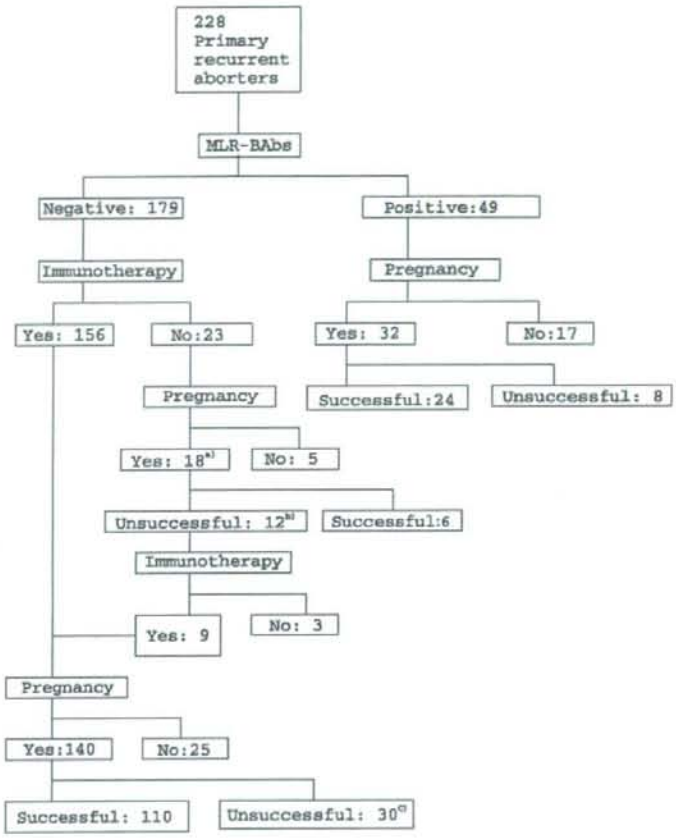

Fig. 1 Accrual of the patients. The study included cases from January 1983 to December 2005. (a) These 18 patients experienced 20 pregnancies. (b) These 12 patients experienced 14 abortions. (c) Two cases of ectopic pregnancy were included.

Table 1 Characteristics of Patients in Group I, Group II, and Group III

	No. of patients	Age (range)	No. of abortions
Group I	165	30.5 ± 4.41 (22–43)	3.22 ± 0.49
Group II	49	31.2 ± 4.53 (22–40)	3.24 ± 0.60
Group III	23	30.1 ± 4.98 (23–40)	3.09 ± 0.29

No significant difference was observed among three groups concerning mean age and mean number of abortions.

abortion in this series was improved compared with that of the control patients who did not undergo immunotherapy. The results of some earlier case-controlled studies of immunotherapy for recurrent spontaneous abortions show that the outcome of subsequent pregnancies is significantly improved by the injection of paternal lymphocytes as compared to the outcome after the injection of autologous cells.[8,9] A worldwide meta-analytic study has concluded that immunization may be highly effective, although for only a small number of patients.[5] Recently, Pandey et al. reported that a double-blind randomized trial of paternal lymphocyte immunization for women with recurrent spontaneous abortion revealed the significant improvement in pregnancy outcome in the patient group.[11,14] Although the current study is not a case-controlled trial, and accordingly has less influence than a randomized trial, the results are still considered to suggest the efficacy of

Table II Comparison of Pregnancy Outcome in Group I, Group II, and Group III

	Group I ($n = 140$)	Group II ($n = 32$)	Group III ($n = 20$)[a]
Normal delivery at week 36 of gestation or later	101	20	6
Light-for-date infant delivery at week 36 of gestation or later	4[b]	1[c]	0
Preterm delivery before week 36 of gestation	4[d]	1[e]	0
Fetus with major anomaly[f]	1	3	0
Spontaneous abortion	28	7	14
Ectopic pregnancy	2	0	0
Number and rate of successful pregnancy	110 (78.6%)[g]	28 (75.0%)[h]	6 (30.0%)[g,h]

[a]Twenty pregnancies in 18 patients.
[b]The pregnancy outcome was; 38 weeks, 1858 g; 38 weeks, 1865 g; 36 weeks, 1865 g; 36 weeks, 2044 g.
[c]The pregnancy outcome was; 38 weeks, 1816 g.
[d]The pregnancy outcome was; 28 weeks, 1088g; 32 weeks, 1766g; 33 weeks, 1524g; 34 weeks, 2118 g.
[e]The pregnancy outcome was; 31 weeks, 1714 g.
[f]The details were described in the text.
[g]Group I versus group III, $P < 0.000001$ by chi-squared analysis.
[h]Group II versus group III, $P < 0.001$ by chi-squared analysis.

Table III Comparison of Pregnancy Outcome in Group I According to the Number of Vaccination(s) Necessary for Positive MLR-BAbs

	One or two vaccinations ($n = 125$)	Three vaccinations ($n = 12$)	No appearance of MLR-BAbs and additional vaccination at the early stage of pregnancy ($n = 3$)
Normal delivery at week 36 of gestation or later	87	11	3
Light-for-date infant delivery at week 36 of gestation or later	4[a]	0	0
Preterm delivery before week 36 of gestation	4[a]	0	0
Fetus with major anomaly	1[b]	0	0
Spontaneous abortion	27	1	0
Ectopic pregnancy	2	0	0
Number and rate of successful pregnancy	96 (76.8%)[c]	11 (91.7%)[c]	100 (100%)[c]

MLR-BAbs, mixed lymphocyte culture reaction blocking antibodies.
[a]The details were described in Table II.
[b]The details were described in the text.
[c]No significant difference was observed among the three groups.

immunotherapy for unexplained primary recurrent spontaneous abortion.

As one of the selection criteria for patients to be considered eligible for immunotherapy, the presence or absence of MLR-BAbs before immunotherapy was considered to be important. This has been emphasized by several investigators, including ourselves.[3,4,6,11,14–16] Park et al. reported that the blocking effect index (BEI), calculated using a modification of our MLR blocking assay, was a reliable indicator of the outcome of subsequent pregnancies in unexplained recurrent aborters following immunotherapy.[16]

As to the origin of naturally acquired MLR-BAbs, there is a possibility that stimulation by paternally derived antigens on the trophoblasts of previous conceptions or by the exposure of semen might generate the MLR-BAbs.

The MLR-BAbs were used in a recent double-blind randomized trial by Pandy et al. as selection criteria for immunized patients.[11] The results obtained in the present study show that the patients negative for MLR-BAbs benefit from immunotherapy with the husband's lymphocytes. In patients with MLR-BAbs, the success rate of pregnancy was significantly higher than that in the control group, while the rate

American Journal of Reproductive Immunology 58 (2007) 530–536

was not significantly different from that in the treated patients. Thus, it can be concluded from this result that immunotherapy was not of additional value for the patients who were positive for MLR-BAbs.

As a negative report concerning the immunotherapy, Porter et al. found that immunotherapy for unexplained recurrent aborters has no beneficial effect, upon analyzing the Chochrane database.[17] The conclusion was critically influenced by the negative results of a randomized trial by Ober et al.[10] As has been pointed out by Clark et al.[18], this trial unfortunately showed considerable flaws in its design. Moreover, the discrepancy between their findings and ours is probably due to the fact that they did not use selection criteria, such as MLR-BAbs, for the recipients of immunotherapy, and it is possible that the immunotherapy was given to patients who did not need it. Another explanation for the discrepancy is that they used lymphocytes stored overnight before the vaccination and we used freshly prepared lymphocytes. Recently, Clark et al. reported that transfusion-related immunomodulation can enhance the growth and survival of the feto-placental unit via CD200, and transfusion-related immunomodulation was lost if the transfused cells were stored overnight.[19,20]

For two decades, the so-called immunotrophic theory, whereby some cytokines produced by maternal cells which recognize fetal antigens, promote the proliferation of trophoblastic cells and sustain a pregnancy, has been thought to be significant for the immunological maintenance of pregnancy.[21] Moreover, some investigators demonstrated the importance of a T-helper 2 (Th2) bias for normal pregnancy, indicating the crucial role of the activation of maternal humoral immunity following recognition of fetal antigens during pregnancy.[22–25] Although the theory that the maternal Th2/Th1 dichotomy plays an important role has been recently challenged by several researchers,[26,27] some studies demonstrated that a predominance of Th2 over Th1 was induced in patients by immunization with the husband's lymphocytes, which was correlated with the efficacy of the therapy.[28,29]

In this study, the appearance of MLR-BAbs was almost ubiquitous (98.2% of vaccinated patients), and the success rate of pregnancy did not differ significantly with the number of vaccination(s) necessary for MLR-BAbs to appear. Such a ubiquitous appearance of MLR-BAbs after immunotherapy may indicate the generation of an appropriate immune reaction in patients, i.e., the induction of a predominance of humoral immunity, which is considered to contribute to a successful continuation of the subsequent pregnancy.

Acknowledgments

This work was partly supported by a research grant from the Ministry of Health, Labor and Welfare of Japan, and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

References

1 Beer AE, Quebbeman JR, Ayers JWT, Haines RF: Major histocompatibility complex antigens, maternal and paternal immune responses, and chronic habitual abortions in humans. *Am J Obstet Gynecol* 1981; 141:987–999.

2 Taylor C, Faulk WP: Prevention of recurrent abortion with leucocyte transfusions. *Lancet* 1981; ii:68–70.

3 Takakuwa K, Kanazawa K, Takeuchi S: Production of blocking antibodies by vaccination with husband's lymphocytes in unexplained recurrent aborters: the role in successful pregnancies. *Am J Reprod Immunol Microbiol* 1986; 10:1–9.

4 Takakuwa K, Goto S, Hasegawa I, Ueda H, Kanazawa K, Takeuchi S, Tanaka K: Result of immunotherapy on patients with unexplained recurrent abortion: a beneficial treatment for patients with negative blocking antibodies. *Am J Reprod Immunol* 1990; 23:37–41.

5 Recurrent Miscarriage Immunotherapy Trialists Group: Worldwide collaborative observational study and metaanalysis on allogeneic leucocyte immunotherapy for recurrent spontaneous abortions. *Am J Reprod Immunol* 1994; 32:55–72.

6 Adachi H, Takakuwa K, Mitsui T, Ishii K, Tamura M, Tanaka K: Results of immunotherapy for patients with unexplained secondary recurrent abortions. *Clin Immunol* 2003; 106:175–180.

7 Kling C, Steinmann J, Flesch B, Westphal E, Kabelitz D: Transfusion-related risks of intradermal allogeneic lymphocyte immunotherapy: single cases in a large cohort and review of the literature. *Am J Reprod Immunol* 2006; 56:157–171.

8 Mowbray JF, Gibbings C, Liddell H, Reginald PW, Underwood JL, Beard RW: Controlled trial of treatment of recurrent spontaneous abortion by

immunisation with paternal cells. *Lancet* 1985; I:941–943.

9 Gatenby PA, Cameron K, Simes RH, Adelstein S, Bennett MJ, Jansen RPS, Shearman RP, Stewart GJ, Whittle M, Doran TJ: Treatment of recurrent spontaneous abortion by immunization with paternal lymphocytes: results of a controlled trial. *Am J Reprod Immunol* 1993; 29:88–94.

10 Ober C, Karrison T, Odem RR, Barnes RB, Branch DW, Stephenson MD, Baron B, Walker MA, Scott JR, Schreiber JR: Mononuclear-cell immunization in prevention of recurrent miscarriages: a randomised trial. *Lancet* 1999; 354:365–369.

11 Pandey MK, Agrawal S: Induction of MLR-Bf and protection of fetal loss: a current double blind randomized trial of paternal lymphocyte immunization for women with recurrent spontaneous abortion. *Int Immunopharmacol* 2004; 4:289–298.

12 Takakuwa K, Higashino M, Yasuda M, Ishii S, Ueda H, Asano K, Kazama Y, Tanaka K: Is an additional vaccination necessary for a successful second pregnancy in unexplained recurrent aborters who were successfully immunized with their husband's lymphocytes before the first pregnancy. *Am J Reprod Immunol* 1993; 29:39–44.

13 Ogawa Y, Iwamura T, Kuriya N, Nishida H, Takeuchi H, Takada M, Itabashi K, Imura S, Isobe K: Birth size standards by gestational age for Japanese neonates. *Acta Neonat Jpn* 1998; 34:624–632.

14 Pandey MK, Rani R, Agrawal S: An update in recurrent spontaneous abortion. *Arch Gynecol Obstet* 2005; 272:95–108.

15 Ramhorst R, Agriello E, Zittermann S, Pando M, Larriba J, Irigoyen M, Cortelezzi M, Auge L, Lombardi E, Etchepareborda JJ, Cotreras Ortiz C, Fainboim L: Is the paternal mononuclear cells' immunization a successful treatment for recurrent spontaneous abortion? *Am J Reprod Immunol* 2000; 44:129–135.

16 Park MI, Edwin SS, Scott JR, Branch DW: Interpretation of blocking activity in maternal serum depends on the equation used for calculation of mixed lymphocyte culture results. *Clin Exp Immunol* 1990; 82:363–368.

17 Porter TF, LaCoursiere Y, Scott JR: Immunotherapy for recurrent miscarriage. *Cochrane Database Syst Rev* 2006; CD000112.

18 Clark DA, Coulam CB, Daya S, Chaouat G: Unexplained sporadic and recurrent miscarriage in the new millennium: a critical analysis of immune mechanisms and treatment. *Hum Reprod Update* 2001; 7:501–511.

19 Clark DA, Yu G, Levy GA, Gorczynski RM: Procoagulants in fetus rejection: the role of the OX-2 (CD200) tolerance signal. *Semin Immunol* 2001; 13:255–263.

20 Clark DA: Shall we properly re-examine the status of allogeneic lymphocyte therapy for recurrent early pregnancy failure? *Am J Reprod Immunol* 2004; 51:7–15.

21 Wegmann TG: Placental immunotrophism: maternal T cell enhance placental growth and function. *Am J Reprod Immunol Microbiol* 1987; 15:67–69.

22 Lin H, Mosmann TR, Guilbert L, Tuntipopipat S, Wegmann TG: Synthesis of T helper 2-type cytokines at the maternal-fetal interface. *J Immunol* 1993; 151:4562–4573.

23 Wegmann TG, Lin H, Guilbert L, Mosmann TR: Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunol Today* 1993; 14:353–356.

24 Marzi M, Vigano A, Trabattoni D, Villa ML, Salvaggio A, Clerici E, Clerici M: Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic human pregnancy. *Clin Exp Immunol* 1996; 106:127–133.

25 Raghupathy R: Th1-type immunity is incompatible with successful pregnancy. *Immunol Today* 1997; 18:478–482.

26 Chaouat G, Zourbas S, Ostojic S, Lappree-Delage G, Dubanchet S, Ledee N, Martal J: A brief review of recent data on some cytokine expressions at the materno-foetal interface which might challenge the classical Th1/Th2 dichotomy. *J Reprod Immunol* 2002; 53:241–256.

27 Trowsdale J, Betz AG: Mother's little helpers: mechanisms of maternal-fetal tolerance. *Nat Immunol* 2006; 7:241–246.

28 Hayakawa S, Karasaki-Suzuki M, Ito T, Ishii M, Kanaeda T, Nagai N, Takahashi-Yamamoto N, Tochigi M, Chishima F, Fujii TK, Oyama J, Kitanaka S, Sato K: Effects of paternal lymphocyte immunization on peripheral Th1/Th2 balance and TCR V beta and V gamma repertoire usage of patients with recurrent spontaneous abortions. *Am J Reprod Immunol* 2000; 43:107–115.

29 Yokoo T, Takakuwa K, Ooki I, Kikuchi A, Tamura M, Tanaka K: Alteration of TH1 and TH2 cells by intracellular cytokine detection in patients with unexplained recurrent abortion before and after immunotherapy with the husband's mononuclear cells. *Fertil Steril* 2006; 85:1452–1458.

American Journal of Reproductive Immunology 58 (2007) 530–536

—Mini Review—

Human Sperm Cryopreservation —Theory and Clinical Application

Hiromichi Ishikawa[1]* and Satoru Kaneko[1]

[1]Reproduction Center, Ichikawa General Hospital, Tokyo Dental College, 5-11-13 Sugano, Ichikawa, Chiba 272-8513, Japan

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).

Received: February 20, 2007
Accepted: March 6, 2007
*To whom correspondence should be addressed.
e-mail: isikawah@tdc.ac.jp

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

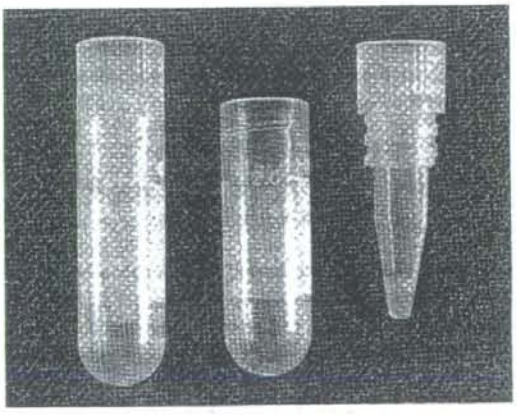

Fig. 1. Double-wall transformable freezing container for human sperm.

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.

References

1) Polge, C. and Rowson, L.E.A. (1952): Fertilizing capacity of bull spermatozoa after freezing at –79°C. Nature Lond., 169, 626.
2) Bunge, R.G. and Sherman, J.K. (1953): Fertilizing capacity of frozen human spermatozoa. Nature, 172, 767.
3) Sherman, J.K. (1955): Temperature shock in human spermatozoa. Proc. Soc. Exp. Biol. Med., 88, 6–10.
4) Masur, P. (1964): Freezing of live cells; mechanism and implications. Am. J. Physiol., 247, C125–C128.
5) Serafini, P.C., Hauser, D., Moyer, D. and Marrs, R.P. (1986): Cryopreservation of human spermatozoa: correlations of ultrastructural sperm head configuration with sperm motility and ability to penetrate zona-free hamster ova. Fertil. Steril., 46, 691–696.
6) Matheson, G.W., Calborg, L. and Gemze, C. (1969): Frozen human semen for artificial insemination. Am. J. Obstet. Gynecol., 104, 495–499.
7) Jeyendran, R.S., van der Ven, H.H., Kennedy, W., Perez-Pelaez, M. and Zaneveld, L.J.D. (1984): Comparison of glycerol and a zwitterion buffer systems as cryoprotective media for human spermatozoa. J. Androl., 5, 1–6.
8) Kaneko, S., Kobayashi, T., Lee, H.K., Won, W.K., Oda, T., Izumi, Y., Ohono, T. and Iizuka, R. (1990): Cryogenic preservation of low-quality human semen. Arch. Androl., 24, 81–86.
9) Kobayashi, T., Kaneko, S., Hara, I. , Park, J.Y., Aoki, R., Ohno, T. and Nozawa, S. (1991): A simplified technique for freezing of human sperm for AIH; cryosyringe/floating platform of liquid nitrogen vapor. Arch. Androl., 27, 55–60.
10) Kaneko, S., Sato, H., Kobanawa, K., Oshio, S., Kobayashi, T. and Iizuka, R. (1978): Continuous-step density gradient centrifugation for the selective concentration of progressively motile sperm for insemination with husband's semen. Arch. Androl., 19, 75–84.
11) Kobayashi, T., Kaneko, S., Hara, I., Park, Y.J., Sato, H., Ohno, T. and Nozawa, S. (1991): Concentrating human sperm before cryopreservation. Andrologia, 23, 25–28.
12) Agarwal, A. (2000): Semen banking in patients with cancer: 20-year experience. Int. J. Androl., 23 (Suppl. 2), 16–19.
13) Puscheck, E., Philip, P.A. and Jeyendran, R.S. (2004): Male fertility preservation and cancer treatment. Cancer Treat. Rev. 30, 173–180.
14) Hallak, J., Sharma, R.K., Thomas, A.J. and Agarwal, A. (1998): Why cancer patients request disposal of cryopreserved semen specimens posttherapy: a retrospective study. Fertil. Steril., 69, 889–893.
15) Schill, W.B. and Trotnow, S. (1984): Verwenedung von Kryospermia fur die in vitro Fertilisation (IVF). Hautarzt, 35, 313–315.
16) Roland, G.F., Cohen, J., Steptoe, P. and Hewitt, J. (1985): Pregnancy following in vitro fertilization using cryopreserved semen from a man with testicular teratoma. Urology, 26, 33–36.
17) Tournaye, H., Camus, M., Bollen, N., Wisanto, A., Van Steirteghem, A.C. and Devroey, P. (1991): In vitro fertilization techniques with frozen-thawed sperm: a method for preserving the progenitive potential of Hodgkin patients. Fertil. Steril., 55, 443–445.
18) Hakim, L.S., Lobel, S.M. and Oates, R.D. (1995): The achievement of pregnancies using assisted reproductive technologies for male factor infertility after retroperitoneal lymph node dissection for testicular carcinoma. Fertil. Steril., 64, 1141–1146.
19) Rosenlund, B., Sjoblom, P., Tornblom, M., Hultling, C. and Hillensjo, T. (1998): In-vitro fertilization and intracytoplasmic sperm injection in the treatment of infertility after testicular cancer. Hum. Reprod., 13, 141–148.
20) Lass, A., Akagbosu, N., Abusheikha N., Hassouneh, M., Blayney, M., Avery, S. and Brinsden, P. (1998a): A program of semen cryopreservation for patients with malignant disease in a tertiary infertility center: lessons from eight years' experience. Hum. Reprod., 11, 3256–3261.
21) Audrins, P., Holden, C.A., McLachlan, R.I. and Kovacs, G.T. (1999): Semen storage for special purposes at Monash IVF from 1977 to 1997. Fertil. Steril., 72, 179–181.

Reproductive Medicine and Biology 2007; 6: 127–131 doi: 10.1111/j.1447-0578.2007.00175.x

Original Article

Cryopreservation of human sperm in patients with malignancy: First 2 years' experience

HIROMICHI ISHIKAWA,[1]* SATORU KANEKO,[2] KEISUKE MIYAJI[1] and KIYOSHI TAKAMATSU[2]

Departments of [1]Urology and [2]Obstetrics and Gynecology, Ichikawa General Hospital, Tokyo Dental College, Ichikawa, Japan

Background: Patients with malignancy ($n = 130$) participated in the sperm cryopreservation program.

Methods: After washing and concentrating, sperm was cryopreserved using KS-VIm cryoprotectant medium. Participant background factors such as age, marital status, underlying disease, presence or absence of previous treatment and semen findings (concentration, motility and morphology) were analyzed to determine parameters associated with the program.

Results: Patients in their 20s were most common (64 cases) and 94 cases were unmarried at the first visit. The main underlying diseases were testicular tumor (53 cases), leukemia (43 cases) and malignant lymphoma (13 cases). The program was completed for 118 cases. For leukemia, all semen parameters were closer to normal in patients without previous treatment (untreated group, UG) compared with the treated group (TG). When semen findings in the UG were classified according to underlying disease, sperm concentration was lower in patients with testicular tumor compared with those who had leukemia or malignant lymphoma. Four couples underwent reproductive therapies with the cryopreserved sperm through assisted reproductive technology, and three babies were born to two couples.

Conclusion: Sperm cryopreservation liberates patients with malignancy from iatrogenic infertility as a consequence of intensive therapy, allowing them to retain reproductive ability. (Reprod Med Biol 2007; 6: 127–131)

Key words: infertility, malignancy, sperm cryopreservation.

INTRODUCTION

TESTICULAR TUMOR, LEUKEMIA and malignant lymphoma occur frequently in young men. Remarkable progress in high-dose chemotherapy and radiation therapy predicts a high chance of complete recovery in such patients. These intensive treatments generally cause the loss of testicular function, especially spermatogenesis, aiming to cure cancer at the expense of sacrificing fertility. High inguinal orchiectomy and castration are often used to treat testicular tumors and prostate cancer, respectively. Given these circumstances, the sperm cryopreservation program is aimed at patients with malignancy as a method to avoid iatrogenic infertility as a consequence of intensive therapies.[1,2] It is thus valuable from the viewpoint of quality of life that successfully treated patients can accomplish the dream of marriage and having children.

Sperm cryopreservation was first reported by Polge and Rowson.[3] Cryopreservation of sperm has been applied to both livestock and humans since the 1950s. To date, we have actively promoted the sperm cryopreservation program for patients with malignancy, and our program has already resulted in three babies being conceived with the cryopreserved sperm. The present study classifies semen findings according to patients' medical histories (underlying diseases and presence or absence of certain previous treatments), and discusses issues associated with the program.

MATERIALS AND METHODS

Subjects

FROM OCTOBER 2002 to April 2005, 130 patients with malignancy visited the Reproduction Center (Ichikawa

*Correspondence: Dr Hiromichi Ishikawa, Department of Urology, Ichikawa General Hospital, Tokyo Dental College, Ichikawa, Chiba 272-8513, Japan. Email: isikawah@tdc.ac.jp
Received 29 September 2006; accepted 12 January 2007.

General Hospital, Tokyo Dental College, Ichikawa, Japan) to participate in the sperm cryopreservation program before chemotherapy and radiation therapy, which were expected to destroy spermatogenesis, were carried out,. The patients were confirmed to be negative for HIV, HCV, HBV and syphilis. All patients with testicular tumor had already undergone orchiectomy before the first visit.

Semen analyses and assessment of semen quality

Ejaculate was obtained by masturbation in the semen collection room at the outpatient clinic. After 30 min liquefaction at room temperature, semen findings (sperm concentration, motility and head morphology) were measured according to the WHO manual.[4] Patients with azoospermia, severe oligozoospermia (less than 1×10^6/mL) and teratozoospermia (normal morphology less than 1.0%) were excluded from the program.

Sperm cryopreservation

Human sperm cryopreservation was carried out as described previously.[5,6] Raw Percoll (1.0 L, Amersham, Uppsala, Sweden) was made isotonic using 10.0 mL of 2.0 mol/L HEPES-NaOH, pH 7.4, and 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) and 10 mL of human serum albumin (25% w/v). The resulting isotonic 98% Percoll solution was sterilized with a Millipore filter (0.45 μm pore size). Then, 5 mL of 98% Percoll was placed in a conical tip test tube and 1.0 mL of Hank's solution was layered on top. The test tube was rotated 10 revolutions at an angle of 30° to make a density gradient. To remove fibers, microcalculi and micinous debris, the ejaculate was diluted twice with Hank's solution, filtered through nylon mesh (ART filter, 20 μm clearance, Nipro, Osaka, Japan), then allowed to stand in a test tube for 10 min to precipitate filterable microcalculi. The resulting suspension was placed on a density gradient, centrifuged at $400 \times g$ for 30 min in a swing-out rotor, and the sediment (0.2 mL) was mixed with an equal volume of KS-VIm cryoprotective medium (20 mmol HEPES-NaOH, pH 7.4, 12% glycerin, 10% egg yolk water soluble fraction, fosfomycin [0.05 g/L] and cepharotin [0.05 g/L] in Hank's solution). The mixture was frozen in liquid nitrogen vapor, stored at −196°C, and thawed in a tap water at 37°C.

Patients' informed consent

Prior to the procedure, patients who satisfied the collateral terms of the contract signed the informed consent form, approved by the Ethical Committee of Tokyo Dental College. In summary, the informed consent form stated that the cryopreserved sperm would be stored only while the patient was alive. The duration of cryopreservation is contracted on a 1-year basis and consent needs to be renewed annually for continuance. The contract is cancelled automatically when a renewal is not made within 3 months after expiration of the agreement, or when the patient is 1 year overdue for the cryopreservation fee. Data can be used anonymously under strict confidentiality. Participants were informed that unavoidable accidents, such as natural calamities, might put the cryopreserved sperm beyond use.

Practice of assisted reproductive technology

After thawing, progressively motile sperm was separated using the swim-up method, then inseminated through intracytoplasmic sperm injection (ICSI) or intrauterine insemination (IUI). ICSI was carried out in four cases (twice in two cases, once in two cases) and IUI was used in one case.

Statistical analyses

The patients' data were analyzed by non-parametric analysis of Mann–Whitney's *U*-test by Stat-View version 5.0 (SAS Institute, Cary, NC, USA). Values are expressed as mean ± standard deviation.

RESULTS

PATIENT AGES WERE widely distributed from 16 to 60 years-of-age (30.1 ± 17.7 years). At the first visit, 94 cases were unmarried (seven of these were engaged) and 33 cases had already received some anticancer treatments (treated group: TG), whereas 97 cases had not (untreated group: UG). In the TG, 76% of cases had leukemia, and all of them had undergone remission induction and post-remission therapy. A short period was usually allowed until the start of the chemotherapy. The ejaculates for cryopreservation in each case were obtained from a maximum of three samples. In the following results, samples having the highest sperm concentration were used in the analyses. Patients' underlying diseases and semen findings are summarized in Table 1. Testicular tumor was the most frequent

Table 1 Classification of underlying diseases and comparison of semen findings between untreated and treated groups

Case	Treated ($n = 33$)		Untreated ($n = 97$)		Total
	Exclude	Preserve	Exclude	Preserve	
Testicular tumor	0	2	4	47	53
Leukemia	3	22	2	16	43
Malignant lymphoma	3	0	0	10	13
Aplastic anemia	0	2	0	1	3
Prostate cancer	0	0	0	3	3
Bladder tumor	0	0	0	2	2
Pharyngeal cancer	0	0	0	2	2
Liposarcoma	0	1	0	0	1
Hepatocellular carcinoma, retroperitoneal tumor, multiple myeloma, thymic tumor, intrapelvic tumor, small intestinal cancer, lingual cancer, colon cancer, rectum cancer, brain tumor	0	0	0	Each 1 (10)	10
	6	27	6	91	130
Sperm concentration ($\times 10^6$/mL)	96 ± 133		87 ± 71		90 ± 90
Motility (%)	29.3 ± 24.4		40.7 ± 23.4		38.0 ± 24.0
Normal morphology (%)	6.5 ± 6.5		9.5 ± 8.9		8.8 ± 8.5

diagnosis, followed by leukemia and malignant lymphoma, accounting for 84% of the patients. Comparing semen findings between the UG and TG, previous treatments significantly decreased sperm motility only ($P < 0.05$). Because the semen quality in 12 cases was below the exclusion criteria, only 118 cases were actually enrolled in the program. In the UG, 6.2% of the patients were excluded, whereas 18.2% of TG patients were excluded from the program.

We compared semen findings among the three major diseases (Fig. 1). For testicular tumor, we found no significant difference between UG and TG in any parameter. However, because this subgroup of the TG comprised only two cases, it remains unclear whether previous treatments had an influence. For leukemia, all parameters were significantly suppressed ($P < 0.05$) by previous treatments. For malignant lymphoma, the percentage of normal morphology in the TG was significantly lower ($P < 0.05$) than in the UG.

For UG, sperm concentrations in the testicular tumor subgroup were significant lower ($P < 0.05$) than in the other diseases. In contrast, no significant difference was found between leukemia and malignant lymphoma. For TG, no parameter was significantly different among the three groups.

To date, cryopreservation has been terminated as a result of patient death in five cases, and four couples underwent assisted reproductive technology (ART) with the cryopreserved sperm, with two couples successfully obtaining three babies with the aid of ICSI (Table 2). Patient I (leukemia) cryopreserved his sperm (75×10^6/mL, 40% motility, in four tubes), then underwent bone marrow transplantation. The couple delivered their first child with the aid of ICSI. Furthermore, this couple delivered a second child in the same manner. After orchiectomy, patient II (testicular tumor) cryopreserved his sperm (110×10^6/mL, 47% motility, in three tubes). After his complete cure, the couple also delivered a baby through ICSI. Patients III and IV underwent ART, without resulting pregnancy.

DISCUSSION

INTENSIVE ANTICANCER TREATMENTS such as chemotherapy, bone marrow transplantation and some adjuvant therapies improve the survival rate in young patients with testicular tumor or leukemia. However, various complications, particularly iatrogenic infertility, are associated with these therapies. Although semen findings normalize after treatment, the possibility of genetic disturbances in sperm cannot be ruled out.[1,2] The ethical committee of our institution recommended that unmarried patients should be excluded from the program. As described in the results, patients in their 20s or younger (72 cases) and unmarried patients (94 cases) were in the majority. Considering overall patient quality of life, we decided to apply the program to unmarried patients as well.

Thirty-three patients had already received anticancer treatments before their first visit. Even in the UG, the patients were allowed a short period to store the sperm.

Table 2 Summary of assisted reproductive techniques with cryopreserved sperm

Case	Age	Underlying disease	Anticancer treatment	Semen findings: concentration ($\times 10^6$/mL)/motility (%)/normal morphology (%)			Method of ART therapeutic result	
				Ejaculate	ART 1st	ART 2nd	1st	2nd
1	24	Leukemia	Radiation chemotherapy bone marrow transplantation	75/40/10.8	10/30/12.6	32/28/10.6	ICSI delivery	ICSI delivery
2	33	Testicular tumor	Radiation	110/46.7/8.9	170/41/20.3	–	ICSI delivery	–
3	50	Malignant lymphoma	Radiation chemotherapy	120/9.4/16.3	81/8.2/19.3	94/16/32.3	ICSI not pregnant	ICSI not pregnant
4	49	Malignant lymphoma	Chemotherapy	70/24/5.7	77/65/19.4	57/40/15.6	AIH not pregnant	ICSI not pregnant

AIH, artificial insemination with husband's semen; ART, assisted reproductive technology; ICSI, intracytoplasmic sperm injection.

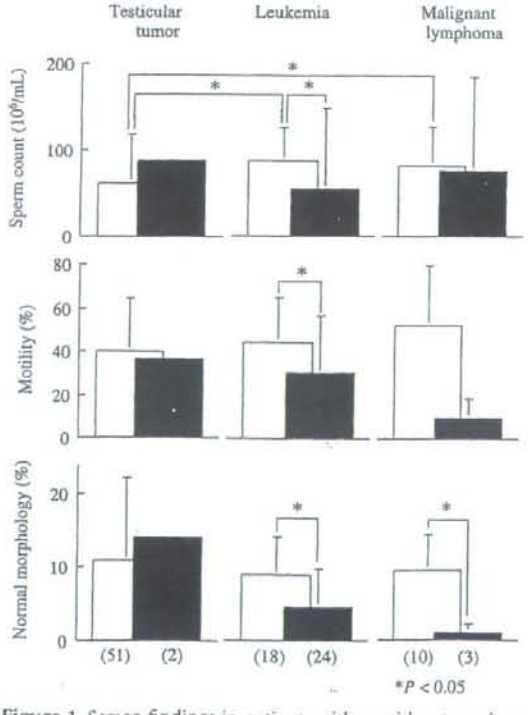

Figure 1 Semen findings in patients with or without previous anticancer treatment. Values in parentheses indicate the number of cases. (□) Untreated group; (■) treated group.

We have to make efforts to publicize the significance of the sperm cryopreservation program to oncologists.

When comparing semen findings between the UG and TG, the percentage of normal morphology decreased for malignant lymphoma patients, and all parameters decreased in leukemia patients. Serious adverse effects on testicular function might occur with the use of alkylating agents (i.e. cyclophosphamide and procarbozine).[7,8] Systemic radiotherapy with subsequent bone marrow transplantation[9,10] is frequently combined with these agents. It is therefore essential to cryopreserve sperm prior to anticancer treatments.

For the UG, sperm concentration was significantly lower for testicular tumor patients compared with leukemia and malignant lymphoma patients. Elevation in intrascrotal temperature[11] and production of antisperm antibodies[12] might suppress spermatogenesis in the contralateral testis. Just after orchiectomy, trauma to the contralateral testis often induced azoospermia, which normalized after one month.[13] If tumor progression were not so rapid, it might be useful to cryopreserve sperm after a certain period following orchiectomy.

It should be emphasized that patients, oncologists and the public should recognize the significance of a sperm cryopreservation program from an aspect of overall quality of life. However, a number of social and ethical problems need to be dealt with, for example, the legal status of cryopreserved sperm after the death of a patient. Recently in Japan, two widows conceived babies using cryopreserved sperm; however, the courts

recognized neither of children as fathered. In the current contract used for this study, cryopreservation would be terminated upon patient death and ART should not be accepted. However, guidelines for administering this program require further debate that includes multiple points of view.

REFERENCES

[1] Agarwal A. Semen banking in patients with cancer: 20-years experience. *Int J Androl* 2000; **23** (Suppl 2): 16–19.

[2] Puscheck E, Philip PA, Jeyendran RS. Male fertility preservation and cancer treatment. *Cancer Treatment Rev* 2004; **30**: 173–180.

[3] Polge C, Rowson LEA. Fertilizing capacity of bull spermatozoa after freezing at −79°C. *Nature Lond* 1952; **169**: 626.

[4] World Health Organization. *Laboratory Manual for the Examination of Human Semen and Semen–Cervical Mucus Interaction*, 4th edn. Cambridge: Cambridge University Press, 1999.

[5] Kobayashi T, Kaneko S, Hara I *et al.* A simplified technique for freezing of human sperm for AIH; Cryosyringe/Floating platform on liquid nitrogen vapor. *Arch Androl* 1991; 27: 55–60.

[6] Kobayashi T, Kaneko S, Hara I *et al.* Concentrating human sperm before cryopreservation. *Andrologia* 1991; **23**: 25–28.

[7] Thachil JV, Jewett MA, Rinder WD. The effects of cancer and cancer therapy on male fertility. *J Urol* 1981; **126**: 141–145.

[8] Costabile RA. The effects of cancer and cancer therapy on male reproductive function. *J Urol* 1993; **149**: 1327–1330.

[9] Sanders JE, Hawley J, Levy W *et al.* Pregnancies following high-dose cyclophosphamide with or without high-dose busulfan or total-body irradiation and bone marrow transplantation. *Blood* 1996; **87**: 3045–3052.

[10] Jacob A, Barker H, Goodman A, Holmes J. Recovery of spermatogenesis following bone marrow transplantation. *Bone Marrow Transplant* 1998; **22**: 277–279.

[11] Weissbach L, Vahlensieck W, Figge M. Diagnostik bei hodentumren. *Urologe B* 1980; **20**: 106.

[12] Guazzieri S, Lembo A, Ferro G *et al.* Sperm anti-bodies and infertility in patients with testicular cancer. *Urology* 1985; 26: 139–142.

[13] Tomomasa H, Oshio S, Amamiya R *et al.* Testicular injury: late results of semen analyses after uniorchiectomy. *Acrh Androl* 1992; 29: 59–63.

特集　造血幹細胞移植の新たな展開

精子形成機能障害対策 —精子凍結保存について*

石川博通**
兼子　智***

Key Words：sperm, cryopreservation, malignancy, assisted reproduction technology (ART)

はじめに

若年男子に好発する精巣腫瘍，白血病，悪性リンパ腫は，大量化学療法，放射線療法などの進歩によって根治が望めるようになってきた．しかし，精巣はこれらの治療に感受性が高く，治療過程において造精機能が犠牲になることも多い．治療後における患者QOL，とくに妊孕性維持を考慮することがもっとも重要な課題となってきた．しかし，治療中に造精機能を保護するよい方法のない現状では，治療前に患者精子を採取して凍結保存することが唯一の対策となる．一方，精子を得る方法として一般的なマスターベーションによるものと精巣組織採取によるものがあるが，後者は本邦ではほとんど行われていない．そこで本稿では通常の採取法を前提として①精子凍結保存の歴史，②精子凍結保存実施法，③精子凍結保存の臨床応用について概説し，かつ文献的考察を行った上で，④治療前精子凍結の実際とその問題点について当院リプロダクションセンターでの成績を中心に述べる．

精子凍結保存の歴史

ヒト精子の凍結保存に関する最初の記載は18世紀中頃にイタリアのLazaro Spallanzaniによる「精子を雪で冷やすと動きがなくなる」というものとされている．また，Mantegazzaは−15℃の環境下で精子を観察しているが，1866年に精子バンクの必要性について言及している．その後1930～1940年にかけてより低温での精子の生存が報告されているが，精子バンクといえるのは1949年のPolgeらの牛精子の報告が最初である．ここで画期的なことは凍結保護剤として偶然に細胞傷害を抑制するグリセリンを用いたことである．また，Polgeらは1952年にこの方法で凍結した精子を用いて人工授精に成功した．さらに1953年には，Shermanらがグリセリンとドライアイスを用いてヒト精子の凍結保存に成功している．これに次いで本邦でも1958年に凍結保存精液による非配偶者間人工授精(AID)により児が得られている[1)2)]．

精子凍結保存の実施法

細胞を凍結する場合，温度低下に伴い細胞外液がまず凍結して，溶質が濃縮するため浸透圧が上昇し，細胞内脱水が起こる．さらに温度が下がると細胞内凍結が起こり，氷晶が形成されてそれが細胞膜，細胞内小器官を物理的に傷害する[3)]．また融解時における昇温過程でも，いったん融解した氷晶の再結晶や細胞内への水の急激な流入などにより細胞の生存性が低下する．

* Sperm cryopreservation in patient with malignancy.

** Hiromichi ISHIKAWA, M.D.: 東京歯科大学市川総合病院泌尿器科〔〒272-8513 市川市菅野5-11-13〕; Department of Urology, Ichikawa General Hospital, Tokyo Dental College, Ichikawa 272-8513, JAPAN
*** Satoru KANEKO, M.D.: 東京歯科大学市川総合病院産婦人科

表1　KS-II精子保存液

溶液A	
Hepes	4.77g
NaCl	6.42g
KCl	0.35g
$CaCl_2 \cdot 2H_2O$	0.295g
$MgSO_4 \cdot 7H_2O$	0.075g
$NaHCO_3$	2.10g
グルコース	0.50g
乳酸Na(98%)	1.73ml
ピルビン酸(99%)	0.02ml
純水	1,000ml

卵黄	50ml
セファロチン	0.005g
ホスミシン	0.001g
プルロニックF68	1.0g
グリセリン	120ml
ショ糖	68.0g

溶液Aで1,000mlとし，
遠心分離上清を濾過滅菌，
凍結保存

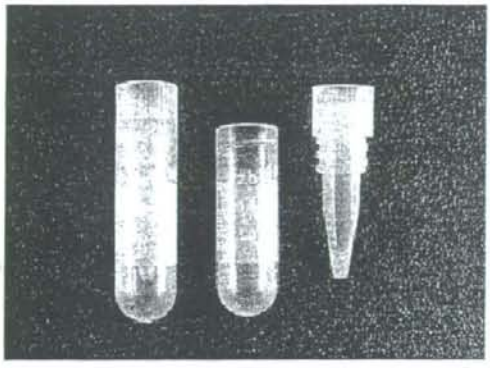

図1　可変性二重腔容器

通常の培養細胞では，融解後の細胞生存率が低くても悪くても増殖により細胞数を回復できるが，分裂能がない精子では高い蘇生率が要求される．このようなことを念頭に入れて精子の凍結保存を行い，さらに授精に供する．一般的な方法，手順を以下に示す．

1．精子洗浄濃縮法

抗生物質を添加した20mM Hepes緩衝化Hanks液(精子培養液，pH7.4)で精液を希釈する．次に等張化99%Percoll液を用いる撹拌密度勾配法で精子洗浄濃縮を行う[4)~6)]．

2．精子凍結保存液

凍結保存液としてKS-II(表1)を用いる．本剤は培養液に凍結保護物質としてグリセリン，卵黄水可溶性分画，ショ糖さらに界面活性剤プロニックF-68(ethyleneoxide-propyleneoxide co-polymer，旭電化工業)を含有する．すなわちグリセリン，ショ糖には脱水および細胞内氷晶形成抑制，卵黄水可溶性分画の作用機序には不明な点が多いが，細胞表面を被覆作用があると考えられており，プロニックF-68は卵黄の可溶化を促進して，作用を増強する[7)~9)]．

3．凍結法

精子凍結法として，①液体窒素直接浸漬法，②プログラムフリーザー法，③ドライアイス錠剤化法，④液体窒素蒸気法がある[10)~12)]．①は精子蘇生率が悪く，②は精子に用いるには凍結速度が遅く，高価なために両法ともあまり行われなくなった．③は動物の精子凍結には頻用されるが，実際のヒト精子保存の現場では個別化が困難であるため利用されない．その結果，高い精子蘇生率が得られ，精子個別が可能で安価な液体窒素蒸気法が多く用いられるようになった．しかし最近，当院では可変型二重腔容器(図1)を開発し，精子蘇生率が保障されたため，より簡便な液体窒素直接浸漬法を用いている．本法では外容器と内容器の間の空気が液体窒素蒸気の代替となり，適当な凍結速度が得られる．

4．融解法

精子は，約30℃の微温湯中で振盪，融解する．融解後ただちにHanks液で緩徐に希釈して撹拌密度勾配法により，凍結保存液を除去する．

精子凍結保存の臨床応用

1．精液所見不良例での備蓄

乏精子症もしくは精子無力症では人工授精または体外受精当日に十分な精子が得られない可能性が高いのでバックアップとして備蓄を行う．

2．非配偶者間人工授精における備蓄および感染症対策

精子提供者の精子を備蓄する．また精子凍結後一定期間をおいて感染症検査を行い，陰性の

表2 年齢分布および結婚年齢

年齢	～19	20～29	30～39	40～49	50～
未婚	9	71	62	5	5
既婚	0	17	24	8	2

表3 原疾患と凍結状況

	凍結可		凍結不可		
	前治療あり	前治療なし	前治療あり	前治療なし	計
精巣腫瘍	4	71	0	8	83
白血病	26	13	9	1	49
悪性リンパ腫	2	16	5	1	24
骨髄異形成症候群	2	8	1	0	11
前立腺がん	6		0		6
再生不良性貧血	6		0		6
多発性骨髄腫	2		1		3
直腸がん	3		0		3
咽頭がん	2		0		2
膀胱がん	2		0		2
その他	13		1		14

場合のみ精子を使用することにより感染症対策とする．

3．射精障害例における備蓄

逆行性射精例では，膀胱内に射出された精子を回収後洗浄濃縮して凍結保存する．脊髄損傷で性交時以外に射精が起こる例では射精時に精子を回収して凍結保存する．

4．悪性腫瘍治療前の凍結保存

悪性腫瘍に対して化学療法もしくは放射線療法を施行すると，精子形成機能が廃絶する可能性がある．そのため治療前に精子の凍結保存を行う．

治療前精子凍結保存の実際とその問題点

2002年4月，東京歯科大学市川総合病院では，夫婦を単位として男性，女性の不妊治療を行う施設としてリプロダクションセンターを開設した．同時に当施設で悪性腫瘍患者の治療前精子凍結保存を行う旨，東京歯科大学倫理委員会に申請した．承認後，2002年9月より実際に凍結保存を開始した．

1．倫理的事項およびインフォームドコンセント

日本生殖医学会および日本癌治療学会の指針に従い，凍結を行う前提として，①腫瘍専門医が生殖医療専門医に必要かつ十分な情報を提供すること，②実施にあたって倫理委員会の承認を得たインフォームドコンセントを作成し，十分な説明の上，同意をとること，③精子を売買の対象としないことなどを確認した．また同意書の中には，①凍結保存は本人の生存中であること，② 1年ごとに契約の更新をすることを記載した．さらに2006年9月1日に作成された日本生殖医学会のガイドライン案に従い，①保存責任，②費用負担などについて検討して，今後同意書(インフォームドコンセント)の中に盛り込む予定である．

2．患者背景[13]

2002年9月から2006年8月までの4年間に治療前精子凍結目的で来院した患者は203例であり，その背景は以下のようであった．

患者年齢は16歳から60歳に及び，20歳代(88例)および30歳代(86例)が多かった．既婚者が51例であったのに対し，未婚者(152例)が多かった(表2)．

精巣腫瘍が83例(40.9%)ともっとも多く，白血病(49例)，悪性リンパ腫(24例)，骨髄異形成症候群(11例)がそれに続いた．203例中精液検査で無精子症もしくは精液所見不良で凍結不可とされたものは，27例(13.3%)であった．受診前に化学療法もしくは放射線療法が行われていたのは，55例であり，そのうち35例の原疾患は白血病であった(表3)．

表4　ARTの成績

症例	原疾患	年齢	結婚	ART	転帰
1	骨髄異形成症候群	24	既婚	顕微授精 顕微授精	妊娠 妊娠
2	悪性リンパ腫	21	未婚	顕微授精	(−) (−)
3	悪性リンパ腫	30	既婚	人工授精 顕微授精	(−) (−)
4	精巣腫瘍	33	既婚	顕微授精	妊娠
5	精巣腫瘍	34	既婚	人工授精	(−)
6	骨髄異形成症候群	22	未婚	人工授精	(−)

(−) 妊娠不成功

3．凍結の可否と前治療との関係

凍結保存しえた177例のうち既治療は34例で，そのうち26例の原疾患は白血病であった．凍結不可26例のうち15例に前治療が行われており，その原疾患は白血病9例，悪性リンパ腫5例であった(表3)．

4．主な原疾患における精液所見

精巣腫瘍，白血病および悪性リンパ腫の精液所見を比較すると，精子濃度は既治療例で精巣腫瘍例が白血病および悪性リンパ腫例に比べて有意に低かった．また精子運動率は白血病例で未治療群が既治療群に比べ有意に高かった．

ART(生殖援助技術)の実施

リプロダクションセンター開設前を含めて6例の妻に顕微授精もしくは人工授精を行った．成績は表4のようであり，症例1および症例4で妊娠が成立し，児を得た(表4)．

治療前精子凍結保存の問題点

1．既治療例が多かったこと

全体の25%以上が治療後に来院しており，凍結不可27例中17例を占めた．また凍結可能であった38例でも精液所見不良の傾向にあった．上述したように，既治療例の63.6%が白血病であり，生命予後を重視した緊急の治療を行う必要があった可能性もあるが，問診時精子凍結保存のことについて説明されていない例も少なからず存在した．このことから，精子の凍結保存を組織的に行っている施設の少ない現状では，われわれが腫瘍専門医もしくは患者に対して啓蒙活動をする必要があると考えられた．

2．未婚者が多かったこと

受診時約75%が未婚であった．原疾患または精子凍結保存ということで若年者が多くなるのは当然のことではあるが，最終的にARTを施行する際に大きな問題となる．実際，凍結開始後に結婚してARTを行う例も存在するが，それはきわめて少ない．積極的に結婚相手を探すよう努力するように勧めているが，パートナーの負担も考えると難しい問題もある．一部には精子凍結保存は既婚者に限るという意見もあり，今後検討を要する課題である．

3．未成年者が精子凍結保存契約をしたこと

未成年者が9例存在した．当施設の説明書には，本人の意思で凍結保存を行うという意味から成人が望ましいという旨が記載してある．しかし，未成年の希望者からの問い合わせも多く，また権利を考えると凍結の契約は避けられないのが現状である．しかしながら，ほとんどが両親の意向で行われているため，いずれは本人の意思を明確にしてもらった上で契約することが必要であろう．

4．高齢者が契約したこと

生殖医療対象としては高齢と考えられる50歳以上の患者が7例あり，かつ2例は未婚であった．これらの症例ではART実施は困難な可能性が高い．初診時にこの点を説明して契約をしないよう説得したが，強い希望で凍結保存をする結果になった．この場合，ART以外の目的を設定すべきであると思われるが，結論を出すのはなかなか難しい．

5．契約者死後の精子の取り扱い

契約者の死亡後，妻が凍結精子を他施設に移

送することを希望するケースが2例あった．契約に凍結保存は本人の生存中に限るという条項があるため，長時間説得した上でこれをあきらめてもらった．また，最近行われた裁判でも死後精子を用いて誕生した児が当事者の子と認知されなかった．これらのことから，この状況下では死後精子の使用はすべきではないことは明白であるが，混乱を避ける意味で法制化されることが望ましい．

文　献

1) Anger JT, Gilbert BR, Goldstein M. Cryopreservation of sperm : Indications, methods and results. J Urol 2003 ; 170 : 1079.
2) Sherman JK. Cryopreservation of human semen. In : Keel BA, Webster BW, editors. CRC Handbook of the Laboratory. Diagnosis and Treatment of Infertility. Boca Raton : CRC Press ; 1990. p. 229.
3) 朝比奈英三．生物細胞の凍結．In：酒井　昭・編．凍結保存―動物・植物・微生物―．東京：朝倉書店；1987．p. 3.
4) 真田利男，小林俊文，兼子　智，ほか．密度勾配用ショ糖重合体を用いた精子洗浄濃縮法のAIHへの応用．日不妊誌 1983；28：267.
5) 真田利男．撹拌密度勾配法による精子洗浄濃縮―とくに配偶者間人工授精への応用―．慶應医学 1989；66：341.
6) 小林俊文，兼子　智，翁　文開，ほか．洗浄濃縮精子の凍結保存に関する研究．日不妊誌 1988；33：639.
7) 枡田博司．精子の凍結保存．In：森澤正昭，星　元紀・編．精子学．東京：東京大学出版会；1992．p. 224.
8) 入谷　明．動物精液の凍結保存．In：酒井　昭・編．凍結保存―動物・植物・微生物―．東京：朝倉書店；1987．p. 131.
9) Fosgate MT, Aschbacher PW. Surface active agents as constituents of diluents for deep freezing of bovine spermatozoa. Daily Science 1987 ; 70 : 1664.
10) 翁　文開．洗浄の宿精子の凍結保存に関する研究―特に非配偶者間人工授精への応用．慶應医学 1989；66：679.
11) 李顕金光．錠剤化凍結法によるヒト精子凍結保存に関する研究―とくに配偶者間人工授精(AIH)への応用．慶應医学 1989；66：845.
12) Devroey P, Silber S, Nagy Z, et al. Ongoing pregnancies and birth after intracytoplasmic sperm injection with frozen-thawed epididymal sperm. Human Reprod 1995 ; 10 : 903.
13) 石川博通，兼子　智，岡崎雅子，ほか．治療前精子凍結保存とその問題点[会]．日生殖医会誌 2006；51：195.

＊　　＊　　＊

平成20年度

Progressed Research Projects Supported by MHLW (Ministry of Health, Labour and Welfare)

Epidemiological Study on Prevalence of HIV Infected Pregnant Women and Evaluation of Trans-Vaginal Delivery Regarding to Prevention of Mother-to-Child Transmission

Tsunekazu KITA, Naoto YOSHINO, Yuki TSUKAHARA, Masao TOGAWA, Noriyuki INABA and Yuichi WADA

Dept. Obstet. and Gynecol., Teikyo University School of Medicine and The National Cooperative Study Group on HIV Infected Pregnant Women and Mother-to-Child Transmission in Japan

Objectives:

In order to update the prevention methods of the mother-to-child transmission (MTCT) of HIV, we evaluated the clinical and virological information from HIV infected pregnant women and their off-springs in Japan. And then we identified the effectiveness of vaginal delivery to prevent MTCT of HIV comparing with elective cesarean section.

Methods:

For 10 years from 1998, a questionnaire about pregnant women infected with HIV was sent annually to more than 1,600 hospitals providing obstetric department and more than 3,500 hospitals or clinics providing pediatrics service. Totally 503 cases of HIV infected pregnancies were reported by 2007. Additionally, we obtained the details of the perinatal information of HIV infected 422 pregnant women from our obstetrics study group and 281 new born babies from pediatrics study group defined from 1987 to 2007 in Japan. After verification of these 703 cases, finally 503 cases by 2007 were evaluated in this study. Epidemioligical, obstetrical, and virological data on mothers and their infants was studied retrospectively.

Results:

Fig.1 shows the rate of pregnant women accepted voluntary HIV screening test in Japan. The screening rate of HIV infection was 95.3% in 2006 and elevated in 22.1% during the recent decade.

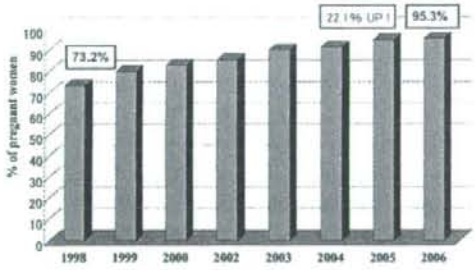

Fig. 1 Rate of pregnant women accepted voluntary HIV screening test in Japan

Fig. 2 Pamphlet informing HIV screening test for pregnant women

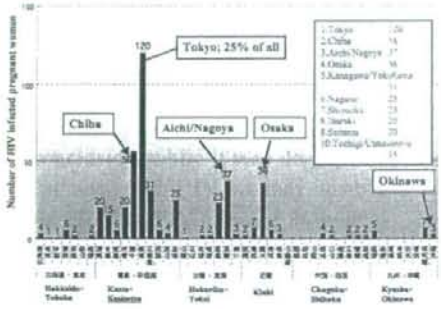

Fig. 3 Distribution of HIV infected pregnant women in provinces

These results are considered to be much indebted to publishing annual report and educational campaign by our study group. Fig.2 is a pamphlet informing HIV screening test for pregnant women established by our study group with supports of the Ministry of Health, Labour and Welfare in Japan.

The prevalence of HIV infected pregnant women is 9 per 100,000 pregnancies in Japan. Fig.3 shows distribution of HIV infected pregnant women in provinces and areas in Japan. About 65% of 503 cases from obstetrics and pediatrics study were