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Technical report

Successful sperm cryopreservation in Egyptian spiny mice *Acomys cahirinus*

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Abstract. The menstruating Egyptian spiny mouse has recently been proposed as a new animal model for reproductive health research. Unfortunately, little is known about reproduction in males. This study compared several characteristics of sperm function before and after cryopreservation. Epididymal spermatozoa were cryopreserved in different concentrations of raffinose and skim milk and tested for motility and membrane integrity (Experiment 1). Further evaluations of motility, plasma membrane and acrosome integrity, mitochondrial membrane potential and DNA integrity were conducted with the addition of L-glutamine to the extender (Experiment 2). The results show that, following cryopreservation, motility and membrane integrity were reduced, but were better maintained in the presence of L-glutamine ($P < 0.05$). Moreover, although all sperm parameters were significantly reduced following cryopreservation ($P < 0.05$), most cryopreserved spermatozoa retained acrosome, membrane and DNA integrity while also maintaining motility and mitochondrial membrane potential. This study provides a new step towards the development of assisted reproductive techniques and archiving the important genetics of the world's only known menstruating rodent.

Keywords: *Acomys cahirinus*, acrosome reaction, cryopreservation, DNA integrity, Egyptian spiny mice, fluorescence microscopy, membrane integrity, mitochondrial potential, sperm, spermatozoa.

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Introduction

Sperm cryopreservation combined with IVF is a commonly used assisted reproductive technique (ART) in laboratory species for colony maintenance, the preservation of strain genetics and reproductive studies.

Until recently, the most commonly used murine sperm freezing extender was a combination of 18% w/v raffinose and 3% w/v skim milk, as defined by Okuyama *et al*. (1990). However, Takeo and Nakagata (2010) reported improved post-thawing sperm parameters in C57BL/6 mice by supplementing Okuyama's extender with 100 mM L-glutamine. Mammalian spermatozoa are particularly susceptible to oxidative stress (Aitken *et al.* 2014) and supplementation with antioxidants such as L-glutamine is known to mitigate oxidative damage following cryopreservation (Gharagozloo and Aitken 2011).

The Egyptian spiny mouse *Acomys cahirinus* is a desert-adapted rodent species that exhibits several human-like reproductive characteristics that are rare traits in laboratory rodents, including precocial young (Brunjes 1990), long gestation (Dickinson and Walker 2007) and a menstrual cycle (Bellofiore *et al.* 2017), the latter being present in <2% of all mammalian species (Emera *et al.* 2012). Because *A. cahirinus* thrives in laboratory breeding colonies (Haughton *et al.* 2016), it has great potential as an animal model to study human reproductive function and dysfunction. The aims of the present study were to examine the functional parameters of cauda epididymal spermatozoa and to develop a robust sperm cryopreservation technique for archiving spiny mouse spermatozoa samples for genetic management and future assisted reproductive studies in the species.

Materials and methods

Experimental design

Two *in vitro* experiments were designed to: (1) evaluate the efficacy of six different extender concentrations in preserving the motility and membrane integrity of spiny mouse spermatozoa following cryopreservation (Experiment 1); and (2) assess the effect of L-glutamine supplementation on sperm motility and membrane integrity while optimising tests for the analysis of acrosome integrity, mitochondrial membrane potential (MMP) and DNA integrity (Experiment 2).

Animals

All experiments were approved by the Monash University/Monash Medical Centre Animal Ethics Committee (MMCA/2017/14). The animals used in this study ($n = 30$) were sourced from the Monash research colony and housed under controlled conditions described by Dickinson and Walker (2007).

Table 1. Extender solutions containing different concentrations of raffinose and skim milk

Treatment group	Cryopreservation solutions (% w/v in Milli-Q water)		Abbreviation
1	18% raffinose	3% skim milk	18R3SM
2	15% raffinose	3% skim milk	15R3SM
3	15% raffinose	5% skim milk	15R5SM
4	10% raffinose	5% skim milk	10R5SM
5	15% raffinose	10% skim milk	15R10SM
6	10% raffinose	10% skim milk	10R10SM

Experiment 1 sperm analysis

Epididymal spermatozoa were cryopreserved in six different extenders containing different concentrations of raffinose and skim milk (Table 1). Sperm extenders were prepared as described by Takeo and Nakagata (2010).

Sperm collection and swim-up procedure

Mice were killed by inhalation of an overdose of isoflurane in a sealed chamber containing 0.2 mL isoflurane per animal before excision of the cauda epididymidis. Each cauda epididymidis was incised several times and placed into 1.5-mL warmed tubes (Thermo Fisher) containing either 500 µL extender before cryopreservation or 500 µL M2 handling media (Sigma-Aldrich). Spermatozoa were allowed to swim up in the medium for 30 min at 37°C to isolate the motile population before analysis.

Sperm freezing and thawing

After selecting spermatozoa using the swim-up procedure, 10-µL aliquots of each sperm suspension (30×10^6 spermatozoa mL^{-1}) were loaded into 0.25-mL cryopreservation straws and sealed with polyvinyl alcohol (PVA). The straws were then held in liquid nitrogen vapour (−150°C) for 10 min before plunging and storage in liquid nitrogen for at least on week prior to thawing. Samples were thawed in air for 5 s and then in a water bath at 37°C for 10 min. Each sample was expelled into a 50-µL drop of warmed M2 handling medium at 37°C and incubated for at least 2 h under oil (Sigma) before analysis (Fig. S1, available as Supplementary Material to this paper).

Experiment 1

A 10-µL aliquot of sperm suspension was added to a prewarmed Makler chamber (Origio) and percentage motility was assessed by scoring the number of motile and immotile spermatozoa in randomly selected microscope fields. At least 200 spermatozoa were counted, and two replicates prepared per sample. A qualitative assessment of sperm motility was also performed (+++, fast progressive motility; ++, slow progressive motility; +, non-progressive motility).

Sperm plasma membrane integrity was assessed using a combination of propidium iodide (PI; final concentration 7.0 µg mL^{-1}; Sigma) and H33342 (final concentration 0.21 µg mL^{-1}; Thermo Fisher) staining. After staining, 10 µL sperm mixture was placed on a microscope slide, coverslipped and examined using an Invitrogen EVOS M7000 imaging system (Fig. S1). Fluorescence was detected using 4′,6′-diamidino-2-phenylindole (DAPI; 500 nm) and red fluorescent protein (RFP; 590 nm) emission filters (Table S1).

Experiment 2

The best extender composition determined from Experiment 1 was chosen for Experiment 2 and supplemented with 100 mM L-glutamine. In addition to motility and membrane integrity, spermatozoa frozen in this extender were evaluated for acrosome integrity, MMP and DNA integrity. At least 200 spermatozoa were counted, and two replicates prepared per sample.

Double- and single-stranded DNA breaks were assessed using terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP–digoxigenin nick end-labelling (TUNEL; Roche Diagnostics) with minor modifications to the manufacturer's protocol. Briefly, a 10-µL aliquot of spermatozoa was dried at 37°C on a microscope slide for 30 min, sprayed with methanol fixative (Leica) and left for 1 h at room temperature. Slides were then washed twice with phosphate-buffered saline (PBS) and permeabilised with 0.1% v/v Triton-X in PBS for 10 min on ice. The slides were then washed twice in PBS and incubated with 50 µL TUNEL reaction mix (TdT enzyme + labelling solution) in a humidified atmosphere in the dark for 1 h at 37°C. Spermatozoa were counterstained with H33342 (final concentration 0.22 µg mL^{-1}; Sigma) for 5 min. Slides were washed in PBS, coverslipped and fluorescence was detected using DAPI (500 nm) and fluorescein isothiocyanate (FITC; 535 nm) emission filters on an Invitrogen EVOS M7000 imaging system (Table S1; Fig. S2).

Plasma membrane integrity, acrosome integrity and MMP were assessed simultaneously using a 'quad' stain of H33342, PI, FITC-conjugated peanut agglutinin (PNA-FITC) and 5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethylbenzimi-dazolylcarbocyanine iodide (JC-1). For staining, H33342 (final concentration 0.21 µg mL^{-1}), PI (final concentration 7.0 µg mL^{-1}), PNA (final concentration 1.7 µg mL^{-1}; Sigma) and JC-1 (final concentration 0.2 µg mL^{-1}; Thermo Fisher) were added to a 10 µL sample from each sperm suspension. The mixture was then incubated in the dark for 15 min at 37°C and fluorescence analysed using an Invitrogen EVOS M7000 imaging system (Fig. 1; Fig. S1; Table S1).

Statistical analysis

All data were analysed using Prism 8 software (GraphPad) and samples were tested for normality using the Shapiro–Wilk test and for homogeneity using the Brown–Forsythe test before further analysis. For Experiment 1, one-way repeated-measure

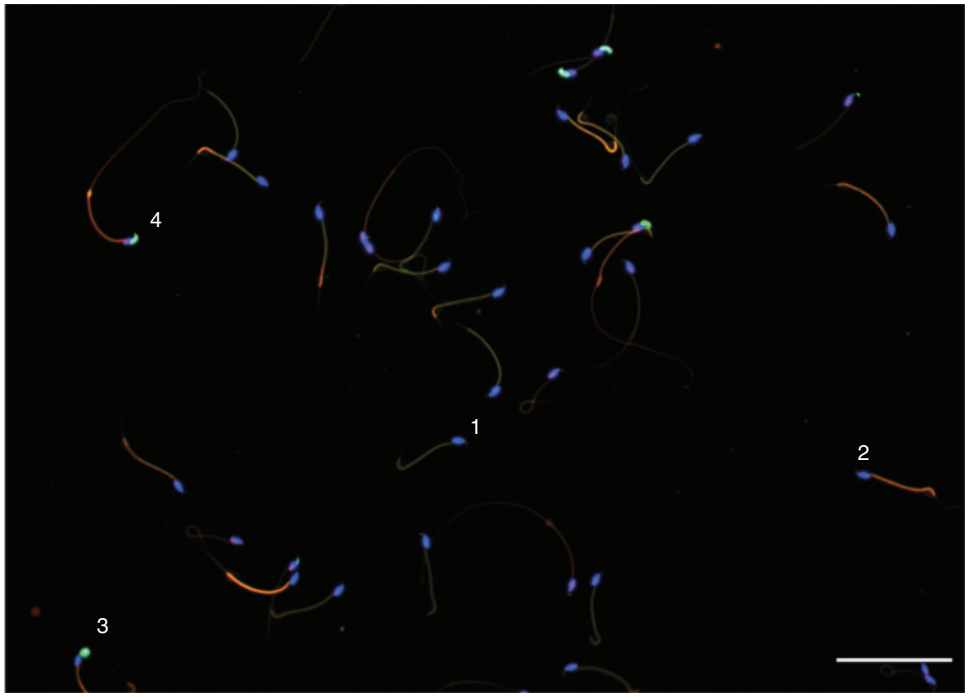

Fig. 1. Fluorescent micrograph showing sperm staining patterns. 1, living spermatozoa with intact acrosomes and low mitochondrial membrane potential (MMP); 2, living spermatozoon with intact acrosome and high MMP; 3, living spermatozoon with a damaged acrosome and high MMP; 4, damaged spermatozoon with a damaged acrosome with high MMP. Scale bar = 50 µm.

analysis of variance (ANOVA) was used to compare data between cryopreservation groups and to determine any inter-subject variability, whereas paired *t*-tests were used to determine any intrasubject variability. For Experiment 2, all sperm parameters were analysed by independent-sample *t*-tests. Statistical significance was set at two-tailed $P < 0.05$ for all experiments.

Results

Experiment 1

No post-thaw motility was observed in spiny mouse spermatozoa cryopreserved in solutions containing 10% skim milk (15R10SM, 10R10SM); this concentration of skim milk was not used in further experimentation. Moreover, post-thaw sperm motility and membrane integrity were low for all treatments when assessed immediately after thawing. However, when samples were allowed to swim-out in M2 for 2 h after thawing, membrane integrity and total motility were significantly improved ($P < 0.0001$) and a qualitative assessment showed an increase in progressive motility (Table S2).

Motility and membrane integrity in the remaining four extenders were significantly reduced compared with fresh controls (Fig. 2*a*; $P < 0.05$). Moreover, motility and membrane integrity in solutions 18R3SM, 15R3SM and 15R5SM were similar ($P > 0.05$), but significantly higher than that in 10R5SM ($P < 0.05$). Inter- and intrasubject variability in all treatments was not significant ($P > 0.05$). Because post-thaw motility was comparable in Treatments 1–3 (18R3SM, 15R3SM and 15R5SM), Treatment 1 (18R3SM) was selected for use in Experiment 2.

Experiment 2

Membrane integrity and total motility were significantly increased in spermatozoa cryopreserved in 18R3SM + L-glutamine compared with 18R3SM alone (Fig. 2*b*; $P < 0.01$). Moreover, mean (±s.d.) motility (67.0 ± 3.7 vs 75 ± 2.3), membrane integrity (74.2 ± 4.3 vs 79.4 ± 1.8), MMP (70.5 ± 4.2 vs 87.5 ± 2.2), acrosome integrity (78.6 ± 1.8 vs 88.2 ± 2.2) and DNA integrity (80.8 ± 2.5 vs 89.2 ± 1.5) remained high after thawing (Fig. 2*c*). (Values here in parentheses represent spermatozoa %, i.e. frozen-thawed % and fresh % respectively.) Qualitative assessment also showed an improvement in progressive motility in spermatozoa cryopreserved in 18R3SM + L-glutamine compared with 18R3SM alone (+++ vs ++). However, all parameters from frozen–thawed spermatozoa were significantly reduced compared with fresh spermatozoa ($P < 0.05$).

Discussion

Cryopreservation has an essential role in the long-term storage of cells and tissues for fertility preservation and for the archiving of important genetic material. Sperm cryopreservation has been routinely performed in many mouse strains (Mochida *et al.* 2014; Taft 2017), as well as in the closely related Mongolian (Sato *et al.* 2000) and Indian (Koshimoto *et al.* 2009) gerbils. However, sperm cryopreservation has not been previously reported for any species in the large murid subfamily Deomyinae.

Optimal conditions for sperm cryopreservation can differ significantly between species due to inherent variability in membrane permeability and osmotic tolerances (Shaw and

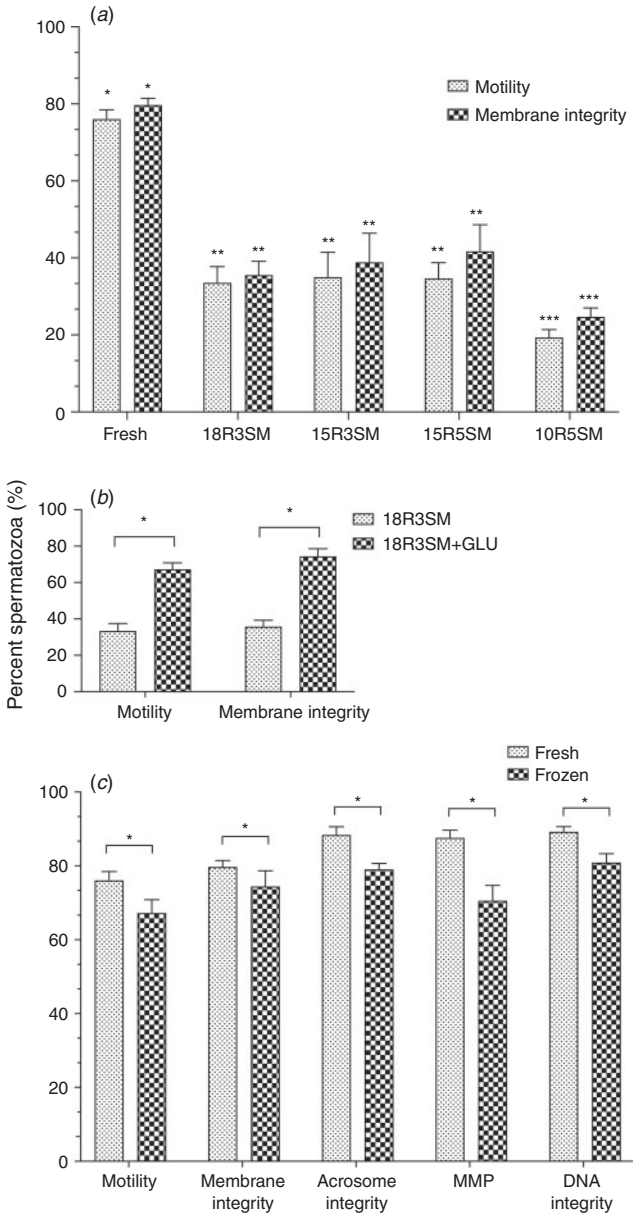

Fig. 2. (*a*) Post-thaw sperm motility and membrane integrity in four extender solutions, (*b*) post-thaw sperm motility and membrane integrity with the addition of L-glutamine to the extender solution and (*c*) various sperm function parameters between fresh spermatozoa and spermatozoa cryopreserved using an extender solution containing 18% raffinose, 3% skim milk (18R3SM) and 100 mM L-glutamine (GLU). Data are the mean ± s.d. $*P < 0.05$. 15R3SM, 15% raffinose and 3% skim milk; 15R5SM, 15% raffinose and 5% skim milk; 10R5SM, 10% raffinose and 5% skim milk; 15R10SM, 15% raffinose and 10% skim milk; 10R10SM, 10% raffinose and 10% skim milk; MMP, mitochondrial membrane potential.

Jones 2003). Despite this, murine spermatozoa are generally well protected against cryodamage using a combination of raffinose and skim milk (Sztein *et al.* 2001), and the results from Experiment 1 in this study demonstrate similar cryotolerance of *A. cahirinus* spermatozoa to those of other mice (Takeo and Nakagata 2010) and gerbils (Sato *et al.* 2000; Koshimoto *et al.* 2009). Moreover, Experiment 2 revealed that, as in mice (Takeo and Nakagata 2010), spiny mouse spermatozoa are successfully cryopreserved in the presence of L-glutamine (Figs 1, 2). L-Glutamine is a known antioxidant and is able to mitigate excessive oxidative damage from reactive oxygen species (Aitken *et al.* 2014). Therefore, the observation of improved functional parameters when spermatozoa are cryopreserved with L-glutamine may be due to the increased resistance of spiny mouse spermatozoa to oxidative stress. Future studies of other parameters, including freezing distances, cryoprotectants and cryopreservation tools, are encouraged to further optimise sperm cryopreservation in this species.

In summary, we have developed a successful protocol for sperm cryopreservation in the Egyptian spiny mouse *A. cahirinus*. An extender consisting of raffinose, skim milk and L-glutamine significantly improved all post-thaw sperm parameters over controls and other extenders used in this study. A reliable and successful cryopreservation protocol and associated assays provide new possibilities for future reproductive research, including assisted reproduction, in spiny mice and a successful method for preserving and archiving the genetics of the world's only known menstruating rodent.

Conflicts of interest

The authors declare no competing interests.

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References

Aitken, R. J., Smith, T. B., Jobling, M. S., Baker, M. A., and De Iuliis, G. N. (2014). Oxidative stress and male reproductive health. *Asian J. Androl.* **16**, 31–38. doi:10.4103/1008-682X.122203

Bellofiore, N., Ellery, S. J., Mamrot, J., Walker, D., Temple-Smith, P., and Dickinson, H. (2017). First evidence of a menstruating rodent: the spiny mouse (*Acomys cahirinus*). *Am. J. Obstet. Gynecol.* **216**, 40.e1–40.e11. doi:10.1016/J.AJOG.2016.07.041

Brunjes, P. C. (1990). The precocial mouse, *Acomys cahirinus*. *Psychobiology* **18**, 339–350.

Dickinson, H., and Walker, D. (2007). Managing a colony of spiny mice (*Acomys cahirinus*) for perinatal research. *ANZCCART News* **20**, 4–11. https://www.researchgate.net/profile/David_Walker10/publication/230717210_Managing_a_colony_of_spiny_mice/links/55dd55d008ae83e420ee66e8/Managing-a-colony-of-spiny-mice.pdf.

Emera, D., Romero, R., and Wagner, G. (2012). The evolution of menstruation: a new model for genetic assimilation. *BioEssays* **34**, 26–35. doi:10.1002/BIES.201100099

Gharagozloo, P., and Aitken, R. J. (2011). The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. *Hum. Reprod.* **26**, 1628–1640. doi:10.1093/HUMREP/DER132

Haughton, C. L., Gawriluk, T. R., and Seifert, A. W. (2016). The biology and husbandry of the African spiny mouse (*Acomys cahirinus*) and the research uses of a laboratory colony. *J. Am. Assoc. Lab. Anim. Sci.* **55**, 9–17.

Koshimoto, C., Watanabe, D., Shinohara, A., and Morita, T. (2009). Maintenance of fertility in cryopreserved Indian gerbil (*Tatera indica*) spermatozoa. *Cryobiology* **58**, 303–307. doi:10.1016/J.CRYOBIOL.2009.02.007

Mochida, K., Hasegawa, A., Otaka, N., Hama, D., Furuya, T., Yamaguchi, M., Ichikawa, E., Ijuin, M., Taguma, K., and Hashimoto, M. (2014). Devising assisted reproductive technologies for wild-derived strains of mice: 37 strains from five subspecies of *Mus musculus*. *PLoS One* **9**, e114305. doi:10.1371/JOURNAL.PONE.0114305

Okuyama, M., Isogai, S., Saga, M., Hamada, H., and Ogawa, S. (1990). *In vitro* fertilization (IVF) and artificial insemination (AI) by cryopreserved spermatozoa in mouse. *J. Fertil. Implant.* **7**, 116–119.

Sato, M., Nishino, N., and Totsukawa, K. (2000). Cryopreservation of Mongolian gerbil spermatozoa. *J. Mamm. Ova Res.* **17**, 115–119. doi:10.1274/JMOR.17.115

Shaw, J. M., and Jones, G. M. (2003). Terminology associated with vitrification and other cryopreservation procedures for oocytes and embryos. *Hum. Reprod. Update* **9**, 583–605. doi:10.1093/HUMUPD/DMG041

Sztein, J. M., Noble, K., Farley, J. S., and Mobraaten, L. E. (2001). Comparison of permeating and nonpermeating cryoprotectants for mouse sperm cryopreservation. *Cryobiology* **42**, 28–39. doi:10.1006/CRYO.2001.2300

Taft, R. (2017). Mouse sperm cryopreservation using cryoprotectant containing monothioglycerol (MTG). *Cold Spring Harb Protoc* **2017**, prot094490. doi:10.1101/PDB.PROT094490

Takeo, T., and Nakagata, N. (2010). Combination medium of cryoprotective agents containing L-glutamine and methyl-β-cyclodextrin in a preincubation medium yields a high fertilization rate for cryopreserved C57BL/6J mouse sperm. *Lab. Anim.* **44**, 132–137. doi:10.1258/LA.2009.009074

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