—Mini Review—

Human Sperm Cryopreservation —Theory and Clinical Application

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Abstract: Since the 1950's, cryopreservation of human semen has been recognized as an efficient procedure for infertility therapy, and research has mainly focused on long-term banking of donor semen for artificial insemination (AID). Because assisted reproductive technology (ART) usually employs fresh ejaculate, it is essential to synchronize ejaculation and ovulation. However, if the sperm is efficiently cryo-accumulated, synchronization would not be necessary and much sperm could be provided for fertilization or insemination. In recent years, survival of young males suffering from some cancers has improved due to advanced treatments including high-dose chemotherapy and radiotherapy. However, testicular functions, especially spermatogenesis, are usually sacrificed temporarily or permanently by these treatments. Sperm cryopreservation liberates these patients from iatrogenic infertility and allows them to retain reproductive capability.

Key words: Human sperm, Cryopreservation, Artificial insemination, Anti-cancer treatment

History of Sperm Cryopreservation

In 1949, mammalian sperm cryopreservation was put into practical use through the accidental discovery that glycerol was superior to a cryoprotectant [1]. At first, cryobanking of semen from livestock animals, especially bovine was in demand because of its economical importance. In 1954, Bunge and Sherman [2] reported that human sperm frozen and stored in dry ice (–78°C) could be used for fertilization, and subsequent development was found to proceed normally. By 1963, the fundamental conditions for freezing and thawing of human semen had been evaluated and the most obvious improvements made were freezing in liquid nitrogen vapor and preservation in liquid nitrogen at –196°C. In the early 1970's, cryobanking became more common with the wider use of artificial insemination using donor semen (AID) as well as artificial insemination using husband's semen (AIH). During the last decade, emphasis has still been on long-term banking of donor semen, which has been recognized as essential for assisted reproductive technology (ART), with particular emphasis on AID. The ejaculate should be cryopreserved for at least 6 months so that the donor can be serologically tested for sexually transmitted diseases to avoid transmission of diseases such as HIV to the recipient. Because it is well known that stimulation of spermatogenesis in oligo-asthenozoospermic patients is often difficult, cryo-accumulation provides sperm sufficient for insemination or fertilization without requiring synchronization with ovulation.

In recent years, high-dose chemotherapy and radiotherapy have improved the survival of young patients with certain cancers, but spermatogenesis is usually sacrificed by these treatments. Sperm cryopreservation allows patients to retain reproductive capability even after these intensive therapies.

Principals of Sperm Cryopreservation

As an aqueous liquid is frozen, the temperature falls steadily to the freezing point (super-cooling), at which point ice appears and the temperature concurrently stabilizes (latent heat) until all the liquid is frozen. Generation of latent heat is due to the release of the heat of fusion necessary to form the molecular lattice of solidified water. The freezing point for water is depressed by 1.86°C for each mole of solute contained in 1.0 kg water (molar depression of freezing point).

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The composition of the medium suspending the cells and the rate of freezing both above and below the freezing point affect the cell survival rate after thawing. Furthermore, immersing cells into low temperatures above the freezing point can also harm cells ("cold shock") [3]. Human sperm is relatively resistant to cold shock with respect to motility and oxygen consumption. At the freezing temperature, the water outside the cell freezes first, and increases the osmolarity through the removal of extra-cellular liquid solvent. Then the intra-cellular water moves along the osmotic gradient, concentrating the intra-cellular components and making them resistant to super-cooling. When cooled too rapidly, this osmotic movement is not sufficient to minimize intra-cellular ice crystal formation and the cells are thereby damaged. Thus, increasing the concentration of extra-cellular solutes acts as a cryoprotectant by regulating extra- and intra-cellular ice formation and osmotic differential, causing cell shrinkage [4].

During the freezing process, damage of the cell membrane also affects the post-thaw survival rate. The most significant injuries to sperm appear to be plasma membrane swelling and acrosomal leakage and breakdown [5]. Mammalian sperm generally have small volume, large surface area, and a small amount of intracellular water, although these features differ among species. To prevent damage to the cells during freezing, the presence of a cryoprotectant is essential. Glycerin is the most commonly used cryoprotectant for mammalian sperm including human sperm [6, 7], and a final concentration of 5–10% glycerin provides adequate protection for the cells. The protective action of this agent may be due to its ability to depress the freezing point and reduce the electrolyte concentration to which the cells are exposed during freeze-thaw procedures.

Human Sperm Cryopreservation

Cryopreservation of oligo- and/or astheno-zoospermic semen has not been widely used because the concentration of motile sperm is reduced during the freeze-thaw process. If this problem could be overcome, cryopreservation would provide various advantages for ART. The strategies to increase the number of motile sperm after thawing are sperm concentration prior to freezing and improvement of post-thaw survival rate by cryoprotectant optimization [8]. Furthermore, ejaculates are usually obtained once or twice a week, and their cryo-accumulation could provide sufficient number of sperm.

Human sperm cryopreservation is performed as described previously [9]. The sperm is concentrated prior to freezing by continuous-step density gradient centrifugation [10]. Twenty millimoles HEPES-buffered Percoll (Amersham, Sweden), pH7.4, is made isotonic by adding powdered ingredients (7.20 g NaCl, 0.32 g KCl, 0.045 g Na_2HPO_4, 0.054 g KH_2PO_4, 0.32 g $NaHCO_3$, 0.84 g glucose, 0.12 g $CaCl_2$, 0.045 g $MgCl_2$, 0.045 g $MgSO_4$, 0.05 g fosfomycin and 0.05 g cepharotin / 1.0 L), and the resulting isotonic 98% Percoll solution is sterilized using a Millipore filter. Five milliliters of 98% Percoll is placed in a conical-tip test tube and 1.0 mL of Hank's solution is layered on top. A continuous-density gradient is made in the test tube by turning it 10 revolutions manually at an angle of 30°. To prepare the semen sample for centrifugation, fibers, micro-calculi and micinous debris are removed. The ejaculate is diluted twice with Hank's solution, filtered through nylon mesh (ART filter, 20 μm clearance, Nipro, Japan), and allowed to stand in a test tube for 10 min to precipitate filterable micro-calculi. The sample is then layered on the density gradient and centrifuged at 400 × g for 30 min in a swing-out rotor. We first reported the use of KSII cryo-medium for washed and concentrated human sperm [11]. Further modifications of this medium gave KSVIm cryo-medium (20 mM HEPES-NaOH, pH7.4, 12% glycerin, 10% egg yolk water soluble fraction, fosfomycin (0.05 g / L) and cepharotin (0.05 g / L) in Hank's solution). The resulting solution is sterilized by filtration with a Millipore filter (0.45 μm pore size). The concentrated sperm in the sediment

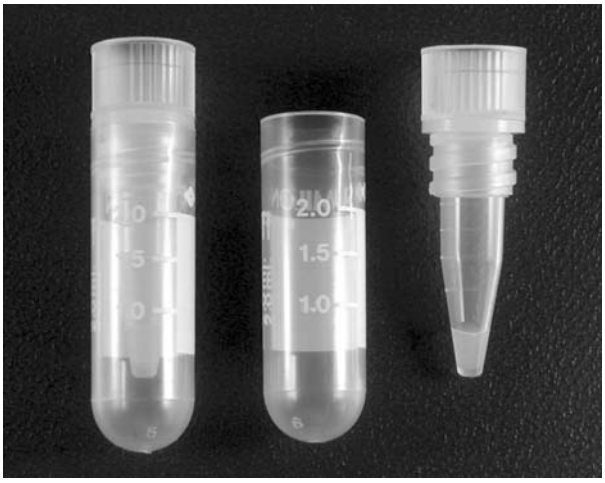

Fig. 1. Double-wall transformable freezing container for human sperm.

Table 1. Summary of reproductive outcomes after ART with cryopreserved sperm

Disease	ART	No. of patients	No. of pregnancies	Reported by	Reference
testicular tumor	IVF	1	1	Schill	15
testicular tumor	IVF	2	2	Roland	16
Hodgkin's	IVF	5	6	Toumaye	17
testicular tumor	ICSI	2	2	Hakim	18
testicular tumor	IVF or ICSI	15	12	Rosenlund	19
Various cancers	IVF or ICSI	10	5	Hallak	14
Various cancers	IVF or ICSI	11	7	Lass	20
Various cancers	IVF or ICSI	18	6	Audrins	21

Table 2. Underlying diseases of the patients who visited our center prior to anti-cancer treatments

Underlying disease	cases
Testicular tumor	84
Leukemia	49
Malignant lymphoma	24
Aplastic anemia	11
Prostate cancer	6
Bladder tumor	2
Pharyngeal cancer	6
Liposarcoma	2
Hepatocellular carcinoma, retroperitoneal tumor, multiple myeloma, thymic tumor, intrapelvic tumor, small intestinal cancer, lingual cancer, colon cancer, rectum cancer, brain tumor	14

(approximately 0.2 mL) is mixed with an equal volume of KSVIm cryo-medium, and the mixture is frozen and thawed in a double-walled transformable freezing container (Fig. 1) composed of inner and outer tubes. The mixture is filled in the inner tube and loaded to the outer tube, frozen in liquid nitrogen vapor and then stored at –196°C. To thaw the sample, the inner tube is taken out from the container and warmed in tap water at 37°C. The thawed sperm is subsequently available for ART.

Cryopreservation of Human Sperm from Patients with Cancer

In recent years, the survival of young males with some cancers has been improved due to advanced diagnostic techniques and better treatments, including high-dose chemotherapy and radiotherapy. However, the damaging effects of chemotoxic agents usually cause serious deterioration in testicular functions; in particular, spermatogenesis can be temporarily or permanently sacrificed. Although the semen findings sometimes normalize after cancer treatments, the possibility of genetic disturbances in sperm cannot be ruled out [12, 13]. Semen quality following cancer treatments depends on many factors: the previous sperm characteristics, the type of cancer, the action mechanisms of cytotoxic agents and the dose and number of treatment cycles. Testicular tumors, leukemia and malignant lymphomas occur frequently in young men, and intensive therapies often have a high prognosis of complete recovery in such patients. High inguinal orchiectomy, a surgical operation, is employed to treat testicular tumors. Given these circumstances, the sperm cryopreservation program for cancer patients is essential to avoid iatrogenic infertility and to give patients the possibility of marriage and having children. The introduction of IVF-ET and ICSI in ART enable pregnancy even with low-quality semen. Since 1984, several authors have reported cases of ART with cryopreserved sperm from patients with cancer. Hallak *et al.* discussed the fertilizing capacity of cryopreserved sperm from 10 patients [14]. Of these, five had Hodgkin's disease, two had testicular tumors, one had

leukemia and two had prostate cancer. The duration of specimen storage ranged from 14 to 135 months. A total of 18 ART cycles were performed on 10 couples with an overall pregnancy rate of 50%, with two deliveries, one ongoing pregnancy and two miscarriages. The overall pregnancy rate was 36.4% per cycle. Table 1 summarizes the published clinical outcomes of ART using cryopreserved sperm.

Approximately 200 patients with cancer visited the Reproduction Center of Ichikawa General Hospital, Tokyo Dental College. Table 2 classifies their underlying diseases. Testicular tumor was the most frequent diagnosis, followed by leukemia and malignant lymphoma, accounting for 79% of the patients. A large group is comprised of young men who were not married when they first visited our facility, and they have to find a spouse after completion of anti-cancer treatments. To date, five couples have tried ART, and three couples have successfully delivered babies (ICSI: two couples and AIH: one couple) in our program.

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