Animal Reproduction Science 115 (2009) 255–266

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Successful cryopreservation of Asian elephant (*Elephas maximus*) spermatozoa

Joseph Saragusty[a,*], Thomas B. Hildebrandt[a], Britta Behr[a], Andreas Knieriem[b], Jürgen Kruse[b], Robert Hermes[a]

[a] *Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke Straße 17, D-10315 Berlin, Germany*
[b] *Zoo Hannover, Adenauerallee 3, 30175 Hannover, Germany*

ARTICLE INFO

Article history:
Received 22 September 2008
Received in revised form 24 October 2008
Accepted 13 November 2008
Available online 20 November 2008

Keywords:
Assisted reproductive technologies
Directional freezing
Gametes cryopreservation
Genome resource bank
Endangered species

ABSTRACT

Reproduction in captive elephants is low and infant mortality is high, collectively leading to possible population extinction. Artificial insemination was developed a decade ago; however, it relies on fresh-chilled semen from just a handful of bulls with inconsistent sperm quality. Artificial insemination with frozen–thawed sperm has never been described, probably, in part, due to low semen quality after cryopreservation. The present study was designed with the aim of finding a reliable semen freezing protocol. Screening tests included freezing semen with varying concentrations of ethylene glycol, propylene glycol, trehalose, dimethyl sulfoxide and glycerol as cryoprotectants and assessing cushioned centrifugation, rapid chilling to suprazero temperatures, freezing extender osmolarity, egg yolk concentration, post-thaw dilution with cryoprotectant-free BC solution and the addition of 10% (v/v) of autologous seminal plasma. The resulting optimal freezing protocol uses cushioned centrifugation, two-step dilution with isothermal 285 m Osm/kg Berliner Cryomedium (BC) with final glycerol concentration of 7% and 16% egg yolk, and freezing in large volume by the directional freezing technique. After thawing, samples are diluted 1:1 with BC solution. Using this protocol, post-thaw evaluations results were: motility upon thawing: 57.2 ± 5.4%, motility following 30 min incubation at 37 °C: 58.5 ± 6.0% and following 3 h incubation: 21.7 ± 7.6%, intact acrosome: 57.1 ± 5.2%, normal morphology: 52.0 ± 5.8% and viability: 67.3 ± 6.1%. With this protocol, good quality semen can be accumulated for future use in artificial inseminations when and where needed.

* Corresponding author at: Department of Reproduction Management, Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Straße 17, D-10315 Berlin, Germany. Tel.: +49 30 5168 233; fax: +49 30 5126 104.
E-mail address: saragusty@izw-berlin.de (J. Saragusty).

0378-4320/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.anireprosci.2008.11.010

1. Introduction

The Asian elephant (*Elephas maximus*) worldwide population is estimated to be at around 50,000–70,000 elephants, of which approximately 15,000 are in captivity (Taylor and Poole, 1998; Wiese, 2000; Sukumar, 2006). Unfortunately, its population in the wild, and to a greater extent in captivity, is not reproducing at a sufficient rate to at least maintain the current population size (Taylor and Poole, 1998; Faust et al., 2006). With the continuous aging of the captive population, low fertility, high infant mortality rate and an elevated occurrence of reproductive tract pathologies, this population might drive itself to extinction within the next few decades (Wiese, 2000). Preserving natural diversity can be done *in situ* by protecting the elephant's habitat, or *ex situ* by creating a Genome Resource Bank (GRB) (Wildt, 1992; Johnston and Lacy, 1995). However, it is also imperative to find ways to improve the elephant's captive fertility and fecundity.

Despite the close relationship between the Asian and the African elephants, their sperm seems to differ greatly in many aspects, including membrane fatty acid composition (Swain and Miller, 2000), size of the head and length of the tail (Jainudeen et al., 1971; Gilmore et al., 1998) and sensitivity to chilling (Leibo and Songsasen, 2002). Finding reliable protocol to cryopreserve Asian elephant spermatozoa will open the way to a variety of possibilities. It will enable us to establish a semen bank that can act as a genome resource bank. It will provide breeding programs with a reliable source of semen where and when it is needed, overcoming the need to collect semen shortly before using it and the risk of quality inconsistency that we face today (Hildebrandt et al., 2000). Cryopreservation of the African elephant's spermatozoa has been reported with post-thaw motility ranging between 30 and 50% (Jones, 1973; Howard et al., 1986). We and others were able to achieve similar results with the Asian elephant spermatozoa (Hermes et al., 2003; Thongtip et al., 2004; Sa-Ardrit et al., 2006). However, attempts to freeze elephant's spermatozoa and thaw it with sufficiently high motility as is achieved in many other species, thus far, failed. Throughout the years, the conventional wisdom was that, unlike most other species, the elephant spermatozoa freezes better when using dimethyl sulfoxide (Me_2SO) as cryoprotectant (Watson, 1995) however, using Me_2SO, only moderate and largely insufficient success has thus far, been achieved. To tackle this problem we adopt a systematic approach to develop an optimal protocol for the cryopreservation of Asian elephant spermatozoa. A novel freezing technology was used in these experiments. This technology is based on multi-thermal gradient (MTG; IMT Ltd., Ness Ziona, Israel) directional solidification (Arav, 1999). In the conventional freezing methods, ice grows at an uncontrolled velocity and morphology and may, therefore, disrupt and kill cells in the sample. By moving the special large volume (2.5 or 8 mL) cryogenic HollowTube™ (IMT Ltd.) with the semen at a constant velocity through a linear temperature gradient, using the MTG apparatus, we are able to control the ice crystal propagation, optimize its morphology, get continual seeding and homogenous cooling rate during the whole freezing process, thereby minimizing damages to the cells. Several attempts using this technique in a variety of species has proved it to be successful (Arav et al., 2002b; Gacitua and Arav, 2005; Saragusty et al., 2006; Si et al., 2006) and superior to conventional freezing techniques (O'Brien and Robeck, 2006; Saragusty et al., 2007; Reid et al., in press).

2. Materials and methods

2.1. Materials

Unless otherwise mentioned, all materials were of reagent grade or higher and were purchased from either:

- Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany.
- Carl Roth GmbH + Co., Karlsruhe, Germany.
- Merck KGaA, Darmstadt, Germany.