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Overcoming reproductive disorders in female greater one-horned rhinoceros (*Rhinoceros unicornis*) to improve artificial insemination and natural breeding success

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Abstract

Reproductive disorders of female greater one-horned rhinoceros (GOHR [*Rhinoceros unicornis*]) contribute to relatively lower (50%) conception success from artificial insemination (AI) with frozen-thawed sperm, in comparison to rhino's closest domestic relative, the mare. Determining the causes of subfertility or infertility can be especially challenging in nondomestic species like rhinoceros. Formative research identified anovulation and intraluminal uterine fluid accumulation as primary factors associated with the established 50% conception rate from GOHR AI. We present an integrative approach taken to identify and treat female GOHR reproductive dysfunctions to improve AI outcome to 66%. Recommended diagnostics for the GOHR practitioner include, but are not limited to, a breeding soundness examination, transrectal ultrasonography of the reproductive tract and ovaries, and urinary hormone analysis. As similar reproductive dysfunctions affect breeding success in their closest living domestic relative, the mare, comparable therapeutic treatment options were explored in female GOHR. Consequently, substantial advancement in GOHR hormone therapies were used to ensure ovulation and resolve uterine clearance issues with Sucromate® and oxytocin treatments, respectively. Managed breeding programs, like that for the GOHR, serve a noteworthy role in the strategy to conserve threatened species. In seeking strategies to address reproductive dysfunction in a cohort of females, we improved the procedural rate of success in AI. Reported strategies can also assist GOHR natural breeding efforts and collectively support the long-term resilience and conservation role of this managed population.

Keywords: Assisted reproductive techniques, ovulation induction, endometritis, anovulation, intraluminal fluid

Introduction

Development and application of reproductive technology in nondomestic species relies heavily on information gained from domestic model species.[1] Rhinoceros are a member of the order Perissodactyla, and their closest living domestic relative is the horse. Similar to other livestock species, horses are used in commercial enterprise where monetary gains and losses are directly tied to reproductive performance. As such, equine reproduction has become a highly focused area of study with results generated from sample sizes unparalleled to those working in nondomestic species field.[2] Research findings in the horse have proved an invaluable resource to those working to conserve rhinoceros. However, as with all model systems that include a domestic and wild counterpart, substantial differences exist between the reproductive physiology of the rhinoceros and horse that preclude making sweeping generalizations. In fact, remarkable differences exist even among various rhinoceros species.[3-9] It is, therefore, imperative to use appropriate methodologies and to draw interpretations based on established species-specific norms (i.e. estrous cycle length, follicular growth pattern, and preovulatory follicle size).

The current size of the North American managed population of greater one-horned rhinoceros (GOHR [*Rhinoceros unicornis*]) is 110 animals distributed across 39 facilities. Substantial genetic skew in the founder GOHR population served as the impetus for development and application of semen collection/cryopreservation and artificial insemination (AI) in this species.[10] GOHR calves have been produced from AI using frozen-thawed sperm with an AI success rate of 50% achieved using a standing sedation approach.[10] Quality of frozen-thawed sperm is generally not a problem in GOHR.[11] Therefore, comparisons to equine AI are made to studies utilizing semen from stallions classified as good for freezing, meaning a postthaw sperm progressive motility of 40 - 60%.[2] Conception rates of 60 - 75% per cycle have been achieved for mares inseminated with > 300 x 10^6 motile frozen-thawed sperm within 24 hours before to 6 hours after ovulation.[12-14] Previous successful GOHR AI procedures utilized semen exhibiting a 60% postthaw motility to inseminate a minimum of 500 x 10^6 motile sperm 24 - 48 hours before ovulation.[10] Through retrospective analysis of ultrasonography and urinary hormone data it was observed that some of these cycles were nonovulatory, decreasing the potential success rate of the AIs.[10] Since that formative work, the authors have developed a comprehensive strategy to identify and treat common reproductive disorders encountered in GOHR to further advance the application of AI technology to more individuals and improve conception success from a single insemination attempt (Figure 1).

Female breeding soundness examination

Subfertility has been identified as a major issue affecting natural and assisted rhinoceros breeding efforts.[4-10,15-23] Its impact has been directly felt by those developing nondomestic assisted reproductive techniques (ART), as young, proven females are rarely afforded for study.[1] Instead, available subjects are frequently those that have failed to succeed via a natural breeding approach.[1] Subfertility of the mare can result in substantial economic losses to equine industry, and equine practitioners implement breeding soundness examination (BSE) as a means to identify and subsequently treat subfertile mares to improve reproductive outcome.[24] It is recommended that GOHR females undergoing AI be subjected to BSE. At minimum, past reproductive history should be reviewed along with recent and concurrently collected ultrasonography information, endocrine and behavior data ahead of any AI attempt. Given an average estrous cycle length of 45 days in GOHR,[8] a 3-month collection timeframe ahead of planned AI is desired. As rhinoceros are nonseasonal breeders, examinations can occur irrespective of time of year.

Ultrasonography

Transrectal examination of the reproductive tract of domestic livestock is routine practice and the authors propose it should be a trained voluntary behavior for all rhino in managed care.[5,7-8,25] The degree of stress caused by operant conditioning and the act of routinely conducting transrectal ultrasonography on GOHR has been studied via fecal glucocorticoid metabolite and behavioral analysis.[26] Results indicate ultrasonographic reproductive assessments conducted using operant conditioning are considered nonstressful events.[26] In general, rhinoceros are very tolerant of transrectal examination if appropriately conditioned. Given the normal follicular growth pattern and size in this species, it is recommended that a low frequency curved array transducer be used for transrectal examination. The left ovary is located more cranial than the right ovary, sometimes out of the practitioner's reach, and an extension tool is needed to facilitate transducer reach to the left ovary and distal horn. It is important that examinations are conducted by one who is familiar with normal ultrasonographic findings at various stages of the GOHR estrous cycle. Notable

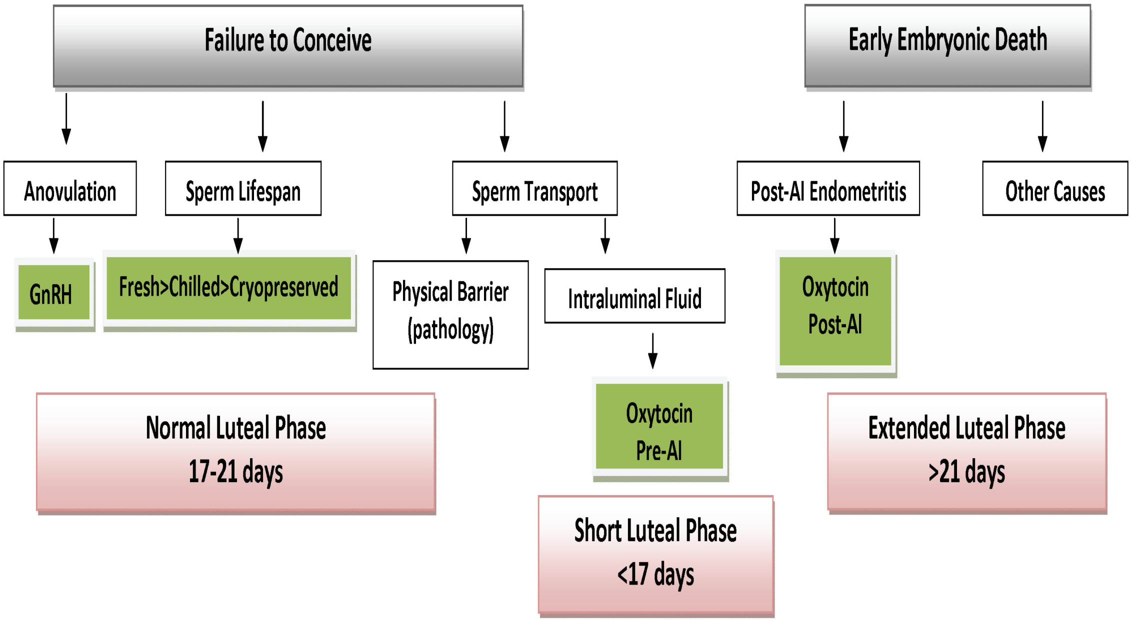

Figure 1. Flowchart of the process used to identify and, when possible, treat reproductive disorders contributing to failure to establish pregnancy in greater one-horned rhinoceros (GOHR) after AI or natural breeding.

differences as compared to the mare include, but are not limited to, the substantial size of the GOHR preovulatory follicle, development of corpora hemorrhagica (CH) versus corpora lutea (CL) and lack of edematous changes in the uterus leading up to ovulation.[8] From the perspective of establishing AI candidacy, it is important to identify any potential barriers for sperm transport and embryo mobility throughout the reproductive tract, including intact hymen. Pathologic conditions such as tumors, cysts and intraluminal uterine fluid can be identified via ultrasonography. GOHR are prone to the development of leiomyomas[20] so their presence should not be unexpected, especially in older nulliparous females (Figures 2a, b). Size and location of cysts within the uterine horns are important to document especially for future referral when conducting postAI examinations to determine early pregnancy. Similar to the mare, GOHR embryonic vesicle movement throughout the uterus is needed for maternal recognition of pregnancy.[27] Depending on the size and location of pathology could prevent signaling and thus release of $PGF_{2\alpha}$ and regression of the CH.

Endocrinology

In comparison to other rhinoceros species, the authors' experience is that GOHR are not as behaviorally compliant to routine blood collection. Therefore, urine has become the gold standard biological matrix for GOHR hormone evaluation as it correlates to systemic hormone dynamics more closely than feces.[8,10] Additionally, urine is easy to collect as female GOHR habitually urinate in specific areas in the enclosure. GOHR are the only rhinoceros species in which estrogens and estrogen metabolites can be reliably measured and accurately reflect ovarian follicle development;[8-9] therefore, the GOHR practitioner has the unique ability to track hormone dynamics throughout the follicular phase. Specifically, urinary estrogen conjugate (EC) and pregnanediol glucuronide (PdG) concentrations and their pattern of excretion can be used to monitor follicular and luteal phases, respectively (Figures 3a, b).[3,8] The GOHR follicular phase averages 14 days and is followed by a 17 - 21 day luteal phase.[8] Mean estrous cycle length is 45 days, but can vary substantially.[8] However, longer estrous cycles in nonpregnant female GOHR are typically due to a delay in emergence of the next dominant follicle,[8] whereas a longer cycle

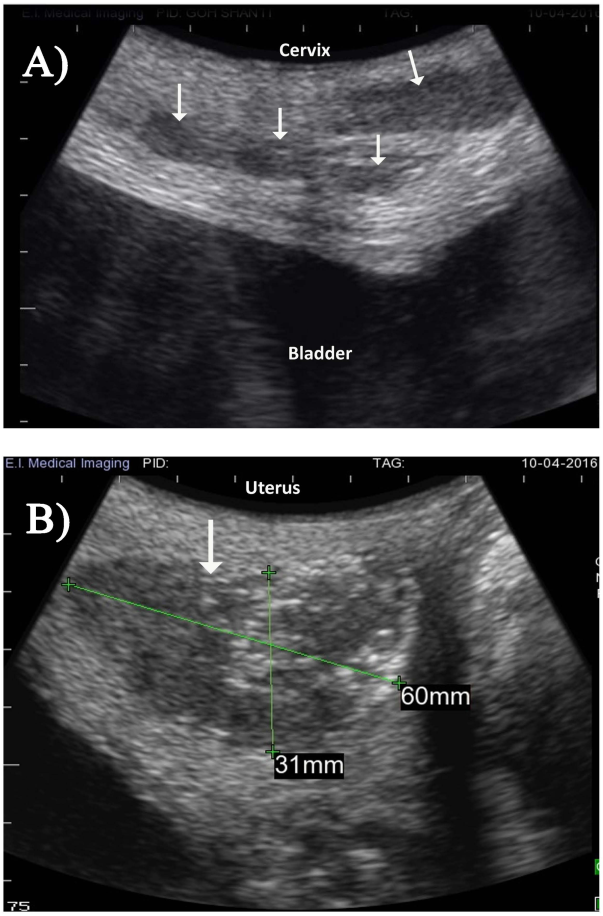

Figure 2. Ultrasonogram of cervix and uterus in a 31-year multiparous greater one-horned rhinoceros (GOHR). Note: multiple A) cervical and B) uterine leiomyomas (arrows).

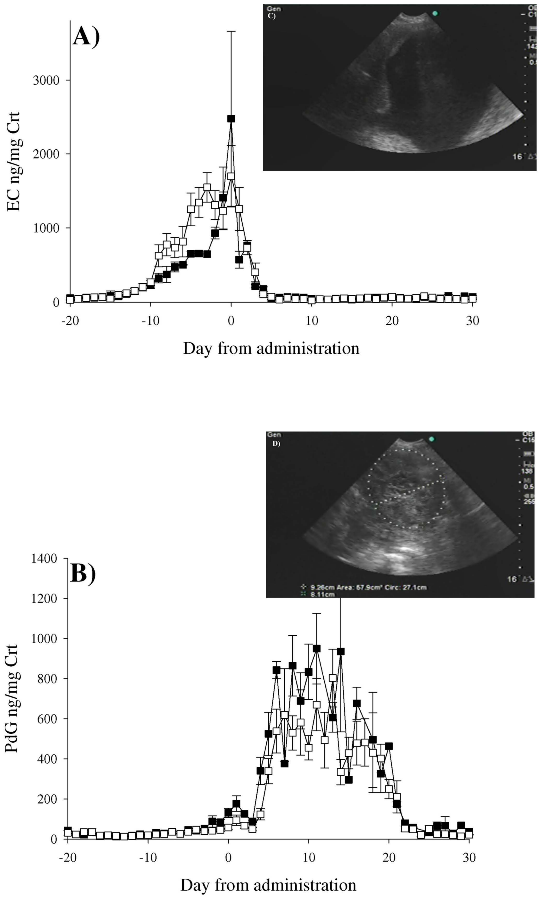

Figure 3. Urinary A) estrogen conjugate (EC) and B) pregnanediol glucuronide (PdG) concentrations and pattern of excretion in greater one-horned rhinoceros (GOHR) that ovulated after deslorelin in saline (■) or Sucromate® (□). Data are aligned by day from ovulation induction agent treatment. Ultrasonograms of C) a preovulatory follicle 47 hours postSucromate® injection and D) resulting CH on day 18 postovulation during a 21-day luteal phase.

length in nonpregnant female African rhinoceros is associated with an extended luteal phase.[6-7] Collecting urinary hormone data permits the GOHR practitioner, through an iterative process, to determine what constitutes baseline EC and PdG concentrations for each female. This information is critical to accurately time AI events that are scheduled according to days after urinary PdG reaches baseline and EC is above baseline.

Behavior

The female GOHR typically exhibits overt estrous behavior. Similar to mares, estrual GOHR have reduced appetite and frequent urination, urine squirting, vulva winking, increased locomotion, and vocalization (whistling and snorting).[8] Peak estrus lasts for 12 - 24 hours; however, GOHR can exhibit cycles with multiple days of some estrual behavior.[8] During these 'split' estrus cycles there are typically several days of quiet in between estrual events making it difficult to determine whether a 'pre' or 'true' estrus has taken place. It is only 'true' estrus that is associated with the LH surge.[8] However, silent estrus has been observed in some older GOHR.[10,21] Estrus intensity can vary among females. Therefore, accurate assessment of estrous behaviors relies on the astute observational skills of rhinoceros care staff and the knowledge they possess regarding 'sometimes-subtle nuances' associated with an individual animal.[10]

Subfertility

Anovulation

All rhinoceros species in managed care have been documented to experience anovulatory cycles on occasion.[5,7,8] This form of ovarian dysfunction has been a major impediment to breeding in the mare[28] and GOHR. Similar to domestic mares, there remains no definitive means to predict early in the cycle whether a given GOHR follicle will be destined for anovulation. In mares the incidence of anovulation increases with age and several causative factors such as insufficient gonadotropin production and elevated estrogen production from the preovulatory follicle have been documented.[28] Timing of previous GOHR AI procedures were based on natural cycles of each specific female.[10] A downfall to this approach was that over a quarter of AI attempts were conducted on anovulatory cycles.[10] Most anovulatory cycles in GOHR are associated with follicles that subsequently undergo luteinization.[8,10] The resulting progestogen production occurs over the course of a normal luteal phase length thus mandating that ultrasonography be performed to confirm ovulation (Figure 4a).[8,10] GOHR practitioners cannot rely solely on urinary PdG concentrations postestrus or postGnRH to serve as confirmation of ovulation. Similar to the mare, only a limited number of anovulation cases in GOHR occur without any significant luteinization following estrogen decline.[8,10]

The previously published GOHR AI protocol relied on behavioral cues from females to time sedation/insemination, that only permitted [24] hour advanced notice for staff, and an ovulation induction agent was not used.[10] As a result, several procedures were not timed correctly due to cycles in which there was a split-estrus, whereas others were hindered by subsequent ovulation failure.[10] Given anovulation was the most frequently encountered disorder during prior GOHR AI efforts, an effective ovulation induction protocol was sought. Preliminary attempts to induce ovulation using gonadorelin diacetate tetrahydrate (Cystorelin™), a GnRH agonist, were unsuccessful due to mistimed injections.[8] Whereas follicle size is the key determinant for timing of ovulation induction in mare[29-30] and white rhino (*Ceratotherium simum simum*),[18-19,22,31] GOHR develop and sustain a large follicle size throughout the majority of their follicular phase,[8] thereby limiting similar application. To correct the timing of injections to induce ovulation, treatment trials using 2 deslorelin synthetic GnRH analogue formulations (Table) were used during natural estrous cycles (n = 15 cycles) in 7 adult female GOHRs (6 - 31 years).

As substantial intra- and inter-individual variability exists in GOHR preovulatory follicle size, timing of all but 1 treatment was based on days urinary EC concentrations were above baseline for each female (Figure 3a). In 1 case, deslorelin treatment timing was based on the number of days from prior estrus and knowledge of behavioral estrus dates for the female's earlier 3 estrous cycles. Deslorelin dosages were extrapolated from those used in domestic mares to induce ovulation during the periovulatory period. Unlike mare that exhibit multiple days of estrus and an extended LH surge, the GOHR estrus lasts only 24 hours and has a narrow LH surge. Compounded deslorelin in saline[29,32] was used earlier but became unavailable and was

Table. Ovulation induction summary in female greater one-horned rhinoceros (GOHR) treated with 2 intramuscular deslorelin formulations

Treatment (year[s] treated)	Dosage (mg)	Number of females	Number of cycles	Age at treatment	Number of days urine EC elevated at treatment	Follicle size (cm) at treatment	Ovulation success rate
Deslorelin in Saline[32] (2009)	3.75	n = 2	n = 4	15.8 ± 1.3	14.3 ± 0.9	14.9 ± 0.4	75% 3/4
Sucromate® (2011 - 2018)	3.15 - 4.95	n = 5	n = 11	16.6 ± 2.5	13 ± 1.1	13.7 ± 0.8	100% 11/11

*verified via ultrasonography and urinary hormone analysis or conception following AI replaced with another deslorelin product, extended release[30] Sucromate®. Fourteen of 15 cycles in GOHR treated with deslorelin resulted in ovulation induction by 48 hours after treatment and was followed by a normal luteal phase length (Table, Figure 3a - d). Retrospective urinary EC analysis of the single unsuccessful ovulation induction trial revealed peak EC concentrations were measured 2 days before treatment with deslorelin in saline and suggests that this treatment was given too early (Figure 4a). The anovulatory follicle (Figure 4b, 4c) subsequently luteinized, resulting in elevated urinary PdG concentration over a ~17 day timeframe (Figure 4a).

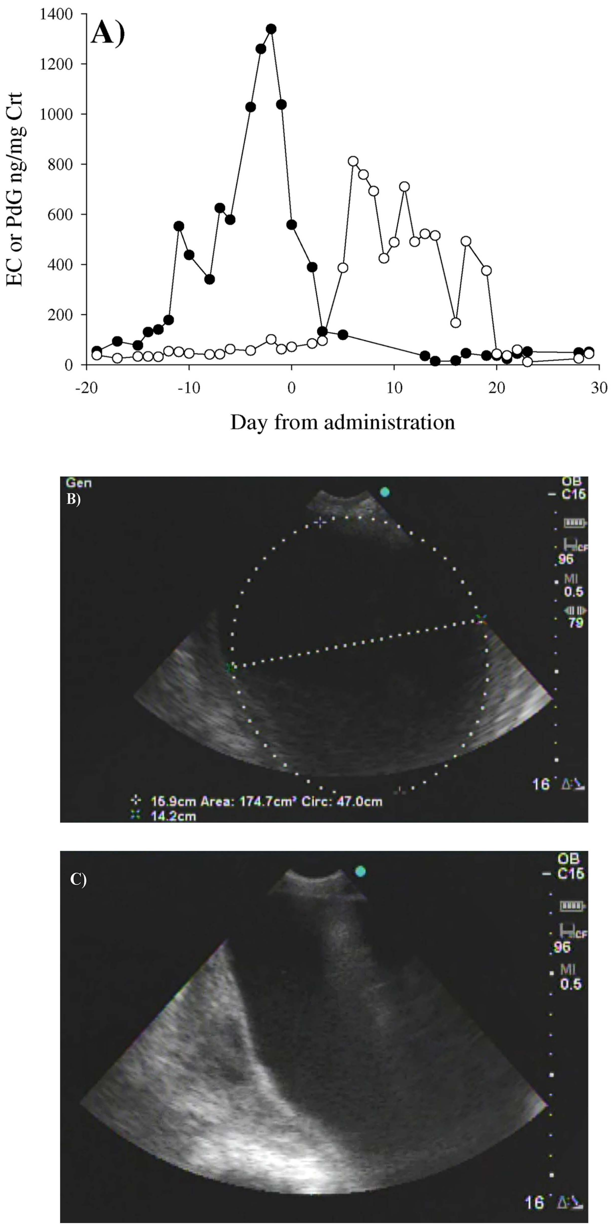

Figure 4. Concentrations of urinary steroid metabolites (A) estrogen conjugate (EC, ●) and pregnanediol glucuronide (PdG, ○) during a failed ovulation induction attempt in a greater one-horned rhinoceros (GOHR) using deslorelin in saline. Data are aligned by day from ovulation induction agent treatment. Corresponding ultrasound images of the follicle on (B) day 0 and (C) day 12.

To date, 2 of 3 GOHR AIs performed in conjunction with Sucromate® treatment at AI resulted in conception and birth of healthy calves. Retrospective urinary PdG analysis confirmed an extended luteal phase was associated with a single unsuccessful induced ovulation AI procedure. This female was not conditioned for routine transrectal ultrasonography and so imaging of the reproductive tract was not possible. Following this event, the female was conditioned for routine ultrasonography that resulted in diagnosis and treatment with a separate disorder (intraluminal uterine fluid accumulation [IFA]) that likely inhibited establishment of viable pregnancy during the aforementioned failed AI. By monitoring urinary EC concentrations to determine initiation of the follicular phase, currently, AI procedures can be timed 1 week ahead and conception rate may be improved with Sucromate® treatment to ensure ovulation, however larger sample size studies are needed.

Intraluminal uterine fluid

The inability to effectively clear uterine fluid that accumulates during the periovulatory period and/or postbreeding may be associated with pre- or post-conception failure events (Figure 1). Instances of early pregnancy loss have been reported in all managed rhinoceros species following natural breeding.[4-5,21] It has also been observed after several conceptive AI procedures in GOHR[10] and white rhinoceros.[33] Occurrences thereof were revealed during ultrasonography by documenting disappearance of a previously positively identified embryonic vesicle/fetus.[4-5,10,21,33] In 3 cases, examination frequency permitted visualization of IFA before and after embryonic demise.[5-10] In each circumstance, IFA was detected during the late diestrus period (Figure 5a). A strong association exists between the identification of IFA in late diestrus and impending early embryonic death (EED) in mares.[34]

In mares, breeding induced uterine inflammation is a natural physiologic reaction to semen and generally a short-lived process.[35-36] However, in a subset of individuals the initial inflammation becomes pathologic and results in an inhospitable uterine environment for the developing embryo.[37] Mares become clinically classified with this disorder if unable to clear inflammation by 48 hours postbreeding.[37] Susceptibility increases with mare age, uterine position, cervical tightness or fibrosis and poor perineal conformation.[37] The authors hypothesize that these same factors may also contribute to development of uterine inflammation in GOHR, although further research is needed to elucidate this association. In mares, delays in uterine clearance translate to persistence of inflammation that can either directly or indirectly result in EED. Therefore, the GOHR practitioner needs to pay close attention to individuals identified via ultrasonography as accumulating intrauterine fluid during the periovulatory period. Analyzing urinary PdG profiles before an AI attempt will also help detect luteal phase

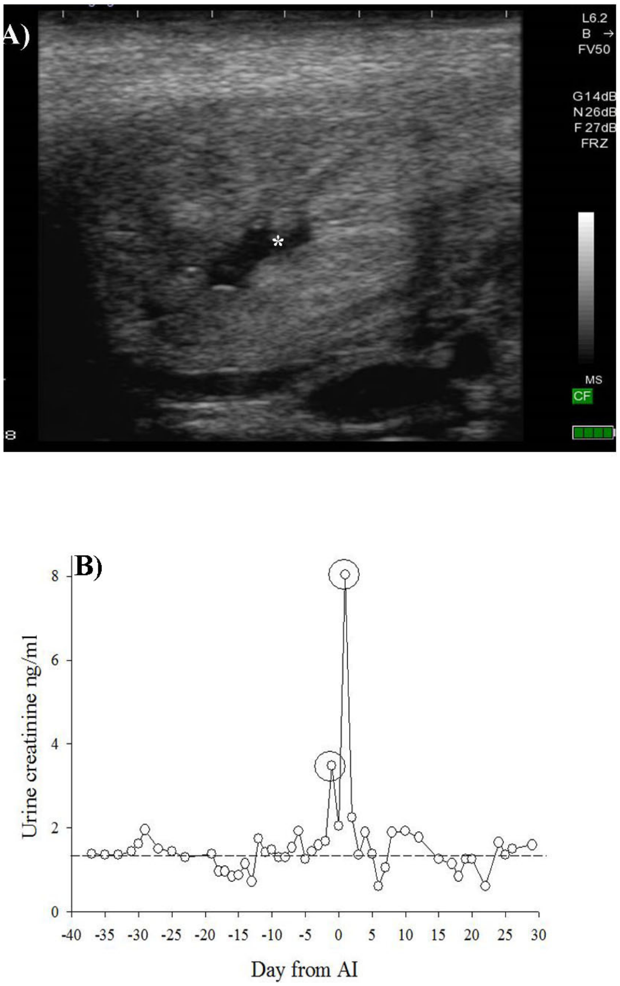

Figure 5. A) Ultrasonogram of intraluminal uterine fluid accumulation (asterisk) during late diestrus in a greater one-horned rhinoceros (GOHR) that experienced early embryonic death (EED) following a conceptive artificial insemination (AI) procedure. B) Urinary creatinine concentrations (ng/ml) before and after oxytocin treatment in a female GOHR. Data are aligned by day from AI. The dashed line reflects mean creatinine concentration before and after oxytocin treatments. Oxytocin (25 IU) was given intramuscularly on days -1 and +1 from AI. Circled data points correspond to creatinine concentrations measured in the first urine collected on the day after each oxytocin treatment.

lengths of shorter than normal duration and therefore indicative of premature luteolysis linked to inflammatory release of $PGF_{2\alpha}$ and reproductive failure (i.e. EED).[38]

Mares inseminated with frozen-thawed semen had an increased incidence of postbreeding intraluminal uterine fluid accumulation and lower pregnancy rates per cycle compared to fresh and chilled semen.[13] Paradoxically, it is standard practice to remove seminal plasma during processing of stallion semen for cryopreservation,[39] but the lack of seminal plasma in extended equine semen has been linked to higher polymorphonuclear neutrophils (PMN) recovered from the uterus postinsemination compared to inseminates that contain this biological fluid.[40-41] In addition, components of seminal plasma have an active role in sperm transport and survival in the reproductive tract of the mare.[42] Given presence of seminal plasma may influence AI success, the authors recommend continued adherence to the practice of maintaining this biological fluid during processing of GOHR ejaculates for cryopreservation.[11]

In addition to maintaining seminal plasma in the inseminate, intervening therapies have helped overcome mechanical aspects of the uterine defense system that fail in mares with clearance issues. Favorable results have been achieved in mares by treating with oxytocin[43-46] that promotes strong, but short-lived myometrial contractions to eliminate intraluminal uterine fluid accumulation via cervical and lymphatic drainage.[43-45] Given oxytocin's short half-life, intramuscular treatment is preferred (supports prolonged systemic circulation) compared to intravenous treatment. We report application of oxytocin treatment to resolve uterine fluid accumulation in a GOHR. Hormone analysis revealed the affected female experienced short luteal phases (< 17 days) during several estrous cycles in which breeding did not occur. A longer than normal luteal phase (23 day) followed her first induced ovulation AI attempt, prompting speculation that EED may have occurred. Ultrasonography was used to confirm occurrence of IFA during the periovulatory period of an unbred cycle. For the second induced ovulation AI attempt (and ultimately conceptive) in this female, intramuscular oxytocin was given (25 IU) a day before and a day after AI. This timeframe correlated to days 3 and 1 before ovulation. In mares, uterine response to oxytocin is greatest on day 2 before ovulation and lowest on day 2 after ovulation.[43,46] In addition to oxytocin treatment, given the potential for frozen-thawed sperm to induce endometritis,[47-49] fresh semen was collected from a proven GOHR bull at another facility, chilled overnight, transported and used for fresh-chilled AI. Many traditional semen extenders, including those used for rhinoceros sperm cryopreservation, contain an animal protein source such as egg yolk or skim milk.[11] Introducing these constituents into a compromised uterine environment has potential to exacerbate an already existing inflammatory process. Modifications were made to the traditional GOHR semen extender that included lowering the percentage of egg yolk down to 2% from the standard 10% volume and adding supplemental antioxidants (ST Genetics, Novasota, TX) to help maintain sperm quality and reduce oxidative stress during overnight transport.

Efficacy of oxytocin treatment was demonstrated by observation of vaginal discharge, ultrasound evidence demonstrating the absence of IFA at AI, presence of an embryonic vesicle after AI, continued absence of IFA, and measured spikes in urinary creatinine (Figure 5b). Oxytocin has antidiuretic properties[50-52] and activity is reflected in urine creatinine concentration. Creatinine is routinely measured in GOHR urine samples to accurately report reproductive hormone results by accounting for variation in hydration status. Significantly higher urine creatinine concentrations were measured the day after each oxytocin treatment in GOHR (Figure 5b). Specifically, creatinine increased 3 and 6 times over baseline (1.36 ± 0.05 ng/ml) following the first and second oxytocin injection, respectively (Figure 5b).

Conclusion

Reproductive disorders of female GOHR decrease the likelihood that a calf is produced via natural and/or assisted breeding. Only a limited number GOHR exist in the North American managed population and the reproductive contribution of each individual is fundamental to maintaining adequate genetic diversity in this population. Unequal GOHR founder representation was the incentive for AI development in this species and its application has expanded the breeding management tools available to help ensure population sustainability. Reproductive dysfunctions identified during formative GOHR AI work limited the conception rate to 50%. By addressing and treating disorders that otherwise result in a failure to conceive or the inability to establish a pregnancy following AI, conception rates have improved to upwards of 66%. Currently, the approach relies on a better understanding of the fundamental species-specific reproductive physiology of the female GOHR and the similarities with that of its closest living domestic relative, the horse. Conducting a BSE that includes a comprehensive reproductive history and the collection of contemporary ultrasonography, endocrine and behavior data permits the GOHR practitioner to assess the probability of a female being able to conceive and carry a calf to term. It enables improved diagnosis of causes of infertility and subfertility and development of an effective treatment plan to optimize AI outcome. Anovulation emerged as the primary reproductive problem throughout original AI trials that led to a large-scale ovulation induction study establishing sustained release deslorelin, Sucromate®, as a highly effective ovulation induction agent in GOHR. Previous successful AI procedures relied on behavioral cues from the female to time sedation/insemination and permitted < 24 hours of advanced notice for staff. Currently, procedures can be timed a week in advance by using urinary hormone analysis to determine initiation of the follicular phase, and ovulation is ensured by treatment with deslorelin. Intraluminal uterine fluid accumulation and/or postbreeding endometritis may be additional disorders that require additional treatments. Ultrasonography of the uterus and urinary hormone dynamics of the luteal phase(s) are key to diagnosing these disorders in GOHR and help delineate whether a pre or postconception failure event has occurred. The first application of oxytocin treatment in a GOHR to resolve intraluminal uterine fluid accumulation was successful and was associated with a healthy full-term pregnancy, when earlier efforts failed. The systematic approach taken to identify and treat reproductive disorders in female GOHR have not only broadened the application of AI to more individuals, many of those of high genetic value for population sustainability, but improved the chance of successful conception following a single insemination attempt.

Conflict of interest

There are no conflicts of interest to declare.

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