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Effects of Various Extenders and Permeating Cryoprotectants on Cryopreservation of Cynomolgus Monkey (*Macaca fascicularis*) Spermatozoa

YA-HUI LI,*†‡ KE-JUN CAI,* ANDRAS KOVACS,§ AND WEI-ZHI JI*

*From *Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan, China; †Graduate School of the Chinese Academy of Sciences, Beijing, China; ‡College of Food Science and Technology, Yunnan Agricultural University, Kunming, Yunnan, China; and §Research Institute for Animal Breeding and Nutrition, Herceghalom, Gesztenyes, Hungary.*

ABSTRACT: The cryoprotective effects of 11 different extenders, TTE, DM, mDM, LG-DM, G-DM, TCG, TEST, TSM, Test-M, Test-H, and LM, on sperm cryopreservation of cynomolgus monkey (*Macaca fascicularis*) have been compared with glycerol as cryoprotectant. Sperm motility, plasma membrane, and acrosomal integrity were examined to evaluate frozen-thawed sperm function. The results showed that TTE, DM, mDM, LG-DM, G-DM, and TCG exhibited the best and similar protective efficiencies for cynomolgus monkey sperm cryopreservation in terms of sperm motility and plasma membrane integrity ($P > .05$). The acrosomal integrity for spermatozoa cryopreserved in TCG was statistically lower than that of TTE, DM, mDM, LG-DM, and G-DM ($P < .05$) but was significantly higher than that of TEST, TSM, Test-M, Test-H, and LM ($P < .05$). The postthaw sperm motility for 5 other extenders (TEST, TSM, Test-M, Test-H, and LM) did not exceed 30%, and the 3 sperm parameters evaluated for them were significantly lower than that of TTE, DM, mDM, LG-DM, G-DM, and TCG ($P < .05$). On the basis of these findings, 5 commonly used permeating cryoprotectants, glycerol, ethylene glycol, dimethyl sulfoxide, acetamide and propylene glycol have further been tested for their effectiveness on sperm cryopreservation in extenders of TTE, DM, mDM, LG-DM, G-DM, and TCG. The results showed that the sperm cryoprotective efficiencies of glycerol and ethylene glycol were similar and best among 5 permeating cryoprotectant treatments ($P > .05$). Dimethyl sulfoxide or acetamide resulted in average cryoprotection for cynomolgus monkey spermatozoa: poorer than glycerol or ethylene glycol but better than that of propylene glycol ($P < .05$). In addition, the action of permeating cryoprotectant appeared to be independent of extenders. The results in the present study demonstrate that 1) TTE, DM, mDM, LG-DM, G-DM, and TCG are excellent extenders and suitable for cynomolgus monkey sperm cryopreservation; 2) the mechanism of action of permeating cryoprotectants are not affected by extender composition; 3) ethylene glycol has a similar cryoprotective efficacy to glycerol that makes it a successful cryoprotectant for sperm cryopreservation in cynomolgus monkeys.

Key words: Sperm, freezing, motility, plasma membrane, acrosome, permeability coefficient.

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Sperm banking provides a useful way to maintain genetic resources for some endangered nonhuman primates (NHP) as well as to improve their breeding in captivity. The first attempt to cryopreserve spermatozoa of NHP was made several decades ago, since then, cryopreservation of spermatozoa has been reported for lemurs (Clavert et al, 1986), marmosets (Holt et al, 1994; Morrell, 1997; Morrell et al, 1998), squirrel (Denis et al, 1976), African green (Roussel and Austin, 1967), patas (Roussel and Austin, 1967), vervet (Seier et al, 1993; Conradie et al, 1994) monkeys; cynomolgus (Cho and Honjo, 1973; Mahone and Dukelow, 1978; Tollner et al, 1990; Sankai et al, 1994; Feradis et al, 2001; Li et al, 2003), Japanese (Sankai et al, 1997), lion-tailed (Cranfield et al, 1988), rhesus (Roussel and Austin, 1967; Leverage et al, 1972; Sanchez-Partida et al, 2000; Si et al, 2000, 2004; Si, 2004), stumptail (Roussel and Austin, 1967), and Tibetan (Chen et al, 1994) macaques; baboons (Kraemer and Vera Cruz, 1969); chimpanzees (Roussel and Austin, 1967; Gould and Styperek, 1989; Younis et al, 1998; Kusunoki et al, 2001); and gorillas (Lambert et al, 1991; Lanzendorf et al, 1992; Pope et al, 1997). However, only 5 of these primate species have been documented to produce offspring when frozen-thawed spermatozoa were

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Correspondence to: Prof Weizhi Ji, Kunming Institute of Zoology, Chinese Academy of Sciences, 32 Jiaochang Donglu, Kunming, Yunnan, 650223, China (e-mail: wji@mail.kiz.ac.cn).

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Table 1. *Extender composition (g/100 mL) and the corresponding glycerol concentration*

	TTE	DM	mDM	LG-DM	G-DM	TCG	TEST	TSM	Test-M	Test-H	LM
Tes	1.2	. . .	. . .	. . .	. . .	. . .	4.324	4.324	4.82	2.23	. . .
Tris	0.2	. . .	. . .	. . .	. . .	3.63	1.027	1.027	1.15	1.88	. . .
Lactose	2.0	11.0	10.0	5.5	. . .	. . .	. . .	. . .	. . .	. . .	. . .
Glucose	2.0	. . .	1.0	2.75	5.5	0.5	1.0	1.0	0.4	. . .	. . .
Fructose	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	0.17	. . .
Raffinose	0.2	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
Egg yolk, mL	20	20	10	10	10	20	30	30	20	20	20
Citric acid·H_2O	. . .	. . .	. . .	. . .	. . .	2.0	. . .	. . .	. . .	. . .	. . .
Sodium citrate·$2H_2O$	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	0.99	1.30
$NaHCO_3$	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	0.55
$K_2HPO_4·3H_2O$	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	0.042
Skim milk	. . .	. . .	. . .	. . .	. . .	. . .	. . .	2.0	. . .	. . .	. . .
Osmolality, mOsm/kg	377	425	407	365	344	376	731	825	728	665	294
Glycerol, mL*	5	4	4	4	4	5	3	3	5	6	7

* Final concentration.

artificially inseminated so far. This low efficiency may be related to the low fertility of cryopreserved spermatozoa, and the decrease is ascribed to the loss of sperm viability and functional impairment as a result of cryopreservation. During sperm freezing, the type of cryoprotective extender is one of the important factors affecting postthaw survival. At least 21 different extenders have been employed in previous studies for NHP sperm cryopreservation (Gould and Styperek, 1989; Chen et al, 1994; Morrell and Hodges 1998; Feradis et al, 2001) and the cryopreservation efficacy varied among laboratories. In addition, only 5 papers reported the simultaneous use of 2 or more different extenders in one study (Sadleir, 1966; Leverage et al, 1972; Tollner et al, 1990; Chen et al, 1994; Si et al, 2000). Until now, there were limited data on extender composition for sperm cryopreservation of primate animals, especially for cynomolgus monkey, one of the most widely used biomedical animal models.

Cryoprotectant is another factor that influences sperm survival during sperm cryopreservation. Although glycerol is a commonly used permeating cryoprotectant in NHP sperm freezing, its toxicity can cause loss in sperm motility and fertility. This encourages searching for other less toxic, effective cryoprotectants for sperm freezing. Recently, other permeating cryoprotectants have been employed in sperm cryopreservation for nonprimate mammalian species. For instance, dimethyl sulfoxide (DMSO) has been successfully used in sperm freezing for rabbit (Vicente and Viudes-de-Castro, 1996), rhinoceros (O'Brien and Roth, 2000), and mouse (Sztein et al, 2001); similar success have been obtained in ram (Molinia et al, 1994) and stallion (Mantovani et al, 2002) with ethylene glycol, as well as in rabbit with acetamide (Arriola and Foote, 2001). As for NHP, dimethyl sulfoxide, propylene glycol, and ethylene glycol have been tested in sperm cryopreservation for gorillas (Sadleir, 1966; Gould and Styperek, 1989), cynomolgus monkeys (Feradis et al, 2001), and rhesus monkeys (Si et al 2004). Even with one such certain cryoprotectant, the results are different between laboratories and species. Since different extenders were used to freeze spermatozoa in those studies, whether extender type affects the efficiencies of these permeating cryoprotectants is yet to be proved. However, no studies have compared the effect of cryoprotectants other than glycerol on sperm freezing using various extenders at the same time. In addition, no reports have been published on NHP sperm cryopreservation using acetamide as a permeating cryoprotectant.

In the present study, the comparison of 11 extenders for sperm cryopreservation has been made using cynomolgus monkey as a NHP model and several optimal extenders for cryopreservation have been sifted. On the basis of this, effects of 5 commonly used permeating cryoprotectants (ie, glycerol, ethylene glycol, dimethyl sulfoxide, propylene glycol, and acetamide) on sperm cryopreservation for this species have been examined.

Materials and Methods

All chemicals were obtained from Sigma Chemical Co (St Louis, Mo), unless indicated otherwise.

Media Preparation

The composition and osmolality of extenders as well as the concentrations of glycerol are presented in Table 1. Penicillin G (6.32 mg) and Streptomycin (sulfate) (5.0 mg) were added separately to each extender (100 mL), and the pH was adjusted to 7.0–7.2. The osmolality of extenders was measured using a freezing-point depression osmometer (Osmette A, 5002, Precision System Inc, Natick, Mass).

The extender was prepared as follows: fresh chicken eggs laid within 8 hours were purchased from a hennery for preparation of extender. Egg yolks were obtained with the procedure of Si et al (2000). For TCG preparation, egg yolks were mixed with a portion of Milli-Q water first, and then the mixture was cen-

trifuged at 7000 × *g* for 1 hour at 4°C to sediment yolk granules. The supernatants were extracted, and the remaining components were dissolved in water complemented accordingly. For other extenders, after dissolving all the ingredients in Milli-Q water, the egg yolk was added and mixed thoroughly and then the yolk granules were separated by centrifugation at 7000 × *g* for 1 hour. The supernatants were adjusted to pH 7.0–7.2 with 1 N NaOH or HCl when needed and used as extenders. The extenders were divided into 4-mL aliquots and stored at −30°C for no more than 2 weeks. Before an experiment, extenders were thawed in a 37°C water bath. The freezing extenders were made by adding cryoprotectants (glycerol, ethylene glycol, dimethyl sulfoxide, acetamide, propylene glycol) to extenders to obtain extenders with certain cryoprotectant concentrations.

Semen Collection

Four sexually mature male cynomolgus monkeys, aged 5–11 years, provided by the Laboratory Animal Center of the Kunming Institute of Zoology, were used for collecting semen. The animals were individually caged and kept with lights on from 0600 to 1800 hours at a temperature of 20°C–25°C. Each animal was anaesthetized with ketamine hydrochloride (Xingang Co, Shanghai, China) using 5 mg/kg body weight intramuscularly and stimulated with penile electro-ejaculation procedure elaborated in our laboratory (Yang et al, 1994). Semen was collected into a disposable plastic test tube containing 2 mL of prewarmed TALP-Hepes (Bavister et al, 1983). The diluted semen was kept at 37°C water bath for 30 minutes to allow the clot to liquefy. After liquefaction, the semen was transferred into a 15-mL disposable plastic tube. A small sample was taken to examine sperm motility, plasma membrane, and acrosomal integrity (see below), the rest was washed twice with 9 mL TALP-Hepes and centrifuged at 200 × *g* for 10 minutes. The supernatant was aspirated and the sperm pellet was dispersed and mixed with a Pasteur pipette before freezing.

Experimental Design

Two experiments were performed within this study. In experiment 1, a total of 10 ejaculates (2–3 ejaculates per male) were studied to compare the efficiency of 11 extenders on sperm cryopreservation in which glycerol was used as cryoprotectant. In experiment 2, a total of 8 ejaculates (2 ejaculates per male) were used in a 6 × 5 factor experiment (6 extenders × 5 cryoprotectants) to investigate effects of permeating cryoprotectant on sperm cryopreservation in which the final concentration of each cryoprotectant was 5% (vol/vol).

Sperm Freezing and Thawing

Before freezing, washed spermatozoa were divided into several aliquots according to experiment design. The procedure of sperm freezing was that described by Sankai et al (1994). Briefly, each portion of spermatozoa was diluted with 1 of the extenders without cryoprotectant and kept at 4°C (water bath) for 2 hours. An equal volume of precooled (4°C) corresponding extender containing cryoprotectant was added stepwise (5 times) at intervals of 6–7 minutes within 30 minutes. Spermatozoa were equilibrated in extenders containing cryoprotectant at 4°C for another 30 minutes. Just before the end of equilibration, spermatozoa were drawn into 0.25-mL plastic straws (IMV, L'Aigle, France) with a syringe, sealed with straw heater (Tew Impulse Sealer, Tish-200, Tew Electric Heating Equipment Co Ltd, Taiwan, ROC). Straws were frozen in liquid nitrogen vapor within 10 minutes and plunged into liquid nitrogen and stored for at least 7 days before thawing. For thawing, straws were placed in a 37°C water bath for 2 minutes.

Examination of Sperm Motility and Motility Recovery

Sperm Motility—With a prewarmed hemocytometer counting chamber, sperm samples were assessed for percentage of progressive forward motility by counting 200 spermatozoa, in duplicates. All samples were evaluated by the same operator, who did not know the identity of the sperm samples offered.

Sperm Motility Recovery Rate—The sperm motility recovery rate was calculated by comparing the motility of prefreeze (M_{pr}) and postthaw (M_{ps}) spermatozoa. If M_{pr} and M_{ps} are the sperm motility percentages before and after freezing, then the recovery rate would be $M_{ps}/M_{pr} \times 100\%$.

Examination of Sperm Plasma Membrane and Acrosomal Integrity

Sperm Plasma Membrane Integrity—Sperm membrane integrity was measured by means of a dual DNA staining technique using Hoechst 33342 (H342) and propidium iodide (PI), through use of flow cytometry and excited simultaneously by a single ultraviolet laser. Briefly, 6 μL H342 (1 mg/mL) and 8 μL PI (1 mg/mL) were added to each sperm sample (1 mL). Each sperm suspension was mixed 3 times by gently pipetting and incubated in a 37°C water bath for 15 minutes. After incubation, all samples were processed for flow cytometric analysis.

Sperm Acrosomal Integrity—Acrosome status was determined by fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA), and the acrosome staining process was that described by Esteves et al (2000). Under a fluorescence microscope, spermatozoa with intact acrosome showed uniform apple-green fluorescence in the acrosomal region of the sperm head, while acrosome-reacted spermatozoa showed little or no green fluorescence in the anterior part of the head. A minimum of 200 spermatozoa were counted for each sample.

Statistical Analysis

All data are expressed as mean ± SD. Data of sperm motility, membrane integrity, and acrosomal integrity were subjected to arcsine square root transformation and analyzed by ANOVA and Fisher protected least significant difference (LSD) test. $P < .05$ was considered as statistically significant.

Results

Experiment 1: Effects of Different Extenders on Cryopreservation

Frozen-thawed sperm motility and motility recovery rate are shown in Table 2. The TTE, DM, mDM, LG-DM, G-DM, and TCG extenders yielded the highest sperm motility and motility recovery rates among 11 groups, and

Table 2. *Postthaw motility and motility recovery rate for cynomolgus monkey spermatozoa cryopreserved in different extenders*

Extender Type	Prefreeze Sperm Motility, %	Postthaw Sperm Motility, %*	Motility Recovery Rate, %*
TTE		50.10 ± 6.35[a]	61.38 ± 3.60[a]
DM		47.10 ± 5.64[a]	57.74 ± 4.06[a]
mDM		47.88 ± 6.88[a]	58.58 ± 3.92[a]
LG-DM		48.21 ± 4.54[a]	59.32 ± 4.74[a]
G-DM	81.51 ± 7.70	46.90 ± 4.97[a]	57.57 ± 3.42[a]
TCG		50.24 ± 5.27[a]	61.65 ± 3.29[a]
TEST		16.14 ± 2.87[b]	20.01 ± 4.28[b]
TSM		8.75 ± 3.18[c]	10.93 ± 4.55[c]
Test-M		13.58 ± 4.72[b]	16.67 ± 5.65[b]
Test-H		23.24 ± 5.49[d]	28.50 ± 5.87[d]
LM		5.96 ± 2.93[e]	7.22 ± 3.32[e]

* Groups with different superscript letters in the same column are significantly different ($P < .05$).

there were no significant differences among these 6 groups ($P > .05$). Sperm cryopreservation efficacy for TEST, TSM, Test-M, Test-H, and LM was poorer than that of TTE, DM, mDM, LG-DM, G-DM, and TCG groups. Additionally, sperm motility with TEST, TSM, Test-M, Test-H, and LM was less than 30%, although significant difference existed among them ($P < .05$) except for TEST and Test-M.

Plasma membrane integrity of frozen-thawed sperm for all treatments is given in Figure 1. The percentages of spermatozoa with intact membrane for TTE, DM, mDM, LG-DM, G-DM, and TCG were significantly higher than other extenders ($P < .05$) and no significant difference was found among them ($P > .05$). Among the lower membrane integrity groups of TEST, TSM, Test-M, Test-H, and LM, the ability of cryoprotection on sperm membrane for these extenders is as follows: Test-H provides greater cryprotection than TEST and Test-M, which provide greater cryprotection than TSM and LM ($P < .05$).

Acrosomal integrity of frozen-thawed sperm for 11 extenders is presented in Figure 2. Spermatozoa cryopreserved with TTE, DM, mDM, LG-DM, and G-DM showed similar and highest acrosomal integrity in all groups. Unlike sperm membrane integrity, cryopreservation in TCG yielded lower percentage of acrosomal intact spermatozoa than in TTE, DM, mDM, LG-DM, and G-DM ($P < .05$), but this acrosome value of TCG was significantly higher than those of TEST, TSM, Test-M, Test-H, and LM ($P < .05$). The TEST, TSM, Test-M, Test-H, and LM resulted in lower percentage of acrosome integrity as compared with TTE, DM, mDM, LG-DM, G-DM, and TCG ($P < .05$). Additionally, there was no statistical difference for acrosomal integrity among TSM, Test-M, and Test-H, neither was there difference between TEST and Test-M ($P > .05$). LM yielded the lowest acrosomal integrity in all treatments ($P < .05$).

Experiment 2: Effects of Permeating Cryoprotectant on Cryopreservation

Postthaw sperm motility, membrane integrity, and acrosomal integrity for spermatozoa cryopreserved in different cryoprotective solutions are shown in Table 3.

The results demonstrated that in general, sperm motility, membrane integrity, and acrosomal integrity for glycerol or ethylene glycol were highest among all groups ($P < .05$). Sperm parameters for dimethyl sulfoxide or acetamide were similar and lower than glycerol or ethylene glycol but higher than that of propylene glycol ($P < .05$). The percentages of the 3 sperm parameters for propylene glycol were lowest in all treatments ($P < .05$). In addition, the percentage of acrosomal integrity for TCG-glycerol was statistically lower than those of TCG-ethylene

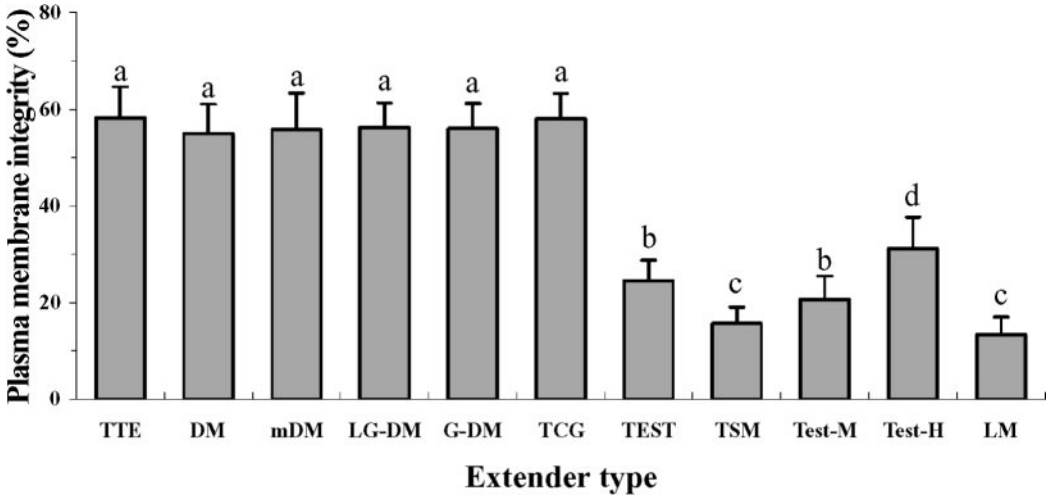

Figure 1. Postthaw plasma membrane integrity for spermatozoa cryopreserved in different extenders (a, b, c, d: Bars with different letters are significantly different [$P < .05$]).

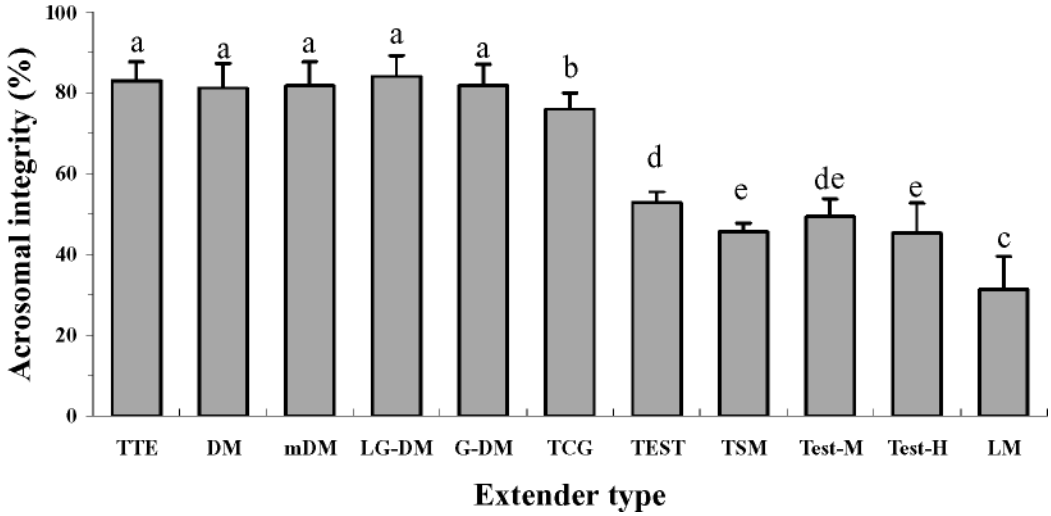

Figure 2. Postthaw acrosomal integrity for spermatozoa cryopreserved in different extenders (a, b, c, d, e: Bars with different letters are significantly different [$P < .05$]).

glycol and other extender groups of glycerol or ethylene glycol ($P < .05$).

Discussion

In previous studies, most of the 11 extenders evaluated in this study were used to cryopreserve spermatozoa for several species of NHP except for Test-H, which was applied to human sperm freezing, and almost all those extenders have been reported to be cryoprotective for spermatozoa. In the present study, however, the efficiencies of these extenders proved to be quite different for cynomolgus monkey spermatozoa. Our results demonstrate that cynomolgus monkey spermatozoa cryopreserved with TTE, DM, mDM, LG-DM, G-DM, and TCG exhibited significantly higher postthaw motility, membrane integrity, and acrosomal integrity than those frozen with TEST, TSM, LM, Test-M, and Test-H, the motilities of which were less than 30%. Additionally, no significant differences were found for postthaw sperm parameters among the groups of TTE, DM, mDM, LG-DM, G-DM, and TCG except that sperm acrosomal integrity for TCG was lower than for the others ($P < .05$). One explanation for good cryoprotection with TTE, DM, mDM, LG-DM, G-DM, and TCG may be the moderate hyperosmolality (344–425 mOsm) that cynomolgus monkey sperm can tolerate. The other explanation may be that some sugars are included in these extenders, which may protect sperm cells by stabilizing their membrane and/or avoiding intracellular ice formation through cell dehydration. The results indicate that TTE, DM, mDM, LG-DM, G-DM, and TCG extenders are recommended for cynomolgus monkey sperm cryopreservation.

Sanchez-Partida et al (2000) obtained 4 offspring of rhesus monkey with spermatozoa cryopreserved in TCG extender and glycerol. They reported as high as 85% of postthaw motility, which was not significantly different compared with fresh sperm. However, the acrosomal integrity and its recovery in frozen-thawed spermatozoa in that study was only 75% and 79.94%, respectively. In our study, the corresponding data for acrosome with TCG were 76% and 81.1%, respectively, consistent with that of Sanchez-Partida et al (2000), but postthaw sperm motility in this study was much lower than that. According to Sanchez-Partida et al (2000), different freezing rates (liquid nitrogen vapor vs dry ice) might be a major reason. There were no significant differences in postthaw motility and membrane integrity for spermatozoa cryopreserved with TCG, TTE, DM, mDM, LG-DM, and G-DM ($P > .05$), but the acrosomal integrity for TCG differed, slightly but significantly, from the other treatments ($P < .05$). In addition, rapid motility, somewhat like hyperactivity, could be observed for spermatozoa frozen with TCG but not with other extenders in this study. Therefore, we infer that a proportion of sperm population undergo capacitation or acrosome reaction after thawing when spermatozoa are frozen with TCG, which will in turn reduce the rate of acrosomal integrity.

Tollner et al (1990) used TEST and TSM to freeze cynomolgus monkey spermatozoa, and postthaw sperm motilities were 56% ± 3% and 67% ± 2%, respectively. Afterward, a baby monkey was born after artificial insemination with spermatozoa frozen in TSM. In our study, the postthaw motility of spermatozoa frozen with these 2 extenders was extremely low (16.1% ± 2.9% vs 8.8% ± 3.2%). The time and temperature of equilibration with glycerol are the 2 main differences between our study and

Table 3. *Postthaw motility, membrane integrity, and acrosomal integrity of spermatozoa cryopreserved in different cryoprotective solutions*

Cryoprotective Solution*	Motility, %†	Membrane Integrity, %†	Acrosomal Integrity, %†
Fresh sperm	76.00 ± 6.11	83.27 ± 5.83	91.09 ± 5.05
TTE			
Gly	48.47 ± 7.65[a]	55.68 ± 7.88[a]	81.52 ± 6.39[a]
EG	45.53 ± 6.11[a]	54.63 ± 7.73[a]	81.92 ± 5.97[a]
DMSO	23.76 ± 7.52[b]	33.73 ± 7.00[b]	68.12 ± 3.08[b]
Ac	22.62 ± 7.58[b]	32.52 ± 8.17[b]	67.04 ± 2.82[b]
PROH	8.55 ± 6.01[c]	22.59 ± 6.43[c]	70.00 ± 4.66[b]
DM			
Gly	48.86 ± 6.22[a]	55.83 ± 6.40[a]	80.59 ± 4.77[a]
EG	46.03 ± 5.34[a]	55.21 ± 6.56[a]	80.93 ± 5.52[a]
DMSO	23.75 ± 6.98[b]	33.74 ± 6.54[b]	67.95 ± 3.16[b]
Ac	23.45 ± 7.52[b]	33.23 ± 7.56[b]	66.84 ± 3.72[b]
PROH	8.41 ± 5.41[c]	22.56 ± 5.85[c]	70.10 ± 4.00[b]
mDM			
Gly	48.14 ± 7.52[a]	55.25 ± 7.67[a]	81.04 ± 5.76[a]
EG	45.84 ± 6.75[a]	54.98 ± 8.03[a]	81.50 ± 5.71[a]
DMSO	23.52 ± 7.53[b]	33.42 ± 7.33[b]	68.06 ± 3.10[b]
Ac	23.14 ± 7.03[b]	32.63 ± 7.69[b]	67.17 ± 3.04[b]
PROH	8.05 ± 5.19[c]	22.00 ± 5.83[c]	69.67 ± 4.25[b]
LG-DM			
Gly	48.93 ± 7.08[a]	55.91 ± 7.38[a]	81.91 ± 6.22[a]
EG	46.33 ± 5.67[a]	55.61 ± 7.02[a]	84.61 ± 5.04[a]
DMSO	24.15 ± 7.10[b]	34.55 ± 6.62[b]	68.32 ± 3.16[b]
Ac	23.33 ± 7.49[b]	33.55 ± 7.96[b]	67.58 ± 4.44[b]
PROH	8.63 ± 5.39[c]	22.84 ± 5.66[c]	70.06 ± 3.59[b]
G-DM			
Gly	47.89 ± 6.31[a]	54.68 ± 6.63[a]	80.52 ± 5.53[a]
EG	45.13 ± 5.23[a]	54.34 ± 6.37[a]	80.74 ± 5.68[a]
DMSO	23.39 ± 8.07[b]	33.15 ± 7.03[b]	68.01 ± 3.48[b]
Ac	22.55 ± 7.75[b]	32.33 ± 8.01[b]	66.84 ± 4.71[b]
PROH	8.41 ± 5.73[c]	22.33 ± 6.06[c]	70.04 ± 4.11[b]
TCG			
Gly	48.61 ± 6.87[a]	55.44 ± 7.18[a]	75.03 ± 5.68[c]
EG	46.03 ± 6.22[a]	54.92 ± 7.46[a]	80.67 ± 5.25[a]
DMSO	23.42 ± 7.61[b]	33.98 ± 7.46[b]	67.73 ± 2.90[b]
Ac	22.86 ± 7.87[b]	33.11 ± 7.96[b]	66.92 ± 4.44[b]
PROH	8.31 ± 5.35[c]	22.20 ± 5.61[c]	69.89 ± 3.95[b]

* Gly indicates glycerol; EG, ethylene glycol; DMSO, dimethyl sulfoxide; Ac, acetamide; and PROH, propylene glycol.

† Groups with different superscript letters in the same column are significantly different ($P < .05$).

that of Tollner et al (1990). In the present study, spermatozoa were equilibrated in glycerol extender for 0.5 hours, while in the study of Tollner et al (1990) the duration was 2 hours. The equilibration temperatures in 2 studies were 4°C vs room temperature, respectively. It seems that these 2 differences may partially account for the conflicting results. What needs to be noted is that the osmolalities of TEST and TSM before addition of glycerol were as high as 731 mOsm and 825 mOsm when prepared according to the reported formula. These considerably high osmolalities might exceed the osmolality limit that monkey sperm cells can tolerate, and this might cause an irreversible damage to these cells. Sperm viability parameters increased dramatically when the osmolalities of these 2 extenders were reduced to around 400 mOsm (data not shown). Therefore, we maintain that hyperosmolality of TEST and TSM may be the major reason for the poor results in the present study.

Using LM extender and programmed freezing procedure, Leverage et al (1972) reported 68% postthaw motility recovery of rhesus monkey spermatozoa. However, the corresponding rate was only 7.22% in this study. Different concentrated salt ions were included in LM, and this may be deleterious to freezing of cynomolgus monkey spermatozoa. In the freezing of bull (Yassen and Foote, 1967), mouse (An et al, 2000), and rhesus monkey (Si, 2004) spermatozoa, postthaw sperm motility decreased dramatically if spermatozoa were exposed to salt ions. Our study confirms these findings, but the mechanisms of this remain unclear, maybe Mazur's 2 factor hypothesis would explain this (Mazur et al 1972). Additionally, the converse result may be partially due to different freezing procedures and primate species used in the 2 studies.

Marmoset and human spermatozoa were diluted and frozen with Test-M and Test-H in previous studies (Weidel and Prins, 1987; Morrell 1997). The results given by Test-M and Test-H in the present study were poorer than those studies. Test-M and Test-H also possessed high osmolalities of 728 and 665 mOsm prepared according to the published formulas. We suggest the hyperosmolality might be a major reason for our poor data; however, differences in species and freezing procedure may also exist.

Although glycerol is most widely used in sperm cryopreservation for primate animals, its cytotoxicity should not be ignored. Recently, some researchers have reported the success of sperm cryopreservation in a few NHP animals with other permeating cryoprotectants, stimulating the study of these for NHP. However, limited information indicated that the efficiencies of nonglycerol cryoprotectants varied in studies of NHP sperm cryopreservation. Using glycerol, dimethyl sulfoxide, and propylene glycol, Feradis et al (2001) cryopreserved epididymal spermatozoa from cynomolgus monkeys and discovered that glycerol had similar effectiveness to that of dimethyl sulfoxide, while the cryoprotection provided by propylene glycol was considerably poorer. On the contrary, Sadleir (1966) found glycerol to be better than dimethyl sulfoxide for cryopreserving chimpanzee spermatozoa. Besides, Gould and Styperek (1989) reported a failure of in vitro fertilization with spermatozoa cryopreserved with dimethyl sulfoxide.

Comparing glycerol, ethylene glycol, dimethyl sulfox-

ide, and propylene glycol for rhesus monkey sperm cryopreservation, Si et al (2004) found that both glycerol and ethylene glycol could offer good protection for this species spermatozoa and there was no significant difference between them, but sperm parameters in case of dimethyl sulfoxide and propylene glycol were significantly worse.

A lot of variability, which is supposed to be attributed to the different results, has been found in these reports, and the type of the extender is probably the main difference that can be readily controlled. To investigate whether extender composition influences the role of permeating cryoprotectant, cynomolgus monkey spermatozoa have been cryopreserved in TTE, DM, mDM, LG-DM, G-DM, and TCG, with glycerol, ethylene glycol, dimethyl sulfoxide, acetamide, and propylene glycol as permeating cryoprotectants.

The results of this study indicate that glycerol and ethylene glycol yield the best and similar cryoprotection for cynomolgus monkey spermatozoa. Dimethyl sulfoxide and acetamide are significantly worse in efficiency than glycerol and ethylene glycol but better than propylene glycol. In addition, no significant difference was found between dimethyl sulfoxide and acetamide. Propylene glycol resulted in the poorest protection for spermatozoa among these 5 cryoprotectants. These findings are basically consistent with those of Si et al (2004). Besides, the efficacy of dimethyl sulfoxide is similar to that described by Sadleir (1966) and Gould and Styperek (1989), but disagrees with the result of Feradis et al (2001). This may be due to different sperm source and equilibration procedures in the 2 studies. Furthermore, our study demonstrates that the effects of permeating cryoprotectants are independent of extender type but probably only related to their own nature, such as their chemical structure and/or physical characters. Hence, spermatozoa from different species and sources may have different responses to these permeating cryoprotectants. Additionally, different freezing techniques may interfere with different cryoprotectants.

From these points, we can see that the results of sperm cryopreservation in monkeys might greatly vary because of variation among semen donor individuals and different semen collection methods. This might be one of the reasons why it is not easy to repeat the results of other teams.

The mechanisms of permeation of cryoprotectants into sperm are still not known. According to Gilmore et al (1997), the optimal cryoprotectant for human sperm is one that can permeate the cell in the shortest period of time, causing the least amount of volume excursion during its addition and removal. Therefore, the cryoprotective action of a cryoprotectant depends on its permeability coefficient. According to the study of Gao et al (1995), approximately 60% of human spermatozoa exposed to glycerol will lose their motility when their volume decreases to 0.68 times or exceeds 1.38 times their iso-osmotic volume.

From the report of Gilmore et al (1997) we can see a great disparity in values of solute and water permeability for dimethyl sulfoxide and propylene glycol. When spermatozoa are exposed to the 2 solutions, great sperm volume excursion, exceeding the limits that human sperm can tolerate, will occur. In this way, the rate of sperm survival reduces considerably. On the contrary, the solute and water permeability for glycerol or ethylene glycol match well enough to avoid great volume excursion, so they can protect sperm cells effectively. Combining our experiment, we suggest that the permeability coefficients of glycerol, ethylene glycol, dimethyl sulfoxide, acetamide, and propylene glycol are similar to cynomolgus monkey and human spermatozoa, so the survival percentage of spermatozoa cryopreserved with glycerol or ethylene glycol is significantly higher than those with dimethyl sulfoxide or propylene glycol. On the other hand, there was no significant difference in motility, membrane integrity, and acrosomal integrity for spermatozoa cryopreserved with glycerol or ethylene glycol. In addition, as a permeating cryoprotectant, acetamide may take its action in the same way as the others, and probably its permeability coefficient is an unmatched value that resulted in poor cryopreservation in our study.

In the present study, plasma membrane was damaged dramatically in spermatozoa cryopreserved with propylene glycol, but this damage was less compared with the damage of motility. This finding implies that apart from head plasma membrane, other compartments of sperm regarding sperm motility, such as tail membrane and mitochondria, may be damaged more severely. Another surprising finding is that there was no significant difference in acrosomal integrity for spermatozoa cryopreserved either with propylene glycol, dimethyl sulfoxide, or acetamide. This finding indicates that the sperm acrosome is the most resistant to freezing compared with motility or plasma membrane; meanwhile, it indicates indirectly that sperm motility is the most convincing parameter for evaluation of sperm function.

In conclusion, the present study confirms the following: 1) TTE, DM, mDM, LG-DM, G-DM, and TCG are excellent extenders and suitable for sperm cryopreservation of cynomolgus monkey. 2) The finding that TEST, TSM, Test-M, and Test-H are unable to protect cynomolgus monkey spermatozoa during freezing may be due to the hyperosmolality these extenders possess, which exceeds the sperm cell toleration limit. Few spermatozoa can survive the freezing with LM; this may result from its concentrated salt ions. 3) Ethylene glycol and glycerol exhibited the best cryoprotective ability for cynomolgus monkey spermatozoa. Dimethyl sulfoxide and acetamide resulted in worse effectiveness than ethylene glycol or

glycerol, and propylene glycol yielded the worst results. This is probably because dimethyl sulfoxide, acetamide, and propylene glycol have unmatched coefficients that make sperm cells shrink or expand so greatly that they are irreversibly damaged.

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