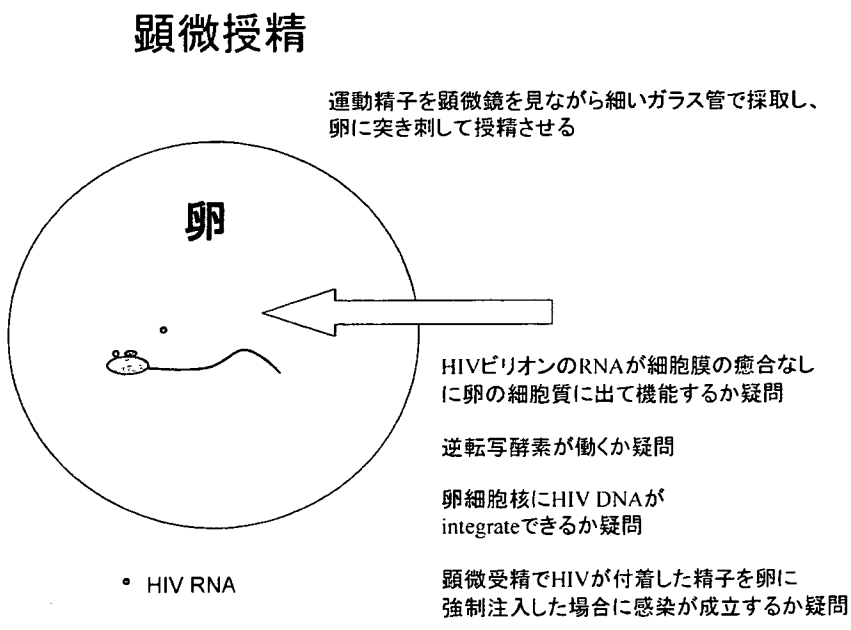

図 6

クリーニング検査を行う。その後，産科施設に紹介して改めて説明を受け，同意書を得た後に実施となる。胚移植後は妊娠の有無に係わらず妻の感染症検査を行い，出産後は母子の検査で2次感染がないことを確認する。

HIV 陽性男性と HIV 陽性女性の場合

HIV 感染者同士が結婚した場合の挙児相談や，夫婦間で感染した場合の相談も寄せられている。今後，HIV 感染者の予後が改善すると共に感染者同士の結婚も増え，挙児相談も多くなると推測される。この場合にどのような対応をとるのかについて，現状では倫理的にも社会的にも幅広い議論が必要である。その妥当性を判断する前提として，superinfection，HIV の変異，薬剤耐性，HIV 感染者の精子機能，HAART の精子への影響など多くの知識が必要であり，感染者夫婦個々の状況に応じた対応が求められる。

Superinfection（図 7）防止目的

HIV は変異速度が速く，宿主の免疫状態によっても感染者個々の HIV diversity は大きく異なる。既に HIV に感染していても別の HIV に再度感染すると，図 7 に示すように，HIV の再構築（recombination）が生じ，AIDS 発症を早めたり薬剤耐性を誘導する危険性がある[10]。また，たとえ夫婦間で感染したとしても夫婦の major clone が異なっていたり，時間と共に大きく変異し，性交渉で再感染が生じる可能性がある。最近，薬物注射による新規 HIV 感染者では約 5% に superinfection が生じているとの報告もあるが[11]，理論上はもっと高い可能性がある。superinfection を防止するためには，HIV 陽性同士でも生殖補助医療が必要となる。

耐性ウィルスの感染防止目的

夫婦のどちらかのウィルスが薬剤耐性となっていると，性交渉で新たに耐性ウィルスに感染する危険性がある。そのために生殖補助医療による予防が必要となる。

夫の精子機能が不良で男性不妊の場合

HIV 感染男性は治療の有無にかかわらず精子機能が傷害されている場合が多い[1]。また，我々は，HAART によって精子のミトコンドリア障害が生じ，精子の運動機能が障害され，男性不妊となっている場合があることを報告した[12]。HIV 感染男性では男性不妊となっている場合も多いと推測される。夫の精子機能が低下している場合は生殖補助医療が必要となる。

症例検討

夫婦共に HIV 陽性といっても多様な状況があり，実際に経験した case の一部を紹介する。

Case 1

夫婦共に凝固因子製剤によって HIV に感染したが，感染から 20 年以上経過しても無治療で免疫が良好に保たれている長期未発症者同士が最近結婚して子供の相談に来た。妻は長年血中 VL＜50 を保ち免疫も良好であるが，夫は最近 VL が 5 万を越えている。性交渉では妻に夫の増殖力の強い HIV が感染して妻の免疫を悪化させる危険性がある。夫が HAART を開始しても結局精液中の HIV DNA は消えず，感染の危険性は残るし，副作用などを総合的に考え，夫婦は HIV 除去精子による体外受精を希望している。

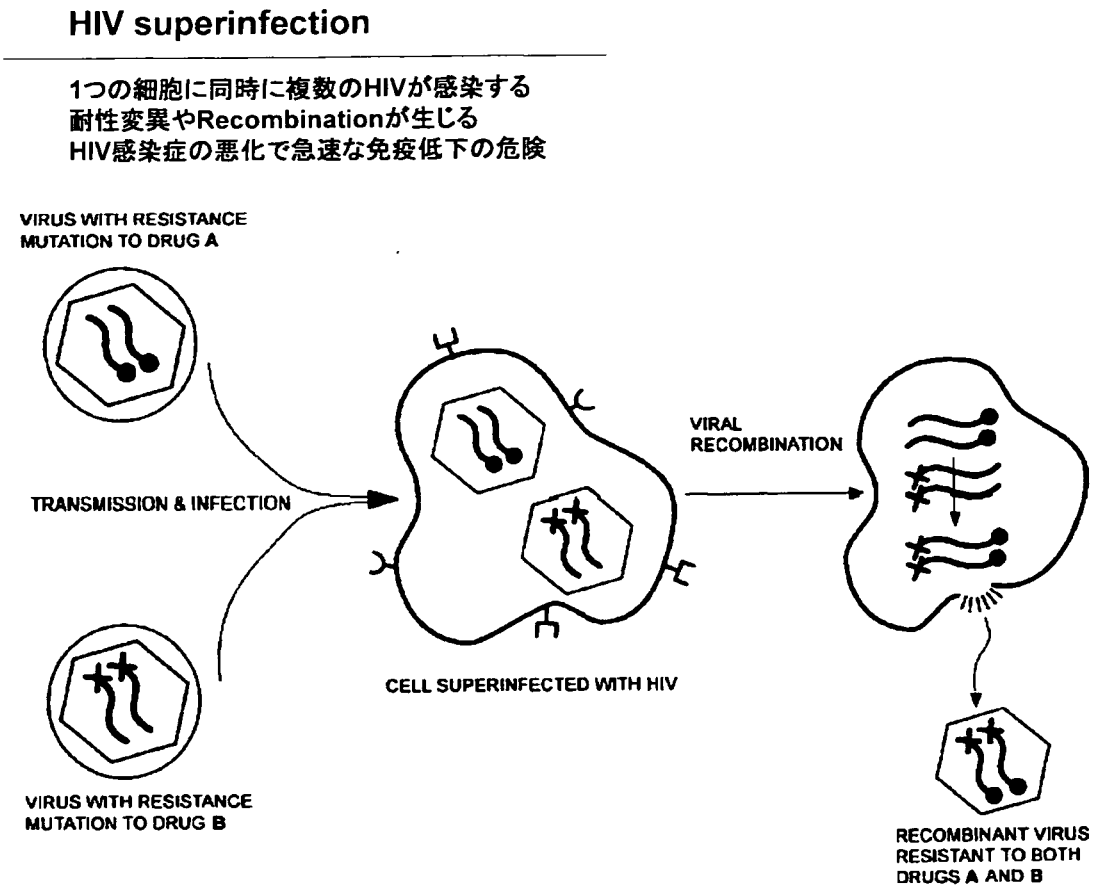

図 7

Case 2

高度の薬剤耐性となっている夫から妻に HIV 感染したが，妻の HIV は薬剤感受性があり治療でウィルスを抑制できている。性交渉で何年も子供ができないため相談に来たが，夫の精子機能障害を認めた。このまま性交渉を続けると薬剤耐性ウィルスが妻に感染する危険性があるので，HIV 除去精子による体外受精を希望している。

Case 3

夫から妻に感染したが，その後夫は治療によって HIV VL＜50 となり，子供の相談に来た。妻のウィルスは薬剤感受性が保たれており，夫の薬剤は有効であった。双方への superinfection の危険性を考えた場合，夫の精子機能が保たれていれば性交渉での妊娠を目指すか，人工授精などを行うかは夫婦とよく相談して決める必要がある。

このように夫婦共に HIV 陽性といっても状況によって対応を検討する必要がある。

倫理的課題

HIV 感染者の生殖補助医療に関して，夫婦共に HIV 陽性の場合は子供を持つべきではないと 2004 年に EU から勧告されている[13]。しかし，その根拠は，夫婦共に HIV に感染していると子供が成人するまでに両親共に死亡する可能性が高く，孤児になると不幸だからと記載されている。この勧告においては，長期未発症患者や superinfection については全く想定されていない。また，HIV 感染症の治療が進歩して予後が改善された場合は見直しが必要としており，夫婦ごとの状況に応じて検討すべきと考えられる。夫婦が共に病気を持っている場合に子供を持つ場合の是非論は HIV/AIDS に限ったことではない。ただ，HIV/AIDS の最新の医療情報をしっかり得た上で議論すべきである。

HIV 陰性男性と HIV 陽性女性の場合

基本的に夫から精液を採取して人工授精を行えば夫に感染することなく，子供を持てるので，妊娠後の母子感染を防止すれば大きな問題はない。ただ，我々は外国人夫婦で男性が精管結紮切除術を行っており，やむなく夫の精巣内精子採取術（Testicular sperm extraction : TESE）を行い体

外受精で妊娠したケースを経験している。

母子感染予防対策

HIV 感染女性が妊娠した場合，母子感染を防ぐための対策が必要である。母親の血中 VL をできるだけ低く抑制する HAART により，胎内感染を防止し，出産時の感染リスクを低くすることが証明されている。さらに陣痛が来る前の選択的帝王切開や，母乳禁止，新生児への抗 HIV 剤の投与などを組み合わせることにより，現在では HIV の母子感染率を 2% 以下に抑制できると報告されている[14]。しかし，耐性ウィルスへの対応方法や母体のウィルス量（RNA, DNA）をどれだけ抑制すれば帝王切開や新生児への抗 HIV 治療の必要性がなくなるのかなど今後検討すべき課題は多い。また，新生児では ZDV の薬物動態の検討も血中濃度測定も殆どされておらず，HAART の検討も十分されていない。ZDV 単独投与後の突然死も報告されており，新生児への投与量や投与期間が妥当かどうか，新生児の腎機能などによる調整の検討も必要と思われる。

成人に比べて小児科領域での新薬の開発は遅く，小児 HIV 感染例の予後もよくない。現状では母子感染を最大限に防止することが必要である。

C 型肝炎の問題

非加熱製剤を使用した血友病患者の 90% 以上が C 型肝炎にも感染し，HIV/HCV 感染者の死因の多くが C 型肝炎の悪化となっている。C 型肝炎の治療として PEG IFN＋ribavirin 併用療法が有効で，早期治療が推奨されている。リバビリンは精子の奇形を高率にもたらし，治療中及び治療終了後半年は避妊が必要とされている。しかし，我々の検討では治療終了後 1 年以上経過しても精子の異常が続く場合や，精子数の減少を認めた場合もある。C 型肝炎の治療を優先させるか，挙児を優先させるかは主治医とよく相談し，場合によっては精子の冷凍保存の必要がある。Swim up 法で精液中の HCV も除去される一方，体外受精で HCV が 2 次感染した報告もあり，生殖補助医療においてもウィルス感染対策が必要である[15]。

最 後 に

EU では，HIV 感染男性と HIV 陰性女性の人工授精や体外受精に対し，2 次感染の危険性を少なくする手段として施行されているが，アメリカ CDC は未だに禁止勧告を解除していない。我が国では HIV 感染男性の精液処理と人工授精は各施設の倫理委員会の承認を得てから実施するように日本産婦人科学会が 2003 年に勧告を出した。夫婦の状況によって，人工授精，体外受精，顕微授精などの適用は異なる。医療従事者が最新情報を提示した上で患者夫婦とよく相談して決定すべきである。しかし，その前に HIV 感染者の生殖補助医療を実施できる医師や施設を増やすことが急務である。

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Original Article

Individual tissue culture system in a disposable capsule with hypoxic atmosphere

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Abstract

In the present study, a novel system for the hypoxic culture of individual tissues was established for the subculturing of cell lines for research as well as for clinical culture of primary cells. To provide a hypoxic environment throughout the process of tissue handling and culture, we designed a clean bench with CO_2 gas circulation and a hypoxic culture incubator containing disposable capsules. The bench top was covered with an acrylic chamber, and an atmosphere of 5.0% CO_2-air was maintained using a sensor control. The cleanliness class of the chamber could easily be improved to 1 within 5 min of circulative filtration, even though it was found to be 10^5 before the unit was operated. Gas buffer solution (220 ml of 20 mM HEPES, 25 mM $NaHCO_3$, pH 7.4) placed in a 500-ml plastic capsule in the unit stabilized the culture environment by functioning as a heat storage and gas pool. The inflow of air that occurred by the cap of the capsule was opened was excluded by the infusion of purging gas (5.0% CO_2 and 95% N_2); the O_2 level returned to 2.0% within 4 min, after which the gas supply automatically switched to the culture gas (2.0% O_2, 5.0% CO_2, and 93% N_2). If this purging process was omitted, restoration of the O_2 level required 120 min, even though the inner volume was only 280 ml.

Keywords: individual tissue culture, gas circulation clean bench, hypoxic culture, disposable capsule

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Introduction

Tissue culture plays an important role in cell biology, and it is mainly focused towards the subculturing of established cell lines. Culture equipments such as the clean bench and CO_2 incubator have been widely employed, and they provide the appropriate settings for the handling of tissues by supplying filtered fresh air and an atmosphere with saturated humidity and 5.0% CO_2, respectively. It is well known that the oxygen tension in the periphery is substantially lower than that of fresh air. Only some mutant variants that tolerate the given culture conditions such as the atmosphere and culture medium can adapt to long-term subculturing. Although media compositions have been discussed in detail, few efforts have been channeled towards ensuring that the oxygen tension at the original growth environments remains unchanged. The development of a hypoxic culture system will enable the subculturing of many more malignant tissues than what is currently possible. Recent advances in regenerative medicine require the culturing of primary cells or stem cells, and advances with regard to transplantation in humans demand more precise duplication of the original growth environments. Recent reports have indicated the influence of hypoxic culture on some cellular functions. BeWo cells - an in vitro model of human trophoblasts - were cultured in 2% O_2, and RT-PCR conducted after the culture indicated that transcription of the organic cation transporter OCTN2 was higher than that after culture in 20% O_2[1]. Hirao et al.[2] observed that when MC3T3-E1 cells and calvariae from 4-day-old mice were cultured in conditions of 20% or 5% O_2, osteoblastic differentiation and the subsequent transformation to osteocytes were promoted by low oxygen tension.

Some structures and mechanisms of conventional culture equipments are not designed to maintain low oxygen tension in the environment when tissues are being handled and cultured. The present study established a new hypoxic tissue culture system for the culturing of cell lines and primary cells in research and clinical settings, respectively.

Materials and Methods

Measurements with regard to particles in air: The degree of air cleanliness was defined in terms of "cleanliness classes" that are specified by the number of particles of size 0.5 μm or more in 1 cubic foot of air[3]. For in-

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stance, cleanliness class 100 indicates less than 100 particles of the specified size in 1 cubic foot of air. Particle size and number were simultaneously measured using a light-scattering particle counter (KC-03A, Rion, Tokyo, Japan). When an intake air stream is passed through a high-intensity laser beam, the particles in the stream scatter light. The particle sizes were divided across 5 categories in terms of the particle diameter (0.3–0.5 μm, 0.5–1.0 μm, 1.0–2.0 μm, 2.0–5.0 μm, and >5.0 μm), and the number of particles belonging to each category was assessed.

Measurement of temperature and O_2 concentration: The temperature sensor in the device (K470; Techno-Seven, Tokyo, Japan) had a resolution of ± 0.02°C. The O_2 concentration was measured using a galvanic current sensor.

Preparation of premixed gas and accuracy control: In the present study, we used commercially supplied premixed gases for culture (2.0% O_2, 5.0% CO_2, and 93% N_2) and for purging the capsules (5.0% CO_2 and 95% N_2). Pure O_2, CO_2, and N_2 gases were mixed according to their weights of corresponding molar ratios and filled in a container; in a pre-shipment review, their composition was assessed using gas chromatography. Post-receipt accuracy control was performed as follows: when the gases were aerated in a solution containing 20 mM HEPES, 25 mM $NaHCO_3$ at 37°C, and the composition was considered accurate if the pH of the solution remained at 7.4 ± 0.05 after gas equilibration.

Results

1. Clean bench with CO_2 gas circulation

In the present study, a new clean bench with CO_2 gas circulation and a built-in microscope was developed. As shown in Fig. 1, the bench top was covered with an acrylic chamber to prevent leakage of the ambient atmosphere; it resembled an infant incubator. Pure CO_2 was infused using a gas sensor control to maintain the composition at 5.0% CO_2-air, and the temperature was maintained at 30–37°C by using a temperature control (Fig. 2). In addition, if the tissue did not allow exposure to 5.0% CO_2-air, the culture dish was isolated in a small chamber placed on the bench top, and humidified culture gas was supplied to it. While fresh air is filtered only once in the conventional clean bench, the enclosed 5.0% CO_2-air was circulated through HEPA filter every 24 s in the present system. As shown in Fig. 3, the cleanliness class of the air was found to be approximately 4×10^5 before operation of the unit was initiated; particles larger than 0.5 μm were not found, and only 10 particles of size 0.3–0.5 μm were observed in 1 cubic foot of air after 5 min of operation. Repeated filtration easily yielded cleanliness class 1. Thus far, if bench tops were contaminated by some infectious material such as body fluid, they were merely wiped clean. In the present system, however, a disposable clear film is placed as a covering shield on the bench top, and it is discarded after each operation.

2. Hypoxic culture of individual tissues in disposable capsules

We developed a disposable capsule for hypoxic tissue culture (Fig. 4). A 500-ml plastic capsule containing 220 ml of the gas buffer solution (20 mM HEPES, 25 mM $NaHCO_3$) was used; it functioned as a heat storage as well as a gas pool. The culture bath had a 16-well aluminum block for heat storage, and the block and the inner space were maintained at 37°C by using a temperature sensor (Fig. 5). First, the gas buffer solution was equilibrated by infusion of a small amount of the culture gas (10 ml/min) at least overnight. When the door was opened for 10 s, a slight temperature change of 0.1°C was observed in the inner space of the capsule, and it was restored within 7 min.

When conducting tissue culture using this capsule, the inflow of air when the cap is opened should be excluded as soon as possible; The purging gas was flushed (500 ml/min) immediately after the cap was closed, and the O_2 level was restored to 2.0% within 4 min; the gas supply automatically switched to the culture gas, which was continuously infused (10 ml/min) to maintain positive pressure (Fig. 6). If this process was omitted, the restoration of the O_2 level required 120 min, even though the inner volume was only 280 ml. In this system, gas control with a CO_2 sensor was unnecessary, and improper control due to the sensor deterioration was not required to be considered. Gas equilibration in each capsule was roughly estimated by the color of phenol red in the gas buffer solution, and the precise control of the culture environments were monitored by measuring the temperature and pH of the gas buffer solution. Although simultaneous culturing of multiple tissues in a single CO_2 incubator is usually possible, the present method that facilitates the culturing of individual tissues in disposable capsules has some advantages: the individual dish can be easily identified without any confusion, and the culture conditions are not disturbed when the door of the unit is opened.

Discussion

The cleanliness class of room air is generally 10^6–10^5. A low-dust environment with a cleanliness class of 10^2 would be provided when the conventional clean bench is operated under the optimum conditions. However, tissues are handled in fresh air, and under these conditions, O_2 may be dissolved in the medium, and the pH could change due to CO_2 removal. The present clean bench with CO_2-air circulation had overcome the above defects, and the functions had been rather similar with the conventional CO_2 incubator.

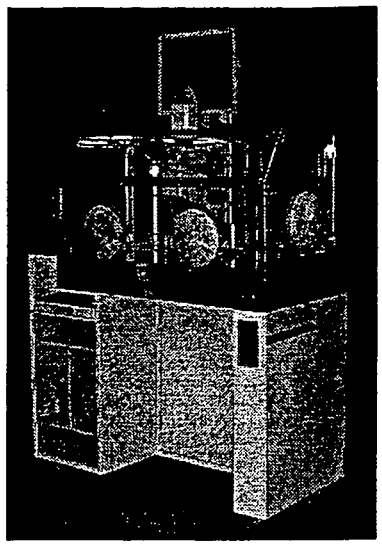

Fig. 1 Clean bench with CO_2 gas circulation

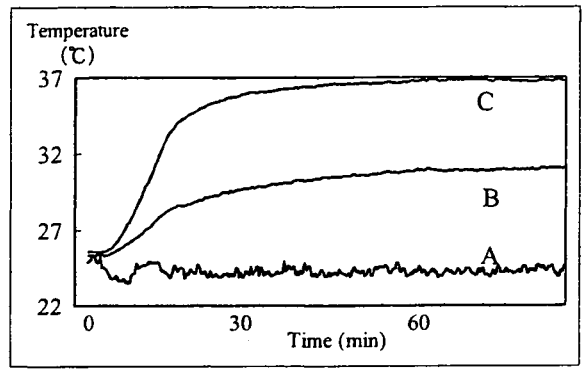

Fig. 2 Temperature of the clean bench
A: fresh air, B: ambient atmosphere, C: bench top

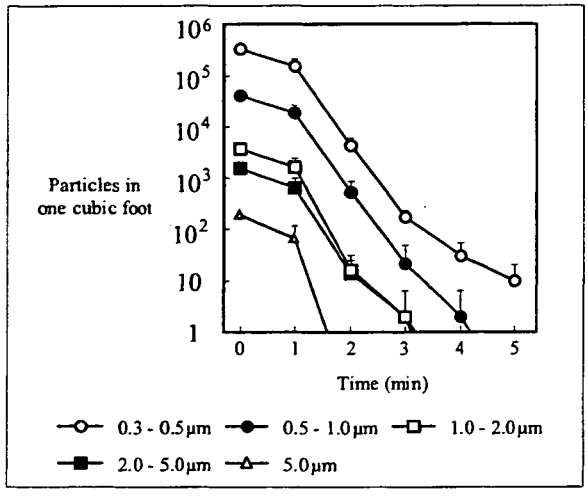

Fig. 3 Change in cleanliness class after beginning of operation
The values are represented as mean ± standard error. They were measured at 5 points on the bench.

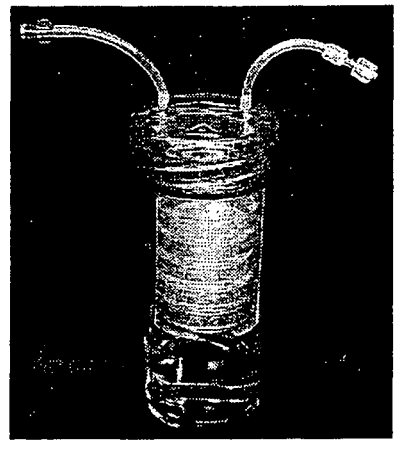

Fig. 4 Disposable capsules for hypoxic tissue culture
A maximum of 5 culture dishes (6.0-cm diameter) can be placed on the tray on the stainless steel stand. The gas buffer solution is placed at the bottom of the stand. The 2 tubes protruding from the cap are the gas inlet and outlet.

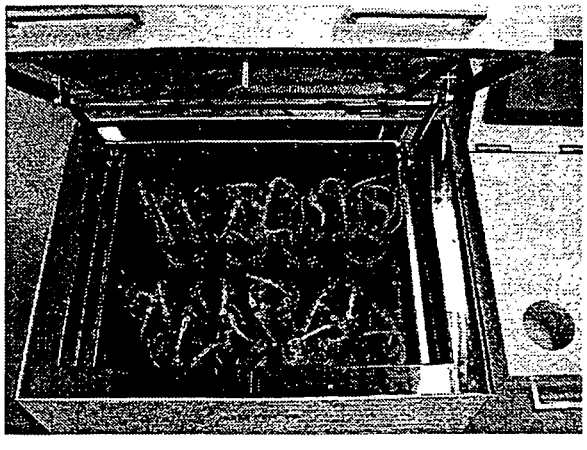

Fig. 5 Culture bath with a 16-well aluminum block

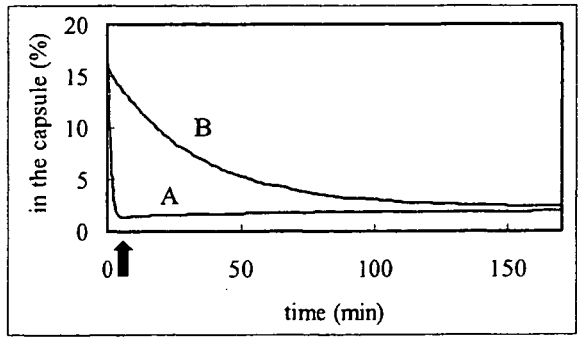

Fig. 6 Effect of gas purging on the restoration of the O_2 level after the cap is closed
A: purging with 5.0% CO_2 and 95% N_2, B: no purging, but 10 ml/min supply of 2.0% O_2, 5.0% CO_2, and 93% N_2. The arrow indicates gas switching.

—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 conventional CO_2 incubator poses a structural problem if hypoxic tissue culture is to be performed. The inflow of fresh air cannot be avoided when the door is opened. The concentration of CO_2 can be restored by infusing pure CO_2 gas. As shown in Fig. 6, it is very difficult to remove O_2 once it enters the chamber, and the tissues may be exposed to a high concentration of O_2. It is well known that the partial pressure of O_2 in fresh air (159 mmHg) is reduced to 100 and 25 mmHg or less, respectively, in arterial blood and at the periphery. When the saturated vapor pressure at 37°C was estimated to be 47 mmHg, those in 5.0% CO_2-air and the culture gas were calculated to be 142 and 14.3 mmHg, respectively. Numerous authors have shown that reactive oxygen species (ROS) exert various harmful effects such as lipid and protein peroxidation and membrane and DNA damage[4-7], however, only a few reports have addressed the effects of O_2 tension in culture environments[1,2,8]. Tissues and cells in body fluids are protected from O_2 and ROS by physiological antioxidants that are limited in artificial culture media, and an extremely high level of O_2 promotes ROS generation. Since the tolerance of cells to O_2 and ROS differs, the optimum O_2 concentration should be established for each cell line in order to minimize cellular damages and subsequent mutation. In the present system, by changing the composition of the premixed gas, the concentration of O_2 can easily be set, in the range of 0%–95%.

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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

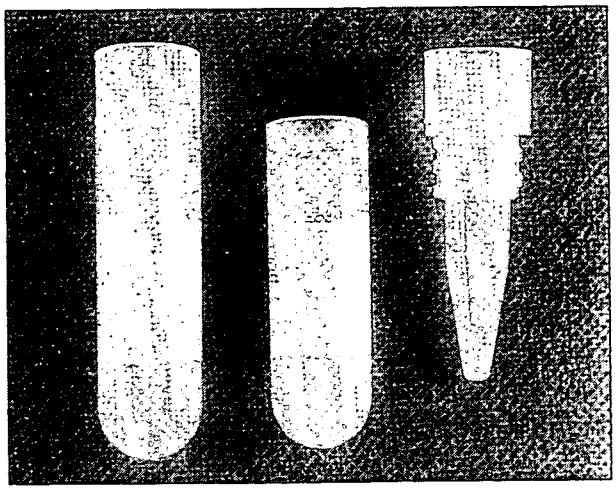

Fig. 1. Double-wall transformable freezing container for human sperm.

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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Original Article

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

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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

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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

	Treated ($n = 33$)		Untreated ($n = 97$)		
Case	Exclude	Preserve	Exclude	Preserve	Total
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

				Semen findings				
				concentration ($\times 10^6$/mL)/motility (%)/ normal morphology (%)			Method of ART therapeutic result	
					ART			
Case	Age	Underlying disease	Anticancer treatment	Ejaculate	1st	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.

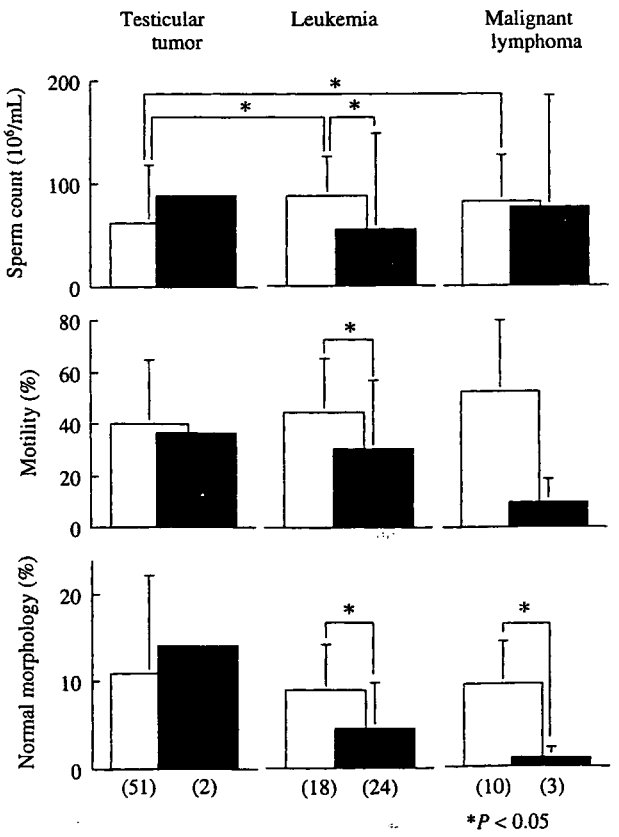

Figure 1 Semen findings in patients with or without previous anticancer treatment. Values in parentheses indicate the number of cases. (□) Untreated group; (■) treated group.

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.

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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*

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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.

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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

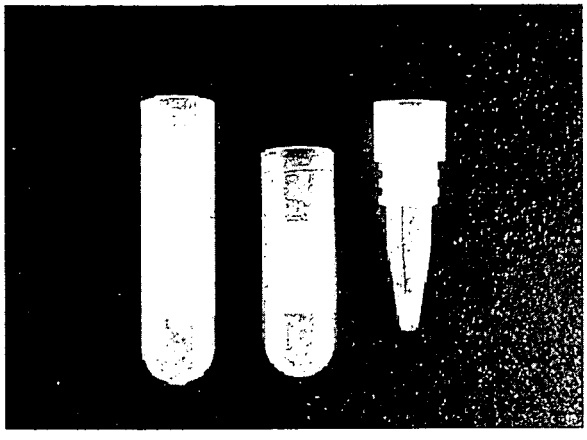

図1　可変性二重腔容器

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 copolymer, 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		計
	前治療あり	前治療なし	前治療あり	前治療なし	
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)．