Cryopreservation of spermatozoa from cynomolgus monkeys (*Macaca fascicularis*)

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Fertilization of eggs from cynomolgus monkeys (*Macaca fascicularis*) was achieved *in vitro* using frozen–thawed spermatozoa. The best sperm survival rate was obtained after freezing spermatozoa in a semen-diluting medium (TTE: Tes, Tris, egg yolk base) containing 5% glycerol with 30 min equilibrium time. Although frozen–thawed spermatozoa could fertilize eggs *in vitro* and some of the fertilized eggs developed into blastocysts or hatched blastocysts, a sperm fertility assay using salt-stored eggs indicated that frozen–thawed spermatozoa had a shorter lifespan in capacitation medium than did fresh, unfrozen spermatozoa. This may be attributable to damage to their plasma membranes.

Introduction

Reproductive technologies such as sperm cryopreservation, artificial insemination and *in vitro* fertilization are all necessary to maintain genetic resources of endangered nonhuman primates as well as to improve reproductive efficiency of these animals in captivity. Although successful freezing of spermatozoa has been reported for rhesus monkeys (Roussel and Austin, 1967; Leverage *et al.*, 1972), baboons (Kraemer and Vera Cruz, 1969), squirrel monkeys (Denis *et al.*, 1976), chimpanzees (Gould and Styperek, 1989) and gorillas (Lambert *et al.*, 1991), there are relatively few reports on the freezing of spermatozoa from cynomolgus monkeys (*Macaca fascicularis*) (Cho and Honjo, 1973; Mahone and Dukelow, 1978; Tollner *et al.*, 1990), despite the wide use of this species in biomedical research. This study reports the effects of various freezing procedures on the survival and fertility of spermatozoa from cynomolgus monkeys. The zona pellucida penetration assay and *in vitro* fertilization were used to assess the efficiency of the freezing procedures.

Materials and Methods

Collection of semen and the preparation of spermatozoa

Fresh semen was collected from sexually mature male cynomolgus monkeys, aged 9–12 years, which had been bred and maintained in our primate centre (Honjo, 1985). Each male was anaesthetized using 10 mg ketamine hydrochloride kg⁻¹ body mass (Ketalar; Sankyo, Tokyo; Cho *et al.*, 1969) and then stimulated with a rectal electrode. Cynomolgus monkey semen immediately coagulates after ejaculation; to counteract this, one

ejaculate (about 0.1 ml) was put in a disposable plastic test tube containing 2 ml of TYH medium (Toyoda *et al.*, 1971). The diluted semen was kept at 37°C for up to 30 min and then gently layered on 2 ml of 90% Percoll (Pharmacia, Uppsala) in a 15 ml disposable tube. The iso-osmotic 90% Percoll was prepared by mixing 1 volume of 9% NaCl (w/v) solution with 9 volumes of Percoll. The tube was centrifuged at 800 *g* for 10 min, and the spermatozoa with high motility were recovered from the bottom of the tube for freezing.

Freezing of spermatozoa

The composition of the primary semen-diluting medium TTE (Tes, Tris, egg yolk base) is shown in Table 1. After mixing all the ingredients, the medium was centrifuged at

Table 1. Composition of TTE medium for dilution of cynomolgus monkey semen

Components	Concentration (g l ⁻¹)
Tes ^a	12
Tris-HCl ^b	2
Glucose ^a	20
Lactose ^c	20
Raffinose ^c	2
Egg yolk	200 (ml)
Penicillin-G	100 000 (iu)
Streptomycin sulfate	50 (mg)

pH: 7.0–7.2; osmolality: 322 mosmol; water: Milli-Q.

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Fig. 1. Effect of glycerol concentration (■: 5%, $n = 9$; ○: 2.5%, $n = 7$; □: 10%, $n = 7$; ●: 0%, $n = 7$; ▲: 20%, $n = 5$; △: 40%, $n = 5$) on survival rate and motility of frozen-thawed spermatozoa from cynomolgus monkeys. *Percentage of motile spermatozoa and intensity of sperm motility was significantly different ($P < 0.01$) from that in 5% glycerol; **intensity of sperm motility was significantly different ($P < 0.05$) from that in 5% glycerol.

7000 g for 1 h to sediment yolk granules. The supernatant was stored, in 15 ml aliquots, at -80°C until use. Just before use, TTE medium was thawed and equilibrated to room temperature. The freezing medium TTE-G was prepared by adding glycerol to the TTE medium to give glycerol concentrations of 2.5–40%. This medium was kept at 5°C until use. The percentage of motile spermatozoa and the intensity of sperm motility were examined before and after freezing. The degree of sperm motility was expressed by the following motility scores: 4, active progressive motility; 3, sluggish progressive motility; 2, mostly non-progressive motility; 1, barely moving; 0, not moving at all (Mahi and Yanagimachi, 1978).

The suspension (100 μl) of washed spermatozoa in TYH medium was diluted slowly in 9 volumes of TTE medium at room temperature, kept at 5°C (water bath) for 2 h and then an equal volume (1 ml) of cool (5°C) TTE-G medium was added, 200 μl at a time, at intervals of 5–10 min. Dilution was complete after 30 min.

The optimal time for the spermatozoa to equilibrate in glycerol was determined using TTE-G medium containing 5% glycerol. The equilibrium times, tested at 5°C , were 0.5, 2, 4, 6 and 8 h. Each test sample was drawn carefully into 0.25 ml cryostraws and sealed with straw powder. This process was carried out at 5°C and completed just before the end of each equilibrium time tested. Sealed straws were cooled for 10 min

Fig. 2. Effect of equilibrium time (●: 0.5 h; ■: 4 h; ○: 2 h; □: 6 h; ▲: 8 h) in 5% glycerol-TTE medium on survival rate and motility of frozen-thawed spermatozoa ($n = 6$ for all groups). *Percentage of motile spermatozoa and intensity of sperm motility was significantly different ($P < 0.01$) from that in 5% glycerol-TTE for 0.5 h.

at 5 cm above the surface of liquid nitrogen. Straws were then plunged into liquid nitrogen for storage.

Zona pellucida penetration assay

The zona pellucida penetration assay was carried out according to Yanagimachi *et al.* (1979) with some modifications. Ovarian oocytes were collected from ovarian follicles of female cynomolgus monkeys that had died from accidental causes or were from females treated with pregnant mares' serum gonadotrophin (PMSG) and hCG (see below). The cumulus cells were removed by treating the oocytes for 10–15 min with 0.1% hyaluronidase (300 U mg^{-1} ; Sigma Chemical Co, St Louis, MO) in Whitten's medium (Whitten, 1971). Cumulus-free oocytes were washed, then placed into a salt solution containing 2 mol ammonium sulfate l^{-1} , 12 mmol NaHCO_3 l^{-1} and 0.5% dextran (pH 7.0–7.2) (Yanagimachi *et al.*, 1993). The oocytes were kept at 5°C for 3–8 days. Before use, salt-treated oocytes were rinsed thoroughly with TYH medium and then heated to 70°C in a water bath for 5 min to increase the ability of spermatozoa to penetrate the zona pellucida (Yanagimachi *et al.*, 1993). The oocytes were then kept in 50 μl of fresh TYH medium under mineral oil.

Five straws containing frozen spermatozoa were thawed by placing them in a water bath at 37°C for 5 min. Thawed sperm suspension (0.25 ml in each of five straws giving a total volume

Table 2. Penetration of fresh and frozen-thawed spermatozoa from cynomolgus monkeys into the perivitelline space of salt-treated oocytes

Source of spermatozoa	Preincubation time (h)	Medium ^a	Number of oocytes examined	Number (%) of oocytes penetrated ^b
Fresh	2	A	10	0
		B	17	5 (29.4)
	4	A	6	0
		B	9	9 (100)
	6	A	9	0
		B	11	11 (100)
	8	A	9	0
		B	18	15 (83.3)
Freeze-thawed	2	A	6	0
		B	17	12 (70.6)
	8 ^c	A	—	—
		B	6	0

^aMedia for sperm preincubation: (A) TYH, (B) TYH supplemented with 1 mmol caffeine l⁻¹ and 1 mmol dibutyryl cyclic AMP l⁻¹.

^bThe number of spermatozoa that penetrated into the perivitelline space of each oocyte was fewer than ten.

^cSpermatozoa were weak after preincubation for 8 h.

of 1.25 ml) was diluted with 2 ml TYH medium or TYH medium with 1 mmol caffeine l⁻¹ (Wako Chemical Co, Osaka) and 1 mmol dibutyryl cyclic AMP l⁻¹ (Yamasa Syoyu, Chiba), adding 0.2 ml medium at a time at 30 s intervals. The diluted sperm suspension was layered on 2 ml of 90% Percoll in a 15 ml disposable tube and centrifuged for 10 min at 800 g. Sedimented spermatozoa with good motility were collected and resuspended in TYH medium. Both fresh and frozen-thawed spermatozoa were incubated for 2 or 8 h in TYH medium supplemented with 1 mmol caffeine l⁻¹ and 1 mmol dibutyryl cyclic AMP l⁻¹. Incubated spermatozoa were added to the TYH medium droplet containing salt-treated oocytes. The final concentration of spermatozoa was approximately 1 × 10⁶ spermatozoa ml⁻¹. After incubation for 4 h, the oocytes were rinsed with TYH medium, and mounted between a slide and coverslip. Penetration of spermatozoa through the zona pellucida was examined using a Nomarski interference-contrast microscope (Nikon Optical Co, Tokyo).

Collection of oocytes for in vitro fertilization

The normal menstrual cycles (28–32 days) of seven sexually mature cynomolgus monkey females were confirmed by examining vaginal bleeding (Honjo *et al.*, 1984) for at least three cycles. Injections of 200 iu PMSG (Teikoku Hormone, Tokyo) were given i.m. eight times on days 3, 5, 7, 9, 11, 12, 13 and 14 of the menstrual cycle. Administration of 4000 iu hCG (Teikoku Hormone) was given i.m. on the morning after the last PMSG injection.

Animals were anaesthetized with 10 mg ketamine hydrochloride kg⁻¹ body mass 28 h after hCG injection. Ovaries were exposed through an abdominal incision and the contents of enlarged follicles (1–5 mm in diameter) were aspirated through a 25 gauge needle connected to a 2.5 ml syringe. The collected follicular contents were diluted immediately with

Fig. 3. A salt-treated cynomolgus monkey egg 4 h after insemination with frozen-thawed spermatozoa. Arrows indicate spermatozoa in the perivitelline space. In this micrograph, two spermatozoa were in focus; three others were out of focus. (Scale bar represents 50 µm.)

30 ml of Whitten's medium containing 2.5 U heparin ml⁻¹ (Novo Nordisk, Bagsvaerd). Heparin was added to the medium to prevent coagulation of contaminating blood. The oocytes were examined under a dissection microscope and mature oocytes with the first polar body or with expanded cumulus cells were selected for insemination. If oocytes were recovered at the germinal vesicle stage, they were cultured for 24–48 h in TCM-199 (Tissue culture medium-199; Flow Laboratories, Virginia, VA) supplemented with 10% fetal calf serum and 10 iu PMSG ml⁻¹. The oocytes were washed and transferred to a drop (50 µl) of Whitten's medium and covered with mineral oil. They were kept at 37°C for about 3 h in a humidified 5% CO₂:95% air atmosphere. Oocytes that had extruded the first polar body were used for *in vitro* fertilization.

Table 3. *In vitro* fertilization by fresh or frozen–thawed spermatozoa from cynomolgus monkeys

Spermatozoa	Preincubation time (h)	Hyperactivated motion of spermatozoa ^a	Number of eggs examined	Number (%) of eggs fertilized
Fresh	2	–	12	0 (0)
	8	+	17	8 (47.1)
Frozen–thawed	2	+	14	8 (57.1)
	8	–	– ^b	

^aOver 90% spermatozoa showed hyperactivated motion (+) or < 10% spermatozoa showed hyperactivated motion (–).
^bNo attempt was made to inseminate eggs because of poor sperm motility at the end of 8 h preincubation.

In vitro fertilization

Frozen spermatozoa were thawed by the method described in the zona pellucida penetration assay. Both fresh and frozen–thawed spermatozoa were incubated in 100 µl droplets of TYH medium under mineral oil at 37°C in a humidified 5% CO₂:95% air atmosphere. The presence or absence of hyperactivated spermatozoa was observed 2 or 8 h later (Yanagimachi, 1970), and 5 µl of the sperm suspension was transferred to 50 µl of Whitten’s medium containing mature oocytes. The final sperm concentration in the medium was approximately 3 × 10⁵ cells ml^{–1}. Five hours later, eggs were examined for the presence of male and female pronuclei as well as a sperm tail within the vitellus, or the second polar body.

Zygotes obtained by *in vitro* fertilization using frozen–thawed spermatozoa that had been preincubated for 2 h were transferred to the Whitten’s medium containing 10% fetal calf serum 18 h after insemination. They were then co-cultured with cumulus cells (Goto *et al.*, 1988) for up to 240 h in a humidified 5% CO₂:95% air atmosphere to allow further development.

Statistical analysis

The penetration rate in the zona pellucida penetration assay and the fertilization rate in the *in vitro* fertilization were analysed statistically by χ² test. The percentage of motile spermatozoa and intensity of sperm motility were analysed by the Kruskal–Wallis test.

Results

Effect of glycerol on survival rate and motility of spermatozoa

The highest percentage of motile spermatozoa and intensity of sperm motility were obtained when the spermatozoa were

Fig. 4. A pronuclear cynomolgus monkey ovum 4 h after insemination with frozen–thawed spermatozoa preincubated for 2 h. Arrows indicate female and male pronuclei; arrowheads indicate first and second polar bodies. (Scale bar represents 50 µm.)

frozen in TTE-G medium containing 5% glycerol (Fig. 1). The effect of equilibrium time in TTE-G medium is shown (Fig. 2). Immediately following the washing after the freeze–thaw procedure, survival and motility of the spermatozoa were excellent at any equilibrium time. However, survival and motility decreased rapidly during 1 h of subsequent incubation when spermatozoa had been equilibrated with glycerol for 2–8 h, but did not decrease when spermatozoa had been equilibrated with glycerol for 0.5 h. These results indicate that cryopreservation of spermatozoa from cynomolgus monkeys is best accomplished by TTE-G medium containing 5% glycerol with 0.5 h equilibrium.

Table 4. Developmental stage of eggs fertilized *in vitro* by frozen–thawed spermatozoa from cynomolgus monkeys examined 240 h after start of incubation

Number of donors	Number of eggs cultured	Number (%) of eggs cleaved	Number of eggs at developmental stage					
			Two-cell	Four-cell	Eight-cell	Morula	Blastocyst	Hatched
5	67	57 (85.1)	4	13	7	20	12	1

Fig. 5. Cynomolgus monkey embryos obtained by *in vitro* fertilization using frozen-thawed spermatozoa. (a) Morula 120 h after insemination; (b) expanded blastocyst 189 h after insemination; (c) hatching blastocyst 213 h after insemination; (d) hatched blastocyst 232 h after insemination. (Scale bar represents 50 μm .)

Zona pellucida penetration assay

Spermatozoa preincubated in TYH medium supplemented with caffeine and dibutyryl cyclic AMP could penetrate the perivitelline space of salt-treated oocytes, regardless of preincubation time (Table 2; Fig. 3). The penetration rates at each preincubation time using this medium were significantly different ($\chi^2 = 44.40$). In the spermatozoa preincubated for more than 4 h, the penetration rates were significantly higher ($P < 0.001$) than those of spermatozoa preincubated for 2 h. When frozen-thawed spermatozoa were used, the penetration rate after 2 h preincubation was higher than that when fresh spermatozoa were used.

In vitro fertilization

Fresh unfrozen spermatozoa that showed hyperactivated motion after 8 h of preincubation fertilized 47% of eggs (Table 3). None was fertilized by spermatozoa that had been preincubated for 2 h and that did not show hyperactivated motion. In contrast, the frozen-thawed spermatozoa showed hyperactivated motion after 2 h of preincubation and fertilized 57% of the oocytes (Fig. 4). No significant difference was

observed between fresh spermatozoa preincubated for 8 h and frozen-thawed spermatozoa preincubated for 2 h with regard to the fertilization rate ($\chi^2 = 0.96$, $P > 0.05$). The ability of frozen-thawed spermatozoa to fertilize eggs after 8 h preincubation was not assessed because the spermatozoa showed only a very low motility after this time.

When 67 fertilized eggs were co-cultured with cumulus cells, 57 cleaved, 12 developed into expanded blastocysts and one hatched (Table 4 and Fig. 5).

Discussion

Tollner *et al.* (1990) succeeded in obtaining live young from a cynomolgus monkey after insemination using cryopreserved spermatozoa. They used a Tes-Tris-buffered medium containing egg yolk, skimmed milk and glycerol as a semen extender. Since the skimmed milk used by these authors was not readily available in Japan, an extender (TTE) with lactose and raffinose was used in this study, with satisfactory results. The most critical freezing factors were glycerol concentration and the equilibration time. A final glycerol concentration of 5%, and an equilibration time of no more than 30 min yielded the best rate

of motile spermatozoa and intensity of sperm motility after freeze-thawing.

The swim-up method (Overstreet *et al.*, 1980) was used by Tollner *et al.* (1990) to collect viable monkey spermatozoa from freeze-thawed sperm samples. The Percoll method used here was very efficient, not only in recovering motile spermatozoa but also in removing glycerol. In both the zona pellucida penetration assay and *in vitro* fertilization, sperm penetration and fertilization never occurred in media without caffeine and dibutyryl cyclic AMP. These reagents seem to promote capacitation and the acrosome reaction of primate spermatozoa (Boatman and Bavister, 1984). Freeze-thawing seems to shorten capacitation time, as judged by high penetration rates by spermatozoa following short preincubation periods. However, some damage to the spermatozoa, perhaps to their plasma membranes, is obvious from the shortened fertile lifespan after thawing. An alternative conclusion is that freeze-thawed semen contains a population of live acrosome-reacted spermatozoa that can bind to and penetrate fresh oocytes. Further improvement is needed to maintain high fertility and prolong the fertile lifespan of spermatozoa after freeze-thawing in cynomolgus monkeys.

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