

## ATTEMPTED IN VITRO MATURATION AND FERTILIZATION OF POSTMORTEM SUMATRAN RHINOCEROS (*DICERORHINUS SUMATRENSIS*) OOCYTES

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**Abstract:** A study was conducted opportunistically to evaluate the potential of rescuing immature oocytes from the ovaries of the Sumatran rhinoceros postmortem. Recovered oocytes ( $n = 30$ ) were placed in maturation culture for 36 hr and inseminated with frozen-thawed homologous spermatozoa. After culture, evaluation of nuclear maturation status revealed that a large number of oocytes were degenerated ( $n = 21$ ), but nine oocytes were assessed at the germinal vesicle ( $n = 3$ ), metaphase I ( $n = 3$ ), and metaphase II ( $n = 3$ ) stages. Frozen-thawed Sumatran rhinoceros spermatozoa were capable of binding to the zona pellucida of in vitro matured oocytes, but no fertilization or cleavage resulted. In conclusion, relatively large numbers of oocytes can be obtained by ovarian follicular aspiration postmortem in the Sumatran rhinoceros, and some of these oocytes are capable of achieving nuclear maturation in vitro. However, additional studies are required to improve maturation success and achieve fertilization in culture.

**Key words:** *Dicerorhinus sumatrensis*, in vitro fertilization, in vitro maturation, oocyte, Sumatran rhinoceros.

### BRIEF COMMUNICATION

The Sumatran rhinoceros is critically endangered, with an estimated 250 inhabiting the fragmented rainforests of Southeast Asia. In response to the rapid decline of wild Sumatran rhinoceroses, a captive breeding program was established in 1984. Unfortunately, small animal numbers and the paucity of information about this species' basic reproductive biology initially limited the program's success. However, more than a decade ago, the discovery of induced ovulation in the Sumatran rhinoceros led to the development of a reproductive management strategy for an otherwise solitary species lacking reliable behavioral signs of estrus.<sup>11</sup> Using precise measures of follicle size and patterns of progesterone secretion to scientifically time introductions for breeding proved successful and resulted in the production of three calves by one pair of Sumatran rhinoceroses.<sup>9,10</sup> Currently, 10 Sumatran rhinoceroses make up the global ex situ population. The application of scientific methodologies to time natural breeding in additional rhinoceros pairs at other locations within the captive population has been demonstrated.<sup>1</sup> Furthermore, developing a cryobiology program that involves the collection, storage, use, exchange, and re-

search of genetic material from founder individuals and their offspring could help augment the global management of this small ex situ population. Importantly, steps toward this goal have already been achieved through the development of semen collection and cryopreservation methodologies in this species.<sup>7</sup> Although there are no other reports of postmortem oocyte recovery in the rhinoceros, transrectal aspiration of oocytes from live hormonally superstimulated African black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses has recently been conducted.<sup>5</sup> The present study was conducted opportunistically to determine the feasibility of collecting Sumatran rhinoceros oocytes postmortem and successfully maturing and fertilizing them in culture using frozen-thawed spermatozoa.

A 21-yr-old multiparous female Sumatran rhinoceros (studbook number [SB No.] 29) at the Cincinnati Zoo & Botanical Garden died as a result of hemochromatosis. Ovaries were removed shortly after death and were maintained at 22°C until gamete rescue could be attempted 6 hr later. Oocytes were recovered by aspirating and scraping surface follicles using a 20-gauge needle and 3-cc syringe. Internal follicles were exposed by carefully slicing through the ovary with a scalpel blade. Follicular contents collected via aspiration were placed into plastic Petri dishes containing TCM-199 Washing Medium (M7653, Sigma Aldrich, St. Louis, Missouri 63103, USA) containing Hank's salts supplemented with 0.1 g/l L-glutamine, 0.35 g/l NaHCO<sub>3</sub>, 0.1% (v/v) bovine serum albumin (3311, Sigma Aldrich), 100 U/ml

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penicillin, and 50 µg/ml streptomycin. Ovarian tissue slices were minced in Petri dishes containing washing media. Oocytes were washed twice in TCM-199 Washing Medium and once in TCM-199 Maturation Medium containing Earle's salts (M7528, Sigma Aldrich) supplemented with 0.1 g/l L-glutamine, 0.1% (v/v) bovine serum albumin (3311, Sigma Aldrich), 100 IU/ml penicillin, 50 µg/ml streptomycin, 2 mM pyruvate, 10% (v/v) fetal calf serum, 1 µg/ml estradiol, 5 IU/ml porcine follicle-stimulating hormone, 10 IU/ml ovine luteinizing hormone, and 50 µg/ml insulin-like growth factor 1. Oocytes were matured 36 hr (500 µl; 2–6 oocytes/drop) under sterile filtered washed mineral oil in plastic Petri dishes (38.6°C in 20.7% O<sub>2</sub> and 5% CO<sub>2</sub>).

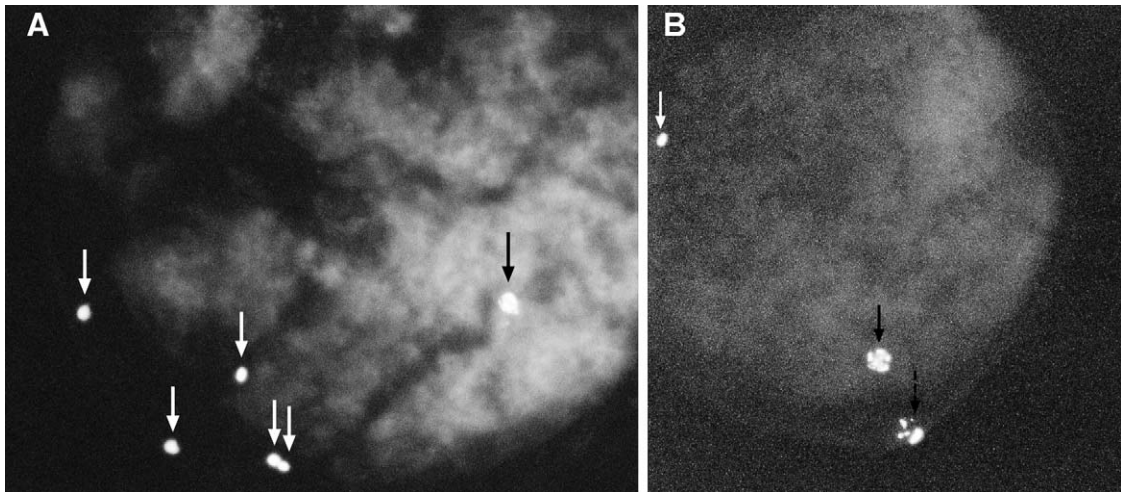
After maturation, oocytes were rinsed and equilibrated 2 hr (38.6°C in 20.7% O<sub>2</sub> and 5% CO<sub>2</sub>) in drops (400 µl) of HEPES-Tyrodé's albumin lactate pyruvate (TALP) under oil in plastic Petri dishes, transferred to 45-µl microdrops of in vitro fertilization (IVF)-TALP,<sup>7</sup> and equilibrated 2 hr prior to insemination. Each fertilization drop, containing 2–4 oocytes, was inseminated with 2.5–3.3 × 10<sup>4</sup> motile spermatozoa. At 24 hr postinsemination (pi), oocytes were stripped of cumulus cells, assessed for cleavage, and placed into new IVF-TALP drops for an additional 24 hr of culture. At 48 hr, pi oocytes were washed through and placed in 500-µl drops (4–11 oocytes/drop) of DMEM-F12 media (D8437; Sigma Aldrich) containing 10% (v/v) fetal calf serum. Every 48 hr, half of the medium in all wells was removed and replaced with fresh medium. At 72 hr pi, all oocytes were fixed in DMEM-F12 containing 5% (v/v) formalin for later fluorescent staining.

Frozen-thawed, postcoital semen from Sumatran rhino SB No. 28 was used for IVF.<sup>8</sup> Spermatozoa were thawed by holding the straw 10 sec in air (22°C) and then immersing the straw into a 38°C waterbath and shaking vigorously for 20 sec. The content of the straw was emptied into a sterile, warmed, 1.5-ml microcentrifuge tube. The sample was examined microscopically (×100 and ×400) for percent motility (0%–100%) and forward progressive status (0–5, with 0 being non-motile and 5 being rapid forward progression).<sup>6</sup> To remove extender and cryoprotectant from the spermatozoal suspension, a 1:1 dilution (v/v; 200 µl) with warm Sperm-TALP<sup>4</sup> medium was conducted, followed by centrifugation (600 g; 2 min). After centrifugation, the supernatant was removed and the sperm pellet was resuspended in sperm-TALP media. Due to poor sperm motility

postcentrifugation, the remaining portion (~300 µl) of the postthaw sample was diluted 1:1 in warm sperm-TALP media, examined for motility, and used to inseminate the IVF drops.

For nuclear staining, oocytes were transferred into 4-well dishes (Nunc, Roskilde 4000, Denmark) containing 500 µl of Hoechst 33342 in citrate buffer (10 µg/ml, 9.0 g/l, NaCl, 4.4 g/l C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>, pH 7.0) and coincubated (20 min; 22°C) while protected from light.<sup>8</sup> Oocytes were transferred onto a clean glass slide and compressed using a coverslip mounted with nail polish. Slides were examined immediately using fluorescent microscopy (× 400–1000) to determine the number of spermatozoa attached to or penetrating the zona pellucida of each oocyte and to assess nuclear maturational status (germinal vesicle, metaphase I, metaphase II, degenerated, fertilized).<sup>2</sup> To determine if numbers of spermatozoa bound to oocytes differed depending on nuclear maturational status, a one-way analysis of variance was performed using SigmaStat software (SPSS; Chicago, IL 60606, USA) with significance at  $P \leq 0.05$ .

The left and right ovaries measured 10.2 × 4 cm and 10.1 × 4.2 cm, respectively. Both ovaries had ≥ 5 surface follicles. The largest follicle measured 12 mm. Follicular aspirate volume ranged 0.5–1.0 cc. A total of 30 oocytes were recovered from the left ( $n = 19$ ) and right ( $n = 11$ ) ovaries. All oocytes were recovered through aspiration of surface or internal follicles and not as a result of mincing ovarian tissue segments. The majority of oocytes were darkly pigmented with expanded cumulus. None of the oocytes cleaved after insemination, and subsequent evaluation of maturational status revealed that 21 of 30 (70%) were degenerate, 3 of 30 (10%) were at the germinal vesicle stage, 3 of 30 (10%) were at metaphase I, and 3 of 30 (10%) were at metaphase II. Postthaw sperm motility was 40% with a 3.0 forward progression but declined to 0% after centrifugation and resuspension in sperm-TALP. However, postthaw spermatozoa diluted in sperm-TALP but not subjected to centrifugation maintained motility and progressive status and therefore were utilized for IVF. Spermatozoa remained motile in culture up to 24 hr pi. In total, 35 spermatozoa were bound to 30 in vitro maturation (IVM) oocytes. The number of spermatozoa bound to oocytes classified as degenerate, germinal vesicle, metaphase I (Fig. 1A), and metaphase II (Fig. 1B) were  $0.81 \pm 1.54$  (range 0–6),  $2.33 \pm 3.215$  (range 0–6),  $2.67 \pm 2.08$  (range 1–5), and  $1.00 \pm 0.00$ , respectively. There was no difference ( $P = 0.10$ ) in numbers of



**Figure 1.** Photomicrographs of Sumatran rhinoceros oocytes collected postmortem and subjected to in vitro maturation followed by in vitro fertilization using frozen-thawed spermatozoa. **A.** Metaphase I (black arrow) oocyte with five spermatozoa (white arrows) bound to the zona pellucida. **B.** Metaphase II (black arrow) oocyte with polar body (black dashed arrow) and one spermatozoon (white arrow) bound to the zona pellucida.

spermatozoa bound and oocyte nuclear maturational status.

Results from this study demonstrate that high numbers of oocytes can be recovered postmortem from the Sumatran rhinoceros. Whereas the majority of oocytes were degenerated, several oocytes did progress to metaphase I and metaphase II in culture. The high number of degenerated oocytes could have been a consequence of the compromised health of the female. However, oocyte recovery efforts in the closest domestic relative, the horse, also result in high numbers of degenerated oocytes, even from donors that were healthy prior to death.<sup>3</sup> Similarly, oocytes obtained after hormonal superstimulation of African black and white rhinoceroses also exhibit variable and low maturation rates in vitro.<sup>5</sup> Although Sumatran rhinoceros ovaries contained many follicles, all measured below the preovulatory size (20–25 mm) for this species.<sup>11</sup> The absence of any large follicles was not surprising given the female's deteriorating body condition and supports the conclusion that the IVM procedure was responsible for stimulating nuclear maturation of recovered immature oocytes. Sumatran rhinoceros spermatozoa exhibited good progressive motility and longevity postthaw when not subjected to centrifugation and retained sufficient functionality in vitro to bind to IVM oocytes. Therefore, it appears postcoital frozen-thawed spermatozoa show promise for use in assisted reproductive procedures and supports

the use of cryobiology as an essential component to the Sumatran rhinoceros ex situ program. Whereas spermatozoa were capable of binding to IVM oocytes, we found no evidence of zona penetration. It is possible that changes in the permeability of the zona pellucida occurred in vitro or as a result of the diseased state of the female prior to death, rendering sperm incapable of penetrating the zona. Although the postthaw sperm suspension was diluted with sperm-TALP to reduce the amount of extender and cryoprotectant introduced into the IVF drops, a small percentage of cryopreservation media had to be introduced during insemination to achieve the desired sperm concentration and may have affected fertilization success. Alternatively, the sperm may have failed to capacitate, a problem often encountered with horse spermatozoa. In conclusion, it was noted that postmortem Sumatran rhinoceros oocytes could be recovered and a limited percentage matured in culture, thereby providing a method to salvage genetic material from this critically endangered species. However, further research is warranted to improve rates of nuclear maturation and to achieve fertilization in vitro.

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