

Cryopreservation of Transgenic Huntington's Disease Rhesus Macaque Sperm: A Case Report

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Abstract

The cryoprotective effects of glycerol in three different semen freezing extenders, Tris-citrate (TRIS), TEST, and Tes-Tris-Egg yolk (TTE), on wild-type (WT) rhesus monkey (*Macaca mulatta*) sperm cryopreservation have been tested. Sperm motility and viability were examined to evaluate the integrity of frozen-thawed sperm, and the best extender was selected to cryopreserve sperm from transgenic Huntington's Disease (HD) rhesus monkey. The results showed no post-thaw motility difference among the freezing extenders tested ($P>0.05$). However, sperm membrane integrity in TEST and TTE were significantly better than in TRIS extender ($P<0.05$). TEST was chosen for HD rhesus monkey sperm cryopreservation. The results showed that post-thawed HD sperm motility and viability was not different compared with the WT control group ($P>0.05$). The present study demonstrates that TEST and TTE were excellent extenders and suitable for rhesus monkey sperm cryopreservation and no detectible differences of post-thaw sperm motility and viability between HD and WT rhesus monkeys resulted from TEST extender.

Keywords: Transgenic monkey model; Huntington's disease; Sperm cryopreservation; Freezing extender

Introduction

Sperm cryopreservation technology is one of the most important Assisted Reproductive Techniques (ART) that is used for genome banking of biomedically important laboratory animals, farm animals and human infertility programs [1]. Since the discovery of glycerol as a Cryoprotectant Agent (CPA) for sperm cryopreservation [2], glycerol have been the major CPA for sperm cryopreservation in many species including; Nonhuman Primates (NHPs) such as lemurs [3], marmosets [4-6] squirrel monkeys [7], African greens [8] vervet monkeys [9,10], cynomolgus monkeys [11-19], Japanese monkeys [20], stump tail monkeys [8], baboons [21] chimpanzees [8,22-24] and gorillas [25-27] among NHPs, rhesus monkeys are the most commonly used research model in the development of sperm cryopreservation due to their high physiologic and genetic similarity with humans [28-38]. There have been extensive studies on rhesus monkey sperm cryopreservation [8,39-45]. However, sperm cryopreservation efficiency varies between laboratories, because different freezing methods, different extenders, and primate species were used. Thus, it is difficult to compare results and the need for an optimal protocol for sperm cryopreservation remains in high demand.

Huntington's disease (HD) is an inherited neurodegenerative disorder resulting from an expanded polyglutamine (CAG) tract in the Exon 1 of the Huntington gene IT15 (*HTT*) [46] that leads to progressive impairment on motor, cognitive and psychiatric functions as an individual ages [4,47-50]. The size of the polyglutamine (CAG) expansion is significantly influenced by gender, as paternal instability has shown to be greater than maternal inheritance in the mouse HD model and human patients [51,52]. While the mechanism of paternal instability in HD remains largely unknown, HD monkey sperm provides a unique resource for investigating CAG stability and paternal inheritance. Besides the understanding of HD pathogenesis and its correlation with CAG instability, cryopreservation of HD monkey sperm preserves the unique genetics for future propagation of the next generation HD monkeys since fertility in HD monkey declines as disease progresses. Thus, the goal of the present study was to develop

and optimize a cryopreservation protocol of HD monkey sperm which will be used for future banking of HD monkey sperm.

Materials and Methods

A total of five ejaculates were collected from a Wild-Type (WT) male rhesus monkey and used to assess freezing efficiency of three freezing extenders (TRIS, TEST, and TTE). A total of four ejaculates were collected from a male HD monkey and compared with a WT control monkey. The extender that offered satisfactory results will be selected for HD monkey sperm cryopreservation.

Animal model

The rhesus monkeys (WT and HD) were housed and maintained at Yerkes National Primate Research Center. All animal procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at Emory University. All studies were conducted in accordance with the guide for *The Care and Use of Laboratory Animals*.

Generation of transgenic HD rhesus monkey

A transgenic HD monkey (rHD1) was generated by Yang and colleagues (2008). In brief, high titer lentiviruses carried exon1 of human *HTT* gene with 84 CAG repeats and were co-injected with lentiviruses carried Green Fluorescent Protein (GFP) gene under the regulation of human polyubiquitin C promoter into the perivitelline space of metaphase II arrested monkey oocytes followed by Intracytoplasmic

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Sperm Injection (ICSI). The resulting embryos were transferred into surrogate females for the generation of transgenic HD monkeys [53].

Sperm cryopreservation extender

Each cryopreservation extender (TRIS, TEST, and TTE) was divided into two parts (with and without glycerol). The composition of each extender is listed in Table 1. The primary semen diluting extender (without glycerol) was centrifuged at $7,000\times g$ at 4°C for one hour to remove yolk granules [18]. Supernatant was filtered with $0.2\ \mu\text{m}$ filter and stored at -80°C . The extender was thawed at 37°C water for 15 min and equilibrated at room temperature (RT) before it was used. The secondary extender was a mixture of the primary extender with 8%, 3%, and 5% glycerol in the TRIS, TEST, and TTE extenders, respectively).

Semen collection procedure

Ejaculates were collected once a week in the morning. All male monkeys were chair trained for semen collection. Monkeys were sedated with a light dose of Telazol (0.7-1.0 mg/kg body weight) by intramuscular injection. One pre-sized defibrillator gel electrode was wrapped around the base of the penis and connected to the negative lead. The second gel electrode was positioned immediately behind the glans and connected to the positive lead. A slow and steady stimulation by increasing the output-adjust dial induced a slight erection, engorgement of the glans, and/or elevation of the testicles into the inguinal region. Typically, engorgement and ejaculation occurred at 10-20 V. When the sample was delivered, the date, time, peak output current, length of time electrical current was delivered, drug administered, behavioral observations, and technician initials were all recorded. The ejaculates were kept at Room Temperature (RT) for 20 min to liquefy. The liquid portion of semen was transferred into 15 mL conical tube and washed with TALP-HEPES medium supplemented with 4 mg/mL bovine serum albumin (BSA) [54], and followed by centrifugation at $112\times g$ at RT for 7 min. The sperm concentration, motility and viability were determined and recorded.

Sperm freezing procedure

Washed WT rhesus monkey sperm was divided into three groups for three different extenders. The sperm was diluted with primary extender in 15 mL conical tube and kept in 500 mL beaker containing RT water and kept in 4°C refrigerator for 2 h. Equal volume of pre-cooled secondary extender (with glycerol) was added and kept for 30 min at 4°C [18]. The final concentration of glycerol was 8%, 3% and 5% in TRIS, TEST and TTE, respectively. Final sperm concentration was 30×10^6 cells mL^{-1} . Sperm was then loaded into 0.25 mL straws by using 1mL syringe and sealed with plug powder. Straws were kept in 4°C water for 30 min. The straws were then laid horizontal on an aluminum rack and then transferred to a styrofoam box containing liquid nitrogen (LN_2) and held for 8min, positioned 4 cm above the surface of LN_2 . LN_2 was increased by 1cm every 2 min until the final distance between surface of LN_2 and straw was 1cm. Straws were then plunged directly into LN_2 and stored for a week before thawing.

For transgenic HD rhesus monkey sperm cryopreservation, the extender that resulted in best post-thawed sperm motility and viability in WT was selected for sperm cryopreservation. The cryopreservation process was preformed following the protocol described above. Sperm samples were evaluated after a minimum of one-week storage in LN_2 .

Sperm thawing

Straws containing frozen spermatozoa were thawed in a 37°C water bath for 1-2 min. Thawed spermatozoa was washed in 15 ml conical

tube with 4mL of TALP-HEPES medium supplemented with 4mg/mL BSA² by centrifugation at $112\times g$ under RT for 7 min. The sperm pellet was re-suspended by gently pipetting, and sperm motility and viability were recorded.

Sperm motility evaluation

10 μL thawed semen were placed onto a 37°C pre-warmed microscope slide and covered with a 22 mm square cover-glass. The slides were visualized and recorded using by an inverted microscope equipped with CellSense Imaging software (Olympus Inc.). Then sperm motility was evaluated by three different individuals with two of them blind evaluators. The sperm motility recovery was calculated by the following formula: (post-thaw motility % \times 100)/ pre-freeze sperm motility %.

Sperm viability evaluation

The sperm viability was assessed by using a LIVE/DEAD[®] Sperm Viability Kit (Invitrogen). The pre-freeze or post-thaw sperm concentration was adjusted at 2.5×10^6 mL^{-1} . Sperm samples were incubated with 100 nM of SYBR 14 dye at 37°C water bath for 5-10 min in a dark room, and then they were incubated with 12 μM of Propidium Iodide (PI) for another 5 min at 37°C water bath in a dark room. After that, stained sperm were placed on a microscope slide coated with 0.1% poly-D-lysine (Sigma) and covered with a 22mm square cover glass. The sperm showing a bright green fluorescence were considered to be alive while sperm with red fluorescence were rated as dead (Figure 1). Sperm viability was calculated by the following formula: (number of

Chemicals	TRIS	TEST	TTE
TES	-	172 mM	47.8 mM
Tris	250 mM	84.7 mM	-
Tris-HCl	-	-	12.7 mM
Glucose	-	55.5 mM	111 mM
Citric acid	87.2 mM	-	-
D-fructose	69.4 mM	-	-
Lactose	-	-	55.4 mM
Raffinose	-	-	3.36 mM
Distill water	50 ml	50 ml	50 ml
pH	7.2-7.4	7.2-7.4	7.2-7.4
Egg yolk	20%	20%	20%
Glycerol	8%	3%	5%
Ref.	Our laboratory	Dong et al., 2008	Sankai et al., 1994

Table 1: The composition of freezing extenders.

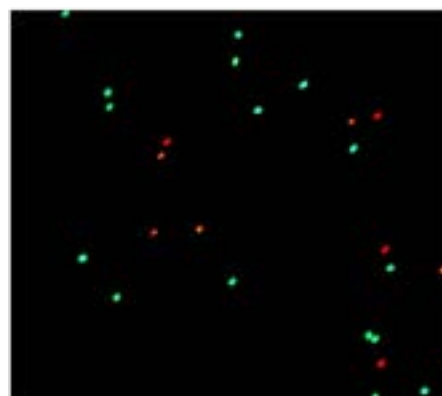


Figure 1: Sperm viability evaluation. Live sperm stained with SYBR 14 dye (green fluorescence) and dead sperm stained with PI (red fluorescence).

live sperm \times 100)/ number of total sperm count. The sperm viability recovery was calculated by the following formula: (viability of post-thawed sperm % \times 100)/ pre-freeze sperm motility %.

Statistical analysis

Sperm motility and viability were analyzed between each treatment group using a general linear model. This test was used to analyze significant differences between freezing extenders in WT frozen sperm and between WT and HD frozen sperm. The analysis was performed using the Statistical Analysis System Software (SAS, version 9.0; SAS, Cary, NC, USA) and a value of $P < 0.05$ was considered as significantly different.

Results

Effect of freezing extender on WT rhesus monkey sperm cryopreservation

The post-thaw motility and motility recovery of WT rhesus monkey sperm is presented in Table 2. The post-thaw sperm motility and motility recovery in TEST group was slightly higher than other groups. However, there was no difference among the three extenders both in terms of motility ($54.78 \pm 3.88\%$, $50.16 \pm 7.52\%$, and $46.99 \pm 4.82\%$, in TEST, TTE, and TRIS respectively) and motility recovery ($60.69 \pm 5.58\%$, $53.99 \pm 6.70\%$, and $52.51 \pm 7.43\%$, in TEST, TTE, and TRIS respectively).

The viability and viability recovery of post-thawed WT rhesus monkey sperm were presented in Table 3. The post-thawed sperm viability in TEST group ($60.75 \pm 2.85\%$) was significant higher than TRIS group ($48.79 \pm 3.46\%$), but no different from TTE group ($54.85 \pm 3.34\%$), while TRIS group was no different from TTE group.

HD rhesus monkey sperm cryopreservation

The comparisons of three extenders using WT rhesus monkey sperm for cryopreservation revealed that TEST extender was slightly better than other extenders. Therefore, TEST was selected for cryopreservation of HD rhesus monkey sperm. The motility and motility recovery of post-thaw HD rhesus monkey sperm was presented in Table 4. The HD sperm motility ($53.67 \pm 8.96\%$) and motility recovery ($56.53 \pm 9.89\%$) showed no significant difference with control group ($58.06 \pm 2.68\%$, in terms of motility and $65.19 \pm 4.29\%$, in motility recovery).

The sperm viability and viability recovery of HD rhesus monkey sperm was presented in Table 5. The post-thawed HD sperm viability was not different from the control group ($58.18 \pm 1.74\%$ and $61.74 \pm 3.46\%$, respectively). However, viability recovery of HD sperm was significantly lower than the control group ($62.97 \pm 1.57\%$ and $78.48 \pm 3.40\%$, respectively).

Discussion

The composition of the freezing extender plays a key role in sperm cryopreservation. Thus, this study investigated the effect of freezing

Extender	Fresh sperm motility, %	Post-thawed sperm motility, % ^a	Motility recovery rate, % ^a
TRIS	87.12 \pm 4.56	46.99 \pm 4.82	52.51 \pm 7.43
TEST		54.78 \pm 3.88	60.69 \pm 5.58
TTE		50.16 \pm 7.52	53.99 \pm 6.70

^a Values within column are not significantly different ($P > 0.05$).

Table 2: The post-thaw motility analysis of WT rhesus monkey spermatozoa in TRIS, TEST, and TTE.

Extender	Post-thawed sperm viability, %	Viability recovery rate, %
TRIS	48.79 \pm 3.46 ^b	60.19 \pm 3.62 ^b
TEST	60.75 \pm 2.85 ^a	75.23 \pm 4.17 ^a
TTE	54.85 \pm 3.34 ^{ab}	67.81 \pm 4.01 ^{ab}

^{a,b} Values within column are significantly different ($P < 0.05$).

Table 3: Post-thaw viability analysis of WT rhesus monkey spermatozoa in TRIS, TEST and TTE extenders.

Sperm types	Fresh sperm motility, % ^a	Post-thawed sperm motility, % ^a	Motility recovery rate, % ^a
HD	95.37 \pm 1.01	53.67 \pm 8.96	56.53 \pm 9.89
WT	86.60 \pm 2.48	58.06 \pm 2.68	65.19 \pm 4.29

^a Values within column are not significantly different ($P > 0.05$).

Table 4: Post-thaw sperm motility comparison of HD and WT rhesus monkey.

Sperm types	Post-thawed sperm viability, %	Viability recovery rate, %
HD	58.18 \pm 1.74	62.97 \pm 1.57 ^b
WT	61.74 \pm 3.46	78.48 \pm 3.40 ^a

^{a,b} Values within column are significantly different ($P < 0.05$).

Table 5: Post-thaw sperm viability comparison of HD and WT rhesus monkey.

extenders on rhesus monkey sperm cryopreservation by comparing three different freezing extenders. Due to the limited number of adult WT and HD monkey sperm donors, we were only able to collect sperm from one WT and one HD monkey in this study to develop a cryopreservation protocol that we could further evaluate when more sperm donors are available. The freezing extenders used in our experiments have been previously employed by other research groups in an effort to freeze NHP sperm. Previous studies obtained satisfactory results with TTE in cynomolgus [18] and rhesus monkey [45], TEST in rhesus monkey [39,40] and TRIS in rhesus monkey (our laboratory, unpublished results). Post-thaw sperm motility was around $\geq 50\%$ for the TEST and TTE in previous studies; however, differences existed among species and freezing methods used. The results of this study showed that three extenders (TRIS, TEST, and TTE) offered good cryoprotection during sperm freezing in rhesus monkey. Although, TEST showed slightly better than other extenders; no significant difference in sperm motility was found among extenders.

Many factors affect successful sperm cryopreservation. These include; freezing and thawing procedures, extenders, types of cryoprotectant, and skill of investigators. The cryoprotectant is one of the major factors that influence sperm survival during sperm cryopreservation. Glycerol is a commonly used cryoprotectant in NHP sperm freezing; however, loss in sperm motility and subsequent fertility can result if optimal concentration was not used [13]. Many reports suggested that 3-5% glycerol offer suitable concentration for cynomolgus [18] and rhesus monkey [13,14,39,40,55], sperm cryopreservation. In the present study, the results showed that 3% TEST and 5% TTE of glycerol concentration provided better cryoprotection than 8% TRIS. This confirms that high concentrations of glycerol as cryoprotectant resulted in higher toxicity to the sperm.

In this study we also conducted transgenic HD rhesus monkey sperm cryopreservation by using TEST extender. Our results showed that post-thaw sperm motility and viability were not significantly different between HD and WT monkey sperm. This suggested that mutant HTT may not have an adverse effect on the quality of sperm and HD monkey spermatozoa can endure stresses during freezing and thawing process.

HD is an inherited neurodegenerative disease. The gender and age of HD parents influences the transmission of CAG repeats to next generation. In paternal transmission there is a propensity toward larger CAG repeat expansion while maternal transmission results in lower CAG expansion rate in progeny [51,56,57]. Although CAG expansion tends to occur more often from the paternal rather than maternal parent, expansion of CAG repeats in the progeny does not always reflect the size of repeats in the sperm of the fathers [52]. While the correlation between CAG expansion and parental inheritance is not fully understood, sperm cryopreservation of HD monkeys at different times as the HD monkeys age provide a unique model for studying germline polyglutamine expansion.

In conclusion, the present study showed that freezing extender TEST and TTE were excellent extenders which are suitable for rhesus monkey sperm cryopreservation, and no detectible differences of post-thawed sperm motility and viability between HD and WT in TEST extender were found. Thus cryopreserved HD monkey sperm that is cryopreserved in this way can be used for future production of HD monkeys for biomedical research and preclinical applications.

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