

Cryopreservation of Rhesus Monkey (*Macaca mulatta*) Epididymal Spermatozoa Before and After Refrigerated Storage

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Running head: Cryopreservation of rhesus monkey sperm

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1 ABSTRACT:

2 Recently, there has been an increased interest in preservation of epididymal sperm as a
3 potential source of material for genetic resource banking; however, cryopreservation of
4 epididymal sperm from the rhesus monkey has not been explored. This study evaluated
5 the effect of prolonged refrigerated storage of the intact cauda epididymides at various
6 conditions on the post-thaw motility of rhesus monkey epididymal spermatozoa, and also
7 tested whether altering cryoprotectants and cooling methods could improve post-thaw
8 motility for epididymal sperm after refrigerated storage. Motility before freezing
9 decreased significantly after refrigerated storage (0°C) for a period of 24 or 48 h.
10 Although post-thaw motility was not significantly different after 24h refrigerated storage,
11 epididymides stored at a higher temperature (4-10°C) yielded better results, but
12 post-thaw motility still decreased significantly after 48h refrigerated storage at 4°C.
13 Comparisons of glycerol and ethylene glycol at 3% and 6% revealed similar post-thaw
14 motility. However, consistently high post-thaw motility was obtained with 3% glycerol
15 throughout all freezing trials regardless of whether samples were collected fresh or after
16 refrigerated storage for 24 or 48 h. Cooling at a higher rate of 220 °C/min was found to
17 yield better post-thaw motility than the slower rate of 29 °C/min. Thawing time
18 duration was evaluated and a minimum of 30 s was required for thawing 0.25ml straws
19 containing 50 µL semen samples. An overall average of 42% post-thaw motility was
20 obtained for rhesus monkey epididymal sperm packed in 3% glycerol and cooled after 24
21 or 48 h refrigerated storage. These post-thaw motility results for epididymal sperm
22 indicate that this method should be practical for use in preserving epididymal sperm,
23 even if tissue must be shipped from sites remote from the cryopreservation laboratory.

24

25 Key words: cryopreservation, epididymal sperm, rhesus monkey, *Macaca mulatta*

26

26 ***Introduction***

27 Non-human primates serve as important research models for human health. With the
28 increased demand for the development of treatments for human diseases, there is more
29 pressure on captive-breeding programs to provide animals for research. In previous years
30 there has been a significant loss of rhesus matrilineal from captive populations (Duggleby,
31 1976; Smith, 1985). Although infant cross-fostering (Smith, 1986) can randomize
32 breeding within a captive population, it cannot reverse the trend toward genetic
33 subdivision of captive populations at different breeding facilities (Kanthaswamy and
34 Smith, 2002). The National Primate Research Centers (NPRCs) lose genetics and rare
35 alleles when animals are lost to disease, trauma or old age and these cannot be replaced
36 because India has long banned further importation of rhesus monkeys. In addition,
37 many non-human primates are endangered species and captive breeding is the only hope
38 for their future survival. Nevertheless, it is important to maintain the genetic diversity
39 within a given population to avoid problems of inbreeding and loss of heterozygosity
40 (Moore, 1992).

41 Establishing a cryobank to preserve the germplasm of non-human primates would help
42 maintain biodiversity as that will allow those genes to be recovered as live, breeding
43 animals. In particular, when combined with assisted reproductive techniques, such as
44 artificial insemination (AI), in vitro fertilization (IVF), intracytoplasmic sperm injection
45 (ICSI), and embryo transfer (ET), sperm cryopreservation provides an effective tool for
46 preserving the genetic diversity of the captive primate populations for biomedical
47 research as well as conservation programs. Semen banking also provides a way to easily
48 transfer genetic materials among colonies of animals because only a few straws of frozen
49 semen must be transported.

50 Recently, there has been an increased interest in preservation of epididymal sperm as a
51 potential source of valuable genes for genetic resources banking (e.g., Foote, 2000;
52 Stilley, 2001; James, 2004; Guerrero, 2006). The ability to bank epididymal sperm from

53 nonreproducing animals, or males that die suddenly (Kusunoki et al., 2001) or are
54 subjects of terminal experiments would prevent the loss of valuable genetic information
55 from the future gene pool. In addition, protocols developed for domestic species or
56 captive populations may apply to their exotic counterparts where semen samples are
57 precious and limited, e.g., domestic cats as a model for wild felids (Pope et al., 1998).
58 These protocols could also be used to aid in solving difficulties with mating performance,
59 and to increase the population of individuals that are currently under-represented or those
60 with desirable genetic traits.

61 Unlike sperm cryopreservation in humans, there have been mostly sporadic attempts at
62 freezing non-human primate sperm over the last 40 years (see Morrell and Hodges, 1998
63 for review). Published reports that directly address sperm cryopreservation in
64 non-human primates number less than 100 and reliable information on the most
65 appropriate methodologies is limited. Compared with the studies of ejaculated sperm,
66 there are fewer reports (9 studies) on the freezing of epididymal spermatozoa from
67 non-human primates (Table 1), and cryopreservation of epididymal sperm from rhesus
68 monkey remains unexplored. The present study evaluated the effect of prolonged
69 refrigerated storage at various conditions on the post-thaw motility of rhesus monkey
70 epididymal spermatozoa, and also tested whether changing of cryoprotectants and
71 cooling methods could improve post-thaw motility for epididymal sperm after
72 refrigerated storage.

73

73 ***Materials and Methods***

74 *Collection of epididymal spermatozoa*

75 A total of 19 pairs of testes were donated from adult rhesus monkeys subjected to
76 necropsy from other research projects at the California National Primate Research Center,
77 and the cauda epididymis was dissected from each testis. For each pair of epididymides,
78 one epididymis was processed immediately for sperm retrieval (hereafter referred to as
79 “without storage”), and the other epididymis (“after refrigeration”) was stored in 8 ml
80 Dulbecco’s phosphate-buffered saline (DPBS) in 15 ml centrifuge tube for various
81 refrigerated storage treatments as described below. To retrieve sperm, the epididymis
82 was rinsed with DPBS, placed into a 35mm Petri dish containing 1ml of TEST-yolk
83 solution (~ 350 mOsm/kg, pH 7.4, detailed recipe see Tollner et al., 1990), cut repeatedly
84 with surgical scissors and sperm were allowed to swim out into the solution for 5 min at
85 room temperature. The resulting suspension of spermatozoa was transferred into a 15
86 ml plastic centrifuge tube prior to use.

87

88 *Preparation, freezing, thawing, and evaluation*

89 A dilution of 1:20 (v/v) of sperm suspension to a modified Tyrode’s medium
90 supplemented with bovine serum albumin (TL-BSA) (VandeVoort, 2004) was used for
91 sperm motility estimation of freshly collected semen, and a second dilution of 1:20 (v/v)
92 of sperm to distilled water was used for hemacytometer counts (Hausser Scientific,
93 Horsham, PA). Sperm suspensions were adjusted to 1×10^8 cells/mL of total motile
94 sperm (count \times motility) with TEST-yolk solution, and were mixed with double-strength
95 cryoprotectant solutions to obtain a final concentration of 5×10^7 cells/mL. All
96 cryoprotectant solutions were prepared within 1 week of use with TEST-yolk at 350
97 mOsm/kg as the diluent and were stored at 4 °C. All chemicals used for preparation of
98 solutions were of reagent grade (Sigma Chemical Corporation, St. Louis, Missouri).

99 Aliquants of 50 μ l sperm suspensions with cryoprotectant (detailed below) were
100 drawn into 0.25-ml French straws (IMV International, Minneapolis) manually with a 1cc
101 syringe. Straws were heat-sealed and placed into a 600-ml glass beaker containing 500
102 ml of room temperature distilled water, and equilibrated at 4°C in a refrigerator for 2 h
103 before initiation of the freezing process. Freezing followed the methods described by
104 Leibo et al., (2007). In brief, straws were placed on a Styrofoam ‘boat’ with the
105 thickness of either 5 cm or 1 cm on top of liquid nitrogen, which was filled to a depth of 4
106 cm in a 33×24×23 cm (inside dimensions) Styrofoam box, and equilibrated for 10 min
107 before being plunged into liquid nitrogen. The average cooling rate measured between
108 -10°C to -70°C was *c.* 29°C/min for the 5 cm boat and *c.* 220°C/min for the 1 cm boat.
109 After a minimum of 12 h, four straws per treatment were thawed in a 37°C water bath
110 (ISOTEMP 102, Fisher Scientific, Pittsburg, PA) to estimate the post-thaw motility.

111 For motility estimation, a 10- μ l drop of pre-freeze or post-thaw semen, covered with a
112 22 mm square coverglass, was visualized with \times 20 positive-phase objective and a
113 condenser setting of 100 (pseudo-dark field) on an Olympus BH-series phase-contrast
114 microscope (Scientific Instrument Co, Sunnyvale, CA). An air curtain incubator (Sage
115 Instruments, Model 279, Orion Research Inc, Cambridge, MA) maintained the
116 microscope stage at 37°C. Motility was expressed as the percent of cells actively
117 moving in a forward direction. Sperm vibrating in place were not considered to be motile.
118 Initial motility refers to the sperm motility after dilution, but before the addition of
119 cryoprotectants to the samples. Post-thaw motility was estimated immediately after
120 thawing without any dilution or washing. Samples were also presented in random order
121 each time so that the operator did not know their identity.

122

123 *Experimental layout*

124 Four experiments (Exp.) were performed (Table 2). Exp. 1 evaluated the effect of
125 thawing time on post-thaw motility, and only the epididymis without storage was used.

126 Exp. 2 evaluated the effect of storage temperature on post-thaw motility, and there were
127 three trials in this experiment. In the first trial, epididymal tissue was stored at 4°C
128 (refrigerator) for 24h. In the second trial, epididymal tissue in a 15 ml centrifuge tube
129 was sealed with parafilm, and packed in a Styrofoam shipping box (same size as the
130 freezing apparatus) at 0°C (ice/water slurry) and processed 24 h later, and in the third
131 trial, epididymal tissue in a 15 ml centrifuge tube was sealed with parafilm, and packed
132 in a Styrofoam shipping box between two ice packs (temperatures varied from 4 to 10°C)
133 and processed 24h later. Exp. 3 evaluated the effect of cryoprotectant on post-thaw
134 motility before and after refrigerated storage (at 0°C ice/water slurry as described in Exp.
135 2.2). Exp. 4 evaluated the effect of cooling and glycerol concentration on post-thaw
136 motility before and after refrigerated storage. There were two trials in this experiment
137 with the first trial evaluating cooling methods and glycerol concentrations, and samples
138 subjected to the same refrigeration treatment as that of Exp. 2.3 (4-10 C for 24h). The
139 second trial evaluated optimal glycerol concentration before and after refrigerated
140 storage at 4°C (refrigerator) for an extended period of 48h.

141

142 *Data analysis*

143 Data were analyzed using two sample independent t-test, one-way and two-way
144 analysis of variance (ANOVA) (Origin 7.0, OriginLab, Northampton, MA). When a
145 significant difference ($P = 0.05$) was observed among treatments, Tukey's Honestly
146 Significant Difference Procedure was used for pair-wise comparisons. All data were
147 arcsine-square root transformed and means of 4 straws per treatment were used for
148 analysis. Levene's test for equal variance showed non-significant difference for all
149 datasets subjected to ANOVA analysis. Values presented are means \pm SD.

150

150 **Results**

151 *Basic characteristics of epididymal spermatozoa*

152 A total of 19 pairs of epididymides were isolated from males that went to necropsy for
153 other research projects (Table 3). Males were between 4 and 14 years old with an
154 average of 7 ± 3 yr ($n = 19$), and their weight ranged from 6.2 to 18.4 kg with an average
155 of 10.6 ± 3.2 kg ($n = 19$). The initial motility of epididymal spermatozoa collected on the
156 same day as necropsy ($83 \pm 16\%$) was significantly higher ($P < 0.001$) than spermatozoa
157 collected after refrigeration for either 24 or 48 h ($59 \pm 22\%$). However, sperm density of
158 samples without storage ($9.2 \pm 6.5 \times 10^8$ cell/ml) was not significantly ($P = 0.351$)
159 different from those after refrigeration ($11.6 \pm 8.9 \times 10^8$ cell/ml).

160

161 *Effect of thawing time on post-thaw motility*

162 Post-thaw motility of sperm samples thawed for different time periods (Figure 1)
163 were significantly different from one another ($P = 0.024$), and the highest post-thaw
164 motility was obtained for time periods of 30 s ($46.7 \pm 2.0\%$) and 60 s ($47.3 \pm 5.8\%$),
165 which were not significantly different from each other, but significantly higher than
166 samples thawed for 10 s ($34.9 \pm 7.4\%$). Therefore, thawing for a period of 30 s was
167 chosen for subsequent experiments because that time gave equally good motility, but
168 required less time per sample than the 60 s period.

169

170 *Effect of storage temperature on post-thaw motility*

171 Refrigerated storage of epididymides for 24 h had no significant effect on post-thaw
172 motility (Figure 2). Despite this, post-thaw motility of sperm samples without storage
173 were generally higher than sperm that were frozen after 24h refrigerated storage at 4°C in
174 the refrigerator ($42.0 \pm 16.8\%$ vs. $35.4 \pm 17.1\%$, $P = 0.565$, Figure 2a) or packed in a
175 Styrofoam shipping box at 0°C ice/water slurry ($44.4 \pm 8.0\%$ vs. $36.8 \pm 13.4\%$, $P = 0.253$,
176 Figure 2b). Interestingly, the opposite was found with the refrigerated storage at a

177 higher temperature of 4 to 10 °C with samples stored between two ice packs in a
178 Styrofoam shipping box ($44.0 \pm 4.3\%$ vs. $48.6 \pm 2.4\%$, $P = 0.117$, Figure 2c). Males
179 were also found to respond to cryopreservation differently with male 13 yielding the
180 highest post-thaw motility of 60% and male 15 yielding the lowest post-thaw motility of
181 10%.

182

183 *Effect of cryoprotectant on post-thaw motility before and after refrigerated storage*

184 No significant difference in post-thaw motility among different cryoprotectants was
185 observed for sperm samples collected from epididymides without storage ($P = 0.292$,
186 Figure 3a) or those after 24 h refrigerated storage ($P = 0.710$, Figure 3b). However,
187 samples cryopreserved with 3% glycerol consistently yielded the highest post-thaw
188 motility for no storage ($45.6 \pm 11.5\%$) or after refrigeration ($36.8 \pm 13.4\%$).

189 Consequently, subsequent experiments focused on further optimization of glycerol
190 concentration. Similar to previous experiments, males responded to cryopreservation
191 differently with post-thaw motility of ~ 60% for some males (e.g., male 18), and less
192 than 10% for others (e.g., male 11).

193

194 *Effect of cooling and glycerol concentration on post-thaw motility before and after* 195 *refrigerated storage*

196 There were two trials in this experiment, for the first trial (Figure 4), glycerol at four
197 concentrations (3, 6, 9, 12%) was cross designed with two cooling rates ($29^\circ\text{C}/\text{min}$ vs.
198 $220^\circ\text{C}/\text{min}$). Interactions of these two factors were found to be non-significant ($P =$
199 0.647), but there were significant differences among glycerol concentrations with 3%
200 and 6% yielding significantly higher post-thaw motility than samples cryopreserved with
201 12% glycerol ($P < 0.05$). Although there was no significant difference between the two
202 cooling rates ($P = 0.054$), higher post-thaw motility was found with samples cooled at
203 $220^\circ\text{C}/\text{min}$, and the highest post-thaw motility was found with 3% glycerol ($55.8 \pm$

204 7.3%).

205 Based on the above results, the second trial employed the cooling rate of 220°C/min.
206 Glycerol concentrations were further evaluated with samples before and after extended
207 refrigerated storage of 48 h (Figure 5). The interaction of glycerol concentration and
208 sample status was not significantly different ($P = 0.746$). However, post-thaw motility
209 of samples without storage was significantly higher than those after 48 h refrigerated
210 storage ($P < 0.001$). Similar to previous experiments, samples cryopreserved with 3%
211 glycerol consistently yielded the highest post-thaw motility, which was significantly
212 higher than samples with 12% glycerol ($P < 0.05$). The highest post-thaw motility (53.9
213 $\pm 4.8\%$) was obtained with 3% glycerol for samples without storage, and the lowest (26.8
214 $\pm 11.0\%$) was with 12% glycerol for samples with refrigerated storage.

215

216 *Discussion*

217 In non-human primates, epididymal sperm have been cryopreserved in the
218 chimpanzee, gorilla, baboon, Japanese and cynomolgus macaque, and marmoset (Table
219 1). Although sperm within the cauda epididymis of a number of species are considered
220 fully mature (Mahony et al., 1996; Yeung et al., 1996; Van der Horst et al., 1999), slight
221 differences in cellular membrane structure and composition, and lack of exposure to
222 seminal plasma have been postulated to make epididymal sperm more susceptible to
223 cryopreservation damage than ejaculated sperm (Nagy et al., 1995; Morrell, 1997;
224 Perchec et al., 1997; Kundu et al., 2000; Feradis et al., 2001; Hori et al., 2004).
225 However, studies with gorilla (Lanzendorf et al., 1992) and porcine (Rath and Niemann,
226 1997) epididymal and ejaculated sperm showed a higher viability or fertilization rate
227 with post-thaw epididymal sperm, while studies with caprine suggested equal tolerance
228 between epididymal and ejaculated sperm (Blash et al., 2000). It appears that whether
229 epididymal sperm is more susceptible to cryopreservation damage than its ejaculated
230 counterpart is species-specific. Despite this, reviewing the literature of non-human

231 primates reveals that freezing protocols for epididymal sperm were essentially the same
232 as those used for ejaculated sperm. For example, most studies used
233 TEST-yolk-glycerol as the freezing medium, and cooled samples either in liquid
234 nitrogen vapor or as pellets on dry ice block. The use of frozen-thawed epididymal
235 sperm has yielded live births in the chimpanzee, cynomolgus and marmoset monkey
236 (Table 1).

237 One of the important aspects involving cryopreservation of epididymal sperm is
238 refrigerated storage and shipping. When valuable males die unexpectedly and facilities
239 for sperm cryopreservation are not immediately accessible, temporary storage of
240 epididymides and shipment to freezing facilities are required. Shipment of fresh
241 epididymides to a genetic resource-banking center would also facilitate standardized
242 sample processing and thus guarantee uniform handling of sperm. As yet no
243 investigation has been done on the possible effects of refrigerated storage on the
244 post-thaw survival of non-human primate sperm. However, it has been shown that
245 epididymides that shipped at 4°C within 24h, and the sperm of which was subsequent
246 frozen-thawed did yield a live birth in the chimpanzee (Kusunoki et al., 2001). In this
247 study, motility before freezing was found to decrease significantly after refrigerated
248 storage for an extended period of 24 or 48 h, while post-thaw motility was not
249 significantly different after 24h refrigerated storage, but epididymides stored at a higher
250 temperature (4-10°C) yielded better results. However, post-thaw motility decreased
251 significantly after 48h refrigerated storage at 4°C. This pattern was similar to studies
252 with refrigerated storage of epididymal sperm from other domestic animals such as
253 canine (Stilley, 2001), caprine, equine, and bovine (James, 2004).

254 Except for non-human primates, refrigerated storage of epididymal sperm has been
255 studied extensively for many other mammalian species. For example, storage of
256 epididymides from non-domestic animals at temperatures near 0°C permits recovery of
257 viable sperm up to 48 h later (Graham et al., 1978). A 41-day pregnancy following AI

258 with cryopreserved epididymal sperm harvested 27 h postmortem was reported for a gaur
259 bull (*Bos gaurus*) (Hopkins et al., 1988). An extreme example is found in mouse, in
260 which pups were produced from epididymal sperm that was subjected to cooled storage
261 for 7 days after death (An et al., 1999; Kishikawa et al., 1999). More recently, it was
262 reported that bovine epididymal sperm stored at 5°C were viable for at least 60 h when
263 used for AI (Foote, 2000). All these studies indicate that epididymal sperm can tolerate
264 refrigerated storage for an extended time period and are suitable for long distance
265 transportation. However, the present study did find male variations in response to
266 refrigerated storage and subsequent sperm cryopreservation. Therefore, some males
267 (e.g., male 11 in this study) may not be suitable for freezing after shipping or extended
268 refrigerated storage. To apply this knowledge for endangered species, it is
269 recommended that sperm from epididymides should be cryopreserved as soon as samples
270 are collected.

271 Glycerol has been the most widely used cryoprotectant in various mammalian species.
272 The choice of appropriate cryoprotectant may be more related to the reproduction mode
273 or sperm characteristics rather than to species because fish that employ internal
274 fertilization (livebearers) and therefore resemble mammals, also showed the best
275 protection with glycerol, which is different from fish with external fertilization (Huang et
276 al., 2004). Glycerol ranging from 2.5% to 10% has been used to freeze epididymal
277 sperm in non-human primates (Table 1). Comparisons among 6% glycerol, dimethyl
278 sulfoxide, and propylene glycol with cynomolgus epididymal sperm indicated that
279 glycerol was superior to others (Feradis et al., 2001). In addition, the survival of
280 marmoset epididymal sperm is not enhanced by the inclusion of dodecylsulphate in the
281 cryopreservation medium, compared to glycerol alone (Holt et al., 1994). Recently,
282 measurement of rhesus monkey sperm membrane permeability coefficients for various
283 cryoprotectants have led to the suggestion that ethylene glycol may be the most
284 appropriate cryoprotectant for this species (Agca et al., 2004). Studies with ejaculated

285 rhesus (Si et al., 2004) and cynomolgus (Li et al., 2005) monkey sperm also indicated
286 similar cryoprotection between ethylene glycol and glycerol when comparing immediate
287 post-thaw motility, membrane and acrosome integrity. Thus, the present study
288 compared glycerol and ethylene glycol at 3 and 6%, and our findings revealed similar
289 post-thaw motility between these two cryoprotectants. However, consistently higher
290 post-thaw motility was obtained with 3% glycerol throughout all freezing trials for
291 samples both cryopreserved immediately after collection and after refrigerated storage
292 for 24 or 48 h, which was also in agreement with the findings for ejaculated rhesus
293 monkey sperm (our unpublished data).

294 It is worth noting that there were male variations in response to different
295 cryoprotectants at different concentrations (Figure 3). This variation in susceptibility to
296 cryopreservation damage is similar to the male-to-male variations previously reported
297 for ejaculated rhesus sperm (Leibo et al., 2007), as well as in other species such as dogs,
298 bulls, boars, stallions, and humans (Leibo and Bradley, 1999; Holt, 2000)

299 Cooling of non-human primate sperm samples was often conducted either in liquid
300 nitrogen vapor or as pellets on a dry ice block. Direct comparisons of these two
301 methods have not been made until more recently, where rhesus monkey ejaculated sperm
302 cooled as pellets on a dry ice block was found to yield a fertilization rate (73%),
303 comparable to fresh controls (Yeoman et al., 2005). However, whether pellet freezing
304 would consistently result in better breeding outcomes still merits future validation. In
305 contrast, freezing in liquid nitrogen vapor using the Styrofoam box has been proven to be
306 cheap, easy, reliable, and repeatable in studies with rhesus monkey sperm across three
307 different laboratories (Leibo et al., 2007). The present study adopted the freezing
308 method described by Leibo et al. (2007), and modified the thickness of the floating boat
309 to produce various cooling rates. Cooling at a higher rate of 220 °C/min yielded better
310 post-thaw motility than the slower rate at 29 °C/min. This may be due to the extended
311 equilibration time period (2h at 4°C) before freezing as dehydration may have occurred

312 in response to osmotic equilibrium. Optimal cooling rate may also vary with
313 cryoprotectants and thawing methods/rates (e.g., Mazur, 1985; Fiser and Fairfull, 1984;
314 1990; Yu et al., 2002). To further optimize cryopreservation protocols for rhesus
315 monkey sperm, future experiments should examine these multiple factors simultaneously
316 and systematically.

317 Temperatures ranging from 5 to 75°C (in air or water bath) have been used for thawing
318 non-human primate sperm with the most satisfactory results obtained from samples
319 thawed at a 37°C water bath (e.g., Younis et al., 1998). However, thawing duration was
320 often neglected in published reports, and the few of those that did specify rate indicated a
321 wide range from 10 s (Leibo et al., 2007), 30 s (Feradis et al., 2001), 45-60 s
322 (Sanchez-Partida et al., 2000; Okada et al., 2001), 2 min (Si et al., 2000), to 5 min
323 (Tollner et al., 1990). To confirm the best results, a comparison of thawing time
324 duration was made in this study, and our findings revealed that a minimum of 30 s was
325 required for thawing 0.25ml straws containing 50 µL samples. In general, fast rates
326 were preferred as this should minimize damage associated with recrystallization upon
327 thawing, and thus aid the recovery of motile sperm (Watson, 1979).

328 Motility has been proven to be a good indicator of sperm quality in frozen-thawed
329 sperm of non-human primates. Post-thaw motility of ejaculated (Tollner et al., 1990)
330 and epididymal sperm (Feradis et al., 2001) from cynomolgus macaque has been found to
331 positively correlate with membrane integrity. Examination of spermatozoa from the
332 same species suggests that motility is the most convincing parameter for sperm function
333 analysis, while sperm acrosome integrity (evaluated with FITC-PNA) was the one most
334 resistant to freezing (Li et al., 2005). This was further evidenced by studies of osmotic
335 tolerance of rhesus monkey sperm, which indicated that sperm motility was more
336 sensitive than membrane integrity to deviations from isotonicity (Rutllant et al., 2003).
337 Positive correlations between motility and live sperm were also found in the howler
338 monkey, *Alouatta caraya* (Valle et al., 2004).

339 Successful live births with frozen-thawed sperm may not only rely on the efficiency of
340 the cryopreservation protocol at preserving the fertilizing ability of sperm, but also on
341 the insemination method. This is especially true with rhesus macaques as they have a
342 tortuous cervical canal, which may restrict the successful application of standard AI
343 involving sperm deposition into the vagina or cervix. However, successful live births
344 were reported through intrauterine insemination with frozen-thawed sperm for
345 cynomolgus (Tollner et al., 1990) and rhesus macaques (Sanchez-Partida et al., 2000).
346 Compared with standard IVF, AI via intrauterine insemination offers a simpler and more
347 cost-effective approach for utilization of frozen-thawed sperm for macaque propagation.
348 The use of transabdominal deposition of sperm into the uterus not only circumvents the
349 tortuous female anatomy, but also places fewer demands on the requirement of highly
350 motile sperm after thawing. In contrast, a standard IVF program is costly, requires
351 expensive equipment and is very complicated as it involves hormone stimulation, oocyte
352 retrieval and in vitro culture, and embryo transfer through surgery, with the potential for
353 failure at each of these steps.

354 Although the absolute dependence of successful live birth on post-thaw motility may
355 vary with the assisted reproduction technique employed, it would be expected that higher
356 post-thaw motility would result in improved assisted reproduction outcomes through the
357 provision of more functional spermatozoa. In fact, a recent study with rhesus monkey
358 sperm has shown that there were no significant differences in chromosome damage
359 between fresh sperm and frozen-thawed sperm when motile sperm were selected for
360 ICSI (Li et al., 2007). In addition to percent motility, recording the forward progression
361 scale (Yeoman et al., 2005) and the sperm longevity may provide a more accurate
362 estimation of sperm quality after thawing. Future studies should evaluate the possibility
363 of developing a composite index that incorporates the progressive scale into the motility
364 estimation as well as the sperm longevity after thawing.

365

366 In summary, the present study demonstrated that sperm from rhesus monkey
 367 epididymides could be cryopreserved with 3% glycerol after extended refrigerated
 368 storage of 24 h, but there were male variations. Future AI trials are needed to confirm
 369 the viability of rhesus monkey epididymal sperm cryopreserved after refrigerated storage.
 370 Nevertheless, techniques developed in this study should help gene banking of
 371 non-human primates including diseased males.

372

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375

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524 Table 1. *Literature review of cryopreservation of non-human primate epididymal sperm*

Species	Cryoprotectant	Cooling	Thawing	Summary of findings	Reference
Chimpanzee	2.5, 5% glycerol	Vapor freezing	37 °C water	40% motility, 69.6% viability, 52.8% intact acrosome, 1 live birth	Kusunoki et al., 2001
Gorilla	3% glycerol	Pellet freezing	37 °C	82.5% motility, 79% intact acrosome	O'Brien et al., 2003
	7% glycerol	Vapor freezing	NA	1 inseminated, no conceive	Beehler et al., 1982
	3% glycerol	Pellet freezing	37 °C air	40.7% normal sperm, 39.5% head abnormality, 0% fertilization (IVF), 1 developed embryo (injection)	Lanzendorf et al., 1992
Japanese monkey	5% glycerol	Vapor freezing	37 °C water	43% fertilization, 19% cleaved, 4 at 8-cell stage (IVF)	Sankai et al., 1997
Cynomolgus monkey	4, 6, 8% glycerol, 6% DMSO, 6% PG	Vapor freezing	37 °C water	36% motility, 45.2% viability, 47% intact membrane	Feradis et al. 2001
	6% glycerol	Vapor freezing	37 °C	2 live birth, 2 abortion (ICSI-TET)	Ng et al., 2002
Baboon	3% glycerol	Pellet freezing	37 °C	42.5% motility, 59.5% intact acrosome	O'Brien et al., 2003
Marmoset monkey	5% glycerol	Controlled-rate	37 °C	31% fertilization (IVF)	Holt et al., 1994
	10% glycerol	Controlled-rate	RT (22-25°C)	6 inseminated, 1 conceived, 1 live birth	Morrell et al., 1998
	3% glycerol	Pellet freezing	37 °C	43.5% motility, 74.5% intact acrosome	O'Brien et al., 2003

525 NA-not available; DMSO-dimethyl sulfoxide; PG-propylene glycol

526 Vapor freezing: cooling samples in liquid nitrogen vapor

527 Pellet freezing: cooling drops of extended semen on the surface of a dry ice block

528 Controlled-rate: cooling with an automated cell freezer

529

529 Table 2. *Experimental layout*

Exp	Parameters	Refrigerated storage treatment	Male number	Cryoprotectant	Cooling	Thawing
1	Thawing	No storage	4, 14, 16, 17	3% glycerol	29 °C/min	37 °C water, 10s, 20s, 30s, 60s
2.1	Refrigeration	(1) No storage vs. 4 °C, 24h	1,13,15,16,17	3% glycerol	29 °C/min	37 °C water, 30s
2.2	Refrigeration	(2) No storage vs. 0 °C, 24h	4,5,7,11,14,18	3% glycerol	29 °C/min	37 °C water, 30s
2.3	Refrigeration	(3) No storage vs. 4-10°C, 24h	2,6,9,10	3% glycerol	29 °C/min	37 °C water, 30s
3	Cryoprotectant	No storage	7,11,18	3%, 6% Gly; 3%, 6% EG	29 °C/min	37 °C water, 30s
		0 °C, 24h	7,11,18,4,5,14	3%, 6% Gly; 3%, 6% EG	29 °C/min	37 °C water, 30s
4.1	Cooling x Gly	(1) 4-10°C, 24h	2,6,9,10	3%, 6%, 9%, 12% Gly	29 vs. 220 °C/min	37 °C water, 30s
4.2	Glycerol con	(2) No storage vs. 4 °C, 48h	3,8,12,19	3%, 6%, 9%, 12% Gly	220 °C/min	37 °C water, 30s

530 Exp-experiment; Con-concentration; Gly-glycerol; EG-ethylene glycol

531 Table 3. *Basic characteristics of epididymal spermatozoa of the 19 males used in this*
 532 *study*
 533

Male number	Age (yr)	Weight (kg)	Initial motility (%)		Sperm density (x 10 ⁸ cell/ml)	
			Without storage	After refrigeration*	Without storage	After refrigeration
1	14	13.8	73	47	4.0	4.0
2	13	17.7	70	70	8.4	9.8
3	10	18.4	60	60	15.6	22.2
4	10	8.6	85	50	9.8	12.4
5	9	9.7	50	30	3.4	5.7
6	7	11.9	95	90	8.6	8.4
7	7	10.7	95	60	11.8	20.8
8	7	12.7	95	50	12.4	19.8
9	6	9.2	90	60	19.6	31.0
10	6	9.8	95	90	8.2	12.4
11	6	8.2	50	20	7.0	1.3
12	6	9.8	95	30	21.8	20.0
13	6	10.6	95	90	19.8	15.2
14	5	8.8	95	60	12.6	10.2
15	5	9.1	72	75	0.9	0.5
16	4	8.4	78	50	6.4	20.8
17	4	6.9	90	90	1.4	1.0
18	13	10.4	90	60	2.2	3.2
19	4	6.2	95	30	1.5	2.0
Mean ± SD	7 ± 3	10.6 ± 3.2	83 ± 16	59 ± 22	9.2 ± 6.5	11.6 ± 8.9

534 *Prefreezing motility after 24 or 48h refrigerated storage

535

535 **Figure legends**

536 Figure 1. Post-thaw motility of rhesus monkey epididymal sperm samples cryopreserved
537 with 3% glycerol and thawed in a 37 °C water bath for 10s, 20s, 30s, and 60s. Values
538 presented were means of 4 straws for each male.

539

540 Figure 2. Post-thaw motility of rhesus monkey epididymal sperm samples cryopreserved
541 with 3% glycerol before (light gray bars) and after (dark gray bars) 24h refrigerated storage
542 at various conditions: a) 4 °C refrigerator; b) 0 °C ice/water slurry in a shipping box; c)
543 4-10 °C between two ice packs in a shipping box. Values presented were means of 4
544 straws for each male.

545

546 Figure 3. Post-thaw motility of rhesus monkey epididymal sperm samples cryopreserved
547 with 3% and 6% glycerol (Gly), as well as 3% and 6% ethylene glycol (EG) immediately
548 after collection (a) and after 24h refrigerated storage at 0 °C ice/water slurry in a shipping
549 box (b). Each bar represents one male, and bars with same patterns identify same males.
550 Values presented were means of 4 straws for each male.

551

552 Figure 4. Post-thaw motility of rhesus monkey epididymal sperm samples cryopreserved
553 with 3%, 6%, 9%, and 12% glycerol after 24h refrigerated storage at 4-10 °C between two
554 ice packs in a shipping box, and cooled at 29°C/min or 220°C/min in liquid nitrogen vapor.
555 Values presented were means of 4 straws for each male.

556

557 Figure 5. Post-thaw motility of rhesus monkey epididymal sperm samples cryopreserved
558 with 3%, 6%, 9%, and 12% glycerol before (without storage) and after 48h refrigerated
559 storage (48h) at 4 °C, and cooled at 220°C/min in liquid nitrogen vapor. Values presented
560 were means of 4 straws for each male.

561

Figure 1

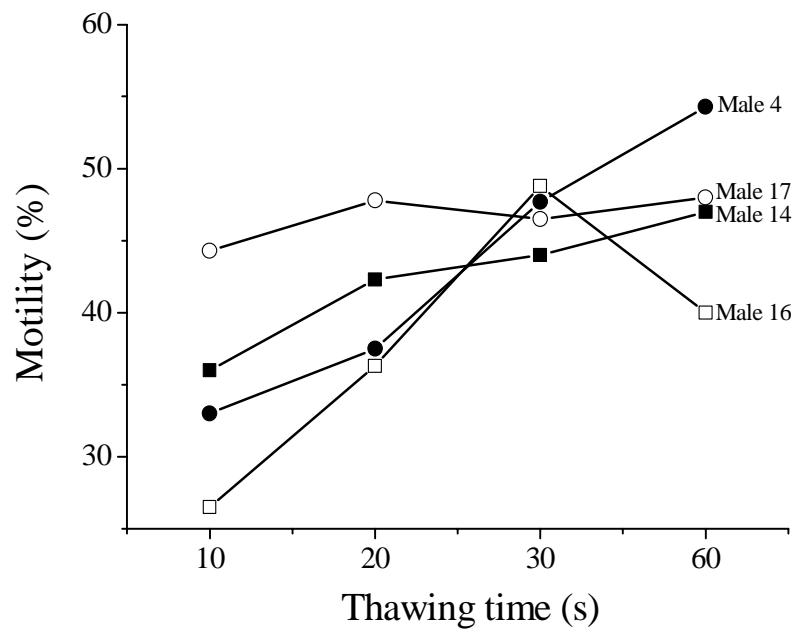


Figure 2

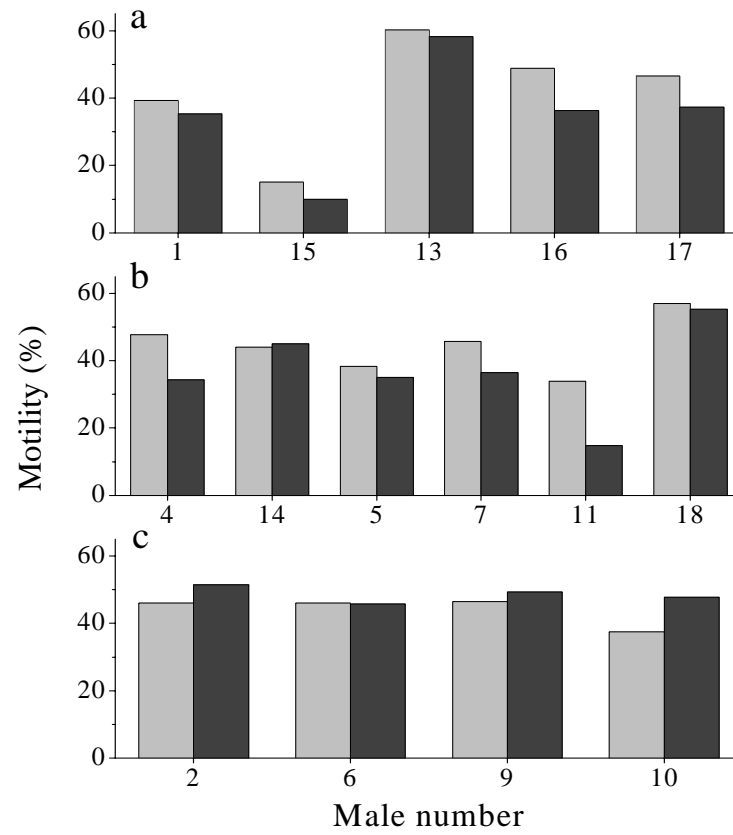


Figure 3

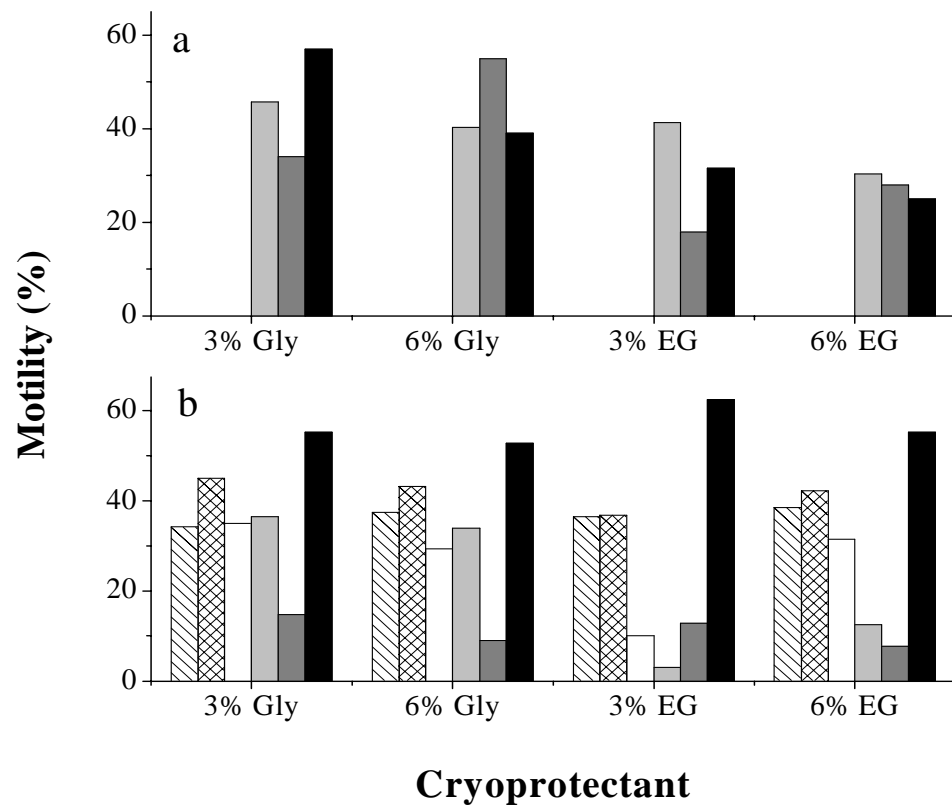


Figure 4

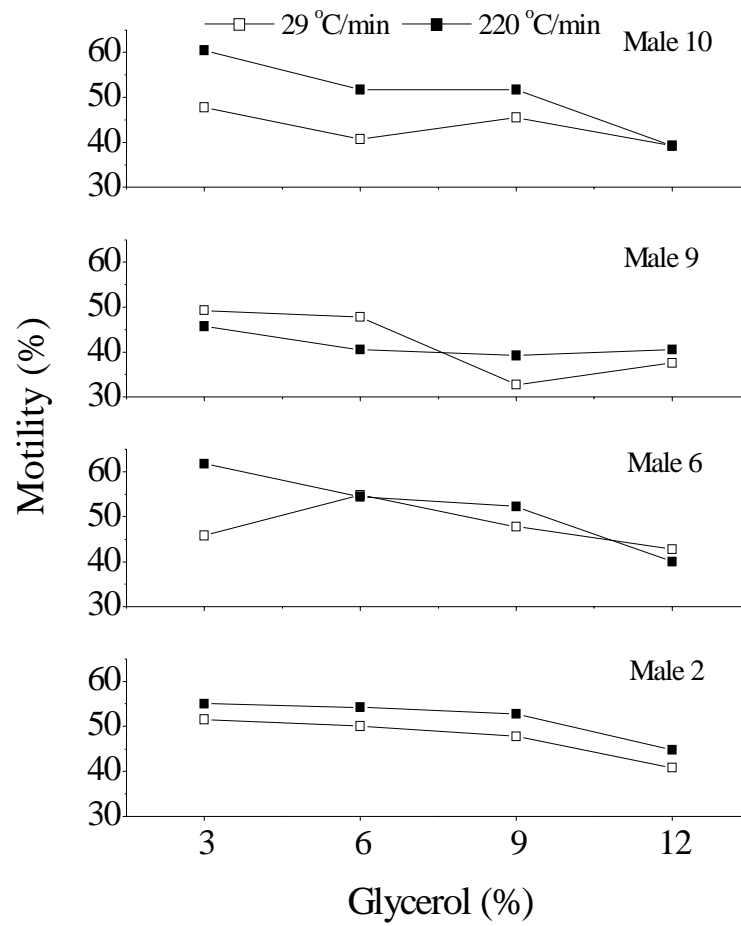


Figure 5

