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Enhancing captive Indian rhinoceros genetics *via* artificial insemination of cryopreserved sperm

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ABSTRACT

The objective of this study was to design an artificial insemination (AI) protocol using cryopreserved spermatozoa to obtain pregnancies in captive Indian rhinoceroses (*Rhinoceros unicornis*). Four methods developed varied by timing and approach, as follows; Method 1: females ($n = 2$) were inseminated pre- and post-ovulation under general anesthesia, Method 2: females ($n = 2$) were inseminated pre-ovulation without anesthetic *via* endoscopy, Method 3: females ($n = 1$) were inseminated pre-ovulation without anesthetic *via* manual insertion of an insemination catheter, Method 4: females ($n = 2$) were inseminated same as Method 3 with the addition of standing sedation. Semen deposition site varied as a result of changes in AI technology and experience. All females conceived following intrauterine AI using three methods. Four pregnancies ($n = 3$ females) produced *via* Method 3 and 4 resulted in term births ($n = 2$ male calves, $n = 2$ female calves) at 481.8 ± 12.8 days post-AI. Unfortunately, two early pregnancy losses were documented in a fourth female conceiving *via* Method 2. Pregnancy rates were 0%, 22%, 17%, and 50% for Method 1–4, respectively. Method 3 and 4 rates improved to 29% and 67%, respectively when accounting for AI's conducted only on ovulatory estrous cycles. Spermatozoa ($n = 5$ males) were cryopreserved 0.3–9.3 y prior to successful AI procedures. The lowest dose of frozen-thawed sperm resulting in conception was 500×10^6 motile sperm. Mean time from AI to ovulation in conceptive and non-conceptive cycles was 26 ± 11.8 h and 66 ± 80.7 h, respectively.

1. Introduction

One of the primary goals for keeping rhinoceroses in zoological collections is to maintain populations that are genetically viable and self-sustaining to guard against extinction in the wild. Unfortunately, the reproductive rate of captive Asian and African rhinoceroses remains insufficient to sustain independent populations, relying on importation of individual animals from facilities overseas or the wild (Eyres et al., 2013; Reiches et al., 2013). Captive rhinoceros populations are not alone in facing a serious loss of critical genetic diversity. Wild rhinoceros populations are experiencing a poaching crisis of unprecedented scale that is endangering their very existence. This insa-

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tiable demand has exponentially increased poaching to a rate that, if left unabated, will render wild rhinoceros populations unsustainable. It is possible that zoos may be the last places these animals exist. Therefore, it is imperative to address and remedy those difficulties associated with reproduction that have limited genetic diversity in captive rhinoceros populations to ensure they are around for many years to come.

The captive North American Indian rhinoceros population has grown since its inception as a result of successful captive reproduction and importation, reaching the current size of 55 animals distributed throughout 23 zoos (Reiches et al., 2013). Indian rhinoceroses are primarily solitary and territorial (Dinerstein, 2003). Males and females are exhibited separately except during introductions for breeding (Lang, 1975). Natural breeding attempts can frequently result in severe aggression between Indian rhinoceros pairs, and this behavioral incompatibility has made genetic management of this species a challenge. Four founder rhinoceroses are responsible for over 50% of all genes found in the present population (von Houwald, 2013). Presently, none of the 23 original founder animals remain capable of contributing *via* natural breeding (von Houwald, 2013).

Behavioral incompatibility is not the only issue impacting the reproductive management of captive Indian rhinoceroses. Fertility declines when female African and Asian rhinoceroses undergo extended lengths of time without carrying a pregnancy (Hermes et al., 2004, 2006, 2014). Female Indian rhinoceroses are prone to the development of leiomyomas within their reproductive tract (Hermes et al., 2014). This pathology may be influenced by changes in reproductive hormones, therefore continuous estrous cycles without intervening pregnancies may exacerbate the pathology leading to infertility (Hermes et al., 2014). The earlier first pregnancies occur and the quicker subsequent pregnancies can be established, the longer the reproductive lifespan should be for captive female rhinoceroses.

Considerable effort has been made to improve timing of natural breeding attempts in captive Indian rhinoceroses and can be attributed to an increased understanding of the species reproductive biology (Kassam and Lasley, 1981; Kasman et al., 1986; Schwarzenberger et al., 2000; Gomez et al., 2004; Stoops et al., 2004, 2014). Concurrent efforts to develop assisted reproductive technology (Stoops et al., 2004, 2007, 2010), specifically artificial insemination (AI), have become necessary to further facilitate their genetic management.

Incumbent for any successful AI program is the ability to collect and cryopreserve spermatozoa. Methods for semen cryopreservation in the Indian rhinoceros have been reported (Stoops et al., 2010). In addition, preliminary evidence from our facility demonstrated the *in vivo* competence of frozen-thawed spermatozoa in this species *via* AI (Stoops et al., 2007). Similar assisted reproductive efforts in captive African white rhinoceroses (*Ceratotherium simum*) have confirmed the utility of using either fresh (Hildebrandt et al., 2007) or frozen-thawed (Hermes et al., 2009) spermatozoa to produce rhino calves through AI. The *in vitro* functionality of fresh and frozen-thawed rhinoceros spermatozoa collected *via* electroejaculation (EEJ) and epididymal gamete rescue has also been shown through heterologous and homologous *in vitro* fertilization (IVF, Hermes et al., 2009; Stoops et al., 2011).

Depending on the species, fertilization rates can be higher when AI is performed just prior to ovulation (Roelofs et al., 2006). Maximal pregnancy rates are achieved in the closest domestic relative, the horse, when pre-ovulatory AI procedures are performed within 12 h prior to ovulation (Katila et al., 1996). As the interval from insemination to ovulation widens the chance of pregnancy declines, especially when frozen-thawed spermatozoa is used (Katila et al., 1996).

The objective of this research was to further develop and apply AI in conjunction with genome resource banking to improve genetic management of Indian rhinoceroses without the need for animal transport. In an effort to enhance utility and application of this technology, AI was conducted on multiple female Indian rhinoceroses of various ages and prior reproductive histories that were located at different zoological facilities and having varying degrees of operant conditioning for reproductive assessment. Specific objectives of the study were to (i) develop technology that would permit intrauterine AI in the Indian rhinoceros; (ii) time AI around the natural reproductive cycle of the female Indian rhinoceros utilizing signs of behavioral estrus and urinary estrogen conjugate (EC) and progesterone metabolite (PdG) concentrations and pattern of excretion; (iii) demonstrate the *in vivo* fertility of frozen-thawed spermatozoa collected from multiple males and cryopreserved for various lengths of time; and (iv) characterize early embryo development and fetal dynamics throughout gestation in this species using ultrasonography.

2. Materials and methods

2.1. Animals

This research was conducted on four female Indian rhinoceroses maintained at three zoological facilities in North America (Table 1).

Cincinnati Zoo & Botanical Garden (CZBG), Cincinnati, OH, USA: Nulliparous female Indian rhinoceros studbook number (SB) 238 was 6.8–10.8 y of age during the study. Nulliparous female SB189 was 13.9–18.9 y of age during the study. During a portion of the study period, a male Indian rhinoceros was housed at the CZBG, but chronic severe arthritis in one of his hips prevented natural breeding.

Montgomery Zoo and Mann Museum, Montgomery, AL, USA: Multiparous female SB274 was 10.5–12.4 y of age during the study. Her prior reproductive history included the successful live birth of two calves as a result of natural breeding at the San Diego Safari Park, San Diego, CA USA. While SB274 had bred naturally at another institution (with a different bull), initial attempts to introduce her with SB239 resulted in significant aggression between the pair. Subsequent introduction attempts were deemed too risky and AI was requested as a means to potentially achieve a pregnancy between the pair.

Buffalo Zoo, Buffalo, NY, USA: Multiparous female SB241 was 16–17.5 y of age during the study, having previously