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SEMINAL EVALUATION AND CRYOPRESERVATION OF SPERMS FROM THE PIG–TAILED MACAQUE, *Macaca nemestrina*

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Abstract: Four wild adult pig–tailed macaques (*Macaca nemestrina*) were captured at Tabin Wildlife Reserve, Sabah, Malaysia, and housed temporarily in cages for electro ejaculation and semen analysis. Testicular volumes ranged from 35.5 to 62.6 and correlated to the total testicular circumference and body weight. Out of 22 ejaculates, five were azoospermic. The volume of ejaculates averaged 447 µl. The low and high total sperm counts averaged 35.5 x 10^6/ml and 310.9 x 10^6/ml respectively. The lowest mean total sperm counts were 34.8 x 10^6/ml and the highest, 237.2 x 10^6/ml. The pH of ejaculates ranged from 6.5 to 9.0. Two macaques showed good sperm motility with 73% normal sperm morphology. The average mean live-dead ratio was 65.7:34.3. The mean sperm abnormalities averaged 29.9%. Coiled and bent tail constituted the highest amount of sperm abnormalities. There was no significant difference between semen volume using high or low voltages. The ejaculates consisted of a liquid fraction followed by a solid rubbery coagulum. The sperms had a strong tendency to agglutinate to small clumps of coagulum. The study is intended to provide information relevant to the application of assisted technology in endangered non-human primates.

Keywords: *Macaca nemestrina*, electroejaculation, sperm, tropical forest, Sabah, Malaysia

Introduction

Preserving germplasm is a means to help safeguard genetic material, and assisted reproductive technologies will likely become an element in the conservation of endangered non–human primate (NHP) species. In Baltimore Zoo, for example, the assisted reproductive technology element of a lion–tailed macaque conservation strategy initially looked to rhesus and pig–tailed macaques as possible surrogate mothers (Wolf, 2009).

The various methodologies associated with semen collection and cryopreservation will need to be refined, and baseline parameters standardized and made readily available. Knowledge of seminal evaluation and sperm cryopreservation in domestic and companion animals is well-established. However, for wild animals, including NHPs, there is a critical lack of information, including species that are considered threatened or endangered with extinction (IUCN Red list of Threatened Species, 2017).

Although macaques have for long been used for biomedical research, including assisted reproductive technologies, their reproductive biology at gamete level is little known. Macaques represent potential models for developing techniques and knowledge of assisted reproductive technology in other endangered NHP species, such as lion–tailed macaque (*Macacasilenus*) and proboscis monkey (*Nasalislarvatus*).

Pig–tailed (*M. nemestrina*) and long–tailed macaques (*M. fascicularis*) are the two most common NHPs in Malaysia. Human – macaque conflicts are becoming a significant problem in many parts of Malaysia, as the macaque population increases locally along with the expansion of human and agricultural habitats. Local abundance in the macaque population provides an opportunity to refine and improve NHP semen collection and preservation techniques.

A favoured semen collection technique is electro ejaculation (EEJ) using a rectal probe (Gould *et al*., 1978) but other options may include penile vibratory stimulation (PVS), electrical penile stimulation (Sarason *et al*., 1991), artificial vagina (AV), masturbation and flushing of the vas deferens. Although semen characteristics have been reported in several species of NHP, including great apes, rhesus monkeys, lion tailed macaques and long-tailed macaques (known in laboratories as cynomolgus monkeys), very little has been recorded for pig-tailed macaques. Rectal probe stimulation is the most commonly used method for semen collection in NHP in zoos and captive facilities. Basic data on semen collection in pig–tailed macaque, long-tailed macaque (also known as crab-eating macaque, *M. irus*) and lion–tailed macaque (*M. silenus*) using rectal probe stimulation has been reported (Schaffer *et al*., 1989a; 1989b). In this study, we describe the testicular biometry and seminal characteristics of wild pig-tailed macaques in Sabah, Malaysia.

Materials and Methods

Animal and Semen Collection

The study was conducted with institutional approval from the Sabah Wildlife Department (via letter with reference number JHL (HQ)400-9/82Jld.9(9)). The macaques were free-ranging mature individuals,

residents on the western edge of Tabin Wildlife Reserve (TWR), situated in eastern Sabah.

Four full-grown male pig–tailed macaques were cage trapped or darted, and housed in temporary cages for semen collection and evaluation. The macaques were weighed, and examination of the external genitalia and andrologic parameters (testis palpation to verify size, consistency, symmetry and mobility) was then carried out.

The cages were under forest canopy and away from human disturbance. The individual cages measured 4 x 1.5 x 2 meters (length, width and height respectively). A 2-hectare perimeter fence surrounded these cages under natural photoperiod and relative humidity (13 hours of light and 11 hours of darkness). The macaques were fed fresh fruits, pumpkins with seeds, vegetables, natural browse, hard boiled eggs and commercial pellets. Clean water was available *ad libitum.*

The selected males (n=4) were healthy, as confirmed by hemogram and serum chemistry. The mean body weight was 13.8 kilograms (13.1 – 14.1 kg; min – max). All the macaques presented testes with normal consistency, symmetry and mobility.

Anesthesia

All macaques were fasted 12-18 hours before drug administration. Animals were kept isolated in individual cages. The macaques received an induction dose of 1mg medetomidine (Medetomidine 40 milligram/millilitre, Kyron®) and 150mg ketamine hydrochloride (Ilium, Ketamil 100mg/ml, Australia®) administered intramuscular (im) in a pelvic limb via blowpipe system (Telinject, GmbH®). This resulted in a total volume of 1.52 ml of Medetomidine/ Ketamine fitted in a 3 ml homemade syringe dart with a modified 18G x 1 ½“ hypodermic needle with side port. Immobilized animals were immediately transferred to a field laboratory nearby and placed in right lateral recumbent position on an examination table.

Following induction, macaques were intubated with a size 5.0 I.D endotracheal tube guided by a size 3a Macintosh laryngoscope. Rectal temperature was taken using a hand – held digital thermometer. Pulse oximetry and pulse rate were measured with a pulse oximeter (H100, Edan®) with the probe placed on the tongue. End-Tidal CO2 and respiratory rate was measured using a mainstream capnometer (EMMA, Masimo®). Indirect blood pressure (MAP) was measured (08A, Contec®) with an appropriate sized blood pressure cuff (40% of limb circumference) placed on the right upper antebrachium. Anesthetic depth was assessed by monitoring jaw tone, anal tone, palpebral reflex, pupillary light reflex and response to noxious stimulus (withdrawal reflex). Supplemental dose of Ketamine was given intravenously to maintain anesthesia if needed. Following general anesthesia, semen collection was done by electro-ejaculation.

After the completion of procedures, macaques were transferred back to their cages and given reversal agent, Atipamezole (Alzane 5mg/ml, Zoetis®) intra muscularly, in the quadriceps at five times the dose of Medetomidine administered and allowed to recover undisturbed.

Electroejaculation

Examination was carried out of the macaques placed in right lateral recumbent position. Both testes were measured for length (cranial – caudal) and width (medial – lateral) using a digital vernier caliper (IP 54, China) and the total testicular circumference calculated. The genital region was thoroughly cleaned with normal saline and wiped dry with paper towel or gauze. The prepuce was retracted with the thumb and index finger and the penis cleaned.

The rectum was emptied of fecal materials and well lubricated with non – spermicidal gel (K- Y Jelly, Johnson & Johnson Co., Arlington Tx, USA). The position of the pelvic brim and the accessory sex glands were palpated to determine the depth of rectal probe insertion (~ 8 centimeters). This is due to the position of the electrodes, 2 cm posterior to the rounded end. The 2 cm diameter rectal probe consisted of three metal plates measuring 2 cm long and 4.7 millimeters wide.

Once the penis had been placed inside a 50 ml conical graduated tube and secured, the rectal probe was smeared with sterile lubricant and gently pushed into the rectum. The dorso – ventral position of the electrodes was ascertained before any stimulations were done using an electro ejaculator with a standard rectal probe measuring 2.5 x 7 cm (diameter and length), consisting of three 4.6 x 56.8mm electrodes (Seager®, Dalzell Medical Systems, USA). The stimulations consisted of three series of 1 – 4 volts. Each series of 30 stimuli was divided into 3 sets of 10 stimuli at 1, 2 and 3 V (Series 1) and 2, 3 and 4 V (Series 2) and 2 sets of 10 stimuli at 3 and 4 V (Series 3). Occasionally, a fourth series consisting of 2 sets of 10 stimuli between five to 10 V was needed to expel the remaining coagulum.

Semen Evaluation

During the electro ejaculation procedures, any fraction of ejaculate obtained in the 50 ml conical tube during the stimulation was transferred and immediately placed in a water bath at 37ºC for 30 minutes to be liquefied. The volume of liquid portion was determined using a micropipette and then transferred into a 2 ml vials and floated on a Styrofoam rack in a water bath at 37°C. Appearance and consistency were assessed subjectively. This includes color (colorless, yellowish or whitish), opacity (transparent or opaque) and appearance (amorphous or filamentary coagulum). A small portion of semen from each fraction of the liquefied semen was then evaluated

individually under light microscope (Leica DME, GmbH Germany) at a 100x magnification. Sperm vigour was evaluated on a scale of 0 – 5, (Denis *et al.*, 1976). Seminal pH was measured periodically to monitor urine contamination, using a pH strip (Merck Pharmaceuticals, Darmstadt, Germany).

No motility was "0"; slight movement with greater than 75% of sperm showing vibrations only was represented by "1"; moderate forward movement in >50% sperm was represented by "2"; progressively forward movement with 70% sperm was as "3". Sperm with 90% and >95% active motile sperm were represented by 4 and 5 respectively. Sperm motility was expressed as % of cells actively moving in a forward movement (Oliveira *et al*., 2015).

The forward progressive sperm motility (%) was measured by placing 10µl of semen on a pre-warmed (37ºC) glass slide with a coverslip and 200 sperms were counted (Oliveira et al., 2011). All fractions of ejaculate with sperm vigour of ≥2 were pooled together. Sperm concentration was determined in a Neubauer counting chamber after dilution of 2µl semen in 38 µl of formal saline (4% formaldehyde in saline solution) a dilution of 20x. Morphologic defects observed in spermatozoa were classified as primary or secondary (Oliveira *et al.,* 2011). Semen was assessed directly after collection (fresh), after dilution and after freezing.

Cryopreservation

A small portion of the semen from the pooled fractions was pipetted into a 2 ml vial (with screw cap) to evaluate the sperm concentration. The rest was immediately added with TYB semen extender (TEST-Yolk Buffer, Irvine Scientific, USA) in a ratio of 1:2 (i.e. 1 part of semen with 2 parts of extender). A study of *Macacafascicularis* semen indicated that 5% glycerol at 30 minutes equilibrium yielded the highest post thaw sperm motility and head membrane integrity (Li *et al*., 2005). In this study, the 12% glycerol in the commercial TYB was accordingly reconstituted to 6%. After determining the sperm concentration, TYB semen extender was further added, if necessary, to give a concentration count of 30 - 50 x 10^6 sperm/ml. The semen and extender mixture were equilibrated for 10 minutes in a water bath at 37ºC and subsequently placed and floated on a styrofoam rack in a beaker (500 ml) of 200 ml warm water at 37ºC and chilled in a refrigerator at 4ºC for 2 hours.

Samples that exhibited a minimum motility of 20%, vigour of ≥2, 30% normal sperm morphology were cooled. Samples were cooled in covered microtubes, following a curve of 37ºC to 4ºC within 2 hours. After cooling, sperms were evaluated as previously described. Eosin-Nigrosin stain (Sigma-Aldrich) was used for percentage of live/dead sperm. The sperm were also stained with Diff-Quik (Medion Diagnostics, Switzerland) to determine the morphology and abnormalities. Cooled samples that showed at least 30% motility, sperm vigour of ≥2 with ≥30% normal sperm morphology was then cryopreserved. Cooled semen was divided into aliquots of 13 - 29 x 10^6 sperm/ml.

Thereafter the aliquots were drawn into the 0.25 ml plastic straws, 100 µl/straw and sealed with polyvinyl sealing powder. Freezing methods followed the procedures described previously (Dong *et al*., 2008). In brief, a Styrofoam box (33 x 24 x 23 cm) was filled with a depth of 4 cm of liquid nitrogen. A Styrofoam rack (1 cm thick) or boat was floated on top of it for 10 minutes and subsequently, the straws were horizontally laid on it for another 10 minutes to equilibrate with the vapour (approximately -120ºC; at a rate of ~220°C/min) before plunging them into the liquid nitrogen (– 196ºC).

Statistical Analysis

All the data were expressed as mean and standard deviation (SD). The testicular biometry and seminal data were evaluated using a one–way ANOVA. The formulation used to calculate the testicular volume (cm^3) = Length (cm) X $Width^2$ (cm) X 0.524.

Results

Testicular biometry

Mean body weight of the four adult males was 13.5±0.4 kg. Rambo was the heaviest (14.1 kg) and Aaron, the lightest (13.1 kg). All the macaques presented testes with normal consistency, symmetry and mobility. The testicular biometry comprised length, width, circumference, and volume was calculated using standard formulation (Table 1).

Table 1: Mean (± standard deviation) of length, width and volume of right and left testes and total testicular volume of pig–tailed macaques (n= 4 males)

ID	Testis	Length (cm)	Width (cm)	Volume (cm^3)	Total volume (cm^3)	Total circumference (cm)
Aaron	Left	4.5±0.5	3.0±0.5	21.3± 9.2	42.7± 19.5	13.1±2.2
	Right	4.4±0.5	3.0±0.6	21.4±10.4		
Ulrich	Left	5.0±0.6	3.4±0.3	30.8±8.5	62.6±19.4	15.2±1.4
	Right	4.9±0.6	3.5±0.4	31.8±11		
KK	Left	4.6± 0.7	3.0±0.5	22.9±10	35.5±22.6	11.9±2.9
	Right	4.7±0.4	2.9±0.3	21.4±7.0		
Rambo	Left	4.9 ± 0.7	3.4±0.3	29.6±9.2	58.6±16	15.1±1.0
	Right	4.7±0.7	3.4±0.2	29.1±7.2		

(KK = King Kong)

The total testicular volume in the adult pig–tailed macaques ranged from 35.5 (±22.6) to 62.6 (±19.4). There is a moderate positive correlation (59%) between the total testicular volume and the total circumference (54%) of the testis with the animals' body weights.

All ejaculations were initiated by a liquid fraction, followed by totally or partially coagulated seminal fraction. The liquid and coagulated fractions were transparent, translucent, opaque or colourless, whitish, milky or yellowish. The ejaculates ranged from watery to thick. The coagulum retrieved from the macaques was either filamentous or amorphous (Figure 1).

From the four males electro stimulated rectally during the study, 100% ejaculates were obtained from 22 electro ejaculations. Five ejaculates (three from King Kong and two from Ulrich) did not contain sperm. The volume of ejaculates ranged from 80 to 1200 µl with a mean of 447 µl ± 338. In general, the sperm concentration ranged from 8 x 10^6/mil to 705 x 10^6/mil. The low total sperm counts (< 100 x 10^6/ml) and the high total sperm counts (> 100x10^6/ml) from all four individuals averaged 35.5 x 10^6/ml and 310.9 x 10^6/ml respectively. However, between individuals, King Kong had the lowest mean total sperm counts of 34.8 x 10^6/ml (± 23 x 10^6/ml), and Ulrich the highest mean total sperm count of 237.2 x 10^6/ml (273.9 x 10^6/ml). Rambo and Aaron had total sperm counts of 51.54±9.9 and 109.6±104.6 x 10^6/ml) respectively. The pH of ejaculates ranged from 6.5 – 9.0 (Table 2).

Figure 1: The coagulum collected from *M. nemestrina* varies from amorphous (a), filamentary or both (b). Semen colour is usually thinmilky (c and d)

Table 2: Mean (±SD) values of ejaculates (No.), sperm motility (%), and vigour grade (VG), normal sperm morphology (NSM: %), sperm concentration (x 10^6/ml) and seminal pH of fresh semen collected

Animal	No.	Motility	VG	Live	NSM	Concentration	pH
Aaron	5	57.6±14.7	2	59.6±23.0	78.1±28.2	109.6±104.7	7.5-9.0
Ulrich	5	78.8±15.8	3	82.14±6.6	73.1±18.8	237.2±273.9	8.0
KK	5	79.0±10.2	3	61.9±16.6	73.5±3.7	34.8±23.0	6.5-8.0
Rambo	2	44.0±14.1	2	64.9±15.0	57.6±6.6	51.54±9.9	8.0

(KK = King Kong)

Ulrich and King Kong showed good sperm motility of about 79% with normal sperm morphology of 73.1±18.8 and 73.5±3.7 respectively. The average mean live – dead ratio observed was 65.7:34.3.

Agglutination of large numbers of sperms to tiny pieces of coagulum was often seen almost immediately after collection and examination under a cover slip. The cluster of agglutinated sperm would appear as mats of sperms with many adhering together at the head (Figure 2).

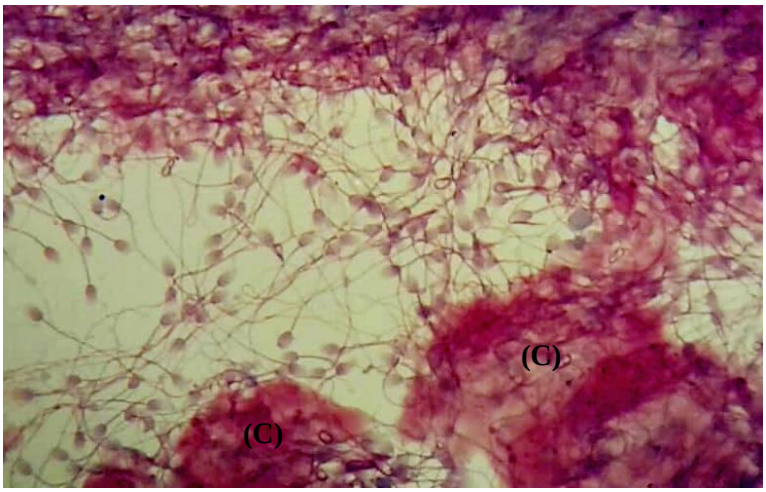

Figure 2: The clustered sperms and pieces of coagulum (C)

In general, the mean sperm abnormalities averaged 29.9%. These include head (0.89±0.89), midpiece (2.35±3.36) and 26.8% tail defects. The tail defects, comprising coiled and bent tail (10.9±7.19 and 15.9±10.78 respectively) constituted the highest amount of sperm abnormalities in these pig–tailed macaques.

Discussion

Semen collection commonly used in non-human primates includes rectal probe stimulation (EEJ) and direct penile electro stimulation or DPES (Gould *et al*., 1978; Mastroianni & Manson, 1963; Sarason *et al*., 1991). These early studies found that the application of DPES achieves better stimulation of the entire reproductive tract, resulting in larger semen volume, less urine contamination and higher numbers of spermatozoa per ejaculate as compared to rectal probe method. However, the most critical part of using DPES is the training and acclimatization of the animal to the chair restraint and subsequent procedure (Vande Voort, 2004). The present study involved wild captured free ranging adult pig–tailed macaques. Training them for DPES would be impossible due to their aggressive nature and wild imprint. The only safe and reliable method is EEJ under complete general anaesthesia. In this study, urine contamination was suspected in only one ejaculate (4.5%) from a total of 22 rectal probe stimulations and ejaculations. Similarly, in the squirrel monkey (*Saimiri collinsi*), the use of EEJ was efficient for semen collection and the tested cooling protocol allowed for recovery of a satisfactory percentage of sperms with intact plasma membrane (Oliveira *et al*., 2015). The voltage stimulations ranged from 1 – 10 V with most of the stimulations focussed on the 1 – 5 V range. In the squirrel monkey (*S. sciureus*), a maximum of 10V was used during EEJ, beyond which, the animal exhibited distress. In an earlier study, ejaculations

were obtained between 5 – 8 volts (Bennett, 1967). In the present study with *M. nemestrina*, most of the high voltage (>5 V) was directed at expelling the remaining coagulum from inside the urethra to avoid post EEJ obstruction. However, no significant difference was observed between semen ejaculation volume using the high (> 5 – 10 V) or low voltages (1 – 5 V).

In the present study, testicular volume in the adult pig–tailed macaques was positively correlated with body weight. Similar studies in non-human primate showed the importance of testis sizes at the inter– and intra–specific levels with the assumption that larger testis produces larger volumes of sperm, important during sperm competition (Bercovitch & Rodriguez, 1993).

The main problem with ejaculates from these pig-tailed macaques was the presence of coagulum which contained motile sperms. In these macaques, the ejaculations were initiated by a liquid fraction prior to the expulsion of the solid rubbery coagulum, which over time undergoes a small amount of liquefaction with the liquid portion containing sperms. However, it was impossible to extract most of the sperms which remained entrapped inside the coagulum. The semen of most monkeys undergoes rapid coagulation after ejaculation due to a reaction between the secretions of the cranial lobe of the prostate gland and the seminal vesicles (Dukelow, 1971). Only a small portion of the coagulum in rhesus monkey will liquefy and the bulk of the sperms will remain in the coagulum (Dukelow, 1971). In the pig-tailed macaques studied, the liquid fraction and coagulum were kept separately to prevent reabsorption of the sperms which subsequently reduces their number and motility. In addition, the sperms of these pig–tailed macaques were found to have a high tendency to agglutinate in large numbers, especially to small clumps of coagulum making it impossible to separate during freezing. Most agglutination occurs as small groups of 2 – 10 sperms with heads adhering to each other (VandeVoort, 2004). The exact cause of the head – head adhesion is unknown but could have been induced by anti-sperm antibodies (Isahakia & Alexander, 1984; Vernekar *et al*., 2004).

The average total sperm count in ~~a~~ ten rhesus monkeys trained for DPES has been reported as 136 x 10^6/ml (VandeVoort, 2004). However, there is a wide range of reported values within and between individual rhesus monkeys both for motility (10 – 85 %) and sperm numbers (100 – 3600 x 10^6 per ejaculate) (Roussel & Austin, 1967; Lanzendorf *et al*., 1990). In Yakushima macaques, the ejaculates collected from masturbation, during the breeding season, yielded a mean sperm concentration of 529.4±265.4 x 10^6/ml (Thomsen, 2013). In this study, the total sperm count in the pig–tailed macaques ranged from 8 – 705 x 10^6/ml (μ=118.3 x 10^6/ml ± 164.7) with motility of 57 – 78%. The retrograde flow of sperms into the urinary bladder was not evaluated. In a fertile group of lion–tailed macaques, the sperm concentration observed ranged from 38 – 800 x 10^6/ml with a motility of 30 – 95%. Out of 15 ejaculates from 6 lion-tailed macaques that were analysed, retrograde flow of semen into the bladder constituted at least half during electro ejaculation. In a pig–tailed macaque, loss to retrograde flow constituted 89% (Schaffer *et al*., 1989a).

The pH of uncontaminated semen of several species of NHP has been reported as 7.4 – 7.6 (Roussel & Austin, 1968). Prolonged, repeated rectal electrical stimulation of cats resulted in higher ejaculate pH in later collections (Dooley & Pineda, 1986). The only reported previous study of pig-tailed macaque showed that the pH ranged from 7.1 with initial fluids to 9.0 in later samples (Schaffer *et al*., 1989a). In Yakushima macaques (*M. fuscatayakui*) the semen collected from masturbatory ejaculations had a pH range of 6.4 – 7.9 (Thomsen, 2013). During this study, all semen samples except one from the pig–tailed macaques showed levels of pH 7.5 – 9.0. Urine contamination does occur occasionally during electro ejaculation and will lower the pH and affect the osmolality of semen. It also induces curling of the sperm cell (McGrady*et al*., 1974).

In general, the mean sperm abnormalities in the pig–tailed macaques reported here were about 5% higher than those from squirrel monkeys (25.3%). The coiled and bent tail defects were similarly higher in the pig–tailed macaques as compared to squirrel monkeys (Oliveira *et al*., 2015).

Post-thawed semen from the pig–tailed macaques will be examined in the future to determine the acrosomal integrity, DNA integrity and sperm motility. In this study, the 12% glycerol was reduced to 6% based on the high success with post- thawed sperm motility and head membrane integrity at 3% or 5% and 30 minutes equilibrium time, as observed in *M. fascicularis* (Li *et al*., 2005). This is to reduce the damage to the cells due to their chemical toxicity and osmotic stress during addition before cooling and removal after thawing (Critser *et al*., 1988; Gilmore *et al*., 1997). In addition, comparison between two permeable cryoprotectants, indicated that there is no significant difference between the sperms cryopreserved with glycerol or ethylene glycol (Chen *et al*., 2017).

In future procedures, particularly if the intention is in vitro fertilization, there is a need to improve and optimise the handling of semen from macaques. There is a need to determine media for washing and incubation of semen that can reduce or prevent agglutination. The likely future use of assisted reproductive technology in NHPs would be in the preservation of endangered NHP species such as the proboscis monkey (*Nasalislarvatus*).

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