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·*Original Article*·

Cryopreservation of cynomolgus monkey (*Macaca fascicularis*) spermatozoa in a chemically defined extender

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Abstract

Aim: To establish a method for cynomolgus monkey sperm cryopreservation in a chemically defined extender. **Methods:** Semen samples were collected by electro-ejaculation from four sexually mature male cynomolgus monkeys. The spermatozoa were frozen in straws by liquid nitrogen vapor using egg-yolk-free Tes-Tris (mTTE) synthetic extender and glycerol as cryoprotectant. The effects of glycerol concentration (1 %, 3 %, 5 %, 10 % and 15 % [v/v]) and its equilibration time (10 min, 30 min, 60 min and 90 min) on post-thaw spermatozoa were examined by sperm motility and sperm head membrane integrity. **Results:** The post-thaw motility and head membrane integrity of spermatozoa were significantly higher ($P < 0.05$) for 5 % glycerol (42.95 ± 2.55 and 50.39 ± 2.42, respectively) than those of the other groups (1 %: 19.19 ± 3.22 and 24.84 ± 3.64; 3 %: 34.23 ± 3.43 and 41.37 ± 3.42; 10 %: 15.68 ± 2.36 and 21.39 ± 3.14; 15 %: 7.47 ± 1.44 and 12.90 ± 2.18). The parameters for 30 min equilibration (42.95 ± 2.55 and 50.39 ± 2.42) were better ($P < 0.05$) than those of the other groups (10 min: 31.33 ± 3.06 and 38. 98 ± 3.31; 60 min: 32.49 ± 3.86 and 40.01 ± 4.18; 90 min: 31.16 ± 3.66 and 38.30 ± 3.78). Five percent glycerol and 30 min equilibration yielded the highest post-thaw sperm motility and head membrane integrity. **Conclusion:** Cynomolgus monkey spermatozoa can be successfully cryopreserved in a chemically defined extender, which is related to the concentration and the equilibration time of glycerol. *(Asian J Androl 2005 Jun; 7: 139–144)*

Keywords: cryopreservation; *Macaca fascicularis*; chemically defined extender; glycerol

1 Introduction

Although spermatozoa of many mammalian species can be frozen successfully, a high proportion of spermatozoa lose their motility or other functions after thawing due to damage during the freezing-thawing process and the recovery of motile spermatozoa is less than 50 % in most mammals [1]. This low freezing efficiency may be due to the lack of information on the mechanisms of sperm injury during cryopreservation and cryoprotective mechanisms of certain compounds.

Several hypotheses such as cold shock, osmotic stress, ice crystal formation [2] and oxidative damage [3] have been put forward to explain the cause of sperm cryoinjury, but the mechanisms of cryodamage largely remain unclear. Therefore, sperm cryopreservation has

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remained empirical and, at present, even with the most advanced equipment and the optimal freezing protocol, only about 50 % of spermatozoa can survive cryopreservation.

Glycerol is a preferable cryoprotectant for sperm freezing in most mammals. Additionally, complex agents such as egg yolk, skim milk, milk and even serum are used in sperm freezing extenders for many different species in order to provide maximal cryoprotection for spermatozoa. As complex components of freezing extenders such as egg yolk contain a large number of undefined molecules, it is difficult to analyze the roles of a particular compound played in sperm preservation. In addition, these complex compounds not only contain potential infectious agents (viruses, bacteria, fungi, prions) but are with difficultly-controlled quality. Therefore, a chemically defined extender would be helpful to understand the mechanism of both sperm cryodamage and cryoprotection. Previous studies have described how the spermatozoa of mouse [4, 5], goat [6] and man [7] were successfully frozen in a chemically defined extender, but not yet those of nonhuman primates.

Sperm motility and functional intact membrane are interrelated and both are required for fertilization [8]. Sperm membranes may be irreversibly damaged by the process of freezing-thawing that leads to a marked decline in sperm motility.

In the present study, efforts have been made to develop a method for the cryopreservation of the cynomolgus monkey sperm in a synthetic extender. Frozen-thawed sperm function was evaluated by sperm motility and sperm head membrane integrity, which are the two important factors related to fertilization.

2 Materials and methods

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

2.1 Dilution and freezing extenders

The extender for dilution of cynomolgus monkey sperm was based on Tes-Tris-egg yolk (TTE) [9], but in this study it did not contain egg yolk and was abbreviated to mTTE (Table 1). The extender was prepared as follows: after dissolving all the ingredients in Milli-Q water and adjusting the pH to 7.0–7.2 with 1 mol/L NaOH or HCl, the extender was divided into 4-mL aliquots and stored at 30 ºC for no more than 2 weeks and thawed in a 37 ºC water bath before use. The freezing extender was made by adding glycerol to the mTTE to reach final concentrations of 1 %, 3 %, 5 %, 10 % and 15 % (v/v), respectively.

2.2 Semen collecting and processing

Four sexually mature male cynomolgus monkeys, aged 5–11 years, were provided by the Laboratory Animal Center of the Kunming Institute of Zoology. The animals were individually caged with lights on from 06:00 to 18:00 at a temperature of 20–25 ºC. Animals were anesthetized using 5 mg of ketamine hydrochloride (Xin-Gang Co. Shanghai, China)/kg body weight i.m. and stimulated with penile electrodes. Ejaculated semen was collected into a disposable plastic test tube containing 2 mL of pre-warmed Tyrode's albumin lactate pyruvate (TALP)-Hepes [10]. The diluted semen was kept at 37 ºC water bath for 30 min to allow liquefaction. After that, the semen was transferred into a 15-mL disposable plastic tube. A small sample was taken to examine the sperm motility and head membrane integrity (see below) and the rest was washed twice with TALP-Hepes at a rate of 1:9 (1 part of semen for 9 parts of TALP-Hepes) and centrifuged at 200 × *g* for 10 min. The supernatant was aspirated and the sperm pellet was dispersed and mixed with a Pasteur pipette. Volume and concentration of sperm were determined, only ejaculates with a concentration of 2.0–4.0 × 10^9 sperm/mL and containing ≥ 70 % motile spermatozoa were used for freezing.

2.3 Freezing and thawing of spermatozoa

The washed spermatozoa were diluted 1:9 slowly in mTTE and kept at room temperature (12–18 °C) for 60 min. An equal volume of mTTE containing glycerol was added in steps at 6–7 min intervals at room temperature within 30 min to reach a final glycerol concentration of

Table 1. Composition of mTTE for dilution of cynomolgus monkey spermatozoa.

Components	Concentration (g/L)
Tes	12
Tris-HCl	2
Glucose	20
Lactose	20
Raffinose	2
Penicillin-G	100 000 IU
Streptomycin sulfate	0.05

1 %, 3 %, 5 %, 10 % and 15 %, respectively.

The spermatozoa were equilibrated in the glycerol extender for some period and the equilibration times tested at room temperature, were 10, 30, 60 and 90 min in mTTE extender containing 5 % glycerol, respectively. After equilibration, spermatozoa were drawn into 0.25-mL plastic straws (IMV, L'Aigle, France), sealed with a straw heater (Tew Impulse Sealer, Tish-200, Tew Electric Heating Equipment Co. Ltd., Taiwan) and then kept in 4 °C for 45 min. Straws were frozen by putting them horizontally on a rack 3 cm above the surface of liquid nitrogen. Ten minutes later, they were submerged directly into liquid nitrogen (LN_2) for storage.

After storage in LN_2 for more than 7 days, straws containing frozen spermatozoa were placed in a 37 °C water bath for 2 min for thawing. Thawed sperm suspension for each straw was diluted in a disposable sterile test tube with 1.25 mL pre-warmed TALP-Hepes, which was added in five parts at 30 s intervals. The diluted sperm was washed twice by centrifugation at 200 × *g* for 10 min, and the sperm pellet was dispersed immediately.

2.4 Examination of sperm motility and head membrane integrity

2.4.1 Sperm motility

Using a pre-warmed hemocytometer counting chamber, fresh and thawed sperm samples were assessed for percentage of forward progressive motility by counting 200 spermatozoa in duplicates. This evaluation was conducted by another operator who did not know the identity of the sperm samples offered.

2.4.2 Sperm motility recovery rate

The sperm motility recovery rate was calculated by comparing the motilities of pre-freeze (M_{pr}) and post-thaw (M_{ps}) spermatozoa. If M_{pr} and M_{ps} are the sperm motility percentages before and after freezing, then the recovery rate would be: $M_{ps}/M_{pr} \times 100$ %.

2.4.3 Sperm head membrane integrity

Sperm head membrane integrity was measured by counting non-damaged spermatozoa, defined as non-stained by Hoechst 33258, as described by Cross *et al.* [11]. Shortly, 1 μL of 0.5 mg/mL Hoechst 33258 was added to 500 μL fresh or thawed sperm specimen (diluted with warm TL-Hepes without protein) and stained at 37 °C for 10 min in the dark. Then, spermatozoa were centrifuged at 250 × *g* for 5 min to remove excess stains. The sperm pellet was resuspended in 100 μL pre-warmed TL-Hepes; 10–20 μL of the suspension was smeared on a microscope slide with a cover slip. Under ultraviolet (UV) light, membrane intact spermatozoa showed little or no blue fluorescence in the head, while spermatozoa with damaged membrane showed bright blue fluorescence. At least 200 spermatozoa were scored for each sample and the identities of the samples were hidden from the observer to obtain an objective assessment.

2.5 Statistical analysis

A total of 16 ejaculates (four ejaculates per male) were studied and experiments were conducted with $n = 16$ replicates. All data are expressed as mean ± SD. Data of sperm motility and head membrane integrity were subjected to arcsine square root transformation and analyzed by ANOVA and Fisher's protected least significant difference (LSD) test. Differences were considered statistically significant at $P < 0.05$.

3 Results

3.1 Effect of glycerol concentration

The effect of glycerol concentration on post-thaw sperm motility and sperm head membrane integrity is shown in Table 2 and Figure 1. With the increase of glycerol concentrations from 1 % to 5 %, the parameters of thawed spermatozoa rose significantly ($P < 0.05$). However, all the examined values of sperm decreased significantly when glycerol concentration increased from 5 % to 15 % ($P < 0.05$). The highest percentages of sperm motility and head membrane integrity were achieved when the spermatozoa were frozen in mTTE extender containing 5 % glycerol ($P < 0.05$).

Table 2. Effect of glycerol concentration on post-thaw sperm motility. Values with different letters(superscripts) within a column are significantly different ($P < 0.05$).

		Sperm motility (%)	Motility recovery rate (%)
Pre-freeze sperm		77.26 ± 3.79	
Glycerol concentration (v/v)	1 %	19.19 ± 3.22[a]	24.83 ± 4.11[a]
	3 %	34.23 ± 3.43[b]	44.34 ± 4.28[b]
	5 %	42.95 ± 2.55[c]	55.58 ± 1.64[c]
	10 %	15.68 ± 2.36[d]	20.32 ± 3.24[d]
	15 %	7.47 ± 1.44[e]	9.64 ± 1.65[e]

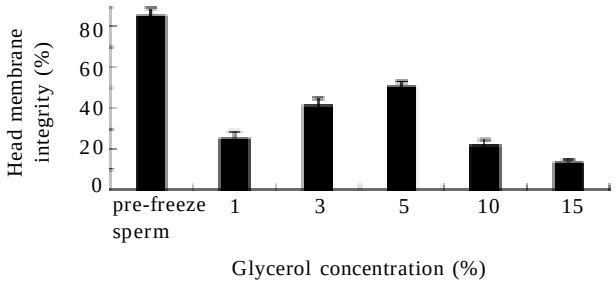

Figure 1. Effect of glycerol concentration on post-thaw sperm head membrane integrity. Bars with different letters are significantly different ($P < 0.05$).

3.2 Effect of equilibration time

The effect of equilibration time on post-thaw sperm motility and head membrane integrity is presented in Table 3 and Figure 2. The highest sperm motility and head membrane integrity data were achieved when the spermatozoa were equilibrated in mTTE extender containing 5 % glycerol for 30 min, more than those of the other groups ($P < 0.05$). There was no significant difference for sperm parameters among the 10, 60 and 90 min groups ($P > 0.05$).

4 Discussion

TTE extender and the simple "straw freezing" procedure were successfully used for cryopreservation of cynomolgus monkey spermatozoa [9]. In the present study, TTE was modified by omitting egg yolk to obtain a chemically defined medium, in which glycerol concentration was tested and the cryopreservation protocol (duration of exposure to mTTE, equilibration temperature in glycerol) had been improved accordingly.

Table 3. Effect of equilibration time on post-thaw sperm motility. Values with different letters (superscripts) within a column are significantly different ($P < 0.05$).

		Sperm motility (%)	Motility recovery rate (%)
Pre-freeze sperm		77.26 ± 3.79	
Equilibration time (min)	10	31.33 ± 3.06[a]	40.62 ± 4.15[a]
	30	42.95 ± 2.55[b]	55.58 ± 1.64[b]
	60	32.49 ± 3.86[a]	42.12 ± 5.01[a]
	90	31.16 ± 3.66[a]	40.37 ± 4.75[a]

Glycerol has been widely used as a cryoprotectant for freezing mammalian spermatozoa to protect them against cryoinjury, but it can also damage sperm cells at high concentration, depending on different species. For example, marsupial spermatozoa can tolerate as high as 10 %–20 % glycerol, whereas mouse spermatozoa will be dramatically damaged if the glycerol proportion exceeds 1.75 % [12]. For nonhuman primate spermatozoa, the concentration of glycerol used in sperm cryopreservation varied in a range of 3 %–14 % [13, 14]. In addition, the optimal glycerol concentration for sperm cryopreservation varies with other factors even within a certain species. For instance, 3 % or 5 % glycerol was found to be best for freezing cynomolgus monkey spermatozoa in straw [9, 13], whereas 5 % or 7 %–10 % glycerol was optimal for pellet freezing on dry ice [15]. Similarly, Morrell [16] observed that the optimal concentration of glycerol for cryopreserving marmoset sperm is lower (2.5 %–5.0 %) for straw freezing than that for pellet freezing (5.0 %–7.5 %). The result of this study showed that the optimal concentration of glycerol was 5 % in the mTTE medium, the same as the results reported by Sankai *et al*. [9], but the temperature of adding extender with glycerol was different. In the study of Sankai *et al.* [9], the extender containing glycerol was added at a lower temperature (i.e. 5 °C) to minimize the toxicity of glycerol. In the present study, however, exposure to glycerol was performed at room temperature (12 °C–18 °C) to match the temperature of incubation in the mTTE medium, avoiding changeful temperature that may damage sperm cells. Previous studies on rhesus [17] and vervet monkey [18] sperm cryopreservation indicated that extenders containing glycerol were added at 37 °C or 32 °C

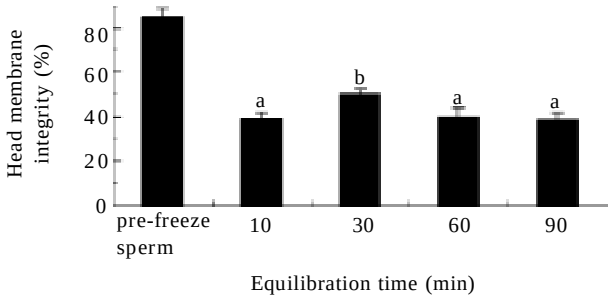

Figure 2. Effect of equilibration time on post-thaw sperm head membrane integrity. Bars with different letters are significantly different ($P < 0.05$).

rather than 5 °C and the recovery of post-thaw motility was more than 60 %. These results suggested that glycerol permeability might have a wide range of temperature dependency.

Equilibration time with glycerol in cryoprotectant extender is another factor to affect the survival of frozen-thawed spermatozoa, because the speed and degree of glycerol penetration of sperm is species-dependent. Glycerol can penetrate into sperm membrane very quickly for some species but slowly for others. Berndtson and Foote [19] found that glycerol was able to permeate bull spermatozoa within 3–4 min either at 25 °C or 5 °C and maximum post-thaw motility occurred when they were exposed to glycerol for as short as 10 s. However, Almlid and Johnson [20] discovered that there was no difference in sperm cryosurvival when boar semen was exposed to glycerol for 0.5, 2, 5, 15, or 75 min. Equilibration time is also quite different in nonhuman primate sperm cryopreservation. Leverage *et al*. [17] and Morrell [16] found that the equilibration time for rhesus and marmoset sperm should be within 10 min to get a maximal sperm survival. Mahone and Dukelow [15] concluded that there was no difference in total sperm motility for 1-, 25- or 45-min glycerol equilibration in cynomolgus monkeys. In Roussel and Austin's study [14], ejaculates from five species of nonhuman primates were added to the glycerol extenders and equilibrated for 30 min at room temperature and an average of 50 % post-thaw survival in the sperm was obtained. However, the result of our study showed that sperm motility and head membrane integrity for 10-, 60- and 90-min equilibration in the chemically defined extender was significantly lower ($P < 0.05$) than that of 30 min, indicating that shorter or longer equilibration time was directly related with the cell survival of cryopreservation. The shorter equilibration time might not allow enough glycerol to penetrate into spermatozoa, but longer exposure to glycerol could be toxic for them. The results suggested that the equilibration time not only depended on the species, but also related with the extenders as well as experimental conditions.

In this study, four male animals used as semen donors were chosen randomly from the Laboratory Animal Center of Kunming Institute of Zoology and it was unclear whether the semen of each individual was suitable for cryopreservation. Additionally, no significant difference was found in susceptibility of spermatozoa to cryodamage among the individual males or between the ejaculates in any donor. Even so, whether there is sperm freezing variability among cynomolgus monkeys and if the protocol described here is suitable for any donor of this species, further investigation is required. Moreover, though the post-thaw motility of spermatozoa is good with the present procedure, their capability of fertilization has to be examined.

In conclusion, our study demonstrates that cynomolgus monkey spermatozoa can be successfully cryopreserved in a chemically defined extender. An extender consisting of 5 % glycerol and 30 min equilibration yielded the highest post-thaw sperm motility and head membrane integrity. The concentration and equilibration time of glycerol proved to be the key factors to successful cryopreservation.

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