for oocyte freezing. Whitten's medium (29) modified with 20 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid and 0.1% polyvinyl alcohol (PVA; M_r 30,000 to 70,000, Sigma-Aldrich, St. Louis, Mo.) (H-WM) was used for gamete handling and sperm injection. Whitten's medium supplemented with 50 μM EDTA (ethylenediaminetetraacetic acid, disodium salt) and 2 μM β-mercaptoethanol (m-WM) was used for embryo culture. H-WM was used in air, and HTF medium and m-WM were used in an atmosphere of 5% CO_2 and 95% air.

Collection and freezing of unfertilized oocytes. C57BL/6J Jcl female mice were induced to superovulate by intraperitoneal injection of 5 IU pregnant mare serum gonadotropin (PMSG, Teikokuzoki Co., Tokyo, Japan) followed by injection of 5 IU human chorionic gonadotropin (hCG; Teikokuzoki Co.) 48 h later. Cumulus–oocytes complexes were collected from oviducts at 13 to 15 h after hCG injection. Oocytes were freed from cumulus cells by treatment for 3 min with 0.1% hyaluronidase in HTF medium. Cumulus-freed oocytes were rinsed and kept in fresh HTF medium at 37°C in 5% CO_2 and 95% air before freezing.

Oocytes were frozen and thawed using the method described by Nakagata (14). Briefly, the oocytes were incubated in HTF medium containing 20% fetal calf serum for 10 min at 37°C in 5% CO_2 and 95% air. The oocytes in a small volume of medium were transferred into 100 μl of PB1 medium containing 1 M dimethyl sulphoxide (DMSO) at room temperature, and then 50 oocytes with 5 μl of solution was put on the bottom of each cryotube (Nalge Nunc International, Rochester, N.Y.) precooled to 0°C. After 5 min, 45 μl of solution containing 2 M DMSO, 1 M acetamide, and 3 M propylene glycol, cooled to 0°C, was added to each cryotube and left for 5 min at 0°C. The tubes were capped and then plunged directly into and stored in liquid nitrogen.

For thawing, cryotubes with opened caps were left at room temperature for 30 sec, and then 1 ml of PB1 medium containing 0.25 M sucrose was added to each cryotube. The morphologically normal oocytes were collected, rinsed in HTF medium, and kept at 37°C in 5% CO_2 and 95% air before sperm injection. Oocytes that had been stored for ≤ 1 month were used. Fresh, unfrozen oocytes served as controls.

Collection and freezing of sperm. Sperm were frozen in 18% (wt/vol) raffinose and 3% (wt/vol) skim milk according to the method described previously (17, 25). The two cauda epididymides were removed from a C57BL/6J Jcl male mouse and transferred into 100 μl of the solution in an organ tissue culture dish (Nalge Nunc International). The cauda epididymides were minced and dispersed well in the solution by using micro-spring scissors. Aliquots of sperm suspension (10 μl) were loaded into a sampling straw (IMV Technologies, L'Aigle, France). Straws were heat-sealed at both ends, frozen in liquid nitrogen vapor for 15 min, and then plunged directly into liquid nitrogen. The epididymal spermatozoa from four lines of transgenic (Tg) mice that originated from C57BL/6J mice [Tg A, B6;D2-Tg(APCS)1lmeg (6); Tg B, C57BL/6J-Tg(MT-hV30M)5lmeg (27); Tg C, B6;CB-Crebbp^{GtAyu3112lmeg} (20); and Tg D, B6;CB-Cdk6^{GtAyu8104lmeg} (1)] also were frozen in liquid nitrogen by the same procedure. In our preliminary study, these transgenic mouse spermatozoa showed low motility after thawing, and they rarely fertilized oocytes by IVF (fertilization rate, 0 to 12%). Freshly prepared, unfrozen spermatozoa served as the control.

Injection of sperm into oocytes. Frozen spermatozoa were thawed for 15 min in 37°C water. Approximately 1 to 2 μl of sperm suspension was mixed in H-WM containing 12% (wt/vol) PVP (Mr 360,000). Motile spermatozoa were selected, and a single spermatozoon was drawn, tail first, into the injection pipette in such a way that the junction between its head and tail was at the opening of the pipette. The head was separated from the tail by applying a few piezo-pulses to the junction. The sperm head was then injected into an oocyte in H-WM by using the method described previously (10, 12). The oocytes injected with spermatozoa were cultured in m-WM at 37°C in 5% CO_2 and 95% air.

Embryo culture and transfer. At 5 h after ICSI, oocytes with two distinct pronuclei and a second polar body were recorded as fertilized. Fertilized oocytes were cultured in m-WM at 37°C in 5% CO_2 and 95% air until they reached the blastocyst stage. In some experiments, embryos that had developed to the two-cell stage by 20 to 24 h after ICSI were collected and transferred into oviducts of pseudopregnant Jcl:ICR female mice that were mated with vasectomized males of same strain on the day before embryo transfer; 14 to 20 two-cell embryos were transferred into oviducts of each female. The transgene integration of offspring derived from embryos with transgenic mice spermatozoa was assessed by a polymerase chain reaction (PCR)-based assay.

Analysis of data. The chi-square test (Microsoft Corp., Microsoft Excel, Redmond, Wash.) with Yate's correction for continuity was used for analysis of all data obtained from this study.

Results

The number of oocytes that survived after sperm injection when frozen-thawed oocytes were injected with fresh or frozen-thawed spermatozoa (83 and 78%, respectively) was significantly increased ($P < .005$) compared with those of fresh oocytes (68 and 51%, respectively; Table 1). For both fresh and frozen-thawed oocytes, > 90% of those that survived after sperm injection were fertilized, and 94 to 100% of fertilized oocytes developed to the two-cell stage. The preimplantation development of the resulting embryos did not differ significantly ($P > .005$) between fresh and frozen-thawed oocytes.

The postimplantation development of embryos derived from injection of frozen-thawed spermatozoa did not differ significantly ($P > .005$) between fresh and frozen-thawed oocytes (41 and 42%, respectively; Table 2). In addition 11 to 40% of the two-cell embryos developed to term when oocytes were injected individually with four lines of transgenic spermatozoa. As confirmed by PCR, some of offspring actually were transgenic mice.

Discussion

We demonstrated frozen-thawed gametes fertilized by ICSI showed normal embryonal development and ultimately developed into normal offspring. Further these offspring had reproductive ability: all of the female mice of six random pairs became pregnant and gave birth to a total of 43 (average litter size, 7.2) normal pups (data not shown).

Moreover, the developmental ability of fresh versus frozen-thawed oocytes after sperm injection was not significantly different (Table 1). This result is consistent with the report of Glenister and colleagues (5) that the in vitro development of frozen-thawed oocytes after fertilization was similar to that of fresh oocytes. The low fertility of frozen-thawed oocytes in vitro has been attributed to the hardening of zona pellucida by the premature release of cortical granules (2). Frozen-thawed oocytes could be fertilized, albeit at low frequency, with spermatozoa in

Table 1. Preimplantation development of embryos derived from fresh or frozen C57BL/6J oocytes

Status of sperm	Status of oocytes	No. of oocytes injected	No. (%) of oocytes that survived[a]	No. (%) of oocytes fertilized[b]	No. (%) of embryos that developed to two-cell stage[c]	No. (%) of embryos that developed to morula stage[d]	No. (%) of embryos that developed to blastocyst stage[d]
fresh	fresh	53	36 (68)[e]	36 (100)	36 (100)	28 (78)	15 (42)
fresh	frozen	111	92 (83)[f]	83 (90)	78 (94)	56 (68)	34 (41)
frozen	fresh	130	66 (51)[g]	61 (92)	61 (100)	47 (77)	30 (49)
frozen	frozen	188	146 (78)[h]	136 (93)	136 (100)	102 (75)	62 (46)

Percentages with different letters were significantly different (e versus f, $P < 0.05$; g versus h, $P < 0.005$).
[a]Percentages from no. of oocytes injected.
[b]Percentages from no. of oocytes survived.
[c]Percentages from no. of oocytes fertilized.
[d]Percentages from no. of two-cell embryos.

Table 2. Postimplantation development of embryos derived from frozen-thawed C57BL/6J oocytes injected with frozen-thawed C57BL/6J or transgenic spermatozoa

Strain of frozen sperm	Status of oocytes	No. of oocytes injected	No. (%) of oocytes that survived[a]	No. (%) of oocytes fertilized[b]	No. (%) of embryos that developed to two-cell stage[c]	No. of two-cell embryos transferred	No. (%) of offspring[d]	No. (%) of offspring with transgene[d]
C57BL/6J	fresh	228	121 (53)[e]	118 (98)	116 (98)	106	43 (41)	NA
C57BL/6J	frozen	223	181 (81)[g]	172 (95)	171 (99)	162	68 (42)	NA
Tg A	frozen	30	23 (77)[f]	21 (91)	21 (100)	20	8 (40)	2 (10)
Tg B	frozen	40	36 (90)[g]	35 (97)	35 (100)	35	7 (20)	4 (11)
Tg C	frozen	140	115 (82)[g]	111 (97)	110 (99)	104	17 (16)	4 (4)
Tg D	frozen	82	68 (83)[g]	63 (93)	63 (100)	63	7 (11)	6 (10)

Tg A, B6;D2-Tg(APCS)1lmeg (6); Tg B, C57BL/6J-Tg(MT-hV30M)5lmeg (27); Tg C, B6;CB-Crebbp^{GtAyu3112lmeg} (20); and Tg D, B6;CB-Cdk6^{GtAyu8104lmeg} (1).
Percentages with different letters were significantly different (e versus f, $P < 0.05$, e versus g, $P < 0.005$).
NA, not applicable.
[a]Percentages from no. of oocytes injected.
[b]Percentages from no. of oocytes survived.
[c]Percentages from no. of oocytes fertilized.
[d]Percentages from no. of two-cell embryos transferred.

vitro when oocytes were exposed to the medium containing fetal calf serum to avoid hardening of zona pellucida before freezing (3, 15). Fertility in vitro between frozen-thawed gametes might be increased if the zona pellucida is dissected partially with a needle (18) or piezo-micromanipulator before insemination (9). However, we overcame the low fertility of frozen-thawed oocytes by using ICSI instead of IVF. Surprisingly, the survival of frozen-thawed oocytes after sperm injection was significantly higher than that of fresh oocytes (Table 1), but the underlying reason remains unclear. Wood and colleagues reported that cytoplasmic modification of oocytes by freezing, such as premature release of cortical granules, did not affect the development of oocytes after fertilization (32). The technique for injecting sperm into mouse oocytes was difficult until the piezo impact micromanipulator was developed, because the mouse plasma membrane has poor wound-healing after sperm injection (10). In the present study, sperm injection was performed by a person well-skilled in the technique, and we used mice that all originated from a colony. We suspect that the wound-healing ability of the plasma membrane of the mouse oocyte increases after freezing at the same time as does hardening of the zona pellucida due to premature release of cortical granules.

Spermatozoa frozen in the solution containing 18% raffinose and 3% skim milk could be used directly to in vitro fertilize fresh oocytes, and the development of these embryos was similar to that of embryos generated from fresh spermatozoa (16). However, frozen C57BL/6J spermatozoa sometimes showed low fertility, and few of those with low motility and none of the immotile spermatozoa could pass unassisted through the zona pellucida. However, when injected into oocytes by ICSI, these suboptimal spermatozoa fertilized normally, and the integrity of sperm chromosomes for normal development of embryos was maintained well after thawing (23). The nuclei of mouse spermatozoa are tolerant to various physical disruptions (4, 33), and normal offspring could be obtained from oocytes injected with freeze-dried spermatozoa (7, 8) or spermatozoa frozen without cryoprotectants (28). In the present study, we consider that the chromosomal integrity and fertilizing ability of the oocytes and spermatozoa were maintained well after freezing because the development of the resulting embryos did not differ between frozen and fresh gametes (Table 1). Furthermore, we obtained normal offspring from frozen-thawed oocytes injected with transgenic spermatozoa (Table 2), all of which demonstrated low fertility in vitro (fertilization rate, 0 to 12%). Our results demonstrate that ICSI contributed to the production of offspring from spermatozoa of valuable strains showing low fertility. In addition, we suggest that using ICSI with frozen gametes led to more effective production of offspring.

The in vitro development of several embryos derived from fresh as well as frozen-thawed oocytes was impaired from the morula to the blastocyst stage (Table 1). Similarly, fresh C57BL/6J oocytes injected with frozen-thawed spermatozoa by ICSI were arrested between the morula and blastocyst stages (23). Our data show that the damage to spermatozoa and oocytes by freezing did not affect the in vitro development of embryos, which did not significantly differ between fresh and frozen-thawed oocytes (Table 1). The decreased rate of development of ICSI-derived embryos from morula to blastocyst might be affected by surrounding physicochemical conditions such as culture medium, gas, and temperature. Further study is needed to determine the optimal conditions for embryo development.

If a large number of unfertilized oocytes are frozen at one time, an appropriate number of the oocytes can be used for ICSI so

that the experiment can be conducted without the time-consuming procurement of oocytes. Furthermore, double-knockout (KO) mice recently have been produced to analyze the functions and genetic interactions of two genes (11). Producing double KO mice from the frozen embryos of two different KO mouse strains can take 3 to 4 months, as two single KO colonies first are produced from each set of frozen embryos and then are interbred to obtain double KO mice. However, if the oocytes and spermatozoa of various KO mouse strains are frozen separately, the desired double KO mouse strains can be produced rapidly after ICSI of various combinations of frozen gametes and transfer of embryos, as the gestation period of mice is just 3 weeks long. In conclusion, our results suggest that producing embryos from cryopreserved mouse gametes contributes to efficient conservation of genetic resources and facilitates making new lines.

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Relation between Storage Temperature and Fertilizing Ability of Freeze-Dried Mouse Spermatozoa

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The advantage of freeze-dried mouse spermatozoa is that samples can be stored in the refrigerator (+4°C). Moreover, the storage of freeze-dried spermatozoa at ambient temperature would permit spermatozoa to be shipped easily and at low cost around the world. To examine the influence of the storage temperature on freeze-dried spermatozoa, we assessed the fertilizing ability of spermatozoa stored at different temperatures. Cauda epididymal spermatozoa were freeze-dried in buffer consisting of 50 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 50 mM NaCl, and 10 mM Tris-HCl (pH 8.0). Samples of freeze-dried spermatozoa were stored at –70, –20, +4, or +24°C for periods of 1 week and 1, 3, and 5 months. Sperm chromosomes were maintained well at –70, –20, and +4°C for 5 months, and oocytes fertilized with these spermatozoa developed to normal offspring. Moreover, the chromosomal integrity of spermatozoa stored at –20 or +4°C did not decrease even after 17 months. In contrast, the chromosomes of spermatozoa stored at +24°C were maintained well for 1 month but became considerably degraded after 3 months. In addition, to investigate the cause of deterioration of sperm chromosomes during storage at +24°C, spermatozoa were freeze-dried in buffer containing DNase I. The chromosomes of spermatozoa freeze-dried with 1 or 0.2 units/ml of DNase I, 100% or 72%, respectively, exhibited chromosomal abnormalities. Our findings suggest that freeze-dried spermatozoa can be stored long-term with stability at +4°C, and the suppression of nucleases present in the buffer or spermatozoa during storage led to the achievement of long-term storage of freeze-dried spermatozoa.

Mouse spermatozoa can be freeze-dried with the maintenance of fertilizing ability, and normal offspring develop from oocytes injected with them (12, 24). Recently, freeze-drying of sperm from other mammals has been attempted (8, 13). We reported previously that the fertilizing ability and chromosome integrity of mouse spermatozoa could be maintained well when spermatozoa were freeze-dried using a simple buffer consisting of 50 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 50 mM NaCl, and 10 mM Tris-HCl adjusted to pH 8.0 (6, 7). Moreover, it was demonstrated that ampoules with freeze-dried spermatozoa could be stored long-term at 4°C, as a high proportion of oocytes developed to normal offspring when the oocytes were injected with freeze-dried spermatozoa stored at 4°C for 1.5 years (25). Traditionally, cryopreservation methods using cryoprotectants and liquid nitrogen have been used to preserve spermatozoa (4, 15, 17, 21, 22). Although fertilization of oocytes with freeze-dried spermatozoa requires intracytoplasmic sperm injection (ICSI) as spermatozoa lose their motility after rehydration, sperm storage at 4°C is more advantageous than is cryopreservation because spermatozoa can be preserved without cryoprotectants and do not require a supply of liquid nitrogen for long-term storage and shipment. Moreover, the final goal of this study is the long-term storage and simple shipment of mouse spermatozoa at ambient temperature. A single report regarding this practice has been published and it shows that their fertilizing ability was reduced when spermatozoa were freeze-dried in embryo culture medium and stored at 25°C (24). However, the factor that affects the fertilizing ability of the spermatozoa has not been described. Moreover, how long spermatozoa freeze-dried in the new simple buffer can be preserved and the maintenance of fertilizing ability at other temperatures other than 4°C have not been studied in detail. Kaneko and colleagues (7) discussed whether an alkaline pH might suppress the activity of nucleases (such as DNase I) present in the buffer or spermatozoa and how the chromosome integrity and developmental ability of spermatozoa are maintained during and after freeze-drying. It is known that nuclease activity depends on buffer conditions such as pH and temperature (10). In the present study, we evaluated whether nucleases were present in ampoules with freeze-dried spermatozoa and determined the optimal temperature to suppress the activity of these nucleases in regard to long-term storage of freeze-dried spermatozoa.

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Materials and Methods

Animals. All animals were purchased from Charles River Japan Inc. (Yokohama, Japan). Crj:BDF1 hybrid male mice older than 11 weeks and female mice 8 to 12 weeks old were used as sperm and oocyte donors, respectively. The recipients of the two-cell embryos were Crj:CD-1 female mice 8 to 16 weeks old. All animals were maintained in an air-conditioned (22°C) and light-controlled room (12:12-h light:dark cycle with lights on at 7 a.m.). Microbial monitoring for mouse hepatitis virus, Sendai virus, *Citrobacter rodentium*, *Clostridium piliforme*, *Corynebacterium*

kutscheri, *Helicobacter bilis*, *Helicobacter hepaticus*, *Mycoplasma* spp., *Pasteurella pneumotropica*, *Salmonella* spp., *Aspiculuris tetraptera*, *Syphacia* spp., *Giardia muris*, *Spironucleus muris*, trichomonads, and ectoparasites was carried out monthly, and all animals had negative test results. The Animal Care and Use Committee of the Kumamoto University School of Medicine approved all procedures performed in this study.

Media. All chemicals were purchased from Sigma-Aldrich (St. Louis, Mo.) unless otherwise stated. The freeze-drying solution for spermatozoa consisted of 50 mM EGTA, 50 mM NaCl, and 10 mM Tris-HCl buffer adjusted to pH 8.0 by addition of 1 M HCl (6, 7). The solution was filter-sterilized and stored at 4°C for less than 1 week before use.

The medium for oocyte collection, subsequent oocyte handling, and micromanipulation including sperm injection was CZB medium (1, 2) modified by the addition of 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 5 mM $NaHCO_3$, and 0.1 mg/ml polyvinyl alcohol (PVA; cold water soluble; M_r 30,000 to 70,000) instead of bovine serum albumin (H-CZB medium) (9). CZB medium supplemented with 5.56 mM D-glucose (m-CZB medium) was used for the oocyte culture after sperm injection. H-CZB medium was used under air, and m-CZB medium was used under 5% CO_2 and 95% air.

Sperm collection and freeze-drying. Freeze-drying of spermatozoa was carried out using the procedure described previously (6, 7). Briefly, 1 ml of the freeze-drying solution was warmed to 37°C in a 1.5-ml polypropylene microcentrifuge tube. A male mouse was killed by cervical dislocation, and its two cauda epididymides were removed using a pair of small scissors. A dense mass of spermatozoa was squeezed out of the each epididymis by using sharply pointed forceps. Sperm masses were placed gently on the bottom of the microcentrifuge tube containing 1 ml of the freeze-drying solution warmed to 37°C. The tube was left at 37°C for 10 min to allow the spermatozoa to disperse into the solution. The 800-μl supernatant was separated into eight 100-μl aliquots, and each aliquot was placed into a long-necked glass ampoule (Wheaton, Millville, N.J.) for freeze-drying. Ampoules were plunged into liquid nitrogen for 20 sec and were then dried under pressure at 37×10^{-3} to 41×10^{-3} hPa with a freeze-drying machine (Labconco, Kansas City, Mo.). Ampoules were flame-sealed after 4 h of drying. In addition, spermatozoa were suspended in a freeze-drying solution containing 0.2 or 1 unit/ml DNase I (Roche Diagnostics Japan, Tokyo, Japan) and were freeze-dried by the same procedure.

Storage of freeze-dried spermatozoa. The ampoules with freeze-dried spermatozoa were stored at –70°C (Sanyo Electric Co., Ltd., Osaka, Japan), –20°C (Toshiba Co., Tokyo, Japan), +4°C (Toshiba Co.) or +24°C (a box placed in room) for periods of 1 week; 1, 3, or 5 months; and longer. These storage temperatures were measured continuously by using a thermometer (Fuso Rikaseihin Co., Ltd., Tokyo, Japan). Eight-hour measurements were performed three times daily, and the maximum, minimum, and mean values were calculated. The ampoules with freeze-dried spermatozoa exposed to DNase I were stored at 4°C for 2 to 3 days.

Oocyte collection. Females were induced to superovulate by intraperitoneal injection of 5 IU equine chorionic gonadotropin (eCG; Teikokuzoki Co., Tokyo, Japan) followed by injection of 5 IU hCG (Teikokuzoki Co.) 48 h later. Cumulus–oocyte complexes were collected from oviducts 13 to 15 h after hCG injection. Oocytes were freed from cumulus cells by treatment with 0.1% hyaluronidase in H-CZB medium. Oocytes were rinsed in fresh H-CZB medium and kept at room temperature before sperm injection.

Table 1. Temperatures at which freeze-dried spermatozoa were stored

Nominal (°C)	Average (°C) [range]	Maximum (°C) [range]	Minimum (°C) [range]
–70	–69.6 [–70.4 to –68.2]	–66.5 [–68.2 to –65.4]	–70.1 [–70.9 to –68.8]
–20	–22.1 [–26.4 to –19.3]	–15.5 [–25.5 to –9.3]	–22.9 [–26.4 to –20.6]
+4	+4.3 [+4.0 to +4.8]	+8.5 [+7.8 to +8.9]	+3.9 [+3.7 to +4.0]
+24	+24.3 [+23.6 to +25.2]	+25.0 [+24.2 to +25.9]	+23.1 [+22.7 to +23.6]

ICSI. An ampoule was opened, and the freeze-dried spermatozoa were rehydrated by adding 100 μl of sterile distilled water. A small volume of the sperm suspension was mixed thoroughly with a droplet of H-CZB medium containing 12% (wt/vol) polyvinylpyrrolidone (PVP; M_r 360,000; ICN Pharmaceuticals, Costa Mesa, Calif.). Spermatozoa that showed a morphologically normal shape were selected and transferred to another droplet of 12% PVP solution. A single spermatozoon was drawn from the tail into the injection pipette. The head was separated from the tail by applying a few piezo-pulses when the junction between the head and tail was at the opening of the pipette. Only heads were injected immediately into each oocyte in H-CZB medium, as described previously (9, 11). Injected oocytes were incubated in m-CZB medium at 37°C under 5% CO_2 and 95% air.

Chromosomal analysis. Chromosomes of oocytes injected with freeze-dried spermatozoa were analyzed using the method described by Kamiguchi and Mikamo (5). Briefly, oocytes containing two distinct pronuclei and a second polar body at 5 h after ICSI were recorded as fertilized. Fertilized oocytes were transferred to m-CZB medium containing 0.006 μg/ml vinblastine to arrest oocytes at the metaphase of the first cleavage. After 19 to 21 h of culture, arrested oocytes were treated for 3 to 5 min with 0.5% pronase (1000 tyrosine units/mg, Kaken Pharmaceuticals, Tokyo, Japan) in phosphate buffered saline pH 7.4 to remove the zona pellucida, and transferred to the hypotonic solution (1:1 mixture of 30% fetal bovine serum and 1% sodium citrate) for 5 min. Oocytes were fixed and air-spread on a glass slide, and their chromosomes were assessed by Giemsa staining. An oocyte with two groups of 20 chromosomes without any structural and numerical abnormalities was recorded as karyotypically normal.

Embryo culture and embryo transfer. Oocytes containing two distinct pronuclei and a second polar body at 5 h after ICSI were recorded as fertilized. Fertilized oocytes were cultured in m-CZB medium until they developed to the two-cell stage. At 20 to 24 h after ICSI, 12 to 19 two-cell embryos were transferred into the oviducts of each surrogate female mouse, which was mated with a vasectomized male mouse of the same strain on the day before embryo transfer. Numbers of implantation sites and normal live offspring were assessed on day 19.5 of gestation. Offspring were raised by lactating foster mothers.

Analysis of data. All data obtained from this study were compared by chi-square test (Microsoft Corp., Microsoft Excel, Redmond, Wash.) using Yates correction for continuity.

Results

Freeze-dried spermatozoa were stored under various temperatures shown in Table 1. At all temperatures, shown in Table 2, there was no striking difference in the rate of oocyte survival after sperm injection, and most of the surviving oocytes were

Table 2. Analysis of oocytes injected with freeze-dried spermatozoa stored at different temperatures

Storage temperature (°C)	Storage term	No. of oocytes injected	No. (%) of oocytes that survived[a]	No. (%) of oocytes fertilized[b]	No. of oocytes analyzed	No. (%) of oocytes with normal chromosomes[c]
−70	1 week	77	58 (75)	54 (93)	39	26 (67)[d]
	1 month	61	50 (82)	48 (96)	44	25 (57)
	3 months	78	54 (69)	52 (96)	45	17 (38)[e]
	5 months	63	46 (73)	41 (89)	39	17 (44)
−20	1 week	79	52 (66)	50 (96)	33	16 (49)
	1 month	58	40 (69)	38 (95)	32	18 (56)
	3 months	80	63 (79)	62 (98)	56	25 (45)
	5 months	75	47 (63)	47(100)	45	16 (36)
+4	1 week	80	61 (76)	57 (93)	29	18 (62)
	1 month	58	43 (74)	39 (91)	34	23 (68)
	3 months	77	43 (56)	43(100)	41	25 (61)
	5 months	70	49 (70)	45 (92)	30	19 (63)
+24	1 week	95	63 (66)	61 (97)	49	22 (45)[f]
	1 month	62	48 (77)	46 (96)	37	19 (51)[h]
	3 months	74	42 (57)	39 (93)	29	6 (21)[i]
	5 months	73	55 (75)	48 (87)	29	1 (3)[g,i]

Percentages with different lowercase superscript letters were significantly different at $P < 0.05$ (d versus e, f versus g, and h versus i).
[a]Percentage from no. of oocytes injected.
[b]Percentage from no. of oocytes that survived.
[c]Percentage from no. of oocytes analyzed.

Table 3. Post-implantation development of embryos fertilized with freeze-dried spermatozoa stored for 5 months at different temperatures

Storage temperature (°C)	No. of oocytes injected	No. (%) of oocytes that survived[a]	No. (%) of oocytes fertilized[b]	No. (%) of two-cell embryos that developed and were transferred[c]	No. (%) of implantation sites[d,e]	No. (%) of normal live offspring[d]
−70	71	55 (78)	54 (98)	51 (94)	26 (51)[f]	12 (24)[h]
−20	88	69 (78)	64 (93)	58 (91)	26 (45)[f]	15 (26)[h]
+4	59	47 (80)	46 (98)	43 (94)	20 (47)[f]	9 (21)[h]
+24	82	66 (81)	64 (97)	53 (83)	0 (0)[g]	0 (0)[i]

Percentages with different lowercase superscript letters were significantly different at $P < 0.005$ (f versus g and h versus i).
[a]Percentage from no. of oocytes injected.
[b]Percentage from no. of oocytes that survived.
[c]Percentage from no. of oocytes fertilized.
[d]Percentage from no. of two-cell embryos that developed and were transferred.
[e]Implantation site = the number of resorption sites + normal live offspring.

Table 4. Chromosomal integrity of freeze-dried spermatozoa stored for 17 months at −20 or +4°C

Storage temperature (°C)	No. of oocytes injected	No. (%) of oocytes that survived[a]	No. (%) of oocytes fertilized[b]	No. of oocytes analyzed	No. (%) of oocytes with normal chromosomes[c]
−20	37	31 (84)	30 (97)	24	10 (42)
+4	31	21 (68)	21 (100)	17	10 (59)

[a]Percentage from no. of oocytes injected.
[b]Percentage from no. of oocytes that survived.
[c]Percentage from no. of oocytes analyzed.

Table 5. Chromosomal integrity of spermatozoa freeze-dried in buffer containing DNaseI

Concentration of DNaseI (units/ml)	No. of embryos analyzed	No. (%) of embryos with multiple chromosomal aberrations[a,b]	No. (%) of embryos with minor chromosomal aberrations[a,c]	No. (%) of embryos with normal chromosomes[a]
1	53	53 (100)	0	0
0.2	53	14 (26)	24 (45)	15 (28)

[a]Percentage from no. of oocytes analyzed.
[b]10 or more chromosome breaks in one set of 20 chromosomes.
[c]9 or fewer chromosome breaks in one set of 20 chromosomes.

fertilized. The chromosomal integrity of freeze-dried spermatozoa was maintained well when the spermatozoa were stored at +4°C or below. In contrast, chromosomes of spermatozoa stored at +24°C were stable for 1 month, but they became increasingly fragmented when stored for 3 or 5 months.

Normal offspring were obtained and raised without abnormality from oocytes fertilized with freeze-dried spermatozoa that had been stored for 5 months at −70, −20, or +4°C (Table 3). However, oocytes fertilized with spermatozoa that were stored at +24°C failed to even implant. Further, spermatozoa stored at −20 or +4°C were extremely stable, exhibiting no deterioration of their chromosomes even after storage for as long as 17 months (Table 4).

Table 5 reports the chromosomal integrity of oocytes injected with spermatozoa freeze-dried in buffer containing a small amount of DNase I. The chromosomes of all spermatozoa freeze-dried with 1 unit/ml DNase I were fragmented, with multiple aberrations (Fig. 1A), and sperm chromosomes exhibited minor damage when spermatozoa had been freeze-dried with 0.2 unit/ml DNase I (Fig. 1B).

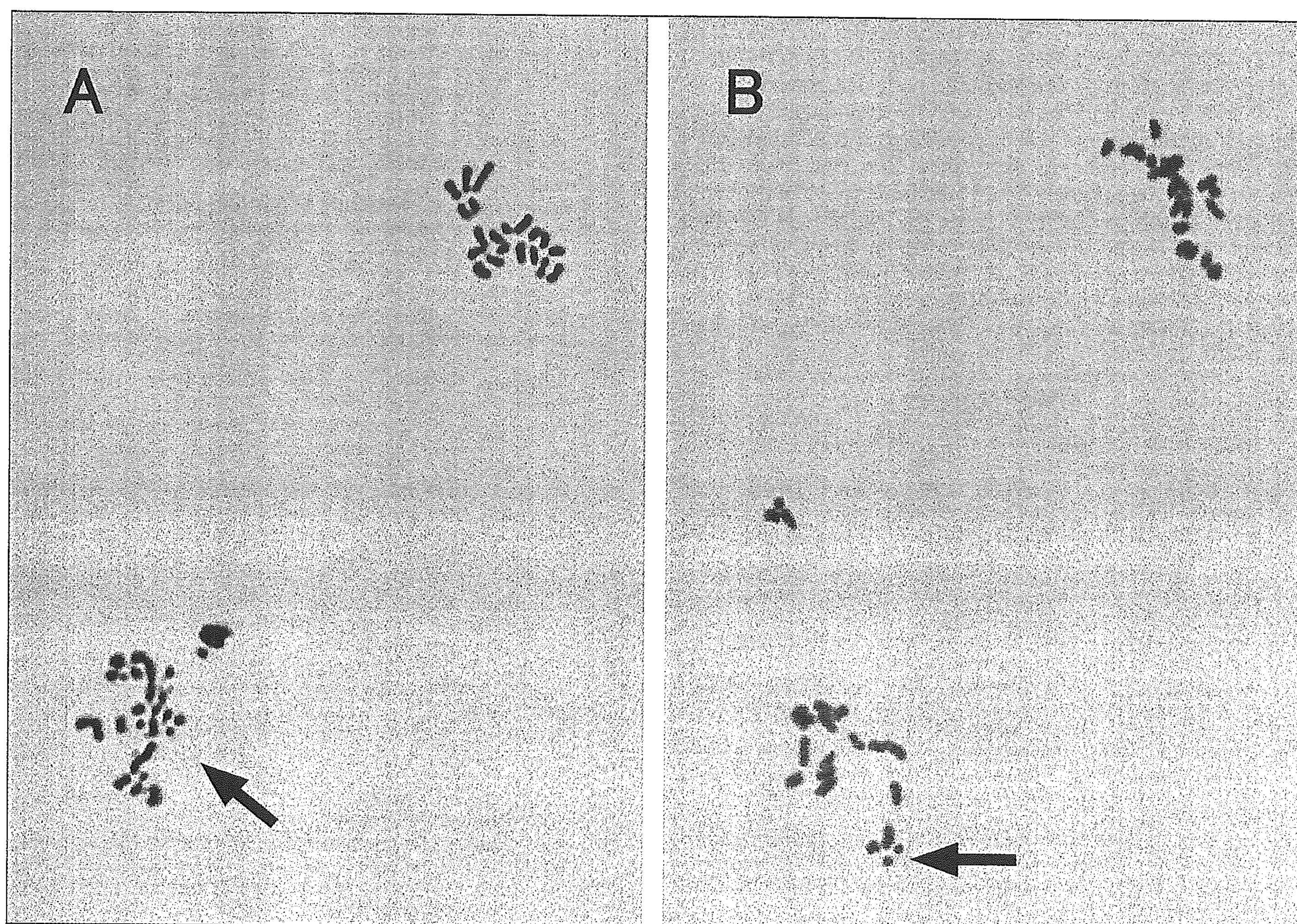

Figure 1. Chromosomes of oocytes injected with spermatozoa freeze-dried in buffer containing 1 (A) or 0.2 unit/ml (B) DNase I. Chromosomes of spermatozoa had multiple aberrations (A, arrow) and minor aberrations (B, arrow). Magnification, ×1000.

Discussion

This study demonstrated that the fertilizing ability of freeze-dried spermatozoa could be maintained completely even after storage for as long as 17 months. However, the chromosomal integrity of spermatozoa stored at –70°C for 3 months was decreased (Table 2). Storage at –70°C is not effective for freeze-dried sperm and does not lend itself to simple storage and shipment of spermatozoa. In contrast, the chromosome integrity of freeze-dried spermatozoa stored at +24°C was maintained well for 1 month, but began decrease gradually thereafter (Table 2). For spermatozoa freeze-dried using the present procedure, long-term storage at +24°C was impossible. However the possibility of simple shipment at ambient temperature remains, as samples can be sent all over the world within several days by aircraft. The achievement of storage and shipment of freeze-dried spermatozoa at ambient temperatures would obviate the need for vapor shipper containers (16), liquid nitrogen (17), and dry ice (18) required for frozen spermatozoa.

Partial degradation of sperm chromosomes was thought to be caused by activation of endogenous nucleases present in the spermatozoa (14, 19, 20). Although we used a commercial DNase detection kit to try to identify endogenous nucleases in buffer with or without spermatozoa, the quantities of enzyme were below the limit of detection (data not shown). However, sperm chromosomes were damaged during or after freeze-drying when DNase I was added to the buffer. Moreover, the incidence of spermatozoa with DNase I-induced chromosomal damage was related to the amount of enzyme present (Table 5). Although the nuclei of mouse sperm are tolerant to physiological disruption (3, 23, 26), because sperm plasma membranes are damaged by freeze-drying, the chromosomes of freeze-dried spermatozoa might be highly susceptible to nucleases present in the buffer or spermatozoa (24).

Chelating agents such as EGTA and ethylenediamine tetraacetic acid (EDTA) suppress the activity of nucleases. It was thought that sperm chromosomes were protected from the activity of nucleases by a high concentration of EGTA in the buffer, as the chromosomal integrity of spermatozoa freeze-dried in such medium was highly maintained compared with that of spermatozoa freeze-dried in embryo culture medium (12). However, despite a high EGTA concentration in the buffer, we noted gradual chromosomal fragmentation in freeze-dried spermatozoa stored at +24°C for longer than 1 month. Moreover EGTA in the buffer could not reduce the effect of exogenous nuclease before or during freeze-drying (Table 5). In comparison, the chromosomal integrity of freeze-dried spermatozoa stored at 4°C did not decrease

over the long-term. This study suggests that, rather than EGTA and EDTA, low temperatures suppress the activity of the small amounts of nuclease present in the buffer or spermatozoa.

The achievement of long-term storage at ambient temperatures of spermatozoa freeze-dried using the present procedure was difficult. However, we were able to show that freeze-dried mouse spermatozoa can be stored long-term at +4°C with maintenance of fertilizing ability. Because the frequency of damaged chromosomes during freeze-drying of spermatozoa varies with the pH condition of the buffer (7), future studies will attempt to improve the freeze-drying method or develop a buffer that can protect sperm chromosomes from damage during freeze-drying.

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JOURNAL OF MEDICAL PRIMATOLOGY

Ovarian follicular development stimulated by leuprorelin acetate plus human menopausal gonadotropin in chimpanzees

Yoshimoto N, Shimoda K, Mori Y, Honda R, Okamura H, Ide Y, Nakashima T, Nakagata N, Torii R, Yoshikawa Y, Hayasaka I. Ovarian follicular development stimulated by leuprorelin acetate plus human menopausal gonadotropin in chimpanzees. J Med Primatol 2005; 34:73–85. © Blackwell Munksgaard, 2005

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Abstract: We attempted ovarian stimulation using gonadotropins in 14 chimpanzees. Subjects were given a single administration of leuprorelin acetate, followed by repeated administration of human menopausal gonadotropin (hMG) for 16–21 days. During the dosing period, the ovarian follicle diameter and count were measured by transvaginal ultrasonography. The hormone administration induced the development of multiple follicles, and multiple oocytes were subsequently retrieved. However, the follicle count was decreased, suggesting atresia, in some subjects. Statistically, the final follicle diameter was dependent on the dosing duration and the hMG dose in the late stage, while the maximum follicle count during hMG administration was dependent on age and the hMG dose in the early stage. Five subjects showed mild ovarian hyperstimulation syndrome (OHSS)-like symptoms with a high serum estradiol (E_2) concentration. These results suggest that leuprorelin acetate plus hMG administration successfully stimulates the development of multiple ovarian follicles for oocyte retrieval and that the serum E_2 concentration is predictive of OHSS-like symptoms in chimpanzees.

Key words: atresia – ovarian hyperstimulation syndrome – *Pan troglodytes* – superovulation – transvaginal ultrasonography

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Introduction

Chimpanzees (*Pan troglodytes*) are the closest evolutionary related species to humans. Approximately 95% of the chimpanzee genome seems to align directly with corresponding regions of the human genome, and sequence divergence is only 1.2% within these aligned segments [31]. Therefore, chimpanzees are expected to have similar physiological and pathological responses to humans and consequently are of great interest in various fields of research [6, 25, 37]. However, numbers living in the wild have decreased considerably due to extensive demolition of natural habitat, and the species has been classified as endangered. In addition, the use of chimpanzees as experimental animals is strictly limited by ethical constrains, and alternative approaches will be required. On the other hand, the number of captive births has gradually increased, and management problems such as costs and limited genetic diversity have become problems in most breeding colonies [16].

Recent progress in reproductive and developmental biology has enabled the artificial

manipulation of embryos, such as *in vitro* fertilization (IVF), cryopreservation and transfer, and these techniques may help to overcome the problems in chimpanzees. IVF and cryopreservation would be helpful for conserving genetic diversity, and embryo transfer would allow birth control for rational colony management. Moreover, embryo-derived cell lines such as embryonic stem (ES) and embryonal carcinoma (EC) cells have been attracting a great deal of attention for regenerative medicine, drug development and basic research in developmental biology [11, 18, 29, 30, 44]. The establishment of chimpanzee embryo-derived cell lines may provide a novel tool for research, and would be alternative to *in vivo* studies of chimpanzees.

As a pre-requisite for the realization of this idea, we need to obtain large numbers of oocytes from chimpanzees. Ovarian stimulation with exogenous gonadotropins for multiple follicular development has become a common technique of reproductive medicine in humans, and has also been reported in primates including rhesus monkeys [46], cynomolgus monkeys [13], squirrel monkeys [7], marmosets [22], lowland gorillas [15] and chimpanzees [14, 45].

In the present study, we attempted ovarian stimulation with leuprorelin acetate, a gonadotropin releasing hormone analog (GnRHa), plus human menopausal gonadotropin (hMG) in chimpanzees for efficient collection of multiple oocytes. The ultrasonographic, physical and endocrine data obtained were statistically analyzed to examine the factors leading to multiple follicular development. In addition, predictive parameters for ovarian hyperstimulation syndrome (OHSS)-like symptoms were examined to minimize suffering.

Materials and methods

Subjects

Fourteen adult female chimpanzees maintained at the Kumamoto Primates Research Park, Sanwa Kagaku Kenkyusho Co., Ltd (Kumamoto, Japan), were used for the present study. They consisted of six nulliparous and eight multiparous individuals, aged 11–32 years and weighing 42.1–76.2 kg (Table 1). They were housed in a facility with a large outside enclosure and indoor bed rooms, and temporarily moved to stainless-steel cages (W140 × D2125 × H1760 mm) in an air-conditioned room (temperature, 18–28°C; lighting, 7:00–18:00 hours) during the experiment. Appropriate amounts of vegetables, fruits and commercial monkey chow (PS, Oriental Yeast Co., Ltd, Tokyo, Japan; Monkey Bit, Nosan Corporation,

Table 1. Female chimpanzees used in the present study

Subject	Age (year)	Body weight (kg)[1]	Parity
Sachi	25	46.5	M
Nacky	27	47.1	M
Sango	26	60.8	M
Cookie	11	46.2	N
Tamae	23	57.4	M
Koiko	26	60.1	M
Yoko	14	53.7	N
Inko	14	45.8	N
Kanae	24	57.4	M
Suzu	20	45.0	M
Niko	29	76.2	M
Betty	32	53.2	N
Chiko	14	44.6	N
Yoshizu	12	42.1	N

N, nulliparous; M, multiparous.
[1]At commencement of the hormone administration.

Yokohama, Kanagawa, Japan) were given twice daily, and tap water was available *ad libitum*. Their appearance and behavior were checked daily for health and evidence of menstruation. All experimental procedures described below were approved by the Animal Ethical Committee of the Kumamoto Primates Research Park, Sanwa Kagaku Kenkyusho Co., Ltd, and were in accordance with the Guide for the Care and Use of Laboratory Animals (ILAR).

Hormone treatment

The schedule of hormone administration is presented in Fig. 1. The dosing regimens were carefully determined for individuals by referring to ultrasonographic, physical and endocrine data so as to avoid adverse events, especially OHSS. Within a couple of days after commencement of menstruation, 3.75 mg/head of leuprorelin acetate (Leuplin® for Injection 3.75; Takeda Chemical Industries Ltd, Osaka, Japan), a sustained active suspension of GnRHa, was subcutaneously administered to the subjects (day 0). This administration induced a transient release and a subsequent continuous suppression of endogenous pituitary gonadotropins for 4 weeks in a preliminary study (data not shown). From day 1, the subjects were given repeated intramuscular administration of 75–300 IU/head of hMG (Humegon®; N.V. Organon, Oss, The Netherlands) once daily for 16–21 days. In most of the cases, hMG administration was terminated when follicles over 17 mm in diameter were found by transvaginal ultrasonography (see below), because the follicle diameter at LH surge was approximately 17 mm in the normal menstrual cycle in a preliminary study

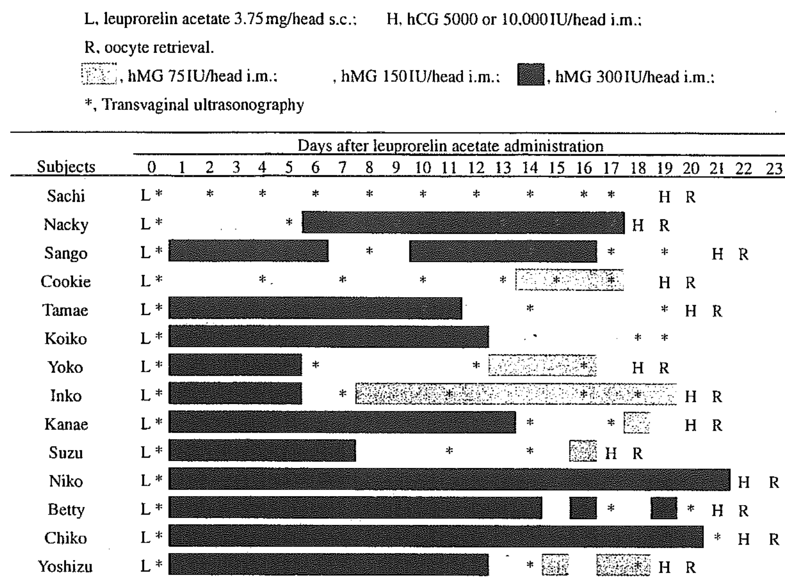

Fig. 1. Schedule of hormone administration, oocyte retrieval and transvaginal ultrasonographic examinations in female chimpanzees.

(data not shown). On the next day or a couple of days after the end of hMG administration, 5000 or 10,000 IU/head of human chorionic gonadotropin (hCG) (Pregnyl®; N.V. Organon) was intramuscularly administered once. One subject, Koiko, was not given hCG and excluded from the subsequent oocyte retrieval because she showed low food-intake and oliguria. She was thereafter under veterinary control and recovered in a week.

Transvaginal ultrasonography

During the hormone administration, time course changes of follicle diameter and count were measured by transvaginal ultrasonography using the Sonovista®-MSC (5.0 mHz probe; Mochida Pharmaceutical Co., Ltd, Tokyo, Japan) by the same observer (N.Y.) under ketamine anesthesia (Fig. 1). Meanwhile, the peripheral region of the ovary was checked to detect a sign of adverse events. For Sachi, Nacky, Sango and Cookie, transvaginal ultrasonography was performed frequently during hMG administration, but ascites was detected only at oocyte retrieval. Therefore, the frequency of the examination was decreased for the other subjects because repeated anesthetization was not preferable for health.

Oocyte retrieval

Follicular fluid was withdrawn between 30 and 35.5 h after hCG administration by ultrasound-guided transvaginal aspiration under ketamine anesthesia. The ascites around the ovary was also aspirated if detected. This method is considered to be much less invasive than laparoscopy, and no adverse events caused by this operation were observed in the present study. Meanwhile, ovarian enlargement and severity of ascites were checked. Cumulus-oocyte complexes (COCs) were collected from the aspirate, and oocytes were denuded with a 0.03% hyaluronidase solution. The oocytes were observed by light microscopy and used in other studies. After the retrieval, the subjects were treated with antibiotics and sent back to the bed rooms. Careful observation was continued until their complete recovery.

Hormone assay

While the subjects were under ketamine anesthesia for the ultrasonographic examinations and oocyte retrieval, blood was withdrawn via the median antebrachial vein. Sera were separated by centrifugation at 1600 ***g*** for 20 min and sent to SRL Inc. (Tokyo, Japan), where the serum concentrations of estradiol (E_2) and progesterone (P) were measured by the solid-phase ^{125}I radioimmunoassay method using a commercial kit (Coat-A-Count® Estradiol-6; Diagnostic Products Corporation, Los Angeles, CA, USA).

Statistical analysis

The dose levels of hMG were converted to values per body weight to minimize the effect of body

Table 2. Total dose of human menopausal gonadotropin (hMG) per body weight (∑hMG) in female chimpanzees

Subject	∑hMG (IU day/kg)			
	D1–final[1]	D1–D6[2]	D7–D12[3]	D13–final[4]
Sachi	54.3	19.3	19.1	15.9
Nacky	90.7	22.3	37.8	30.7
Sango	75.6	29.8	22	23.8
Cookie	47.5	19.4	18.8	9.3
Tamae	76.0	31.4	28.9	15.8
Koiko	73.8	29.8	29.2	14.8
Yoko	52.7	30.7	16.5	5.5
Inko	57.2	36.0	11.4	9.8
Kanae	77.7	31.4	31.1	15.2
Suzu	70.1	40.0	23.5	6.7
Niko	82.8	23.6	23.7	35.4
Betty	100.5	33.8	33.8	32.9
Chiko	132.9	40.4	38.6	53.9
Yoshizu	52.7	30.7	16.5	5.5

The day of leuprorelin acetate administration was designated day 0.
[1]From day 1 to the final ultrasonographic examination.
[2]From day 1 to day 6.
[3]From day 7 to day 12.
[4]From day 13 to the final ultrasonographic examination.

size [17]. To examine the relationship between hMG regimen and follicular development, the dosing period of hMG was divided into three stages: day 1 to day 6 as the early stage; day 7 to day 12 as the intermediate stage; and day 13 to the final ultrasonographic examination as the late stage. Then, the total hMG dose per body weight (ΣhMG) from day 1 to the final examination ($\Sigma hMG_{D1\text{-}final}$) and the doses in the respective stages ($\Sigma hMG_{D1\text{-}6}$,$\Sigma hMG_{D7\text{-}12}$ and $\Sigma hMG_{D13\text{-}final}$) were calculated (Table 2). The dependency of follicle diameter and count on age, dosing duration and each ΣhMG were estimated by correlation coefficient and univariate linear regression analysis to examine the factors leading multiple follicular development in subjects differently treated for individuals. For comparisons between subjects apparently in normal health and those showing OHSS-like symptoms, Student's *t*-test was applied when variances were found to be homogenous with the *F*-test, or Aspin–Welch's test was applied when variances were found to be heterogeneous with the *F*-test.

Results

Follicle diameter

Multiple ovarian follicles were formed by stimulation of leuprorelin acetate and hMG (Fig. 2). Time course changes in mean follicle diameter measured by transvaginal ultrasonography are shown in Fig. 3. There was no great difference in follicle growth rate among most of the subjects. At the final ultrasonographic examination between day 16 and day 21, the mean diameter was >10 mm, and multiple follicles reached >15 mm in diameter. On the other hand, Koiko showed slower follicle growth than the others. At the final examination on day 18, the mean diameter was only 6.7 mm, and no follicles reached 15 mm in diameter.

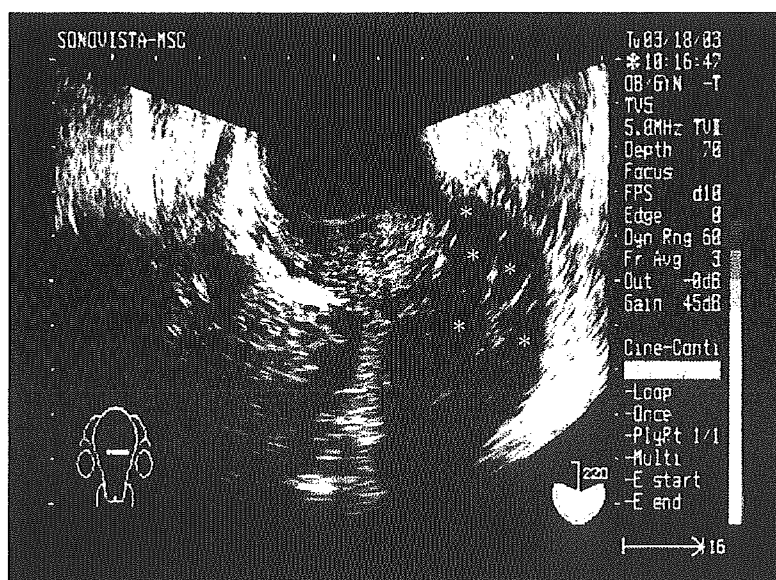

Fig. 2. A transvaginal ultrasonograph showing the ovary possessing multiple follicles. Asterisks indicate ovarian follicles.

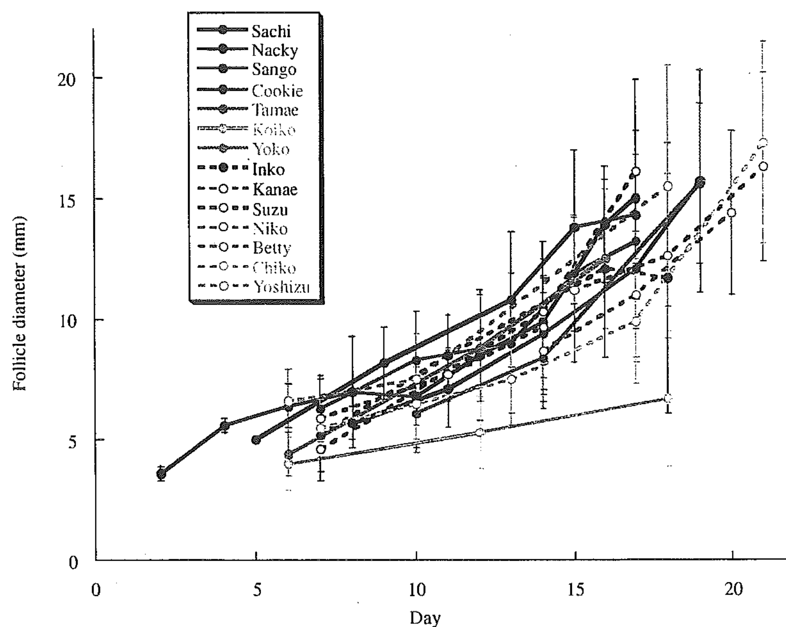

Fig. 3. Mean diameter of ovarian follicles measured by transvaginal ultrasonography in female chimpanzees treated with leuprorelin acetate plus human menopausal gonadotropin. Vertical bars represent standard deviations.

Follicle count

Time course changes in the follicle count measured by transvaginal ultrasonography are shown in Fig. 4A,B. The administration of leuprorelin acetate plus hMG increased the number of developing follicles, and the follicle count reached >30 in two subjects (Suzu and Chiko), 20–29 in six subjects (Cookie, Koiko, Yoko, Inko, Betty and Yoshizu), 10–19 in five subjects (Sachi, Sango, Tamae and Kanae) and <10 in one subject (Niko). However, four subjects, Sachi, Inko, Kanae and Betty, had subsequently lost >5 follicles by the final examination. The decrease in follicle count started at various time points (Fig. 4B).

Serum E_2 and P concentrations

Time course changes in serum E_2 concentrations are presented in Figs 5A,B and 6. There were great differences among the subjects. The peak E_2 concentration was >4000 pg/ml in three subjects (Nacky, Betty and Yoshizu), 2000–3000 pg/ml in three subjects (Cookie, Yoko and Suzu), 1000–2000 pg/ml in four subjects (Sango, Tamae, Kanae and Chiko) and <1000 pg/ml in four subjects (Sachi, Koiko, Inko and Niko). The E_2 concentration decreased rapidly after hCG administration in most of the subjects, but the decline started during hMG administration in Inko.

Time course changes in serum P concentrations are presented in Fig. 5A,B. The concentration was maintained at a very low level during hMG administration and rapidly increased after hCG administration. At oocyte retrieval, the serum P concentration reached >100 ng/ml in one subject (Nacky), 50–100 ng/ml in two subjects (Chiko and Yoshizu) and <50 ng/ml in 10 subjects (Sachi, Sango, Cookie, Tamae, Yoko, Inko, Kanae, Suzu, Niko and Betty).

Oocyte retrieval

Numbers and stages of oocytes retrieved are presented in Table 3. Five oocytes or more were retrieved from the aspirate of follicular fluid in nine subjects. In particular, >10 oocytes were obtained from Tamae, Betty and Chiko. In Inko and Kanae, COCs were collected from the aspirate of follicular fluid, but they did not contain any oocytes. Sachi and Niko possessed only a few or no follicles in the ovaries at the time of oocyte retrieval (35.5 h after hCG administration), respectively. In Sachi, only one oocyte was obtained from the aspirate of follicular fluid, and two oocytes were obtained from the aspirate of ascites in the pouch of Douglas. In Niko, ascites in the pouch of Douglas was aspirated, but no COCs were collected.

Most oocytes retrieved (92%) already had a first polar body and were estimated to be at the

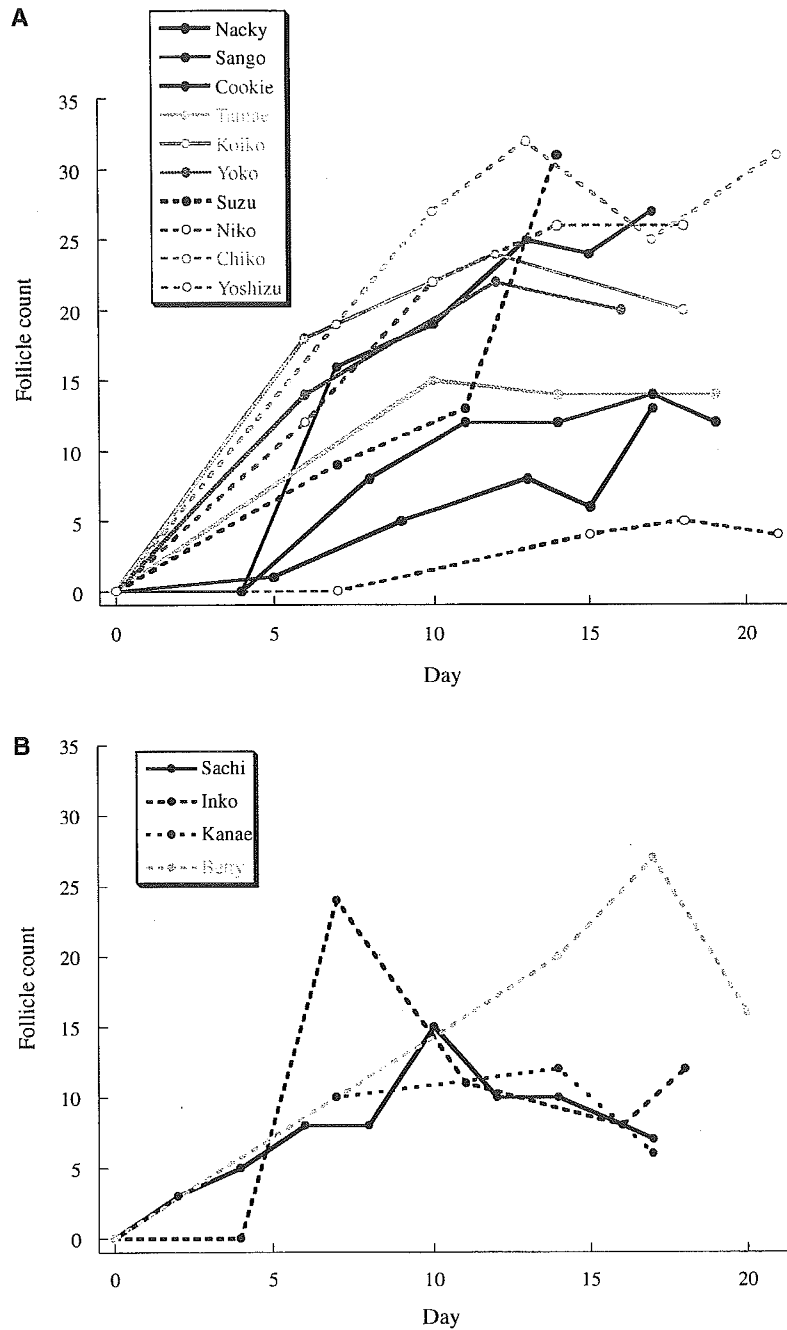

Fig. 4. Ovarian follicle counts measured by transvaginal ultrasonography in female chimpanzees treated with leuprorelin acetate plus human menopausal gonadotropin. Subjects who lost <5 follicles (A) or ≥5 follicles (B) during the dosing period.

metaphase II (M II) stage. Four oocytes (5%) did not have a polar body and were estimated to be at the stage of germinal vesicle breakdown (GVBD) or metaphase I (M I). Fragmentation was noted in only two oocytes (3%).

Statistical analysis on follicle development

The final follicle diameter was significantly dependent on the dosing duration and $\Sigma hMG_{D13\text{-}final}$ ($P < 0.05$; correlation coefficient = 0.546 and

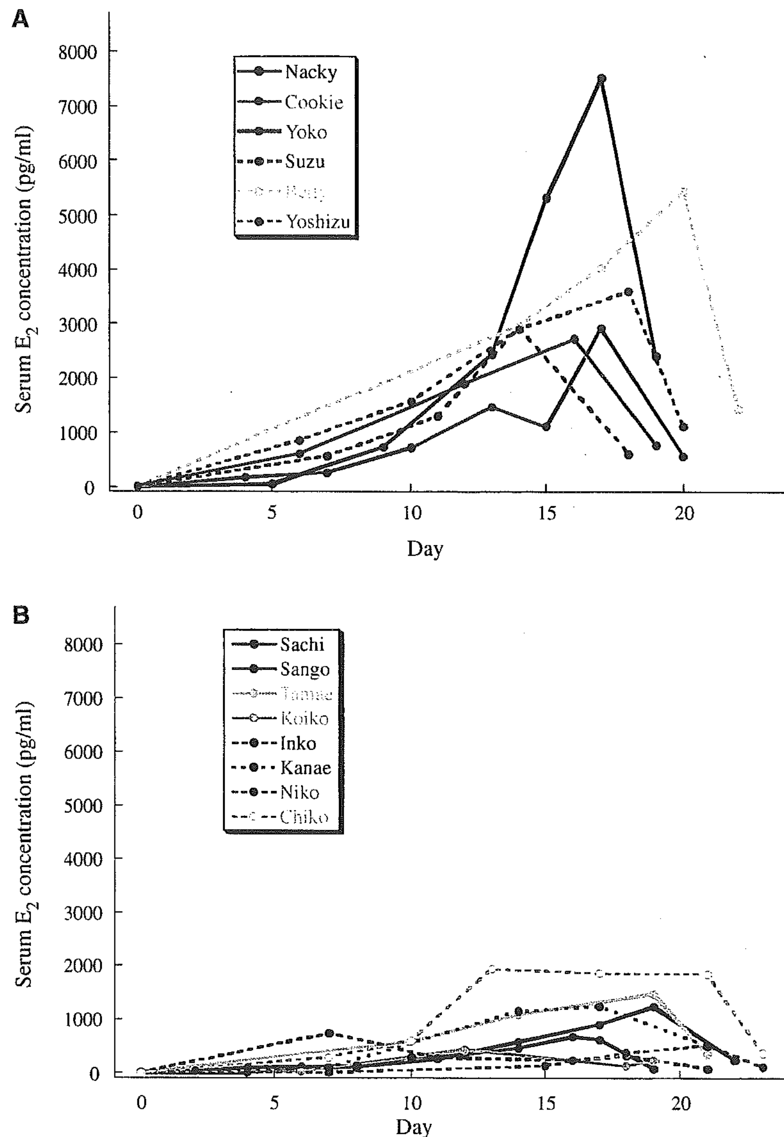

Fig. 5. Serum estradiol (E_2) concentrations in female chimpanzees stimulated with leuprorelin acetate plus human menopausal gonadotropin, followed by human chorionic gonadotropin. Subjects in which the maximum E_2 concentration reached ≥2000 pg/ml (A) or <2000 pg/ml (B) during the dosing period.

0.588, respectively), but not on age, $\Sigma hMG_{D1\text{-final}}$,$\Sigma hMG_{D1\text{-}6}$ or $\Sigma hMG_{D7\text{-}12}$ (Table 4). The maximum follicle count was significantly dependent on age and $\Sigma hMG_{D1\text{-}6}$ ($P < 0.05$; correlation coefficient = −0.561 and 0.567, respectively), but not on the dosing duration, $\Sigma hMG_{D1\text{-final}}$,$\Sigma hMG_{D7\text{-}12}$ or $\Sigma hMG_{D13\text{-final}}$ (Table 5). The decreased follicle count, which was defined as the difference between the maximum follicle count and the final follicle count, was not dependent on age, the dosing duration or any ΣhMG (Table 6).

OHSS-like symptoms

Five subjects (Nacky, Cookie, Yoko, Suzu and Betty) showed very mild OHSS-like symptoms, despite careful determination of the hMG regimen. At oocyte retrieval, they showed apparent ascites in the pouch of Douglas (Fig. 7). In 5 days, a decrease in food intake and sometimes in locomotion was observed. These signs disappeared within 2 weeks, and no abnormality was seen thereafter.

Endocrinologically, the mean peak E_2 concentration of these subjects was 4314 pg/ml, which was

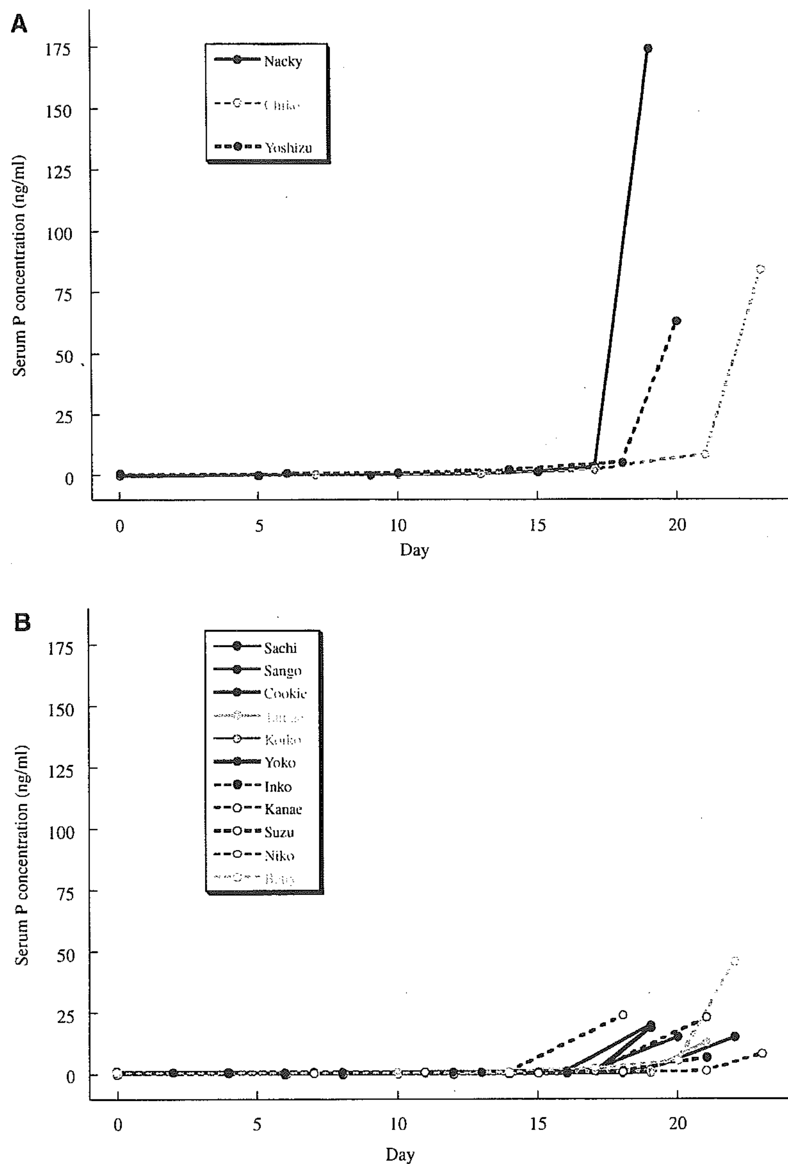

Fig. 6. Serum progesterone (P) concentrations in female chimpanzees stimulated with leuprorelin acetate plus human menopausal gonadotropin, followed by human chorionic gonadotropin. Subjects in which the P concentration at oocyte retrieval reached ≥50 pg/ml (A) or <50 pg/ml (B) during the dosing period.

significantly higher than that of the subjects apparently in normal health ($P < 0.05$). In contrast, there were no significant differences in the final follicle count or serum P concentration at oocyte retrieval between the two groups (Table 7).

Discussion

In the present study, we attempted ovarian stimulation with leuprorelin acetate plus hMG in 14 female chimpanzees and successfully induced the development of multiple follicles. The mean follicle diameter reached >10 mm at the final ultrasonographic examination in most of the subjects. The maximum follicle count during hMG administration was >30 in two subjects, 20–29 in six subjects, 10–19 in five subjects and <10 in one subject. Statistical analysis revealed the effect of the hMG regimen on follicular development. The final follicle diameter was dependent on the dosing duration and $\sum hMG_{D13\text{-}final}$, while the maximum follicle count was dependent on $\sum hMG_{D1\text{-}6}$. This

Table 3. Numbers and stages of oocytes retrieved in female chimpanzees stimulated with leuprorelin acetate plus human menopausal gonadotropin, followed by human chorionic gonadotropin (hCG)

Subject	Retrieval time (h)[1]	Number of oocytes	Stage		
			M II	GVBD – M I	Fragmented
Sachi	35.5	3[2]	3	0	0
Nacky	31	5	4	0	1
Sango	31	4	4	0	0
Cookie	30.5	5	4	0	1
Tamae	30	10	10	0	0
Koiko[3]	–	–	–	–	–
Yoko	30.5	9	9	0	0
Inko	30.5	0	–	–	–
Kanae	30	0	–	–	–
Suzu	33	5	4	1	0
Niko	35.5	0	–	–	–
Betty	30	16	16	0	0
Chiko	30	10	9	1	0
Yoshizu	30	8	6	2	0

GVBD, germinal vesicle breakdown; M I, metaphase I; M II, metaphase II.
[1]Time after hCG administration.
[2]Two oocytes were collected from ascites in the pouch of Douglas.
[3]Oocyte retrieval was not performed due to low food-intake and oliguria.

Table 4. Correlation coefficient and univariate linear regression analysis of the final follicle diameter (mm) in female chimpanzees stimulated with leuprorelin acetate plus human menopausal gonadotropin (hMG)

Factor	Correlation coefficient	Univariate linear regression analysis	
		r[1]	*P*-value
Age (year)	0.156	0.064	0.595
Dosing duration (day)	0.546	0.794	0.043*
$\sum hMG_{D1\text{-}final}$ (IU day/kg)[2]	0.424	0.033	0.646
$\sum hMG_{D1\text{-}D6}$ (IU day/kg)[3]	−0.145	−0.062	0.621
$\sum hMG_{D7\text{-}D12}$ (IU day/kg)[4]	0.304	0.104	0.291
$\sum hMG_{D13\text{-}final}$ (IU day/kg)[5]	0.588	0.120	0.027*

[1]Regression coefficient.
[2]Total hMG dose per body weight from day 1 to the final ultrasonographic examination.
[3]Total hMG dose per body weight from day 1 to day 6.
[4]Total hMG dose per body weight from day 7 to day 12.
[5]Total hMG dose per body weight from day 13 to the final ultrasonographic examination.
*Significant *P*-values ($P < 0.05$).
The day of leuprorelin acetate administration was designated day 0.

Table 5. Correlation coefficient and univariate linear regression analysis of the maximum follicle count in female chimpanzees stimulated with leuprorelin acetate plus human menopausal gonadotropin (hMG)

Factor	Correlation coefficient	Univariate linear regression analysis	
		r[1]	*P*-value
Age (year)	−0.561	−0.655	0.037*
Dosing duration (day)	−0.223	−0.913	0.444
$\sum hMG_{D1\text{-}final}$ (IU day/kg)[2]	0.097	0.034	0.743
$\sum hMG_{D1\text{-}D6}$ (IU day/kg)[3]	0.567	0.683	0.034*
$\sum hMG_{D7\text{-}D12}$ (IU day/kg)[4]	−0.036	−0.035	0.902
$\sum hMG_{D13\text{-}final}$ (IU day/kg)[5]	−0.097	−0.058	0.741

[1]Regression coefficient.
[2]Total hMG dose per body weight from day 1 to the final ultrasonographic examination.
[3]Total hMG dose per body weight from day 1 to day 6.
[4]Total hMG dose per body weight from day 7 to day 12.
[5]Total hMG dose per body weight from day 13 to the final ultrasonographic examination.
*Significant *P*-values ($P < 0.05$).
The day of leuprorelin acetate administration was designated day 0.

Table 6. Correlation coefficient and univariate linear regression analysis of the decreased follicle count in female chimpanzees stimulated with leuprorelin acetate plus human menopausal gonadotropin (hMG)

Factor	Correlation coefficient	Univariate linear regression analysis	
		r[1]	*P*-value
Age (year)	0.109	0.064	0.711
Dosing duration (day)	−0.096	0.199	0.744
$\sum hMG_{D1\text{-}final}$ (IU day/kg)[2]	−0.134	−0.024	0.649
$\sum hMG_{D1\text{-}D6}$ (IU day/kg)[3]	0.144	0.088	0.624
$\sum hMG_{D7\text{-}D12}$ (IU day/kg)[4]	−0.271	−0.133	0.348
$\sum hMG_{D13\text{-}final}$ (IU day/kg)[5]	−0.128	−0.039	0.663

[1]Regression coefficient.
[2]Total hMG dose per body weight from day 1 to the final ultrasonographic examination.
[3]Total hMG dose per body weight from day 1 to day 6.
[4]Total hMG dose per body weight from day 7 to day 12.
[5]Total hMG dose per body weight from day 13 to the final ultrasonographic examination.
The decreased follicle count was defined as the difference between the maximum follicle count and the final follicle count.
The day of leuprorelin acetate administration was designated day 0.

analysis suggests that sufficient exposure to hMG in the early stage is needed to increase the number of developing follicles and that sufficient and continuous exposure to hMG in the late stage is needed to increase the size of growing follicles. In addition, the maximum follicle count was conversely correlated with age. In contrast, the decreased follicle count was independent of age, dosing duration or any ∑hMG.

It has been reported that a single administration of hMG or follicle stimulating hormone (FSH) in the early follicular phase increases the number of small follicles and stimulates proliferation of granulosa cells in humans [12, 36], suggesting that

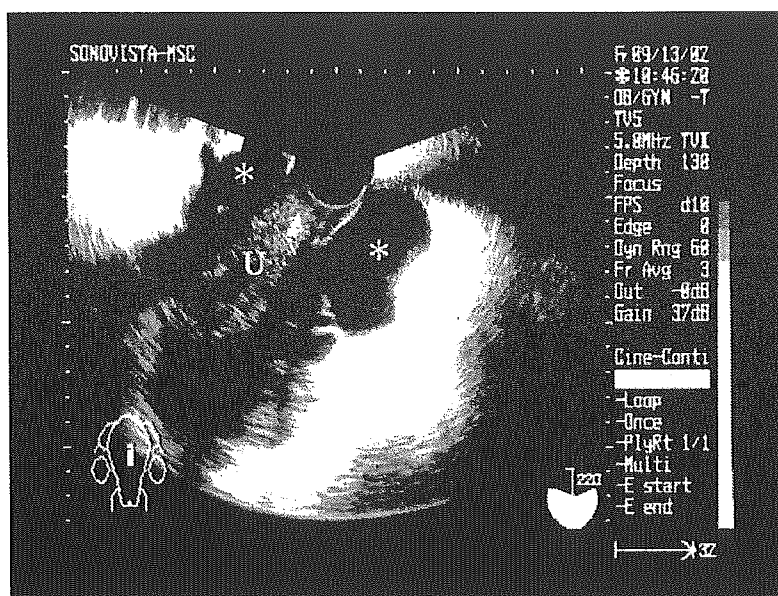

Fig. 7. A transvaginal ultrasonograph at oocyte retrieval in a subject showing OHSS-like symptoms (Nacky). Asterisks indicate ascites in the pouch of Douglas. U indicates the uterus.

Table 7. The final follicle count, serum peak estradiol (E_2) concentration during hMG administration and serum progesterone (P) concentration at oocyte retrieval in female chimpanzees apparently in normal health and those showing ovarian hyperstimulation syndrome (OHSS)-like symptoms

Group	Normal[1]	OHSS-like[2]
n	9	5
Final follicle count	15 ± 11	21 ± 8
Peak E_2 (pg/mL)[3]	1323 ± 988	4314 ± 2114*
P (pg/mL) at oocyte retrieval	29.0 ± 28.5	40.7 ± 54.9

[1]Subjects apparently in normal health.
[2]Subjects showing OHSS-like symptoms (see text for details).
[3]Peak concentration during the dosing period.
*Significantly different from the normal group with Aspin–Welch's test ($P < 0.05$).
Values shown are mean values ± SD.

a short, but distinct, increase in the hMG or FSH level can accelerate follicle recruitment in the early follicular phase. Therefore, the dependency of the maximum follicle count on $\Sigma hMG_{D1\text{-}6}$ in the present study may indicate that the increased hMG exposure in the early stage accelerates follicle recruitment also in chimpanzees.

In addition, a converse correlationship between the maximum follicle count and age was detected in the present study. It has been reported that the number of growing follicles reflects the total number of follicles present in the ovary, which is the major factor determining the response to exogenous gonadotropins [4]. Therefore, this result might reflect the decrease in the total number of follicles with age in chimpanzees.

In contrast to early follicle development, advanced follicle development requires continuous stimulation by gonadotropins. In the normal menstrual cycle, the FSH concentration reaches a maximum in the early follicular phase. Maturating follicles secrete estrogens and inhibins, which suppress pituitary FSH release via a feedback mechanism. The maturing follicles are very sensitive to FSH, and they can keep growing after pituitary FSH suppression. In contrast, the other immature follicles cannot avoid undergoing atresia [3, 21]. Exogenous gonadotropin treatment in the mid- to late follicular phase compensates for the endogenous pituitary FSH suppression and rescues immature follicles from atresia [36]. Thus, the continuous hMG administration in the intermediate and late stages may have helped to maintain multiple follicle development until oocyte retrieval in the present study.

Despite continuous hMG administration, however, four subjects, Sachi, Inko, Kanae and Betty, lost >5 follicles, indicating that the follicles underwent atresia. The reason why follicle atresia occurred in these subjects is unclear. The decreased follicle count was not affected by age, dosing duration or any $\sum hMG$, and the decrease started at various time points. Further study will be needed to detect the factors responsible for atresia.

Recent studies have suggested that the follicle atresia can be explained, at least in part, by the apoptosis of granulosa cells [19, 40]. It has been reported that cell-death ligand and receptor systems, such as the Fas ligand and Fas system, the tumor necrosis factor (TNF)-α and TNF-receptor system, and the TNF-α-related apoptosis-inducing ligand (TRAIL) and TRAIL-receptor system, are involved in the regulatory mechanisms of granulosa cell apoptosis in mice and pigs [28, 33, 35, 41, 42]. Although there are species-specific differences in the apoptosis of granulosa cells [27], some cell-death ligand and receptor systems could be involved in the regulation of follicle atresia in chimpanzees.

The final diameter was statistically dependent on the dosing duration and $\sum hMG_{D13\text{-}final}$, despite no great inter-individual difference in the follicle growth rate. It has been reported that continuous exposure to FSH is needed for follicle maturation and that the fully matured follicle possesses high aromatase activity in granulosa cells. In addition, the amount of E_2 synthesed by granulosa cells is correlated with the size of large follicles [21]. Thus, prolonged and increased hMG exposure in the late stage might promote final follicle maturation, resulting in the increased final follicle diameter in the present study.

We obtained multiple oocytes from most of the subjects by ultrasound-guided transvaginal aspiration without significant adverse events. However, the oocyte retrieval was unsuccessful in certain subjects. In the cases of Sachi and Niko, a few follicles or less remained at the time of oocyte retrieval, indicating that ovulation had already occurred. In these subjects, the aspiration was performed at 35.5 h after hCG administration, which was later than in the others (30–33 h after hCG administration). This result indicates that oocyte retrieval should be performed within 33 h of the administration of hCG in chimpanzees. On the other hand, no oocyte was collected from Inko and Kanae, because the COCs collected from them did not contain any oocytes. Interestingly, these subjects showed relatively low serum E_2 levels and lost >5 follicles during hMG administration. It is speculated that the remaining follicles at oocyte retrieval were already atretic and that the oocytes were degenerative.

OHSS is an iatrogenic and potentially life-threatening complication of ovulatory stimulation. It has been reported that OHSS is associated with symptoms such as abdominal bloating, nausea, vomiting, enlarged ovaries, ascites, pleural and pericardial effusions, hemoconcentration, hypercoagulation and serum electrolyte imbalance [10, 26]. A high serum E_2 concentration and an increased number of follicles are among the risk factors for OHSS, and most studies in humans have selected 3000 pg/ml of E_2 as a safe value for hCG administration [1]. In the present study, five subjects showed apparent ascites with a decrease in food intake and locomotion, suggesting the occurrence of very mild OHSS. They showed a significantly higher peak serum E_2 concentration than the others, and their mean concentration (4314 pg/ml) was above the safe value for humans. This result suggests that the peak serum E_2 concentration was predictive of OHSS-like symptoms in chimpanzees. On the other hand, there was no significant difference in the final follicle count between the two groups, suggesting that the increased number of follicles was not an indicator of OHSS-like symptoms in chimpanzees.

Although a rise in the E_2 concentration precedes OHSS, E_2 itself is not a causal factor of this disease [24]. After hCG administration, matured follicles are ruptured and transformed to corpora lutea. During luteinization, a basket-like capillary wreath surrounding the follicular basement membrane is reconstructed to form a capillary network in the corpus luteum [5, 20, 38]. This drastic change in the microvasculature is accompanied by angiogenesis and capillary hyperpermeability, which is induced by vascular endothelial cell growth factor (VEGF) [9, 39, 43]. In mice, luteinization, neovascularization and edema simultaneously occur in theca interna before ovulation [38]. VEGF is also considered to be the primary molecule involved in the pathogenesis of OHSS, because capillary hyperpermeability is the major initial change leading to the full appearance and maintenance of this disease [2, 34, 43]. Thus the occurrence of OHSS is closely related with angiogenesis and capillary hyperpermeability during luteinization. However, there was no significant difference in serum P concentrations at oocyte retrieval between subjects in normal health and those showing OHSS-like symptoms in the present study. This contradiction might be explained by the notion that angiogenesis and capillary hyperpermeability before ovulation are independent of the secretion of P from luteinizing theca cells.

Although we statistically detected the dependence of follicular development on hMG dose, a great variation was noted in response to hMG. It has been reported that the response to exogenous FSH therapy is quite variable and associated with the polymorphism of ovarian FSH receptor in humans [23]. Therefore, the variation in response to hMG could be caused by the polymorphism of ovarian FSH receptor in the present study.

We succeeded in obtaining multiple oocytes from chimpanzees. Most of them were at the M II stage, suggesting that the dosing regimen of the present study was appropriate for oocyte maturation in follicles. However, further investigation will be needed for their application. It has been reported that a rise in the E_2 concentration after administration of hMG or leuprorelin acetate is predictive of IVF success in humans [8, 32]. Therefore, the relationship between the serum E_2 concentration and the potential of retrieved oocytes should be examined to optimize the procedure of hormone treatment. In addition, one should undertake the optimization for each subject, because there are great inter-individual differences in pituitary and ovarian hormone dynamics in chimpanzees.

In conclusion, we successfully stimulated ovaries with leuprorelin acetate plus hMG administration for multiple follicle development and multiple oocyte retrieval in chimpanzees. The present study also suggested that the dose of hMG affected the number of developing follicles in the early stage and follicle maturation in the late stage. In addition, the peak serum E_2 concentration was shown to be predictive of OHSS-like symptoms. The results of the present investigation should help to resolve various problems arising from the circumstances of this endangered species.

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