QOL after childhood cancer therapy — Cutting-edge researches on fertility preservation —

Collaboration | Super-Microsurgery * QOL after cancer therapy

Super-Microsurgery for testis organ transplantation and cryopreservation

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Abstract

Recently, research on the freezing of unfertilized eggs and ovaries aimed at preservation of fertility has been advanced as a way to avoid iatrogenic infertility in young girls, whereas research in young boys is less emphasized due to the development of techniques the freeze sperm. A treatment for the preservation of fertility, however, has not been developed because sperm cannot be collected from prepubertal boys due to the immaturity of spermatogenesis. We developed an experimental model of vascularized testis transplantation with the aim of preserving fertility in prepubertal boys with childhood cancer and successfully achieved vascular anastomosis of a rat testicular artery and vein. We also found that when mouse testicular tissue was frozen under a magnetic field, tissue destruction is reduced as compared to the existing method of freezing. Combined application of super-microsurgery and a novel technique for freezing testis opened the way to long-term preservation of the testis and transplant studies.

Keyword | Super-Microsurgery, Testis transplantation, Organ cryopreservation, QOL after cancer therapy

Introduction

It has been some time since the infertility of patients with childhood cancer became a subject at issue. Research on freezing of the ovum and the ovaries has advanced for young girls. Whereas, the necessity of gamete preservation has not been fully discussed in relation to preserving fertility in prepubertal boys with childhood cancer (Table 1). In prepubertal boys with childhood cancer, spermatogenesis is immature, and the cryopreservation of spermatogonia may be the only treatment option for preservation of fertility. We initiated two trials to investigate this treatment option. In the first trial, we perform autotransplantation of a vascularized whole testis in an attempt to preserve spermatogonia in the testis. In the second trial, we froze the testicles in consideration of in vitro maturation-in vitro fertilization (IVM-IVF). Both a whole testis and the fragments of testicular tissue are assumed in the transplantation with the aim of autotransplantation after cancer therapy by freezing the immature testis.

Male sexual dysfunction due to cancer chemotherapy

The testis is highly sensitive to anticancer agents, and germ cells become damaged in proportion to the dose of anticancer agents. Anticancer agents have a direct influence on normal spermatogenesis of the testis leading to testicular atrophy, oligozoospermia, azoospermia and infertility. In anticancer agents, alkylating agents may cause severe testicular dysfunction with the frequent occurrence of oligozoospermia if the radiation exceeds 18 Gy. Concomitant use of alkylating agents and low-dose radiotherapy, as well as other combination therapies, have been investigated because the use of multiple drug therapy of anticancer agents can reduce the level of dysfunction. The risk of teratospermia, however, has not been avoided by any therapies. Infertility caused by cancer therapy is based on the damage inflicted on spermatogonial stem cells and Sertoli cells that support spermatogenesis. Therefore, preservation of the testis is required in order to preserve fertility in patients whose sperm cannot be preserved.

Cryopreservation of sperm/testicular tissue

Polge et al. reported in 1949 that the cryopreservation of bovine sperm can be performed by using glycerin as a protective agent, pioneering in a sperm bank. In 1953, Bunge and Sherman succeeded in stabilization of human sperm cryopreservation using glycerin and dry ice. The survival rate of sperm after melting is approximately 50%, but sperm can maintain proper functions due to advances in reproductive technology. Taking advantage of these advances, sperm cryopreservation has been conducted as a measure for infertility caused by cancer therapy. Sperm, however, cannot be collected from prepubertal boys, and there is the additional difficulty of collecting sperm from adults because their treatment is essentially prioritized within a limited amount of time. In addition, cryopreservation may not be performed with current technology in some cases, such as male patients with germ cell tumors, which is said to be 10 times as many as that of females, or patients with oligozoospermia or azoospermia who do not have many sperm left in the first place. The history of testicular cryopreservation is short, and...
Problems of testicular freezing

While sperm is suitable for freezing because it is the smallest cell in humans and does not contain much moisture, larger tissue has the problem of tissue destruction when frozen due to the difficulty of penetration of cryoprotective agents. In ovary transplantation, cryopreserved vascularized ovary, instead of the fragments of ovarian tissue, are used for transplantation in order to avoid ischemic injury, but the clinical application has not been reported with low feasibility, except a few birth cases reported in animal experiments.

The fragments of ovarian tissue is frozen using either a vitrification procedure that uses a high concentration of cryoprotective agent and liquid nitrogen or a slow freezing procedure that uses a low concentration of cryoprotective agent and program freezer. The former is becoming mainstream in recent years. Sperms, ova, fertilized eggs, and ovarian tissue have been frozen with the vitrification procedure, and the clinical application has also been developed. However, no consensus has been achieved as to which procedure is better for the freezing of testicular tissue.

Major problems in organ freezing and transplantation are tissue destruction by freezing and ischemic injury due to lack of blood flow. We believe that these problems can be solved by the combined application of super-microsurgery in the plastic surgery and reconstructive field and freezing under a variable magnetic field, a novel freezing technology.

Table 1. The present techniques of fertility preservation

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Figure 1. Rat testis transplantation

Pentobarbital was administered intraperitoneally to 2 or 10-weeks-old rats to give general anesthesia, and the rats were placed in a supine position. Subsequently, laparotomy was performed by epigastric midline incision, and the testicular artery and vein were identified. The testicle was removed while the vessels were preserved and cross-inbred strain rats. The time period from the removal of the donor rat's testicle to the transplantation to the recipient

Figure 2. Freezing of mouse testis

Mouse testis was removed without cutting and was frozen by liquid nitrogen (A) or under variable magnetic field in embedding materials (B). The frozen section of the testis was stained with HE and histologically evaluated.
rat followed by reperfusion was 2 hours. Regarding anastomotic vessels of the recipient, end-to-end anastomosis was performed at the divergence of the femoral artery and vein, and survival of testis was identified after vascular anastomosis.

**Cynomolgus monkey testis transplantation**

Epigastric midline incision was performed in cynomolgus monkeys (body weight 3–5 kg) under general inhalation anesthesia to expose the abdominal aorta. After visually identifying the abdominal aorta, the renal artery diverging from the abdominal aorta was identified. Subsequently, the kidney was exposed in order to identify the abdominal aorta and the ureter.

**Freezing experiment**

Pentobarbital was administered intraperitoneally to mice to administer general anesthesia, and mice were placed in a supine position. Subsequently, a laparotomy was performed by epigastric midline incision to remove the testicle. The testicle was washed by physiological saline and embedded by OCT compound. It was frozen by liquid nitrogen or in a program freezer (ABI) that generates variable magnetic field. The brine bath of the freezer was filled with 60% ethylene glycol, and a testicle enclosed in a nylon bag (minimum amount of air within the bag) was placed into a gage with weight and sunk into the bath. Subsequently, it was cooled to -30°C in magnetic field strength of 0.1–0.2 mT. Frozen tissue was sliced into 4–5 μm by cryostat, followed by HE staining.

**Testis transplantation**

We developed a rat model of vascularized testis transplantation. The diameters of testicular artery and vein of adult rats were approximately 0.5–0.6 mm, which were large enough for vascular anastomosis by the application of super-microsurgery (Figure 1). Vascularized ovary transplantation was also performed using young rats assuming a transplantation of immature testicular tissue. The results showed that the diameters of testicular artery and vein were approximately 0.1–0.2 mm, which were very small, suggesting that the current vascular anastomosis technique is not sufficiently advanced for experiments. Further study should be carried out using larger animals in addition to adult mice and rats.

The diameters of testicular artery and vein of cynomolgus monkeys were approximately 0.5–0.8 mm, which confirmed that vascular anastomosis can be performed (Figure 3).

**Testicular freezing**

Mouse testis was frozen by liquid nitrogen or under variable magnetic field, and the testis was histologically evaluated using the frozen section. Results indicated that the level of tissue destruction was reduced in cases under variable magnetic field than those of liquid nitrogen (Figure 2).

Development of sperm freezing techniques has been a prospective measure for male infertility. A sperm has very small size cells and permits having a baby by micro-insemination as long as DNA can be collected. From these perspectives, preservation of fertility of boys with childhood cancer had not often been treated as a major issue. Given that sperm cannot be collected in prepubertal boys, the focus of the current study was the collection and maturation of spermatogonia. We tried to develop an organ cryopreservation method of vascularized testicle and tissue cryopreservation method of the testis with the aim of preserving fertility in prepubertal boys with childhood cancer. The current study revealed that vascularized testicle organ transplantation is possible in adult rats and that testicular cryopreservation is effective under a magnetic field. We assumed both transplantation of vascularized testis and the fragments of testis tissue transplantation; the strengths and weaknesses of these transplantations are described as follows.

(1) Vascularized whole testis freezing method

Strengths: A number of spermatogonia in testis organ can be collected; ischemic injury after freezing and retransplantation can be avoided by vascular anastomosis.

Weaknesses: Surgical technique is particularly difficult and requires general anesthesia; scientific explanation for freezing technique has not been provided.

(2) The fragments of testis tissue cryopreservation method

Strengths: Collection under local anesthesia is possible; multiple sperm cells can be produced from a spermatogonium.

Weaknesses: The possibility of ischemic injury after retransplantation is high; development of sperm IVM-IVF technique may be recommended.

The IVM-IVF method in which maturation of gametes occurs outside the body has been generally developed in immature...
oocytes. IVM-IVF of ova has been clinically applied in Japan since 1999, but less than 10 institutions have actually continued the clinical application. With regard to IVM-IVF of immature spermatogonia the development is limited to basic research. Further study may be required in the aim of preserving fertility in prepubertal boys with childhood cancer.

Establishment of testicular freezing and transplantation protocols

We are convinced that research on testicular freezing and transplantation will become more active, but the protocol needs to be established first as this field is currently in the primitive stage. Transplant the testis to the outside the abdominal cavity (in the scrotum) and perform chronologically deliberate echo testing. In particular, testing is performed regarding changes of spermatogonia and blood flow state in the testis, increase/decrease of the amount of tissue, etc. Perform the transplantation of the testis thawed in the abdominal cavity of cross-inbred strain rats. Try to preserve blood flow by anastomosis to the branch of the femoral artery and vein. We plan for pregnancy and childbirth by in vitro fertilization. No case of childbirth by in vitro fertilization has been reported in any country.

Cryopreservation of the testis organ and testicular tissue can be less invasive due to exist outside the abdominal cavity compared to the collection of ovarian tissue in girls who have genitalia in the abdominal cavity, and we believe that early clinical feedback may be expected. In addition, when cryopreservation of spermatogonial stem cells and spermatogenesis in vitro is available, cryopreservation of testis appears to be clinically useful even if low survival rates of spermatogonia are found after freeze-thaw.

The experimental model of rat testis transplantation was developed in the application of micro-vascular anastomosis. The diameters of rat testicular artery and vein were 0.5 mm and 0.7 mm, respectively, which proved that vascular anastomosis can be performed. The maturation process of spermatogonia after freeze-thaw and autotransplantation should be evaluated by performing testicular freezing in the future.

<Issue 1 Possibility of sperm maturation>
Further investigation is required as to whether immature sperms should be matured and fertilized in vitro or spontaneously allowed to mature after autotransplantation.

<Issue 2 Possibility of occurrence of malformation after transplantation>
Sperms with malformation are eliminated during in vitro fertilization using frozen sperm. There is a possibility that sperm influenced by freezing and freeze-thaw can be fertilized.

<Issue 3 Comparisons between organ and tissue freezing of the testis>
It is arguable as to whether the testis should be frozen as an organ or tissue.

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