donnez et al.(2) have reported that in a 25-yr-old female patient with Hodgkin's lymphoma whose several strips of ovary were removed and cryopreserved before anticancer chemotherapy and then autotransplanted, normal pregnancy and delivery through a normal sexual life was possible. This cryopreservation of ovarian tissue has been investigated as a useful method to restore reproductive potential in cases of ovarian failure due to senile changes or the sequelae of antineoplastic chemotherapy or progression of the disease. Cryopreservation and autotransplantation

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will be the only useful method to restore reproductive potential before the establishment of the ovum culture technique. It has been documented that in mice, mating with subsequent normal delivery is possible after autotransplantation of cryopreserved ovaries.*(3)* Meirow et al.*(4)* reported that ovarian failure occurred in 48% of patients treated with alkylating agents such as cyclophosphamide (Cy). A histologic study assessing the effect of anticancer agents on the human ovarian tissue demonstrated that the end results of antineoplastic chemotherapy are the atrophy of the ovary and the decrease of primordial follicles.*(5)* The methods of preventing ovarian failure and reproductive potential include cryopreservation of the embryo, cryopreservation of the matured oocyte, simultaneous administration of the anticancer agents and the gonadotropin-releasing hormone analogue (GnRHa), cultures of the ovarian tissue, and autotransplantation of frozen ovaries. Although it has been used as an in vitro fertilization technique since 1983, the cryopreserved embryo is not satisfactory to preserve reproductive potential.*(6)* This technique is subject to 2 limitations. First, it is impossible to confirm whether the embryo is alive. Second, this technique can not be used for single unmarried female adults. Chen et al.*(7)* performed cryopreservation of the ovum in order to solve the aforementioned problems and reported that normal delivery was possible by using this technique. However, it was found that only 4 of approximately 300 cryopreserved matured ova that were thawed and fertilized during a period of approximately 8 yr were successfully delivered. The result of this report indicates that the probability of normal delivery for thawed ovaries only 1%. Although in mouse experiments, the decrease of follicles induced by Cy can be prevented by the GnRHa, in primates including humans, the effect of GnRHa has not been yet determined.*(8, 9)* It is thought that autologous transplantation of cryopreserved ovarian tissues may be the most useful method for preventing ovarian failure in female cancer patients before the establishment of an feasible ovarian tissue culture technique. Advancements in freezing technology have expedited cryopreservation of ovaries, and it has been verified that simple transplantation without vascular anastomosis can restore normal menstrual cycles. Cyclophosphamide has been used to treat breast cancer patients for a long time. Recently, in addition, long term administration of it with another prodrug of 5-fluorouracil has been attempted for the metastatic breast cancer patients.*(10)* This study was conducted to determine whether autotransplantation of frozen ovaries in mice pretreated with cyclophosphamide could restore reproductive potential.

METHODS

Female mice aged 10-12 weeks (weight; 28-33 g) and male mice aged 12-18 weeks purchased from Biolink Co. (Eumsung, Korea) were placed in housing cages in the housing facility at the Clinical Research Institute of Daejeon St. Mary Hospital, Catholic University (temperature, 22±40℃; relative humidity, 65±5%). Natural lighting was used, and water and food were provided ad libitum. A total of 30 female mice were included in the study.

1. Experimental group

1) Positive control group (n=5)

Right total oophorectomy was performed, and then sham operation was performed one week later.

2) Negative control group (n=5)

Bilateral total oophorectomy was performed.

3) Comparison group (n=10)

Right total oophorectomy was performed, greater than or equal to 90% of the left ovarian tissue towards the fallopian tube was removed with lesser than 10% subsequently being preserved. After the third postoperative day, Cy at a dose of 750 mg/kg (a daily dose of 250 mg/kg for 3 days) (Alkyloxan for injection; Choongwae Pharmaceutical Co., Seoul, Korea) was intraperitoneally administered.

4) Experimental group (n=10)

The resected right ovary was cryopreserved, and after

the third postoperative day, Cy at a dose of 750 mg/kg (a daily dose of 250 mg/kg for 3 days) was intraperitoneally administered. Ten days after the last administration of Cy, ≥90% of the left ovary was resected, and the cyropreserved right ovary was autotransplanted to the remaining left ovary.

2. Oophorectomy

Mice were anesthetized by intraperitoneal injection of ketamine (Ketara, 50 mg/kg; Yuhan Pharmaceutical Co., Seoul, Korea) and xylazine (lumpum, 2.5 mg/kg; Bayer Korea Co., Seoul, Korea), and an approximately 1.5 cm horizontal skin incision was made in the lateral portion of the abdomen. A horizontal incision was made in the peritoneum, and the gonadal fat pad (GFP) was exposed. A small incision was made in the GFP, and all ovarian tissue was removed en bloc. In the comparison and experimental groups, the left ovary was removed with less than 10% of the ovary towards the fallopian tube being preserved through the same skin and peritoneum incisions. The peritoneum and skin incisions were sutured layer by layer using a 3-0 black silk. In the experimental group, the resected right ovary was cryopreserved for autotransplantation.

3. Sham operation in positive control group

Under the same anesthesia as described above, an approximately 1.5 cm horizontal skin incision was made in the lateral portion of the left abdomen. After an incision was made in the peritoneum, the peritoneum and skin incisions were sutured in the same manner as above.

4. Cy dosage schedule in the comparison and experimental groups

In the comparison and experimental groups, Cy at a daily dose of 250 mg/kg (a total dose of 750 mg/kg) was administered for three days after the third postoperative day.

5. Cryopreservation and thawing in the experimental group

The resected right ovary was cryopreserved using a modification of the vitrification method suggested by Nake et al.*(11)* By mixing 0.3 g of BSA (Bovogen Biologicals, Keilor East, Australia) with 100 mL of PBS (Gibco Laboratories, New York, NY, USA), 100 mL of PB1 was produced and used as a buffer solution. The ovary was plunged in a 2.0 mL cryovial. After adding 50 μL of 1M cryoprotectant DMSO (Sigma-Aldrich Co. Ltd, St. Louis, MO, USA), which was produced by PB1 stored at room temperature, the cryovial was stored for approximately 5 min in the ice water so that DMSO can smear well into the ovary. 450 μL of DAP213 (2 M DMSO; 1 M acetamide (Sigma-Aldrich Co. Ltd); 3 M propylene glycol (Sigma-Aldrich Co. Ltd) which was produced by PB1 stored in the ice water was added in the cryovial containing the ovary and stored for approximately 5 min in the ice water, which was plunged in liquid nitrogen for quick cryopreservation.

For autotransplantation, the cryovial containing the ovary was taken out and thawed at room temperature for approximately 3 min. Immediately after the cryovial containing the thawed ovary was diluted with 500 μL of 0.25 M sucrose (Sigma-Aldrich Co. Ltd) that was produced by PB1 stored at room temperature and was rinsed with PB1 4 to 5 times, autotransplantation was performed.

6. Autotransplantation of frozen ovaries in the experimental group

Ten days after the last injection of Cy, the cryopreserved ovary was transplanted. In the experimental group, after the mice were anesthetized using the same method as described above, greater than or equal to 90% of the ovarian tissue of the left ovary was removed with lesser than 10% being preserved. The thawed ovary was autotransplanted to the remaining left ovarian tissue and its surrounding GFP using 2 or 3 stitches of a 6-0 black silk. The incisions were sutured layer by layer using a 3-0 black silk.

7. Assessment of mating and pregnancy (Table 1)

1) The positive control group

Mice freely mated with male mice in the same cages for 21 days from the 21st day after sham operation.

2) The negative control group

Mice freely mated with male mice in the same cages for 21 days after the 21st day after bilateral total oophorectomy.

3) The comparison group

Mice freely mated with male mice in the same cages for 21 days from the 21st day after the last peritoneal injection of Cy.

4) The experimental group

Mice freely mated with male mice in the same cages for 21 days from the 21st day after autotransplantation.

Pregnancy was estimated as a 7-day weight gain of 5-7 g with a daily weight gain of 0.8-1.0 g. Mice with estimated pregnancy were killed by cervical dislocation, all remaining mice were killed immediately after the lapse of 21 days by cervical dislocation. After examining the status of the ovary, the absence or presence of pregnancy, and the number of fetuses through a horizontal incision in the central portion of the abdomen were observed, the ovary was resected. The resected ovary was fixed in 10% formalin. After paraffin embedding, 3-6 microsections were made for hematoxyline and eosin (H&E) stain.

8. Statistical analysis

Data were analyzed using the Descriptive Statistics procedure of the SPSS software, version 12.0 (SPSS Inc, Chicago, IL, USA). The number of pups of each group was presented as mean±SD. Comparisons were conducted through the least significant difference approach. A value of $p<0.05$ was considered statistically significant.

RESULTS

In the positive control group, all 5 mice became pregnant, and the mean number of fetuses was 4.60±0.55. In contrast, in the negative control group, none of the mice became pregnant. In the comparison group, 5 of

Table 1. Time-frame of the experiment

	D1	D3	D4	D5	D7	D15	D21	D28	D37
Positive	RT.OX				Sham			Mating	
Negative	BO.OX						Mating		
Comparison	NT.OX	CY 250	CY 250	CY 250				Mating	
Experimental	RT.OX, CP	CY 250	CY 250	CY 250		LT.OX, AT			Mating

D=postoperative day; RT.OX=Right total ovariectomy; BO.OX=Both total ovariectomy; NT.OX=Right total and left near-total ovariectomy; CP=Cryopreservation of resected right ovary; CY250=Intraperitoneal injection of cychlophosphamide 250 mg/kg; Sham=sham operation; LT.OX=Left neartotal ovariectomy; AT=Autotransplantation of cryopreserved ovary.

Table 2. The number of the offsprings of successfully pregnant mice

Number	Positive		Negative		Comparison		Experimental		
	P	Pups	P	Pups	P	Pups	P	Pups	Atrophy
1	○	4	X	0	○	2	○	9	X
2	○	5	X	0	○	1	○	3	X
3	○	4	X	0	○	1	○	6	X
4	○	5	X	0	○	1	○	4	X
5	○	5	X	0	○	2	○	1	○
6					X	0	○	6	X
7					X	0	○	4	X
8					X	0	X	0	X
9					X	0	X	0	○
10					X	0	X	0	○
P rate (%)	5/5 (100%)		0/5 (0%)		5/10 (50%)		7/10 (70%)		

P=Pregnancy (○) or non-pregnancy (X); Pups=the number of the offsprings of successfully pregnant mice; Atrophy=atrophy auto-grafted ovary (○) or not (X).

10 mice became pregnant (pregnancy rate; 50%), and the number of fetuses was 1 to 9 (Table 2). In the experimental group, a mouse with 1 fetus (animal no. 4) and 2 of 3 mice without any fetuses (animal nos. 9 and 10) showed the atrophy of the transplanted ovary. One of 3 non-pregnant mice (animal no. 8) showed no atrophy of the transplanted ovary, and one possible cause of this result is tubal occlusion due to adhesion despite correct implantation of the ovary (Fig 1).

In the experimental group, the mean number of fetuses

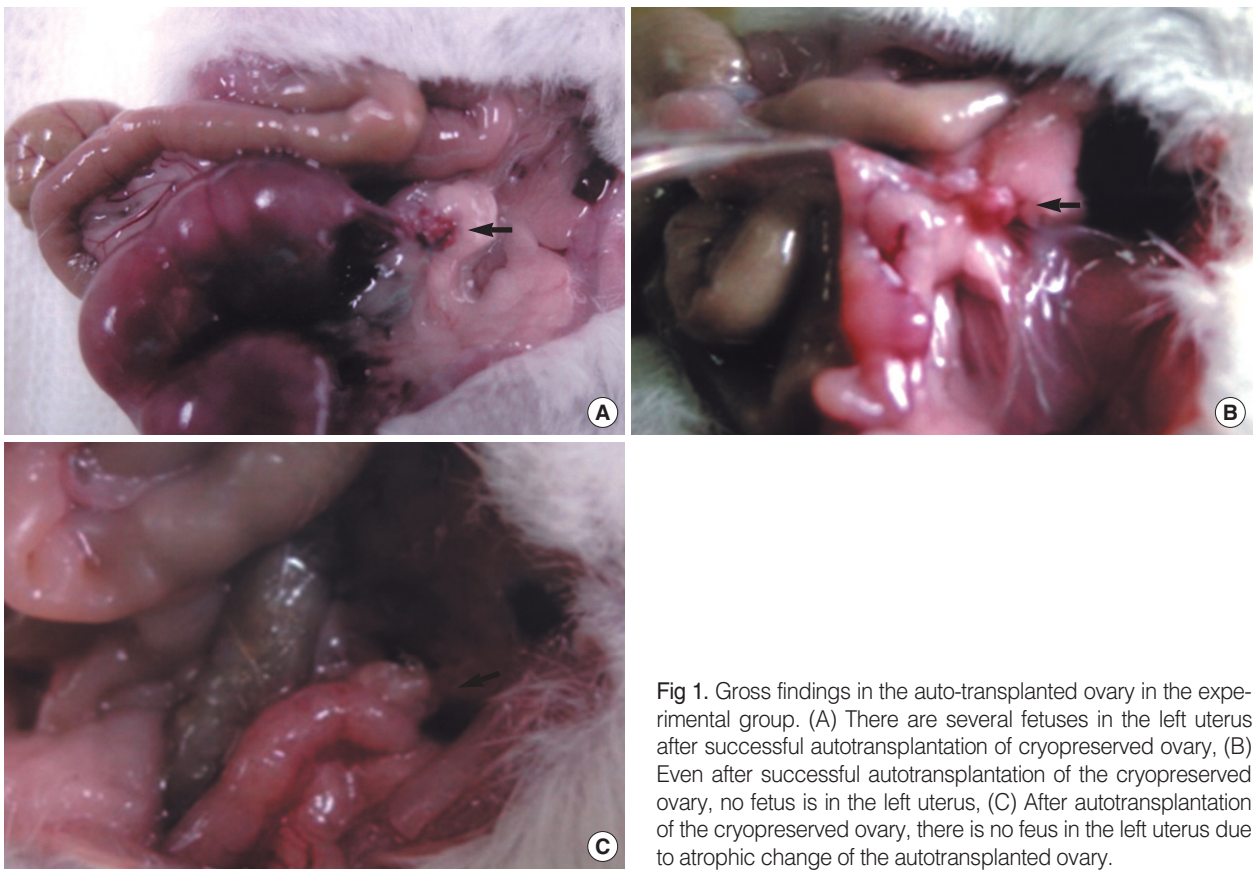

Fig 1. Gross findings in the auto-transplanted ovary in the experimental group. (A) There are several fetuses in the left uterus after successful autotransplantation of cryopreserved ovary, (B) Even after successful autotransplantation of the cryopreserved ovary, no fetus is in the left uterus, (C) After autotransplantation of the cryopreserved ovary, there is no feus in the left uterus due to atrophic change of the autotransplanted ovary.

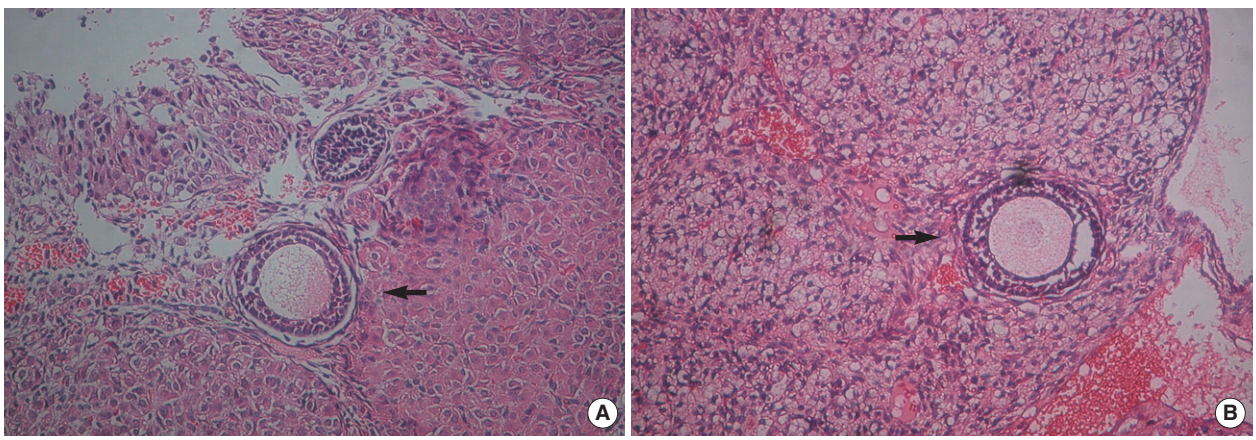

Fig 2. A primary follicle in the the positive control group (A) and the experimental group (B) after transplantation (H&E stain, original magnification ×200). A primary follicle of the experimental group is alive and there is no significant difference in the structural components between the two groups.

was 4.71±2.56. There was no significant difference in the number of fetuses between the positive control and experimental groups (p=0.093) (Table 3), but there was a significant difference between the comparison and experimental group (p=0.019) (Table 4). In the experimental group, normal primary follicles were identified by histologic examination using H&E stain, and the appearances of them were not different between the positive control and experimental group (Fig 2).

In the positive control group, many oocytes were found, whereas in the experimental group, the left ovary received Cy before autotransplantation showed a severe atrophic appearance with few oocytes. The transplanted cryopreserved ovary in the experimental group showed a moderately atrophic appearance with several oocytes (Fig 3).

Table 3. Number of pups of successfully pregnant mice

	Positive control (Unilateral ovariectomized group)	Comparison group (Partial ovariectomized, Cy [750 mg/kg] IP)	Experimental group (Cy [750 mg/kg] IP, cryopreserved ovary transplant)
Number of pups of successfully pregnant mice (Mean±SD)	4.60±0.55	1.40±0.55 (p<0.001)	4.71±2.56 (p=0.093)

There was a significant difference between the comparison and the experimental group (p=0.019).

Table 4. The difference in the number of pups of successfully pregnant mice between the comparison group and the experimental group comparison group

	Comparison group (Partial ovariectomized, Cy [750 mg/kg] IP)	Experimental group (Cy [750 mg/kg] IP, cryopreserved ovary transplant)
Number of pups of successfully pregnant mice (Mean±SD)	1.40±0.55	4.71±2.56 (p=0.019)

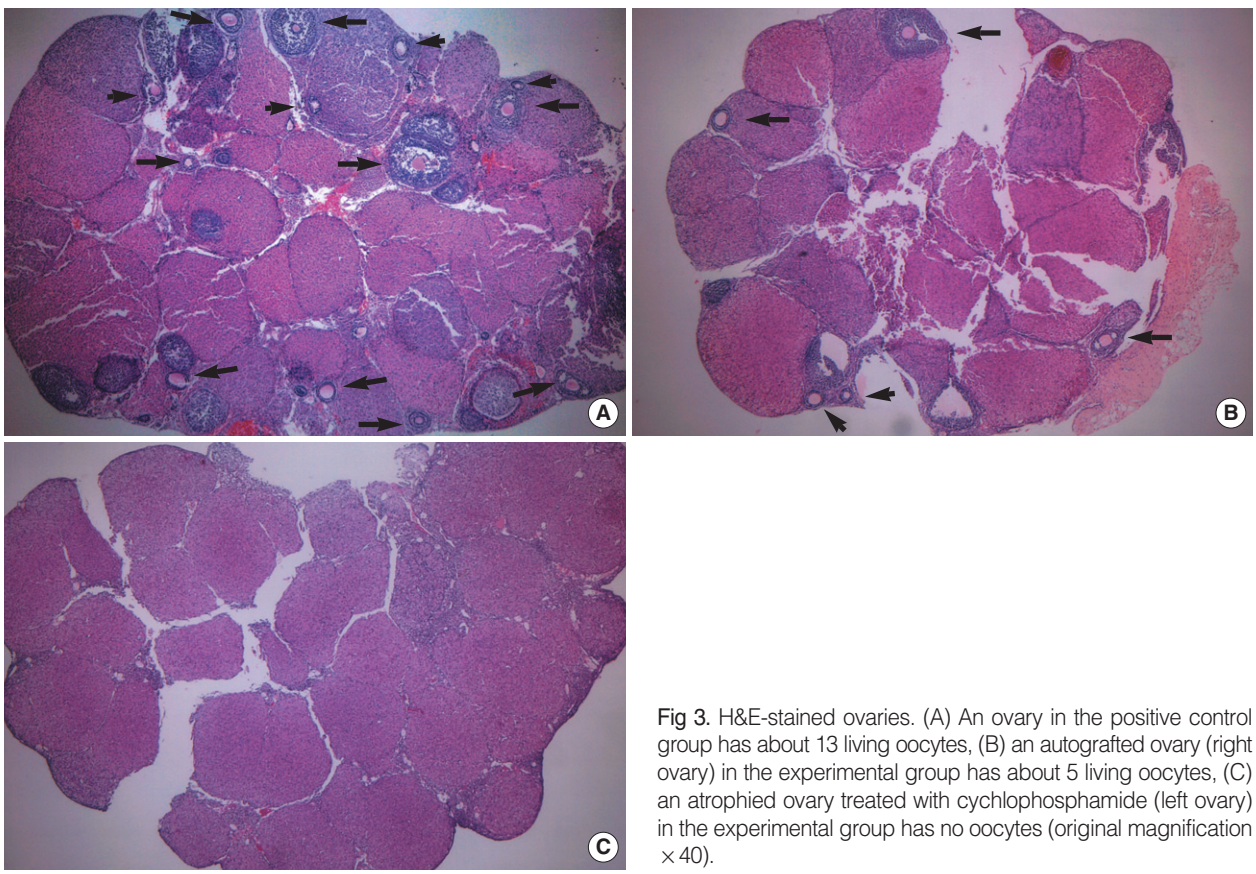

Fig 3. H&E-stained ovaries. (A) An ovary in the positive control group has about 13 living oocytes, (B) an autografted ovary (right ovary) in the experimental group has about 5 living oocytes, (C) an atrophied ovary treated with cychlophosphamide (left ovary) in the experimental group has no oocytes (original magnification ×40).

DISCUSSION

With advancements in combined antineoplastic chemotherapy and radiotherapy, the long-term survival rate of cancer patients has increased, and many adolescent and pediatric patients with cancer have been successfully treated. Although combined antineoplastic chemotherapy has been attempted to reduce the side effects of individual agents, a long-term loss of reproductive potential has occurred. Cytotoxic antineoplastic agents may induce only mild temporary menstrual irregularity, but permanent infertility in some cases. Since the survival rate of female cancer patients in the reproductive age has increased, the prevention of infertility induced by antineoplastic chemotherapy and radiotherapy has become an important consideration. Since Parrott*(12)* reported a normal delivery by using autotransplantation of a cryopreserved ovary in mice in 1960, this technique has been attempted with larger animals, such as sheep or other primates.*(13)* In 1999, Oktay et al.*(14)* found that after several cryopreserved strips of ovarian tissue were transplanted in the pelvic cavity in a 29-yr-old female patient, ovulatory cycles were restored by stimulating the ovarian tissue with human menopausal gonadotropin (hMG) 3 months after the transplantation. Finally, in October 2004, Donnez et al.*(2)* reported that normal pregnancy and delivery through an normal sexual life were possible after autotransplantation of the human cryopreserved ovarian tissue to the pelvic cavity. Although autologous transplantation of frozen ovaries can restore fertility, previous studies have been directed only to the technological aspects of freezing and thawing of the ovary or the ovarian toxicity of antineoplastic agents.*(14-16)*

There have been few studies as to whether reproductive potential can be restored by autotransplantation of frozen ovaries after pretreatment with antineoplastic agents. This is the reason why this study has been attempted. This study investigated whether reproductive potential can be restored in the frozen ovary that has been resected and stored before Cy administration and then has been transplanted after a certain period of time. The number of primordial follicles decreases in proportion to the dose of injected Cy, but such decrease does not represent the decrease of reproductive potential.*(15)* Thus, this study was performed using the maximum dosage that did not cause death and exhibited a maximum occurrence of ovarian toxicity. In the preliminary experiment, all mice expired within 1-3 days after the single administration of 1,000 mg/kg of Cy, and 50% of the mice were expired during the same period of time after the single administration of 800 mg/kg of Cy. Based on these results, we administered 250 mg/kg per day for 3 days (a total dose of 750 mg/kg) in this study. All mice survived at this dose of Cy throughout the experimental period.

The cryopreservation method recommended by Harp et al.*(16)* has the disadvantage of requiring more cost and time because the ovary should be slowly frozen using an automated freezer and be quickly thawed. In this study, a modification of the vitrification method suggested by Nakao et al.*(4)* was used to cryopreserve the ovary. The keys to a successful autotransplantation of the frozen ovary in terms of surgical procedures are performing successful implantation of the transplanted ovary at GFP and avoiding the adhesion of the fallopian tube. Infertility may occur if the fallopian tube is not patent due to adhesion, although normal menstrual cycles are restored. In the preliminary experiment where right total oophorectomy was performed and the resected right ovary was transplanted to the left GFP after complete resection of the left ovary, 1 of 5 (20%) mice became pregnant. In this study, in order to resolve this problem, a small part or lesser than 10% of the left ovary was left intact and then the cryopreserved ovary was transplanted, and thus 7 of 10 (70%) mice in the experimental group was confirmed to have become pregnant. The positive control group with experimental conditions similar to the experimental group where right total oophorectomy was performed showed that the mean number of fetuses was 4.60 ± 0.55, and the experimental group revealed that the mean number of fetuses was 4.71 ± 2.56, but the difference was not statistically significant ($p=0.093$). From this result, it is verified that the cryopreserved

ovary in the experimental group restore normal ovarian function.

In this study, in order to determine whether the normal function of the remaining left ovary was related to the successful pregnancy, the comparison group was established, where right total oophorectomy was performed, greater than 90% of the left ovary was removed, and then the same amount of Cy as was administered in the experimental group was administered. The mean number of fetuses was 1.40±0.55 in the comparison group and 4.71±2.56 in the experimental group, and the difference was statistically significant (p=0.019).

CONCLUSION

The results of this study suggest that if one ovary is cryopreserved before Cy administration and is transplanted to the small part or lesser than 10% of the ovary and its surrounding GFP on the contralateral side, the autotransplanted ovary may restore its normal reproductive potential. Based on these results, autotransplantation of frozen ovaries can overcome ovarian failure due to antineoplastic chemotherapy. However, it is conceivable that human ovaries which are much bigger than those of mice are difficult to implant using the aforementioned method. Further studies on the freezing technology will be able to cryopreserve human ovaries efficiently.

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