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Ovarian superstimulation, transrectal ultrasound-guided oocyte recovery, and IVF in rhinoceros

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Abstract

Numerous reports on reproductive pathology in all rhinoceros species illustrate the abundance of female infertility in captive populations. In infertile rhinoceroses, oocyte collection and embryo production could represent the best remaining option for these animals to reproduce and to contribute to the genetic pool. We report here on superstimulation, repeated oocyte recovery, and attempted in vitro fertilization (IVF) in white and black rhinoceroses. Four anestrous rhinoceroses (two white, two black) with unknown follicular status were treated with gonadotropin-releasing hormone analogue, deslorelin acetate, for 6 to 7 d. Number and size of follicles in superstimulated females was significantly higher and larger compared with those in nonstimulated anestrous females (n = 9). Ovum pick-up was achieved by transrectal ultrasound-guided follicle aspiration. Up to 15 follicles were aspirated per ovary. During six ovum pick-ups, a total of 29 cumulus-oocyte complexes (COCs) were harvested with a range of 2 to 9 COCs per collection. No postsurgical complications were noted on the rhinoceros ovaries using this minimally invasive approach. Various in vitro maturation (IVM) and IVF protocols were tested on the collected COCs. Despite the low total number of COCs available for IVM and IVF in this study, we can report the first rhinoceros embryo ever produced in vitro. The production of a 4-cell embryo demonstrated the potential of transrectal ultrasound-guided oocyte recovery as a valuable tool for in vitro production of rhinoceros embryos from otherwise infertile females.

Keywords: Assisted reproduction technology; 4-cell embryo; GnRH analogue; IVM; Ovum pick-up

1. Introduction

Rhinoceros conservation faces great challenges with a growing human population and increasing loss of

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habitat. Despite efforts by wildlife authorities, governments, the zoo community, and nongovernmental organizations, the extinction of a number of species and subspecies of these charismatic megavertebrates, a group of big mammals that have always captured the imagination of mankind, seems to be inevitable. In situ populations of the Northern white rhinoceros (*Ceratotherium simum cottoni*), the Eastern Sumatran rhinoceros (*Dicerorhinus sumatrensis harrissoni*), or the Javan rhinoceros (*Rhinoceros sondaicus*) are down to 4, 13, and less than 50 individuals, respectively. Each of these populations faces a similar fate to that of the Western black rhinoceros (*Diceros bicornis longipes*), which was declared extinct in 2006 [1,2]. The uncertain future of rhinoceros species and subspecies in situ illustrates the importance of ex situ breeding programs. Whereas protecting natural habitat and eliminating other human-caused threats remain the highest priority, parallel development and application of assisted reproduction technology to produce viable, healthy offspring in captivity with maximum genetic diversity is imperative.

The ultimate goals of managing a population of endangered species ex situ are the retention of maximum genetic diversity and a reproduction rate that equals or surpasses all losses of the population. Captive populations of the black rhinoceros (*Diceros bicornis* subsp.) or the greater one-horned rhinoceros (*Rhinoceros unicornis*) have high enough reproduction rates to make them self-sustaining [3,4]. Yet, some genetically valuable founder animals have not contributed to the genetic pool due to reproductive disorders and infertility. What seems to be a minor problem in black and greater one-horned rhinoceroses is of great concern to the captive populations of white and Sumatran rhinoceroses. In these two rhinoceros species, reproduction has been poor ever since their arrival in modern zoos, and female infertility is a common problem contributing to the low reproductive rate [5,6]. Long nonreproductive periods in captive females result in asymmetric reproductive aging. It occurs when conception, pregnancy, and lactation remain absent. It induces uterine pathology and ovarian exhaustion and results in early, nonreversible infertility of the females [7,8]. The consequence for the Northern white rhinoceros subspecies, for instance, is that only two of five females kept in captivity remain fertile [7]. Artificial insemination (AI), one of the basic assisted reproduction tools, can help to prevent this asymmetric aging and to reproduce rhinoceroses when natural breeding is absent or intercalving intervals are too long. Moreover, AI could enhance the genetic diversity with the use of fresh or frozen-thawed sperm from a diverse group of males including those in distant populations or even those in the wild [8,9].

A prerequisite for these ‘‘simple’’ assisted reproduction technologies are reproductively healthy females. However, reports on reproductive pathology in all rhinoceros species illustrate the presence of many infertile females in captive populations [5,10–12]. Female rhinoceros with pathologic conditions of the vagina, cervix, uterus, or oviduct are consequently unable to reproduce and contribute to the genetic pool. Anecdotal reports on the occurrence of uterine tumors in isolated Sumatran rhinoceroses in the wild demonstrates that the consequence of a lack of reproduction and infertility is not restricted to captivity but occurs also in small, secluded populations in the wild where reproduction is irregular.

Currently, there are no means to rescue the genetic potential of female rhinoceroses if these pathologies are the cause of their infertility. In infertile rhinoceroses, oocyte collection and in vitro embryo production represent the last remaining option to rescue genetic diversity. In some rhinoceros subspecies, female gamete recovery from infertile animals would be of tremendous importance in reducing the immense inbreeding factor. For example, in the Northern white rhinoceros, 60% of the remaining female captive population is infertile. Without in vitro technologies, the Northern white rhinoceros population will be based on closely related animals only: mother and daughter/grandfather and half brother [4,7].

The in vitro production of embryos by means of in vitro maturation (IVM), gamete intrafallopian transfer (GIFT), in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), and subsequent embryo transfer (ET) are solutions to female infertility and are well-established assisted reproduction technologies (ARTs) in domestic species and humans [13–15]. In human medicine, in vitro embryo production helps to produce offspring for otherwise infertile patients. In domestic species, it boosts reproductive rate and output of highly valuable animals. Furthermore, in mares, IVF, ICSI, and ET are proposed means of overcoming various causes of subfertility and infertility, including lesions of the reproductive tract, low embryo collection and survival rates, embryo succumbing before the first pregnancy check, or deficient oviductal environment [16]. However, the adaptation of these techniques to wildlife species faces a number of barriers.

The thick integument and extended ribcage hinders the classic and direct laparoscopic approach to the ovary in the rhinoceros. The length of the reproductive tract and the inability of manual fixation of the ovary oppose

a transvaginal ovum pick-up (OPU) for harvesting gametes in these species. Transvaginal follicle aspiration, similar to in vivo OPU in the cow and the mare, has been attempted in black and white rhinoceroses but failed to give access to the ovaries [17]. A transrectal ultrasound-guided follicular aspiration would overcome anatomic and technical obstacles encountered with a laparoscopic or transvaginal follicular aspiration.

The aims of this study were to examine a hormone treatment protocol to induce superstimulation and develop an in vivo technique for harvesting oocytes from infertile female rhinoceroses.

2. Materials and methods

2.1. Superstimluation

From a group of 13 anestrous females (9 white rhinoceroses, *Ceratotherium simum simum*; 4 black rhinoceroses, *Diceros bicornis minor*), 4 females (2 white, 2 black rhinoceroses) were diagnosed infertile due to uterine or para-ovarian lesions identified in pretrial assessments. As these infertile animals were excluded from mating, they were selected for this study. Each female was superstimulated two or three times with at least 6 mo between treatments. Animals were treated three times with deslorelin acetate, a synthetic gonadotropin-releasing hormone (GnRH) analogue (4.2 mg deslorelin implant; Ovuplant; Peptech, Melbourne, Australia; or 4.5 mg im BioRelease Deslorelin; BET Pharm, Lexington, KY, USA). Deslorelin was given on Days 0, 2, and 4. The implants were administered by an implant dart (TS-3D 100; Telinject, Römerberg Germany). The injectable GnRH was given in a standard blow dart. Both dart types were delivered using a pressurized dart pistol or rifle. The ovarian status was evaluated by ultrasound in all females before and on Day 6 or 7 of treatment (SonoSite 180PLUS, C60 5–2/10–5 MHz probe; Product Group International, Inc., Lyons, CO, USA; Voluson 'i,' 2–5/5–8 MHz probe; GE Healthcare, Zipf, Austria). During two superstimulations, the follicular development was monitored by ultrasound on Days 1, 4, 6, and 10 of treatment. Ultrasound examination of all females required either standing sedation or general anesthesia [7,18,19]. The fecal progesterone metabolite pregnane was monitored throughout and after treatment during nine stimulations [20,21].

2.2. Oocyte recovery

For transrectal ultrasound-guided follicular aspiration, one white and two black rhinoceroses were used as oocyte donors. Oocytes were collected via OPU at three different collections times (Collections 1, 2, and 3). The number of rhinoceros cows aspirated for Collections 1, 2, and 3 were 1, 2, and 2 respectively. The 120- to 180-min procedure required inhalation anesthesia [19]. After induction of anesthesia with an opioid-based drug combination, the rhinoceroses were intubated and maintained on isoflurane in oxygen. Intubation was achieved by endoscopic visualization of the epiglottis. In the smaller black rhinoceroses, the use of a sling system allowed for the maintenance of sternal position and thus equal access to both ovaries [19]. The white rhinoceros was kept in lateral recumbency. Inhalation anesthesia allowed for sufficient analgesia and restraint throughout the procedures. After conclusion of the procedure, the isoflurane was discontinued, the rhinoceroses were extubated, and the opioid component was antagonized with 150 mg naltrexone hydrochloride iv (Naltrexone; Kyron Laboratories, Benrose, South Africa). The rhinoceroses were ambulatory within 5 to 8 min and remained moderately ataxic for a further 3 to 5 min after reversal of anesthesia.

To prepare for the transrectal follicle aspiration, the rectum was manually cleansed. Multiple enemas were given until the flushed water became clear of feces. Immediately prior to rectal puncture, the perineal area was scrubbed, and the rectal ampulla was soaked and flushed with Betadine (Braunol®, Braun, Melsungen, Germany). After all fluids had been removed, a sterile towel was placed cranially into the rectum to block possibly remaining fecal material from advancing into the puncture area.

The follicles were aspirated on Days 6 to 7 of GnRH analogue treatment. A 100-cm-long, custom-made, 14-gauge, single-lumen needle was mounted onto a needle-holding device with integrated micro-convex ultrasound transducer (9–5 MHz, Sonosite; Product Group International Inc.). A sterile lubricant was applied, and the transducer together with the needle-holding device were placed into the rectum. Prior to puncture, the ovary was visualized and positioned directly adjacent to the rectal wall. Under ultrasound guidance, the needle was inserted through the rectal wall directly into the follicle with a maximum penetration depth of 4 cm. The follicular fluid was aspirated (DC15 Endo-S; Asskea Medizintechnik, Greussen, Germany) into Emcare Complete Flushing solution (ICP Bio Ltd., Auckland, New Zealand) supplemented with 0.3% (vol/vol) bovine serum albumin (BSA) and 50 IU mL^{-1} heparin or HEPES-buffered TCM199 supplemented with 2% (vol/vol) fetal calf serum (FCS) and 100 IU mL^{-1} heparin, with both solutions containing 100 μg mL^{-1} streptomycin sulfate and 100 IU mL^{-1} penicillin G. Each aspirated follicle

was repetitively flushed under ultrasound control. Postsurgical wound management consisted of rectally placed antibacterial foam sticks (Terramycin®-Uterusschaumstabletten, Pfizer, Karlsruhe, Germany), oral trimethoprim (5 g), and sulfonamide (25 g) (Trimidine Powder, Pty Ltd, Mascot, Australia) twice daily for 5 d. COCs were recovered from the aspirate by filtering through EmCon filters and identified using a stereomicroscope (×40 magnification). Unless noted, all chemicals were from Sigma (Sigma Chemical Co., St. Louis, MO, USA).

Number of follicles and COCs are presented as means ± SEM. Values were tested for normality before significance was calculated with an unpaired *t*-test. For populations with different SDs, the test was Welch corrected. Significance is reported as two-tail P values <0.05.

2.2.1. In vitro maturation

All COCs recovered that had unexpanded cumulus were classified as immature and placed in maturation medium. Briefly, for Collections 1 and 2, COCs were rinsed three times in aspiration medium (HEPES-buffered TCM199 supplemented with 2% [vol/vol] FCS [CSL Ltd, Parkville, Australia]), 100 IU mL^{-1} heparin (David Bull Laboratories, Melbourne, Australia), 100 μg mL^{-1} streptomycin sulfate, and 100 IU mL^{-1} penicillin G and then twice in maturation (IVM) medium. In vitro maturation medium contained sodium bicarbonate–buffered TCM199 with 20% (vol/vol) heat-inactivated pooled estrous (white) rhinoceros serum (ERS), 5 μg mL^{-1} follicle-stimulating hormone (FSH; Folltropin; Bioniche Inc., Caufield North, Australia), 5 μg mL^{-1} 1 LH (Lutropin; Bioniche Inc), 1 μg mL^{-1} estradiol, 50 ng mL^{-1} IGF-I (GroPep Pty Ltd, Adelaide, Australia), and 100 μM cysteamine. Maturation took place in culture wells (Nunc Inc., Naperville, IL, USA) containing 600 μL IVM medium covered with 300 μL mineral oil at 38.6 °C in a humidified atmosphere of 5% CO_2 in air for between 23 and 56 h. For Collection 3, COCs were rinsed three times in G-Mops (Vitrolife; Göteborg, Sweden) supplemented with 5.0 mg/mL human serum albumin (HSA) and then twice in maturation (IVM-2) medium (G-Mat; supplemented with 2.5 mg/mL HSA). Maturation took place in culture wells (Nunc Inc.) containing 50 μL IVM-2 medium covered with 800 μL Ovoil (Vitrolife). Oocytes were matured at 38.6 °C in a humidified atmosphere of 6% CO_2 in air for 28 h.

2.2.2. Semen preparation

Fresh semen was obtained and diluted 1:1 (v:v) with egg-yolk extender (buffer solution containing 2.41% [wt/vol] TES, 0.58% [wt/vol] Tris, 0.1% [wt/vol] fructose, and 5.5% [wt/vol] lactose supplemented by 20% egg yolk [final concentration 15.6%, vol/vol], and 20 IU (–tocopherol/mL) in a 14-mL tube (Falcon; Becton Dickinson, Melbourne, Australia) [22]. The tube with the diluted semen was then placed into a 50-mL beaker containing water (36 °C) and chilled (4 °C) for a minimum 4 h.

To determine the preferred IVF medium, ''swim-up'' procedures were conducted using synthetic oviduct fluid (SOF) medium supplemented with heparin (20 μg/mL) and either 5% (vol/vol) ERS, 0.5 mg/mL BSA, or 5% (vol/vol) FCS. Briefly, chilled semen was warmed (36 °C) and then 200 μL layered under 1 mL of each medium in a 14-mL tube. After 1 h, sperm in the upper fraction of each medium was assessed for motility and morphology.

2.2.3. In vitro fertilization

For Collections 1 and 3, excess cumulus cells were gently removed after maturation using a Gilson pipette (leaving the corona radiata intact), washed three times in the preferred IVF medium (SOF medium containing heparin and 5% [vol/vol] ERS), and either cultured in groups (Collection 1) or individually (Collection 3) in culture wells containing 450 μL IVF medium covered with 300 μL mineral oil. Motile sperm were obtained from the diluted chilled semen using the preferred swim-up procedure (as described earlier). The concentration of sperm in the upper fraction was determined after 1 h, and approximately 0.5×10^6 sperm were added to the culture well. COCs were co-incubated with sperm at 38.6 °C in a humidified atmosphere of 5% CO_2 in air.

2.2.4. Intracytoplasmic sperm injection and activation protocol

For Collection 2, cumulus cells were removed after maturation by pipetting oocytes in 12.5 mg/mL sodium hyaluronidase. Injection of oocytes was performed in HEPES SOF containing 6 mg mL^{-1} BSA and amino acids at ovine oviductal fluid concentrations [23]. Fresh or frozen semen prepared by the preferred swim-up procedure was placed into 10% (v:v) polyvinyl pyrrolidine (PVP; Irvine Scientific, Hawthorne East, Australia). A single spermatozoon was selected, immobilized, and injected into each oocyte. After ICSI, oocytes were activated with 10 μM calcium ionophore for 5 min and then washed three times in HEPES SOF containing 20% (vol/vol) FCS. Oocytes were then cultured in SOF culture medium containing 1.9 mM 6 dimethylaminopurine (6-DMAP) for 3 h before being transferred to conventional SOF culture medium (see later).

2.2.5. In vitro culture

For Collections 1 and 3, remnant cumulus cells were removed approximately 26 h after the commencement of IVF by gentle pipetting. Presumptive zygotes were then washed three times in In Vitro Culture (IVC) medium. For Collections 1 and 2, presumptive zygotes were cultured in 600 μL SOF (covered with 300 μL mineral oil) containing 6 mg mL^{-1} BSA and amino acids (IVC medium) at 38.6 °C in a humidified atmosphere of 5% CO_2, 5%O_2, 90% N_2. For Collection 3, presumptive zygotes were individually cultured in 50 μL G-1 Plus (Vitrolife) covered with 800 μL Ovoil (Vitrolife) at 38.6 °C in a humidified atmosphere of 6% CO_2.

3. Results

3.1. Superstimulation

Gonadotropin-releasing hormone analogue treatment (n = 11) stimulated continuous follicular growth in all anestrous females. Ultrasound examination on Days 1, 4, 6, and 10 of treatment documented a continuous increase in follicular size and number. The follicular development in treated females well exceeded that of anestrous females (Table 1). The number and size of follicles in superstimulated females was significantly higher and larger compared with that in anestrous females. In anestrous females, a maximum of one to two dominant follicles were present on one of the ovaries at any one time, whereas multiple large follicles were present on both ovaries in the superstimulated animals (Fig. 1). In 73% of the cases (n = 8), a hemorrhagic structure was visible at OPU 24 to 48 h after last GnRH analogue treatment. A rise in fecal progesterone concentration was measured in 7 of 9 females 2 wk after GnRH treatment indicating that ovulation had followed the superstimulation treatment (Fig. 2).

Table 1
Follicle number and size in anestrus and superstimulated white and black rhinoceroses.

Species	Number of follicles ≥5 mm/ovary	Size of largest follicle/ovary (mm)
White rhinoceros		
Anestrus (n = 10)	3.1 ± 0.4^{a}	12.9 ± 1.8^{a}
Superstimulated (n = 4)	16.5 ± 2.2^{b}	26.8 ± 1.6^{b}
Black rhinoceros		
Anestrus (n = 6)	4.9 ± 0.7^{c}	16.3 ± 2.6^{c}
Superstimulated (n = 7)	13.7 ± 0.6^{d}	31.0 ± 0.7^{d}

[a–d]Values within a column and species with different superscripts are significantly different.

3.2. Oocyte recovery

The transrectal ultrasound-guided oocyte recovery technique facilitated follicle aspiration in all rhinoceroses. In the black rhinoceroses, where OPU was carried out in a sternal position, both ovaries were well accessible. On the other hand, the larger physical dimensions and the further cranial anatomic position of the left ovary in the white rhinoceros made puncture and

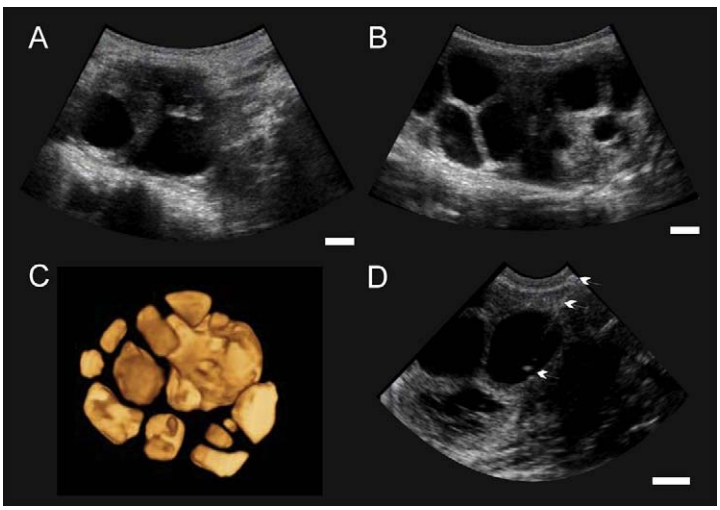

Fig. 1. Ultrasound images of anestrous black rhinoceros and superovulated black rhinoceros. (A) Ovary in anestrus with two large follicles. (B) Ovary with multiple large follicles after stimulation with GnRH analogue. (C) Inverse mode three-dimensional ultrasound reconstruction of the ovary. Grey-colored area represent fluid-filled follicles. (D) Aspiration needle has penetrated the rectal wall and ovarian tissue and is situated in the follicular lumen (arrows).

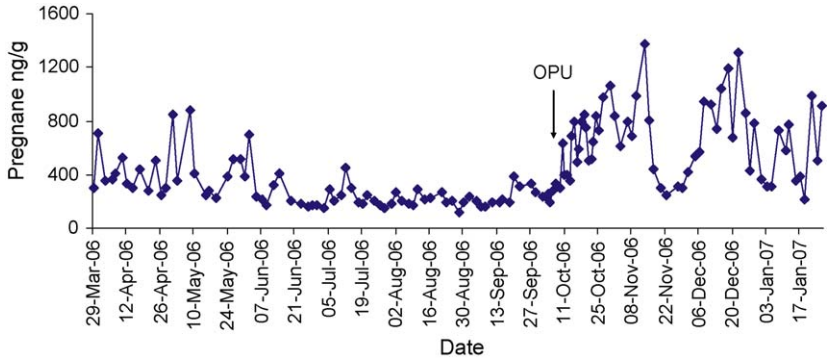

Fig. 2. Fecal pregnane profile of an anestrous black rhinoceros. Note the rise in fecal pregnane after GnRH analogue stimulation and OPU (↑). Two estrous cycles occurred after the hormone treatment (↔), defined by 3 to 4 wk of elevated luteal concentrations and 1 wk of baseline luteal activity in between cycles.

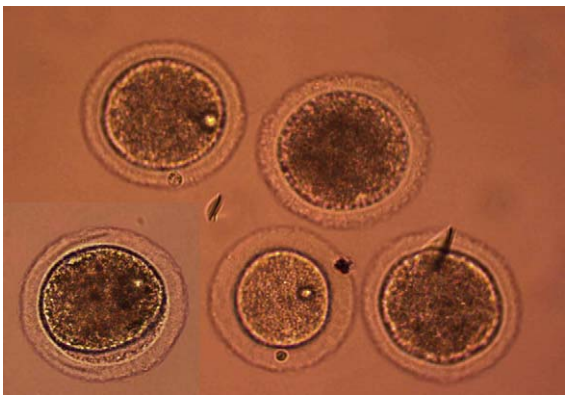

Fig. 3. Total harvest of five oocytes from a transrectal ultrasound-guided oocyte recovery in a white rhinoceros.

aspiration of follicles from the left ovary challenging and was therefore no longer attempted. Up to 15 follicles were punctured, aspirated, and flushed on each ovary (Fig. 3). During 6 oocyte recoveries, a total of 29 COCs were harvested (Table 2). A range of two to nine COCs were harvested per collection. No postsurgical complications were noted in any of the OPU candidates. Upon repeated OPU in the black rhinoceroses, no intra-abdominal adhesions, cysts, or visible ovarian scarring tissue was observed. Oocytes recovered from two black rhinoceroses were used in IVM, IVF, and ICSI trials.

3.2.1. IVF medium and semen evaluation

Visual assessment after 1 h swim-up indicated that SOF supplemented with ERS gave the highest percentage of progressive movement (70%) compared with that for SOF + BSA (20%) and SOF + FCS (20%).

3.2.2. OPU Collection 1

Three unexpanded COCs were recovered from the aspirate. After IVF, two oocytes had a polar body (confirmed metaphase II with Hoechst 33342 staining). The third oocyte appeared to have three extrusion bodies. No cleavage was recorded. Hoechst 33342 staining showed six distinct prophase nuclei.

3.2.3. OPU Collection 2

Five compact cumulus COCs and one expanded COC and nine compact COCs were recovered from black rhinoceros Females 1 and 2, respectively. In Female 1, one oocyte with expanded cumulus 22 h after the commencement of IVM had an extruded polar body and underwent ICSI with frozen-thawed semen, followed by activation. After 48 h IVC, no cytoplasmic division was observed, and staining with Hoechst 33342 showed a stained sperm head but no other nuclear material. At 26, 36, and 56 h, no polar bodies were observed in the remaining five oocytes. Hoechst 33342

Table 2
Oocyte collection in one white and two black rhinoceroses.

Species	Individual no.	OPU attempts	Follicles >10 mm/ovary	Follicles aspirated/OPU	COCs with compact cumulus	COCs with expanded cumulus	Denuded oocytes
White rhinoceros	1	1	16.5	10	5	–	–
Black rhinoceros	1	3	13.1 ± 0.74	23.0 ± 2.0	13	1	–
	2	2	15.3 ± 0.85	22.5 ± 1.5	9	–	1

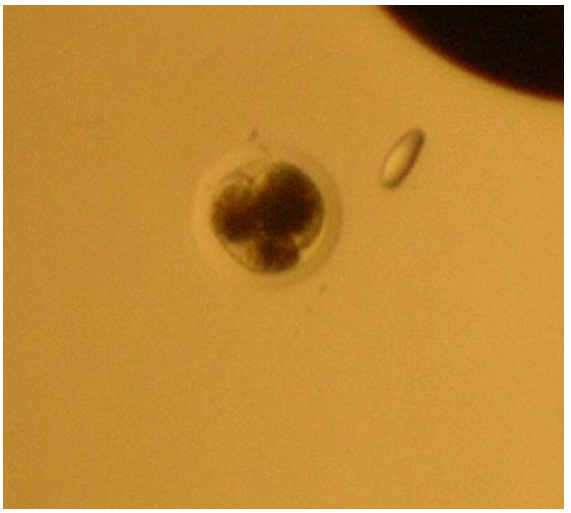

Fig. 4. Black rhinoceros 4-cell embryo produced by IVF.

staining indicated one oocyte at anaphase/telophase and four oocytes at metaphase I.

In Female 2, nuclear maturation staining with Hoechst 33342 indicated that only one of the nine COCs was at metaphase II (MII). No cleavage was observed.

3.2.4. OPU Collection 3

In Black Rhinoceros 1, five compact COCs and one nonviable oocyte were recovered from the aspirate. Hoechst 33342 staining of one compact COC indicated germinal vesicle breakdown (GVBD) had commenced and confirmed the nonviable oocyte as degenerating. Of the four remaining COCs, three were placed into IVM, and one was dedicated to proteomic metabolism studies. After 28 h, two oocytes underwent IVF, and the third was earmarked for proteomic metabolism studies. At Day 2 (Day 0 = day of IVF), observations indicated one 4-cell embryo (Fig. 4). Hoechst 33342 staining showed two distinct polar bodies (PBs) and three cells with distinct prophase nuclei.

In Black Rhinoceros 2, two COCs were recovered from the aspirate. One of the oocytes was completely denuded and immediately stained with Hoechst 33342 and was at MII. The remaining oocyte underwent 28 h IVM and IVF. No cleavage was observed, and Hoechst 33342 staining showed sperm bound to the zona and four chromatin structures.

4. Discussion

We report on the successful follicle stimulation and the repeated, in situ oocyte recovery from infertile rhinoceroses using a minimally invasive technique. Unlike domestic species where OPU is performed either by laparoscopy or transvaginal follicle aspiration, oocyte recovery in the rhinoceros was performed transrectally under ultrasound guidance. Puncture of the rectal wall and subsequent aspiration of multiple follicles resulted in the recovery of two to nine oocytes per collection. Various protocols for IVM, IVF, and ICSI were tested on this low number of ‘‘valuable’’ oocytes. As a result, we can report on the first rhinoceros embryo ever produced in vitro. In domestic species, a vast number of gametes is available for the development of IVM, IVF, or ICSI protocols. This resource is limited in large and endangered wildlife species like the rhinoceros. However, the transfer of modern ART from domestic animals to wildlife species can lead to fast results even if the resources are limited. Yet, the production of embryos as means to preserve genetic diversity of postreproductive female rhinoceroses needs further investigations.

The development of the OPU in itself is a necessary step on the way to developing a reliable in vitro embryo production technique for rhinoceros. The obvious question that arises is whether the rhinoceros really needs it and what benefits will be gained from such an ART? In our view, the answer is definitely yes for several reasons. In the absence of such ART techniques, as many as 50% of the female rhinoceroses in captivity will probably never conceive and will be permanently excluded from the reproductive population. Some individuals may not be able to conceive, yet if their oocytes can be used to produce embryos that can be transferred to reproductively healthy females, these females will not be lost to the gene pool. The establishment of gene resource banks (GRBs) was proposed almost two decades ago [24]. Among their many advantages are the collection of genomes, gametes, and embryos, fulfilling their function as a means to extend the reproductive life span of individuals beyond their biological lives. To set up a useful GRB for the rhinoceros species/subspecies, methods should be developed for the collection, preservation, and utilization of tissues, gametes, and embryos. The collection, cryopreservation, and use of rhinoceros spermatozoa for this purpose has already been demonstrated [25,26]. However, the cryopreservation of oocytes has proved challenging from a wide range of species [27], and it appears that frozen embryos may respond better to challenges introduced by the chilling, freezing, and thawing processes. Thus, it is our intention to develop a procedure that will include OPU, IVM, IVF, and/or ICSI and ET with and without a

cryopreservation step. These can be used when natural breeding or AI are not possible or when attempting to salvage oocytes from deceased animals and routine storage. Having such techniques available will make it possible to put these embryos into safekeeping rather than waiting until these species are extinct.

Rhinoceroses are monovulatory mammals. Follicles grow in waves, from which one dominant follicle develops to become the preovulatory follicle [5,9,28]. The fate of this preovulatory follicle might be atresia, hemorrhage, or rupture with the release of the oocyte and the formation of a corpus luteum. In captive white rhinoceros, approximately 50% of females are in permanent anestrus [20,29]. The ovarian activity of anestrous rhinoceroses is similar to that of mares during the transitional period in the prebreeding season. The ovaries show continuous follicular development, yet dominant follicles do not rupture but become hemorrhagic or atretic [7,8,28,30]. Whereas only one large preovulatory follicle is present at the end of each follicular wave, the aim of this study was to promote additional follicular growth to increase the population of follicles available for follicular aspiration.

Common superovulation protocols in the mare, the closest domestic relative to the rhinoceros, rely on the administration of equine FSH twice per day [31,32]. However, remote delivery of equine FSH by dart twice daily for up to 5 d would be unnecessarily traumatic and stressful in a captive rhinoceros.

The sustained release of the GnRH analogue deslorelin acetate has been extensively used to hasten ovulation in cycling mares [33]. In white rhinoceroses, this analogue treatment has been effective in hastening the occurrence of ovulation during transitional anestrus and during first postpartum estrus in females [9]. Unlike these studies, where ovulatory-sized follicles were present at the time of treatment, the GnRH analogue treatment in this study was extended to 6 to 7 d to stimulate follicular growth in ovaries with minimal follicular activity. The treatment generated a three- to fourfold higher number of follicles on each ovary and a twofold increase in size of the follicles compared with that of nontreated anestrous rhinoceroses. Up to 15 follicles per ovary were available for aspiration at the time of OPU. Hemorrhagic structures at the day of OPU and the rise in fecal pregnane concentrations in 80% of the treated animals suggests that deslorelin was suitable to induce superovulation in black rhinoceros and white rhinoceros with unknown follicular activity at the onset of treatment. Similar results were reported in mares after prolonged administration of GnRH. In randomly selected transitional mares with a lack of substantial follicular development, prolonged GnRH analogue treatment for up to 12 d has been reported to induce an ovulatory response [34,35]. However, the number and size of follicles present on the mares' ovaries after this treatment have not been reported.

The long-term administration of a GnRH analogue normally induces pituitary desensitization and decreased gonadotropin release. For instance, contraception has been achieved in female giraffe using long-term GnRH agonist treatment [36]. However, the susceptibility to the downregulatory effect of long-term GnRH differs between species. Specifically, in the mare, even long periods of GnRH analogue treatment cannot reliably induce reproductive shutdown [37]. When using short-term deslorelin implants in the mare, suppressive effects are limited to a prolonged interovulatory interval [38,39]. The prolonged GnRH analogue treatment in our study for 6 d seemed not to induce a suppressive effect on the pituitary-ovarian axis ceasing reproductive activity. On the contrary, in both black and white rhinoceroses, follicle growth was stimulated. The 6-d GnRH analogue treatment produced a broad ovarian response with large follicular output.

To access the ovary for oocyte recovery, two classic approaches exist: laparoscopy as in the sheep and pig and transvaginal follicle aspiration as in cattle and horses. In the rhinoceros, the thick integument and extended ribcage hinders an easy and direct laparoscopic approach to the ovary. Laparoscopic uterine biopsy and ovariectomy from the flank have been reported for the rhinoceros [40,41]. However, risk of wound dehiscence, peritonitis, scar tissue formation in the integument, and intra-abdominal adhesions after flank laparoscopy would rarely allow for repeated oocyte recovery. Transvaginal follicle aspiration, similar to OPU in the cow and the mare, has been attempted in black and white rhinoceroses but failed to give access to the ovaries [17]. Despite a minimal incision in the vaginal roof and use of flexible endoscopy, technical problems such as insufficient insufflation of the large abdominal cavity, difficult visualization of the ovaries, and resorption of CO_2 with subsequent intestinal emphysema and emphysema of the abdominal wall have limited its use in repeated OPU in the rhinoceros [17]. Consequently, transrectal ultrasound-guided follicular aspiration was attempted to overcome anatomic obstacles and technical problems experienced with both the laparoscopic and transvaginal follicular aspiration techniques. The risk of abdominal contamination with fecal bacteria was restricted to a single penetration through the rectal

wall. The preparation of the rectum with water and Betadine enemas and local and systemic antibiotics, similar to standards in human medicine prior to colon resection, further reduced the risk of infection [42]. The transrectal follicular aspiration provided direct access to the ovary and rendered critical surgical intervention or abdominal insufflation unnecessary. No postsurgical complications were noted. Moreover, the minimally invasive technique was repeatable, and even upon repeated OPU, no signs of ovarian scar tissue or adhesions were noted. Nevertheless, transrectal follicular aspiration, necessitated by the animal's size in the rhinoceros, is not a sterile approach and has some risk of wound contamination from remaining fecal material and bacteria in the rectum. However, given the complications associated with other approaches to the rhinoceros ovary, the complications associated with the transrectal approach remain minimal. The absence of postsurgical complications and the repeatability of the results indicate that this approach is equivalent to transvaginal OPU in the horse and cow. Although the rhinoceroses selected for this study were unable to conceive, it is likely that the minimally invasive transrectal OPU would not adversely affect the fertility in healthy donors as demonstrated in the mare [43].

The oocyte recovery rate per rhinoceros reported here will require further improvement in order to contribute substantial oocyte numbers for the development of IVM, IVF, or ICSI. The recovery rate of oocytes reported here is satisfactory compared with that of the closest related domestic species, the horse. However, follicular stimulation protocols will need to be refined to recover a larger number of mature oocytes, which would eliminate the need for IVM. However, in vivo transrectal oocyte recovery is a necessary prerequisite for the development of advanced ARTs for infertile, valuable, female rhinoceroses. In rhinoceros species and subspecies in captivity, where the number of infertile females is high and the potential loss of genetic diversity is alarming, in vitro embryo production is likely to be the last resort in maintaining genetic diversity or even survival of the species.

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