Journal of the American Association for Laboratory Animal Science
Copyright 2016
by the American Association for Laboratory Animal Science

Vol 55, No 6
November 2016
Pages 744–748

Cryopreservation of Cynomolgus Macaque (*Macaca fascicularis*) Sperm by Using a Commercial Egg-Yolk–Free Freezing Medium

Yaping Yan,[1,2,†] Lei Ao,[3,†] Hong Wang,[1,2] Yanchao Duan,[1,2] Shaohui Chang,[1,2] Bingbing Chen,[1] Dalong Zhi,[1,2] Sujuan Li,[1,2] Yuyu Niu,[1,2] Weizhi Ji,[1,2] and Wei Si[1,2,*]

Conventional TRIS–egg yolk (TEY) freezing medium for the cryopreservation of NHP sperm has the risk of contamination due to widespread zoonotic diseases. This study was aimed at determining the optimal glycerol concentration, freezing rate, and holding time in liquid N_2 vapor for the cryopreservation of cynomolgus macaque sperm by using a commercial egg-yolk–free freezing medium (SC medium) designed for human sperm cryopreservation. Sperm motility and acrosomal integrity after freezing were assessed. Sperm in SC medium (dilution ratio, 3:1) frozen at cooling rates of –67° and –183°C/min in liquid N_2 vapor showed higher post-thaw motility than did samples frozen at –435 °C/min. At the cooling rate of –183 °C/min and dilution in SC medium at a 3:1 ratio, post-thaw motility was higher after a holding time of 10 min than after 30 min (recommended by the manufacturer). In addition, post-thaw motility of sperm frozen in SC medium was higher with dilution ratios of 3:1, 4.5:1, and 6:1 compared with 9:1, 10.5:1, and 12:1, and the sample diluted 12:1 showed the lowest percentage of thawed sperm with intact acrosomes. Sperm showed higher post-thaw motility after freezing in TEY than in SC medium; acrosomal integrity did not differ between the 2 media. Our results indicated that cynomolgus macaque sperm can be cryopreserved successfully by using a commercial egg-yolk–free freezing medium, which provides an option for genetic preservation with decreased zoonotic risk in this important NHP species.

Abbreviations: LN_2, liquid N_2; SC medium, commercial egg-yolk–free freezing medium; TEY, traditional egg-yolk–containing cryopreservation medium

Cynomolgus macaques are one of the most widely used NHP species in biomedical research. Because of their genetic, physiologic, behavioral, and neurologic similarities to humans, cynomolgus macaques provide excellent translational validity in preclinical studies.[23] Substantial increases in the numbers of NHP models of human disease and health can be anticipated in the near future due to the recent successes in macaque models of human diseases with targeted mutation.[17] Meanwhile, the maintenance and breeding of NHP are extremely costly. Sperm banking provides an effective way to preserve valuable genetic resources and relieve the heavy financial burden of maintaining colonies.

Sperm cryopreservation of cynomolgus macaques is associated with a reduction in sperm motility, cell viability, and fertilizing capacity.[8,13,25] Osmotic stress, cold shock, ice-crystal formation, and oxidative injury are the main causes of sperm cryoinjury during freezing and thawing. TEY freezing medium has been the medium most widely used for sperm cryopreservation of cynomolgus macaques, and usually 20% egg yolk is added as an extender.[13,25] The beneficial effects of egg yolk on sperm cryopreservation have been demonstrated in many species including NHP.[5,7] The LDL in egg yolk are thought to provide cryoprotection to sperm,[6,11] but the exact mechanism remains unclear. Egg yolk contains numerous undefined compounds, thus complicating the analysis of the mechanisms of cryoprotection and cryoinjury. In addition, because of its animal origin and potential contamination with bacteria, fungi, viruses and prions, egg yolk carries a risk of pathogen introduction into cryopreserved sperm samples. Subsequently, cryopreserved sperm can not only be damaged by the endotoxins produced by microbes in egg yolk but also might serve as a source of widespread zoonotic diseases such as avian influenza.[4,22] Commercially available egg-yolk–free mediums for sperm cryopreservation have been developed for several species, including rabbits and humans,[24,27] but not NHP.

One particular commercial egg-free medium (SpermCryo Allround [SC medium], Cryos International Sperm Bank, Aarhus, Denmark) has been widely used in human sperm banks and in-vitro fertilization centers. Given the high similarity of NHP to humans, we hypothesized that SC medium might also be useful (after optimization) for cryopreservation of cynomolgus macaque sperm. Optimal cooling rates and concentrations of penetrating cryoprotectants, such as glycerol, differ among cell types and species.[10,28,30] For example, the widely used glycerol concentration for human sperm cryopreservation is 7.5%,[9,18] but 5% glycerol is preferable for cynomolgus macaque sperm.[25] Therefore, before using commercial SC medium for NHP sperm cryopreservation, various freezing parameters including cooling rate and glycerol concentration need to be optimized. This study was therefore designed to determine: 1) the optimal concentration of glycerol by diluting the sperm sample with SC medium at different ratios; 2) the optimal freezing rate by varying the

Received: 19 Feb 2016. Revision requested: 20 Apr 2016. Accepted: 13 May 2016.
[1]Yunnan Key Laboratory of Primate Biomedical Research, Institute of Primate Translational Medicine, Kunming University of Science and Technology, Kunming, Yunnan, China; [2]National Engineering Research Center of Biomedicine and Animal Science, Kunming, Yunnan, China; and [3]Kunming Sino–UK Angel Women's and Children's Hospital, Kunming, Yunnan, China.
[†]These authors contributed equally to the study.
**Correspondence author. Email: siwei76@hotmail.com*

distance between the loaded cryostraws and the surface of the liquid N_2(LN_2); and 3) the optimal time the sperm sample is held in the vapor phase of LN_2 during the freezing procedure. In addition, we compared the cryoprotection efficiency of the SC medium by using the optimized process with that of the conventional TRIS–egg yolk (TEY) medium and corresponding freezing method. Sperm motility and acrosomal integrity after thawing were used to assess the efficiencies of the various freezing regimes.

Materials and Methods

Animals. Sexually mature cynomolgus macaques (n = 4; age, 7 to 10 y) provided by Kunming Biomed International were used as semen donors in this study. All of the animals were individually caged in an animal room with a 12:12-h light-dark cycle. The room temperature was maintained between 18 and 26 °C and with a relative humidity of 40% to 70%. All procedures were approved by the IACUC of the Institute of Primate Translational Medicine (Kunming University of Science and Technology) and were performed in accordance with the *Guide for the Care and Use of Laboratory Animals*.[12] The animal facility is AAALAC-accredited.

Sperm freezing medium. All chemicals were obtained from Sigma Chemical (St Louis, MO), unless otherwise indicated. The SC medium was purchased from Cryos International Sperm Bank and stored at 4 °C until use. The TEY medium contained 0.2% Tris, 1.2% TES, 2% glucose, 2% lactose, 0.2% raffinose, and 20% (v/v) fresh egg yolk was prepared in house as previously described.[25] The TEY medium was stored at –80 °C. Before each experiment, TEY medium was thawed, and 10% glycerol was added.

Semen collection and processing. Semen samples were collected from the 4 cynomolgus macaques by using electric penile stimulation. The semen was kept at 37 °C for 30 min to allow for liquefaction. About 10µL of each ejaculate was used as a nonfrozen control and evaluated for sperm motility and acrosomal integrity. The rest of the liquefied semen was used in the various cryopreservation experiments.

Sperm functional evaluation assays. The percentage of sperm showing forward progression was assessed by light microscopy. A sample (10 µL) of each fresh or frozen-thawed sperm sample was placed on a prewarmed hemocytometer and covered with a coverslip. The motility of each sperm sample was assessed by an experienced evaluator who was blinded in regard to sample identity and who evaluated approximately 200 sperm in duplicate.

Sperm acrosomal integrity was determined by using the Alexa Fluor-488–peanut agglutinin assay (Molecular Probes, Eugene, OR).[30] Sperm with an intact acrosome showed uniform apple green fluorescence in the acrosomal region of the sperm head, whereas sperm with acrosomal damage showed little or no green fluorescence. At least 200 sperm were scored for each sample.

Effect of cooling rate on cryosurvival of cynomolgus macaques-perm. To determine the optimal cooling rate for cynomolgus sperm cryopreservation by using the egg yolk free freezing medium SC, a total of 6 ejaculates from 4 cynomolgus macaques were used. Each ejaculate was diluted with TALP–HEPES medium[1] to a concentration of 2×10^6 sperm per milliliter. Each diluted sperm sample was divided into 4 aliquots, one of which was a nonfrozen control; the other 3 aliquots were mixed 3:1 with SC medium as recommended by the manufacturer. Briefly, one part of SC medium was slowly added dropwise to 3 parts of sperm samples. The diluted sperm samples were mixed incubated for 10 min at room temperature (according to the manufacturer's instructions), and then were loaded into 0.25-mL cryostraws (about 200 µL per straw; IMV, L'Aigle, France). The filled straws were laid horizontally held for 10 min at 1, 4, or 7 cm above the surface of the LN_2, and then submerged directly into LN_2 for storage. The freezing rates corresponding to the various distances from the LN_2 surface were measured by using a type-T bare-wire thermocouple (5SRTC-TT-T-36-36, Omega Engineering Stamford, CT) and analyzed in a data acquisition program (TC-08 Recorder, Omega Engineering) as described in our previous study;[30] the average cooling rates at 1, 4, and 7 cm above the LN_2 surface were –435, –183, and –69 °C/min (measured between –10 and –70 °C), respectively. Straws were thawed rapidly by plunging them directly into a 37 °C water bath for 1 min. The sperm motility and acrosomal integrity of the nonfrozen control and 3 frozen groups were examined after thawing.

Effect of holding time in liquid N_2 vapor on cryosurvival of cynomolgus macaque sperm. To determine the effect of holding time in LN_2 vapor on cell dehydration during freezing, we used the optimal freezing rate determined in the previous experiment. A total of 6 ejaculates from 4 cynomolgus macaques were cryopreserved as done previously. The straws loaded with sperm samples were frozen in LN_2 vapor for 10 or 30 min at the previously determined optimal cooling rate before they were submerged directly into LN_2 for storage. Sperm motility and acrosomal integrity of the nonfrozen control and the 2 experimental groups were examined after thawing.

Effect of the dilution ratio in SC medium on the cryosurvival of cynomolgus macaque sperm. The SC medium is a commercial medium, and the glycerol concentration of the medium is proprietary information. Varying the dilution ratio at which SC medium is used with the sperm changes the glycerol concentration of the frozen samples, and an optimal glycerol concentration is important for the cryosurvival of sperm. To determine the optimal ratio between SC medium and sperm sample for the cryopreservation cynomolgus macaque sperm, each of 7 ejaculates from 4 cynomolgus macaques was diluted with TALP-HEPES[1] to a concentration of 2×10^6 sperm per milliliter and then divided into 8 aliquots. One aliquot was used as a nonfrozen control, and the other 7 aliquots were further mixed with SC medium at a ratios of 3:1, 4.5:1, 6:1, 7.5:1, 9:1, 10.5:1, and 12:1 (v:v). The mixed sperm samples were incubated for 10 min at room temperature (as directed by the manufacturer), loaded into 0.25-mL cryostraws, and frozen at the optimized cooling rate and holding time determined in the earlier experiments. The straws were then submerged directly into LN_2 for storage. The sperm motility and acrosomal integrity of the nonfrozen control and the 7 experimental groups were examined after thawing.

Cryosurvival of cynomolgus macaque sperm in SC compared with TEY freezing medium. A total of 4 ejaculates from 4 cynomolgus macaques were used in this experiment. Each ejaculate was diluted with TALP-HEPES[1] to a concentration of 2×10^6 sperm per milliliter and then divided into 3 aliquots. One aliquot was used as a nonfrozen control, another was cryopreserved with SC medium at the optimized freezing parameters determined previously, and the remaining aliquot was cryopreserved with TEY medium as previously described.[25] Briefly, sperm samples were diluted with TEY freezing medium (without glycerol) in a sterile test tube at room temperature, and then the tube was transferred into a beaker containing 200 mL water. Sperm samples were chilled from room temperature to 4 °C over 2h by placing the beaker inside a refrigerator. The 4 °C sperm samples were diluted with an equal volume of precooled (4 °C)

TEY containing 10% glycerol (v:v) by stepwise addition (6-min intervals) to reach the final glycerol concentration of 5% (v:v). Diluted sperm samples were loaded into 0.25-mL precooled (4 °C) cryostraws (200 µL per straw). Filled straws loaded with sperm samples were laid horizontally, frozen at 5 cm above the LN_2 surface for 10 min, and then submerged directly into LN_2 for freezing and storage. The motility and acrosomal integrity of fresh sperm and sperm cryopreserved with TEY and SC were examined.

Statistical analysis. All data are expressed as mean ± 1 SD. The percentage data for sperm motility and acrosomal integrity underwent arcsine square root transformation before statistical analysis. ANOVA and the Fisher protected least-significant difference test (SPSS 16, SPSS, Chicago, IL) were used to analyze differences among groups. A P value less than 0.05 was considered to be statistically significant.

Results

Effect of cooling rate on cryosurvival of cynomolgus macaque sperm. The motility and acrosomal integrity of sperm cryopreserved at different freezing rates are summarized in Table 1. Compared with fresh sperm, cryopreservation at both cooling rates significantly ($P < 0.05$) decreased sperm motility and the percentage of sperm with intact acrosomes. Sperm frozen at 4 cm (–183 °C/min) and 7 cm (–69 °C/min) above the LN_2 surface showed ($P < 0.05$) higher post-thaw motility compared with that of sperm frozen at 1 cm (–435 °C/min). However, motility did not differ between the 4-cm and 7-cm groups. In addition, acrosomal integrity did not differ among the 3 groups (Table 1).

Effect of holding time in LN_2 vapor on cryosurvival of cynomolgus macaque sperm. Sperm frozen at 4 cm above the LN_2 surface for 10 min showed higher post-thaw motility compared with those frozen for 30 min after freezing and thawing ($P < 0.05$; Table 2). However, holding time did not affect acrosomal integrity.

Effect of the dilution ratio in SC medium on the cryosurvival of cynomolgus macaque sperm. The motility and acrosomal integrity of sperm cryopreserved at 4 cm above the LN_2 surface for 10 min were assessed for different dilutions of SC medium (Table 3). Post-thaw motility was significantly ($P < 0.05$) higher for sperm frozen at dilution ratios of 3:1, 4.5:1 and 6:1 (sperm:medium) than at the ratios of 9:1 and 10.5:1. Sperm frozen at a ratio of 12:1 showed the lowest post-thaw motility among the 7 groups ($P < 0.05$). Regardless of the dilution ratio, acrosomal integrity in frozen–thawed sperm samples was significantly ($P < 0.05$) decreased compared with that of fresh sperm, and sperm diluted with SC medium at a ratio of 12:1 showed the lowest percentage of sperm with intact acrosomes ($P < 0.05$).

Cryosurvival of cynomolgus macaque sperm in SC compared with TEY freezing medium. The post-thaw motility and acrosomal integrity of sperm cryopreserved with either TEY or SC medium were decreased significantly compared with those of fresh sperm ($P < 0.05$; Table 4). Post-thaw motility was higher ($P < 0.05$) for sperm cryopreserved in TEY than in SC medium, but acrosomal integrity did not differ between mediums.

Table 1. Motility and acrosomal integrity of cynomolgus macaque sperm cryopreserved with SC medium at different cooling rates

Distance to LN_2 surface	Cooling rate (°C/min)	Sperm motility (%)	Acrosomal integrity (%)
1 cm	–435	9.7 ± 5.0^b	86.0 ± 1.9^b
4 cm	–183	25.0 ± 8.4^c	88.2 ± 1.7^b
7 cm	–69	18.2 ± 9.9^c	86.8 ± 2.0^b
Fresh sperm	—	84.2 ± 11.3^a	91.7 ± 1.6^a

All samples were held in LN_2 for 10 min. Different superscripted letters within a column indicate significant ($P < 0.05$) differences between values.

Table 2. Motility and acrosomal integrity of cynomolgus macaque sperm cryopreserved at different holding times

Holding time (min)	Sperm motility (%)	Acrosomal integrity (%)
10	25.0 ± 8.4^b	88.2 ± 1.7^b
30	14.2 ± 4.7^c	86.3 ± 2.3^b
Fresh sperm	84.2 ± 11.3^a	91.7 ± 1.6^a

All samples were held 4 cm above the LN_2 surface. Different superscripted letters within a column indicate significant ($P < 0.05$) differences between values.

Table 3. Motility and acrosomal integrity of cynomolgus macaque sperm cryopreserved at different ratios of SC medium:sperm

Dilution ratio	Sperm motility (%)	Acrosomal integrity (%)
3:1	23.7 ± 6.4^b	84.0 ± 4.6^b
4.5:1	21.1 ± 6.7^b	84.0 ± 3.3^b
6:1	23.0 ± 6.9^b	85.1 ± 3.8^b
7.5:1	$17.4 \pm 7.0^{b,c}$	84.6 ± 4.0^b
9:1	$12.7 \pm 5.4^{c,d}$	81.3 ± 4.5^b
10.5:1	$11.3 \pm 6.4^{c,d}$	81.1 ± 3.6^b
12:1	9.4 ± 7.3^d	76.4 ± 4.5^c
Fresh sperm	83.4 ± 11.0^a	90.0 ± 3.1^a

Different superscripted letters within a column indicate significant ($P < 0.05$) differences between values.

Discussion

Egg yolk has been the most widely used nonpenetrating cryoprotectant for the cryopreservation of NHP sperm.[21] Because of its ready availability, hen egg yolk is traditionally used in sperm freezing extenders. The proposed mechanisms of cryoprotection of egg yolk include stabilizing cell membrane and preventing lipid efflux from sperm membrane.[2] However, the addition of avian-derived egg yolk into sperm freezing medium carries sanitary risks, and the wide variability in the composition of egg yolk is disadvantageous for studying the mechanisms of cryoprotection and cryoinjury.[3,4] In addition, harvesting hen egg yolk is a cumbersome process, and skill is required to separate the yolk from albumin and chalazae. Therefore, a commercially available egg-yolk–free freezing medium would be beneficial, in practical terms, for the cryopreservation of NHP sperm. Because an NHP-specific sperm freezing medium is not yet commercially available, we used the commercial egg-free SC medium designed for human use to cryopreserve cynomolgus macaque sperm in the present study. Although both humans and macaques are primates, the parameters for their sperm cryopreservation differ widely. Therefore, the cooling rate and glycerol concentration need to be optimized before SC medium can be used for cryopreserving the sperm of cynomolgus macaques.

In the present study, 3 freezing rates (fast, moderate, and slow) determined by the distance between the cryostraws and the LN_2

Table 4. Motility and acrosomal integrity of cynomolgus macaque sperm cryopreserved with TEY compared with SC medium

	Sperm motility (%)	Acrosomal integrity (%)
SC medium	25.5 ± 3.5[b]	86.5 ± 1.3[b]
TEY medium	44.0 ± 5.8[c]	88.7 ± 1.3[b]
Fresh sperm	83.0 ± 6.2[a]	91.7 ± 1.7[a]

Different superscripted letters within a column indicate significant ($P < 0.05$) differences between values.

surface were assessed. During freezing, ice nucleates initially in the extracellular spaces and causes an osmotic gradient between the freeze-concentrated extracellular solution and the intracellular solution. Cells are not dehydrated sufficiently when the cooling rate is too fast, and intracellular ice formation leads to cryoinjuries in the cytoplasm. Conversely, when the cooling rate is too slow, cells are injured due to solution effects caused by severe dehydration and exposure to toxic concentrations of electrolytes.[19] The optimal freezing rate for sperm cryosurvival should be low enough to avoid intracellular ice formation but fast enough to minimize solution effects.[30] The freezing rate is an important factor that affects the cryosurvival of cells, and different cell types have different optimal cooling rates.[19] Studies have found that high survival rates can be obtained by freezing sperm at their optimal freezing rate.[28-30] In our study, sperm frozen at the slow and moderate cooling rates (4 and 7 cm above LN_2) showed higher cryosurvival. The detrimental effects of faster cooling (–435 °C/min) on sperm of cynomolgus macaques are probably due to the formation of intracellular ice resulting from too-rapid freezing. Our previous study on the cryopreservation of rhesus macaque sperm revealed that samples frozen at 4 cm above the LN_2 surface (–183 °C/min) showed the highest post-thaw motility.[30] However, in addition to species-associated differences, ethylene glycol rather than glycerol was used as penetrating cryoprotectantand a TRIS–egg yolk freezing medium rather than egg-yolk–free product were used in the previous study.[30]

Conventionally, cynomolgus sperm diluted with TEY freezing medium usually are frozen by holding filled cryostraws in LN_2 vapor for either 10 or 15 min before plunging them into LN_2 for storage.[8,15] The user's guide for the SC freezing medium states that cryostraws loaded with human sperm samples should remain in LN_2 vapor for 30 min before immersion into LN_2. Therefore, we compared 2 different holding times (10 and 30 min) in LN_2 vapor on the cryosurvival of cynomolgus sperm frozen in SC medium. Our results indicate that a 10-min hold time led to a higher cryosurvival rate, according to postthaw sperm motility and acrosomal integrity. At the initiation of ice nucleation, the extracellular osmolarity of freezing medium rises approximately 10-fold.[20] When held for 30 min in LN_2 vapor, sperm cells might be injured through solution effects due to severe dehydration of cells, osmotic injury, or prolonged exposure to toxic concentrations of electrolytes.

SC medium uses glycerol as the penetrating cryoprotectant. According to the user's guide, the typical dilution ratio between sperm and SC medium is 3:1, which achieves the preferred concentration of glycerol for freezing human sperm. Glycerol is the most widely used cryoprotectant for primate spermatozoa cryopreservation.[21] Previous studies proved that freezing human sperm with 7.5% glycerol achieved desired post-thaw survival and fertility.[9,18] Similarly, the optimal glycerol concentration for cynomolgus macaque sperm is 5% when TEY medium was used.[25] However, because the concentration of glycerol required for optimal survival and fertilizing capacity of frozen sperm is dependent on cryopreservation medium,[10] we evaluated several dilution ratios for SC medium. Our results indicated that ratios of 3:1 to 6:1 (sperm:SC medium) maximized the post-thaw sperm motility. Below the threshold concentration, the penetrating cryoprotectant has no protective effect, and the threshold of efficacy varies among species.[10] Accordingly, when we increased the dilution ratio to 9:1, post-thaw motility decreased. In addition to the penetrating cryoprotectant, nonpenetrating cryoprotectants such as sugar and amino acids contribute to the cryosurvival of macaque sperm,[14,27] and the high dilution ratios we evaluated (>9:1) diluted the concentrations of nonpenetrating cryoprotectants, including sucrose, glucose, and glycine, and consequently decreased sperm cryoprotection overall.

In our study, to apply a commercial sperm freezing medium to cynomolgus macaque sperm, we optimized the freezing rate, the holding time in LN_2 vapor for cell dehydration, and the appropriate ratio of sperm sample to SC medium. It should be noted that the parameters were determined through separate experiment, and interactions between parameters might exist. In addition, the protocols for cryopreservation of cynomolgus sperm differed between the 2 mediums we used, and we followed the most effective protocol for each medium. Egg yolk has long been recognized to protect sperm cells from cold shock, and macaque sperm cryopreserved in an egg-yolk–free medium with a 2-h equilibration at 4 °C before being frozen in LN_2 vapor had an extremely low post-thaw survival.[5] Therefore, we omitted this equilibration at 4 °C for the SC medium. Compared with the conventional method for cryopreservation of macaque sperm, the post-thaw motility of sperm preserved by using SC medium with the optimized freezing parameters was lower (44.0% compared with 25.5%). The instant transfer of sperm samples diluted with the egg-yolk–free SC medium into LN_2 might have caused cryoinjury and affected the overall cryosurvival of sperm. In a previous study, the motilities of human sperm frozen with conventional TEY freezing medium and SC medium in LN_2 vapor decreased from 43.4% (nonfrozen) to 22.8% and 14.0%, respectively,[24] similar to our current results. In other words, after being frozen in SC medium and LN_2 vapor, about a third of both the human and cynomolgus macaque sperm samples retained motility, thus confirming that the commercial SC medium can successfully be used for cynomolgus sperm cryopreservation after the optimization of freezing parameters. Previously, we used a chemically defined freezing medium to freeze cynomolgusmacaque sperm.[16] However, the process was complicated, cumbersome, and took hours to complete, which is not practical. The present study is the first in which a commercial medium developed for freezing human sperm was used successfully for the cryopreservation of NHP sperm.

In conclusion, we optimized the ratio of sperm to SC freezing medium, the freezing rate, and holding time for cryopreservation of cynomolgus macaque sperm. Sperm diluted with the commercial egg-free sperm freezing medium at the ratio of 3:1 and frozen for 10 min at 4 cm above LN_2 surface before immersion in LN_2 achieved the highest cryosurvival rate. The cryosurvival rate of cynomolgus macaque sperm frozen according to our optimized protocol was equal to that of human sperm with the same freezing medium reported previously.[24] Our study provides an alternative method for the genetic preservation of this important species that reduces associated health risks.

Acknowledgments

This work was supported by grants from the National Basic Research Program of China [2012CBA01305], Yunnan young leaders in academic and technical talents project [2012HB040], Yunnan technology innovation talent project [2014HB090], Kunming science and technology planning project [2014-04-A-S-02-3074] and Personnel training project of Kunming University of science and technology [KKZ3201526003]. We thank Mr. Jianpeng Liao and Chenyang Si from Yunnan Key Laboratory of Primate Biomedical Research for the help of semen collection.

References

1. **Bavister BD, Leibfried ML, Lieberman G. 1983**. Development of preimplantation embryos of the golden hamster in a defined culture medium**. Biol Reprod 28:**235–247.
2. **Bergeron A, Manjunath P.** 2006. New insights towards understanding the mechanisms of sperm protection by egg yolk and milk. Mol Reprod Dev **73:**1338–1344.
3. **Blanch E, Tomás C, Hernández M, Roca J, Martínez EA, Vázquez JM, Mocé E.** 2014. Egg yolk and glycerol requirements for freezing boar spermatozoa treated with methyl β-cyclodextrin or cholesterol-loaded cyclodextrin. J Reprod Dev **60:**143–149.
4. **Bousseau S, Brillard JP, Marguant-Le Guienne B, Guérin B, Camus A, Lechat M.** 1998. Comparison of bacteriological qualities of various egg yolk sources and the in vitro and in vivo fertilizing potential of bovine semen frozen in egg yolk or lecithin based diluents. Theriogenology **50:**699–706.
5. **Dong Q, Correa LM, VandeVoort CA.** 2009. Rhesus monkey sperm cryopreservation with TEST-yolk extender in the absence of permeable cryoprotectant. Cryobiology **58:**20–27.
6. **Dong QX, Rodenburg SE, Hill D, Vandevoort CA.** 2011. The role of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) in comparison with whole egg yolk for sperm cryopreservation in rhesus monkeys. Asian J Androl **13:**459–464.
7. **Dong Q, VandeVoort CA.** 2009. Effect of egg yolk on cryopreservation of rhesus monkey ejaculated and epididymal sperm. J Androl **30:**309–316.
8. **Feradis AH, Pawitri D, Suatha IK, Amin MR, Yusuf TL, Sajuthi D, Budiarsa IN, Hayes ES.** 2001. Cryopreservation of epididymal spermatozoa collected by needle biopsy from cynomolgus monkeys (*Macacafascicularis*). J Med Primatol **30:**100–106.
9. **Hammadeh ME, Greiner S, Rosenbaum P, Schmidt W.** 2001. Comparison between human sperm preservation medium and TEST-yolk buffer on protecting chromatin and morphology integrity of human spermatozoa in fertile and subfertile men after freeze-thawing procedure. J Androl **22:**1012–1018.
10. **Hammitt DG, Walker DL, Williamson RA.** 1988. Concentration of glycerol required for optimal survival and in vitro fertilizing capacity of frozen sperm is dependent on cryopreservation medium. Fertil Steril **49:**680–687.
11. **Iaffaldano N, Di Iorio M, Rosato MP, Manchisi A.** 2014. Cryopreservation of rabbit semen using non-permeable cryoprotectants: effectiveness of different concentrations of low-density lipoproteins (LDL) from egg yolk versus egg yolk or sucrose. Anim Reprod Sci **151:**220–228.
12. **Institute for Laboratory Animal Research**. 2011. Guide for the care and use of laboratory animals, 8th ed. Washington (DC): National Academies Press.
13. **Li Y, Cai K, Li J, Dinnyes A, Ji W.** 2006. Comparative studies with 6 extenders for sperm cryopreservation in the cynomolgus monkey (*Macaca fascicularis*) and rhesus monkey (*Macaca mulatta*). Am J Primatol **68:**39–49.
14. **Li Y, Si W, Zhang X, Dinnyes A, Ji W.** 2003. Effect of amino acids on cryopreservation of cynomolgus monkey (*Macaca fascicularis*) sperm. Am J Primatol **59:**159–165.
15. **Li YH, Cai KJ, Kovacs A, Ji WZ.** 2005. Effects of various extenders and permeating cryoprotectants on cryopreservation of cynomolgus monkey (*Macaca fascicularis*) spermatozoa. J Androl **26:**387–395.
16. **Li YH, Cai KJ, Su L, Guan M, He XC, Wang H, Kovacs A, Ji WZ.** 2005. Cryopreservation of cynomolgus monkey (*Macaca fascicularis*) spermatozoa in a chemically defined extender. Asian J Androl **7:**139–144.
17. **Liu H, Chen Y, Niu Y, Zhang K, Kang Y, Ge W, Liu X, Zhao E, Wang C, Lin S, Jing B, Si C, Lin Q, Chen X, Lin H, Pu X, Wang Y, Qin B, Wang F, Wang H, Si W, Zhou J, Tan T, Li T, Ji S, Xue Z, Luo Y, Cheng L, Zhou Q, Li S, Sun YE, Ji W.** 2014. TALEN-mediated gene mutagenesis in rhesus and cynomolgus monkeys. Cell Stem Cell **14:**323–328.
18. **Mahadevan M, Trounson AO.** 1983. Effect of cryoprotective media and dilution methods on the preservation of human spermatozoa. Andrologia **15:**355–366.
19. **Mazur P.** 1984. Freezing of living cells: mechanisms and implications. Am J Physiol **247:**C125–C142.
20. **Mazur P, Kleinhans FW.** 2008. Relationship between intracellular ice formation in oocytes of the mouse and Xenopus and the physical state of the external medium—a revisit. Cryobiology **56:**22–27.
21. **Morrell JM, Hodges JK.** 1998. Cryopreservation of nonhuman primate sperm: priorities for future research. Anim Reprod Sci **53:**43–63.
22. **Nishijima K, Kitajima S, Koshimoto C, Morimoto M, Watanabe T, Fan J, Matsuda Y.** 2015. Motility and fertility of rabbit sperm cryopreserved using soybean lecithin as an alternative to egg yolk. Theriogenology **84:**1172–1175.
23. **Ogawa LM, Vallender EJ.** 2014. Genetic substructure in cynomolgus macaques (*Macaca fascicularis*) on the island of Mauritius. BMC Genomics **15:**748.
24. **Paras L, Freisinger J, Esterbauer B, Schmeller N, Szlauer R, Jungwirth A.** 2008. Cryopreservation technique: comparison of Test yolk buffer versus SpermCryo and vapour compared with computerised freezing. Andrologia **40:**18–22.
25. **Sankai T, Terao K, Yanagimachi R, Cho F, Yoshikawa Y.** 1994. Cryopreservation of spermatozoa from cynomolgus monkeys (*Macaca fascicularis*). J Reprod Fertil **101:**273–278.
26. **Si W, Hildebrandt TB, Reid C, Krieg R, Ji W, Fassbender M, Hermes R.** 2006. The successful double cryopreservation of rabbit (*Oryctolagus cuniculus*) semen in large volume using the directional freezing technique with reduced concentration of cryoprotectant. Theriogenology **65:**788–798.
27. **Si W, Wang H, Reid C, Hildebrandt TB, Ji W.** 2006. Effect of sugar type on the survival of frozen-thawed rhesus monkey (*Macaca mulatta*) sperm. Am J Primatol **68:**103–108.
28. **Stacy R, Eroglu A, Fowler A, Biggers J, Toner M.** 2006. Thermal characterization of Nakagata's mouse sperm freezing protocol. Cryobiology **52:**99–107.
29. **Sumigama S, Meyers S.** 2012. Cooling rate affects rhesus monkey sperm survival. J Med Primatol **41:**278–283.
30. **Yang S, Ping S, Si W, He X, Wang X, Lu Y, Ji S, Niu Y, Ji W.** 2011. Optimize ethylene glycol concentrations, freezing rates and holding times in liquid nitrogen vapor for sperm cryopreservation of rhesus macaque (*Macaca mulatta*). J Vet Med Sci **73:**717–723.