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Improvement of sperm cryo-survival of cynomolgus macaque (*Macaca fascicularis*) by commercial egg-yolk–free freezing medium with type III antifreeze protein

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ABSTRACT

When nonhuman primate sperm undergoes cryopreservation in an egg yolk medium there is an increased risk that the egg yolk might adversely affect the sperm due to containing of avian pathogens. Although commercial egg-yolk-free medium for human sperm cryopreservation has been used for macaque sperm, the cryo-survival remains less than optimal. The present study, therefore, was conducted to determine the optimal concentration of antifreeze protein (AFP) III supplemented in a commercial egg-yolk-free medium for cynomolgus macaque (*Macaca fascicularis*) sperm cryo-survival. The function of frozen-thawed sperm was evaluated by post-thaw sperm motility, acrosome integrity, and mitochondrial function. Results indicate that the sperm motilities were greater when 0.1, 1, and 10 μg/ml of AFP III were supplemented into the sperm freezing medium ($P < 0.05$). In addition, the mitochondrial membrane potential was greater in the sperm cryopreserved with the medium that was supplemented with 0.1 μg/ml of AFP III ($P < 0.05$). The addition of AFP III at any of the concentrations, however, did not have any cryoprotection effect on the sperm acrosome, and the greatest concentrations of AFP III at 100 and 200 μg/ml had detrimental effects on acrosomal integrity ($P < 0.05$). Results of the present study indicated the methods used are effective for the cryopreservation of cynomolgus monkey sperm while reducing associated health risks due to avian pathogens being present in egg yolk-based extenders.

1. Introduction

As the number of species being affected by the heightened amount of deforestation and human encroachment increases, the need to preserve the genetic material of endangered species is also increasing. Nonhuman primates are especially affected by this habitat destruction and unsustainable human activities (Estrada et al., 2018). Many ranges of New World primates such as the black-headed

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squirrel monkey are restricted to a small area and, therefore, the genetic diversity is at risk of being too little for maintenance of a viable genetic pool (Oliveira et al., 2016). The maintenance of monkeys in captivity can be very expensive and sometimes, depending on the species, natural reproduction can be unsuccessful. Additionally, for areas with small populations, an outside introduction of diverse genes are required for maintenance of such regional populations.

Sperm cryopreservation can be a cost-effective way of storing valuable and unique genetic material (McCarthy and Meyers, 2011; Nichi et al., 2016). The cynomolgus macaque (*Macaca fascicularis*) has a habitation range that is third largest in the world compared to all other primates (Zhang et al., 2017), can be used for development of effective sperm cryopreservation methods to conduct the initial studies for preserving genetic material for other nonhuman primate species for which there is great need for preservation. The current survival rates of cryopreserved sperm, however, are less than optimal due to sperm cryoinjury (Grotter et al., 2019; Yeste, 2016). The main causes of sperm cryoinjury, osmotic stress, cold shock, oxidative injury, and ice crystal formation, occur during sperm cryopreservation (Mahadevan and Trounson, 2010). This cooling process reduces sperm viability and fertilizing capacity.

Conventionally, when egg yolk is supplemented to the freezing medium, this can result in protection of sperm against cold shock, and contribute substrates for sperm metabolism, and provide a buffer against pH changes (Watson, 2000; Mocé et al., 2003). One of the disadvantages of the use of egg yolk is that a large number of undefined compounds found in the egg yolk may complicate the analysis for the mechanisms of cryoinjury and cryoprotection (Dong et al., 2011; Iaffaldano et al., 2014). Other disadvantages include the possibility that egg yolk can contain bacteria, fungi, viruses, prions, and other unknown pathogens, which may be a risk factor for sperm damage caused by microbic endotoxins and the spread of zoonotic diseases (Paras et al., 2010; Si et al., 2010). Instead, the use of an egg-yolk-free freezing medium for sperm cryopreservation can overcome these disadvantages. There have been more than 20 commercial egg-yolk-free media developed for mice, rabbit and human sperm cryopreservation, but none for non-human primates. In a previous study, there was cryopreservation of cynomolgus macaque sperm using a commercial egg-yolk-free freezing medium developed for humans, but the cryo-survival was less compared to using the traditional egg yolk based medium (Yan et al., 2016).

Antifreeze proteins (AFPs), polypeptides that can inhibit the development of ice crystals and re-crystallization by means of a non-colligative mechanism, are found in fungi, bacteria, plants, insects and fish, and have been used as a non-penetrating cryoprotectant for sperm cryopreservation (Rubinsky et al., 1991; Carpenter and Hansen, 1992). The addition of AFPs in the freezing medium increases the survival rate of frozen-thawed sperm of different species, such as sheep, chimpanzees, and cattle, as well as mouse and rabbit embryos, and a species-specific optimal AFP concentration exists for each of species (Payne et al., 1994; Younis et al., 1998; Prathalingam et al., 2006; Jo et al., 2011). The mechanism(s) through which proteins function in cryoprotection remains largely unknown. The benefit of AFP on nonhuman primate sperm cryopreservation has only been reported in chimpanzees and the supplementation of the AFP III was in an egg-yolk-based freezing medium. To the best of our knowledge, the effects of AFPs on macaque sperm cryopreservation, however, have not been reported. The present study was, therefore, conducted to determine the optimal concentration of AFP III for sperm cryopreservation of cynomolgus macaques and improve the sperm cryo-survival using a commercial egg-yolk-free sperm medium designed for human sperm. Frozen-thawed sperm motility, acrosome integrity, and mitochondrial membrane potential were used to evaluate the function of the cryopreserved sperm.

2. Materials and methods

2.1. Animals and ethics

Four male cynomolgus macaques (ages from 6 to 10 years old) provided by the Yunnan Key Laboratory of Primate Biomedical Research (Kunming, Yunnan, China) were used as semen donors. Sperm samples were collected from the males by penile electroejaculation (Gould and Mann, 1988). All procedures were approved by the Institutional Animal Care and Use Committee Kunming University of Science and Technology (authorization code: LPBR201701001) and were executed in accordance with the Guide for Care and Use of Laboratory Animals (National Research Council, 2011).

2.2. Sperm freezing medium

All chemicals used in this study were from Sigma Chemical (St Louis, MO, USA) unless otherwise stated. The egg-yolk-free sperm freezing medium (consisting of calcium chloride, gentamicin sulphate, glucose, glycerol, glycine, HEPES, human albumin solution, magnesium chloride, Milli RX water, potassium chloride, sodium bicarbonate, sodium L-lactate, sodium phosphate, SSR® (Synthetic Serum Replacement), and sucrose) was purchased from ORIGIO (Knardrupvej, Malov, Denmark) (https://fertility.coopersurgical.com/products/sperm-freezing-medium/) and was stored at 4 °C until use.

2.3. Type III AFP expression and purification

A plasmid containing a type III AFP with His tag, QAE isoform from ocean pout was transformed using heat shock into a BL21-DE3-PlysS *E.coli* strain (Eickhoff et al., 2019). The plasmid was obtained from Peter L. Davies (Queen's University, Canada; Chao et al., 1994; Phippen, 2017). A stirred tank fermentor of 1 l (Applikon Biotechnology, Netherlands) was used for a fed-batch fermentation process to produce the protein. Bacteria were grown in controlled physical and chemical conditions. Type III AFP production was induced by 1 mM of IPTG. Cells were harvested, precipitated using centrifugation (Sorvall RC-5C) at 4 °C at 3075 *g* (4500 rpm, rotor SLA 1500, 136 mm) for 45 min, and stored at −80 °C until purification. Five grams of wet cell pellet were re-suspended in 50 ml of 50 mM sodium phosphate and 300 mM NaCl (Sonication lysis buffer; pH 7.5) supplemented with protease inhibitors, pre-

cooled to 4 °C. Cells were lysed using sonication (Sonics Vibra-Cell VCX 750 W Ultrasonic Homogenizer with CV33 Transducer placed in a refrigerator) for 15 min in 100 ml flask at 4 °C. The lysed cells were centrifuged for 45 min at 27,000 *g* (15,000 rpm, rotor SS-34, 107 mm) and the supernatant that contained the proteins was collected. Type III AFP was purified using nickel affinity chromatography. Briefly, type III AFP was bound to Ni-NTA resin, washed with lysis buffer, and then eluted with an elution buffer containing 200 mM imidazole in addition to the lysis buffer. All fractions containing type III AFP were dialyzed against PBS. The SDS-PAGE processing of the purified proteins resulted in a single protein band at the expected molecular weight. The concentration was quantified using a Micro BCA Protein Assay (Thermo Scientific, USA). Thermal hysteresis activity was determined using procedures that have been previously described (Braslavsky and Drori, 2013). Samples were dialyzed into 50 mM of ammonium bicarbonate and lyophilized for storage. Thermal hysteresis activity measurement of the proteins after resuspension from the lyophilized state was performed and compared to the resulting values reported in the literature for AFP III (Chao et.al. 1994) to validate the ice binding activity of the proteins (Thermal hysteresis of 0.45 °C at 0.3 mM).

2.4. Semen collection and freezing

A total of 14 ejaculates from the four male cynomolgus macaques was collected using penile electroejaculation procedures (Gould and Mann, 1988) for this study. Each semen sample was collected into a 50 ml centrifuge tube and was allowed to liquefy in a 37 °C water bath for 30 min. A sample of fresh sperm was set aside for evaluation of the following variables: motility, acrosome integrity, and mitochondrial membrane potential. The other portion of the sperm sample was divided into six aliquots and then diluted with TALP-Hepes medium containing 0.3% BSA (TH3; Niu et al., 2010) supplemented with AFP III to reach the concentrations of 0, 0.2, 2, 20, 200 and 400 μg/ml of AFP III. The samples were subsequently mixed 1:1 with ORIGIO sperm freezing medium and kept at room temperature for 10 min before being loaded and sealed into 0.5 ml cryostraws (IMV, L'Aigle, France) as recommended by the manufacturer. The final AFP III concentrations in the six sperm samples were 0, 0.1, 1, 10, 100 and 200 μg/ml. The straws were horizontally placed at 0.5 cm above the surface of the liquid nitrogen (LN2) for 30 min and then were immediately immersed directly into the LN2 for storage. The freezing rates corresponding to the distances from the LN2 surface were determined using a type-T bare-wire thermocouple (5SRTC-TT-T-36-36, Omega Engineering Stamford, CT) and analyzed using a data acquisition program (TC-08 Recorder, Omega Engineering) as previously described (Yan et al., 2016). The average cooling rates at 0.5 cm above the LN2 surface was -71.2 °C/min (measured between –10 and −70 °C). For thawing, the straws were rapidly thawed by plunging the samples into a 37 °C water bath with vigorous shaking for 1 min.

2.5. Evaluation of sperm motility, acrosomal integrity, and mitochondria membrane potential

Fresh sperm and thawed sperm samples (10 μL) were placed on a pre-warmed Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) for evaluation of sperm motility. The motility was assessed by counting approximately 200 sperm in duplicate using light microscopy by an experienced evaluator who was blind to the identity of the samples. Motility was evaluated after liquefaction for fresh sperm and after thawing for samples supplemented with the following concentrations: no AFPIII or 0.1, 1, 10, 100 and 200 μg/ml of AFP III. The sperm normalized post-thaw motility was calculated by using the following formula: (post-thaw motility % × 100)/fresh sperm motility %.

The sperm acrosome integrity for fresh and thawed samples was determined using the Alexa Fluor-488-peanut agglutinin conjugate assay (Molecular Probes, Eugene, OR, USA) (Yang et al., 2011). Fresh and frozen-thawed sperm samples (10 μl each) were smeared onto a microscope slide, air-dried and fixed with anhydrous ethanol at room temperature for 30 min. The smear was then incubated with 10 μg/ml Alexa Fluor-488-peanut agglutinin at 37 °C for 30 min in a darkened area and immediately observed afterward using a fluorescence emission ratio of 530 nm utilizing an epifluorescence microscope with an excitation wavelength of 488 nm. Sperm with an uniform apple green fluorescence in the acrosomal region of the head was considered to have an intact acrosome, whereas sperm with little or no green fluorescence staining in the anterior part of the head was considered to have a damaged acrosome. There were at least 200 sperm evaluated for this staining per each semen sample.

The mitochondrial membrane potential of fresh and thawed sperm samples was detected using the fluorescent cationic dye (5, 5′, 6 6′ - tetrachloro - 1, 1′, 3, 3′ tetraethyl benzimidazole carbocyanine - iodide) that is a component of the JC-1 assay kit (Solarbio, Beijing, China) following the manufacturer's instructions (Smiley et al., 1991). Each sample with a final concentration of about 2×10^5 was incubated with JC-1 for 20 min in a 37 °C water bath. The samples were washed and then immediately analyzed using a fluorescence emission ratio of 590 and 530 nm utilizing an epifluorescence microscope with an excitation wavelength of 488 nm. For the sperm with intact mitochondria, the JC-1 reagent aggregates in the mitochondria and fluoresces **orange**and yellow. In contrast, for the sperm with damaged mitochondria, the JC-1 reagent is dispersed and fluoresces green. At least 200 sperm of each sample were evaluated for mitochondrial potential using the fluorescence staining procedure.

2.6. Statistical analysis

All of the data were presented as means ± SD. The results of the test for homogeneity of variance indicated the variance within each of the groups was equal. The percentage data for sperm motility, acrosomal integrity, and mitochondria membrane potential were analyzed using ANOVA and the Fisher protected least-significant difference test to detect the statistical differences (SPSS 16, SPSS, Chicago, IL), and a *P* value of less than 0.05 was considered statistically significant.

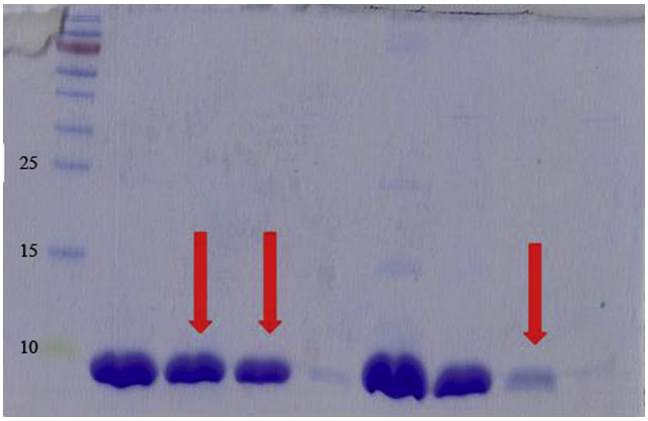

Fig. 1. SDS-PAGE of eluted fraction selected for the experiment; All selected fractions (red arrows) were mixed and lyophilized before restoration (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3. Results

3.1. Production of recombinant AFP III

The protein activity as indicated by the TH measurement of type III AFP and a sample of the protein from the same fraction after lyophilization and restoration was assessed using procedures previously described (Braslavsky and Drori, 2013). By assuming full dissolving of the restored sample, the sample concentration was approximately 0.31 mM with a TH of 0.45 ± 0.01 and 0.43 ± 0.01, respectively. The results are consistent with previously published results for the isoform of this protein (Chao et al., 1994). The SDS-PAGE eluted fraction selected for the experiment is shown in Fig. 1.

3.2. Evaluation of sperm motility

The data for motilities of fresh sperm and frozen-thawed sperm cryopreserved with ORIGIO medium supplemented with no AFPIII or 0.1, 1, 10, 100 or 200 μg/ml of AFP III are included in Table 1. The motilities were less in the frozen-thawed sperm of all samples as compared with the fresh sperm ($P < 0.05$). The percentage of motile sperm and motility recovery rate were greater in the samples supplemented with 0.1, 1 and 10 μg/ml of AFP III than in samples without AFP III ($P < 0.05$), and there was no difference among the samples that were cryopreserved with ORIGIO medium supplemented with 0.1, 1 and 10 μg/ml of AFP III ($P > 0.05$).

3.3. Evaluation of acrosome integrity

The data for acrosome integrities of the fresh sperm and the sperm cryopreserved withORIGIO medium supplemented with no AFPIII or 0.1, 1, 10, 100 and 200 μg/ml of AFP III are summarized in Fig. 2A. Cryopreservation had a detrimental effect on the acrosomal integrity of the sperm cryopreserved with the ORIGIO medium not supplemented or supplemented at the concentrations of 0, 1, 10, 100 and 200 μg/ml of AFP III compared to the fresh sperm ($P < 0.05$). Sperm cryopreserved with the medium supplemented with 0.1 μg/ml of AFP III had a similar percentage of sperm with intact acrosomes compared with the fresh sperm ($P > 0.05$). The samples with the greatest concentrations of AFP III (100 and 200 μg/ml) had less sperm acrosomal integrity compared to

Table 1
Means ± SEM of motility in the groups fresh cynomolgus macaque sperm and post-thawed cynomolgus macaque sperm cryopreserved with ORIGIO medium supplemented with no (Control) or with the following concentrations of antifreeze protein III (AFP III): 0, 0.1, 1, 10, 100, and 200 μg/ml; Normalized post-thaw motility rate was calculated by the formula (post-thaw motility % x 100)/fresh sperm motility %.

Group	Sperm motility (%)	Normalized post-thaw motility (%)
Fresh sperm	86.0 ± 2.4 ^{a}	–
0	36.0 ± 2.1 ^{b}	42.0 ± 2.1 ^{a}
0.1	45.0 ± 2.6 ^{c}	53.0 ± 3.1 ^{b}
1	45.0 ± 2.9 ^{c}	52.0 ± 3.3 ^{b}
10	44.0 ± 2.3 ^{c,d}	52.0 ± 2.6 ^{b}
100	41.0 ± 2.7 ^{b,c}	49.0 ± 3.0 ^{a,b,c}
200	37.0 ± 2.5 ^{b,d}	44.0 ± 2.8 ^{a,c}

Data are presented as mean ± SEM; Different superscripts within a column indicate a difference ($P < 0.05$); Same superscript letters within a column represent no statistical difference between those samples.

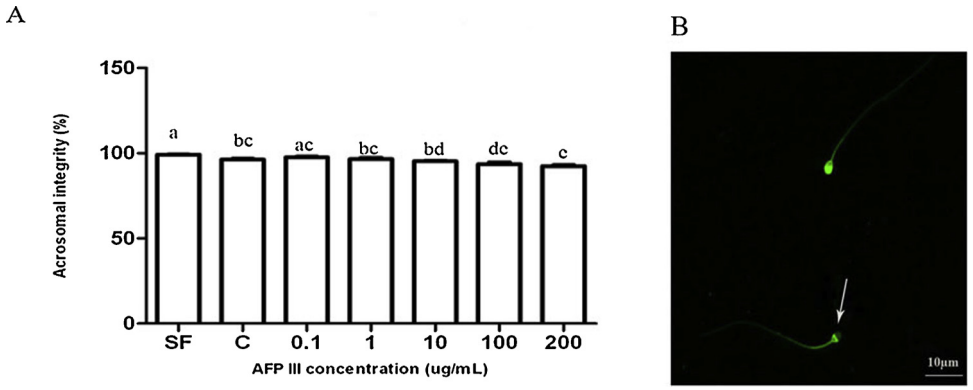

Fig. 2. Acrosome integrity: A) Sperm cryopreserved with ORIGIO medium not supplemented or supplemented with 0.1, 1, 10, 100 or 200 μg/ml of AFP III was compared to that of fresh sperm; B) Frozen−thawed sperm cells with intact and reacted acrosomes; Arrow indicates an acrosome damaged sperm; All data expressed as the means ± SEM; Different superscript letters represents a differences ($P < 0.05$).

the samples without AFP III ($P < 0.05$). Examples of the acrosome-intact and acrosome-damaged sperm are shown in Fig. 2B.

3.4. Mitochondrial membrane potential evaluation

The data for mitochondrial membrane potential of the fresh sperm and the sperm cryopreserved with ORIGIO medium that were not supplemented with or supplemented with 0.1, 1, 10, 100 and 200 μg/ml of AFP III are depicted in Fig. 3A. Cryopreservation decreased the proportion of sperm that had a relatively greater mitochondrial membrane potential in all of the six groups compared to the fresh sperm ($P < 0.05$). The mitochondrial membrane status was greater in the sperm cryopreserved with the medium supplemented with 0.1 μg/ml of AFP III compared with the frozen-thawed sperm from the other five groups ($P < 0.05$). Sperm with relatively greater or lesser mitochondrial membrane potentials are shown in Fig. 3B.

4. Discussion

Combined with the application of assisted reproductive techniques, such as artificial insemination and *in vitro* fertilization, sperm cryopreservation has been successfully used as one of the techniques to produce offspring of macaques (Tollner et al., 1994). Normally, the egg yolk based medium, containing glycerol as the penetrating cryoprotectant is the most widely used sperm freezing medium for cynomolgus sperm cryopreservation (Li et al., 2006.). Egg yolk supplemented added to the sperm-freezing medium provides anti-cold shock and cryoprotective effects for most mammalian sperm during freezing (McCarthy and Meyers, 2011). Numerous commercial egg-yolk-free sperm freezing media are available for humans. In a previous study, the commercial egg-free freezing medium SpermCryo All-round designed for humans was used to cryopreserve cynomolgus macaque sperm and the freezing protocol was optimized. Even so, compared with the conventional egg yolk-based sperm-freezing medium, the post-thaw cryosurvival of sperm either from humans or macaques was less (Yan et al., 2016). In the present study, the chemically defined egg-yolk-free sperm-freezing medium from ORIGIO, which is claimed by the manufacturer to maximize sperm survival, was used to cryopreserve sperm from cynomolgus macaques as a substitute. The average motility recovery rate of macaque sperm cryopreserved using the Sperm Freezing Medium from ORIGIO is 41.9%, which is greater than that reported previously (30.7%) when the SpermCryo All-round medium was used (Yan et al., 2016).

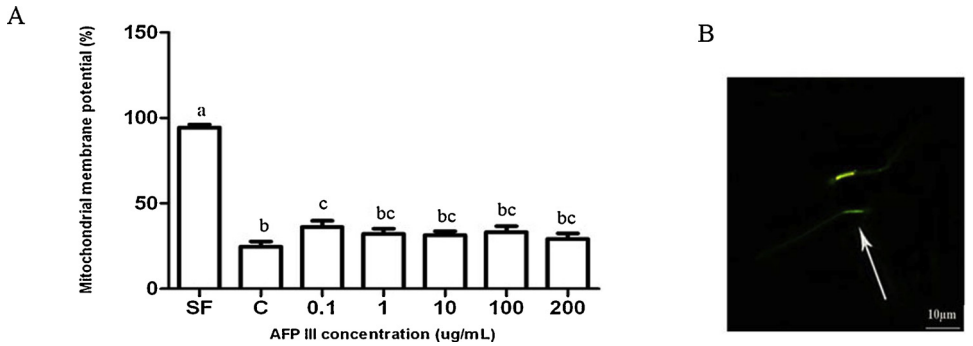

Fig. 3. Mitochondrial membrane potential: A) Sperm cryopreserved with medium supplemented with 0.1 μg/ml of AFP III compared to fresh sperm; B) Frozen-thawed sperm with intact membranes and small amounts of mitochondrial function; Arrow indicates a sperm cell with relatively lesser mitochondrial membrane potential; All data expressed as the means ± SEM; Differences in superscript letters represent differences ($P < 0.05$).

Antifreeze proteins (AFPs) are produced by certain fish, insects, plants, fungi and bacteria and can inhibit ice growth in organisms living in cold environments and permit their survival by cold acclimatization in subzero environments (Crevel et al., 2002). The AFPs bind to small ice crystals to prevent the recrystallization and formation of large and fatal ice crystals (Carpenter and Hansen, 1992). In addition, there is evidence that supplementation of extender with AFPs resulted in a lesser freezing temperature and that this compound interacts with cell membranes to protect membranes from cryoinjuries. The specific mechanisms of action, however, have been difficult to determine. The supplementation with AFP III has been confirmed to be beneficial for seabream, rabbit, bull, and chimpanzee sperm cryopreservation, and depending on the species, an optimal concentration of AFP III exists for sperm cryopreservation (Payne et al., 1994; Younis et al., 1998; Prathalingam et al., 2006; Jo et al., 2011). The post-thawed motility of sperm frozen by using egg-yolk-free medium is less in both Old World primates (cynomolgus monkeys; Yan et al., 2016) and New World primates (squirrel monkeys; Takehito, 2015). The development of an egg-yolk-free medium that improves the cryo-survival of cryopreserved sperm of cynomolgus macaques, therefore, is necessary. In the present study, to further increase the sperm cryo-survival of cynomolgus macaques after freezing and thawing, there was assessment of the cryoprotective effects of AFP III and determination of the optimal concentration based on the chemically defined ORIGIO Sperm Freezing Medium.

In a previous study on chimpanzee sperm cryopreservation, the addition of a relatively larger amount of AFP III to result in a concentration of 100 μg/ml in the glycerol-egg yolk based medium resulted in a greater cryoprotection of sperm motility than a lesser concentration of AFP III (1 and 10 μg/ml), and the post-thaw motility recovery rate increased from 28.9% (without AFP III) to 67.8% (with 100 μg/ml AFP III). Unlike in the previous studies, the current study is apparently the first where there was assessment of AFP III in an egg-yolk-free medium. Results of the present study indicate the post-thawed sperm motility of cynomolgus macaques was greater when there were relatively lesser concentrations of AFP III (0.1, 1, and 10 μg/ml) in the ORIGIO Sperm Freezing Medium. Results from the present study are similar to those from a previous study with rabbit sperm cryopreserved with an egg yolk based medium containing small concentrations of AFP III (0.1 and 1 μg/ml) which led to improvement of the post-thaw sperm motility. Also, the addition of 0.1 μg/ml AFP III in an egg yolk based medium improved the motility and plasma membrane integrity of cryopreserved buffalo bull sperm. The varying results of AFP III might be attributed to different mediums, cooling rates, and different cell membrane compositions in different species (Prathalingam et al., 2006). In the current study, there also was no use of a controlled-rate freezer per the manufacturers of the sperm freezing medium instructions and because the use of nitrogen vapors are known to induce cryodamage. The results from the present study might, therefore, have been different if another method was used.

In addition to the loss of motility, the damage to sperm acrosomes is another of the major cryodamage outcomes in nonhuman primates (Mclaughlin et al., 1993). The acrosomal reaction is an exocytotic calcium-dependent process that is considered the major pre-requisite for sperm penetration through the oocyte membranes (Gerardo et al., 2012). Accompanying the reduction in sperm motility, post-thawed sperm lose acrosome integrity, which might be indicative of the decrease in its fertilizing capacity. In a previous study with chimpanzee sperm cryopreservation where there was the addition of a large amount of AFP III that resulted in a concentration of 100 μg/ml of extender and also supplementation with insulin transferrin selenium, there was a relatively greater percentage of sperm with intact acrosomes after the freezing and thawing process than with the other groups included (Younis et al., 1998). In contrast, when there was no AFP III supplementation or supplementations resulting in concentrations of 0.1, 1, and 10 μg/ml there was no increase in the acrosome integrity of cynomolgus sperm after the freezing and thawing process. Furthermore, supplementations at the greatest concentrations of AFP III (100 and 200 μg/ml) had detrimental effects on acrosome integrity in the present study. Results of the present study are consistent with those from previous studies where there was supplementation of 0.01, 0.1, 1 and 10 μg/ml of AFP III without improvement in buffalo sperm with intact acrosomes (Qadeer et al., 2014). The negative effect of the relatively greater concentrations of AFP III on acrosomal integrity indicates that AFP III is cytotoxic to cryopreserved sperm depending on the concentration in the media. Further study, however, is required to clarify what is leading to the cytotoxic effects of AFP III on the sperm of cynomolgus macaques.

Functional mitochondria are considered a potential indicator of sperm motility and fertility. This study is apparently the first from which it has been reported the effect of antifreeze proteins on the mitochondrial membrane potential of cryopreserved sperm. Lipid peroxidation during sperm freezing and thawing processes, which increases the generation of mitochondrial active oxygen, is believed to be the reason for the loss of the mitochondrial membrane potential in sperm (Kadirvel et al., 2009). In the present study, the mitochondrial membrane potential of cryopreserved sperm that were stored in egg-yolk-free medium supplemented with a relatively small concentration of AFP III (0.1 μg/ml), was increased from 25% to 36%. Cell membranes are vulnerable to damage by the active oxygen produced by lipid oxidation produced during sperm cryopreservation. The stabilization of the cell membrane by AFPs involves the binding of this protein to the biphospholipid-layer of the cell membrane. Thus, the relatively lesser concentration of AFP III in the present study might have resulted in binding of this compound to the mid-piece where the mitochondria are located. There would, therefore, have been less damage to the mitochondrial membrane by ice crystallization during freezing and thawing.

In summary, in the present study the effect of different concentrations of AFP III for cynomolgus macaque sperm cryopreservation using a commercial egg-yolk-free medium was determined. The sperm motilities were greater when there was supplementation of the ORIGIO sperm freezing medium with 0.1, 1, and 10 μg/ml of AFP III. In addition, the mitochondrial membrane potential was greater for sperm cryopreserved with the ORIGIO medium supplemented with 0.1 μg/ml of AFP III. The addition of AFP III at either concentration did not contribute to cryoprotection of the acrosomal membrane of sperm, and the relatively greater concentration of AFP at 100 and 200 μg/ml had detrimental effects on acrosomal integrity. Results of the present study indicate there is a new and effective method for genetic preservation of cynomolgus macaques with a reduction in associated health risks.

Declaration of Competing Interest

The authors would like to declare that there is no conflict of interest in the publication of this work.

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