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ORIGINAL RESEARCH

The effect of a warmed enema during ovum pick-up on subsequent in vitro oocyte maturation in Southern white rhinoceros (*Ceratotherium simum simum*)

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Background: Rhinoceros are currently one of the most threatened mammal species globally. Slow population growth, increased poaching and habitat destruction have led to increased conservation efforts for each species. Assisted reproductive technologies (ART) have been implemented in an attempt to aid reproductive outputs for the conservation of these endangered species. Developing species-specific ART programmes for wildlife have been challenging. Temperature control during oocyte recovery is essential for ensuring *in vitro* success.

Objective: This study is the first to investigate the effect of enema warming prior to trans-rectal ovum pick-up (OPU) on *in vitro* oocyte maturation in Southern white rhinoceros (*Ceratotherium simum simum*).

Methods: OPUs were performed on 20 rhinoceros cows from three different game farms in South Africa; oocytes were submitted to one of two *in vitro* fertilisation laboratories for culture. The enema fluid was either warmed to 32 °C or not warmed prior to the OPU. Location of the farm, the different laboratories, ambient temperature, season, aspiration probe temperature, media type and enema temperature were investigated as predictor variables for oocyte maturation success.

Results: After considering all other potential covariates, warming of the enema fluid was the only independent predictor of *in vitro* oocyte maturation success during this study.

Conclusion: Oocytes retrieved from rhinoceros cows that received an enema warmed to 31 °C were 2.3 times more likely to mature *in vitro* compared to oocytes from cows that received an unwarmed enema; the findings can be implemented in other rhinoceros ART programmes and in conservation efforts of other endangered mammalian species.

Keywords: aspiration, assisted reproductive technology, enema, ovum pick-up, reproduction, rhinoceros

Introduction

All five extant rhinoceros species are currently threatened according to the International Union for Conservation of Nature's Red List of Threatened Species (IUCN 2023). Poaching for their horns and habitat destruction are the primary factors driving the decline in these species (Ververs et al. 2015; International Rhino Foundation 2023). Three of the five species, the black rhinoceros (*Diceros bicornis*), Javan rhinoceros (*Rhinoceros sondaicus*) and Sumatran rhinoceros (*Dicerorhinus sumatrensis*) are all classified as critically endangered (IUCN 2023). The greater one-horned rhinoceros (*R. unicornis*) is considered vulnerable while the white rhinoceros (*Ceratotherium simum*) is classified as near threatened (IUCN 2023). The Northern white rhinoceros subspecies (*C. s. cottoni*) is considered functionally extinct with only two living females remaining (Emslie 2020; International Rhino Foundation 2023). The fast decline and slow increase in population numbers have highlighted the need to investigate alternative methods to natural reproduction in order to save these iconic species from extinction.

Male white rhinoceros become sexually mature between seven and nine years of age with females reaching the same developmental stage between four to six years of age (Owen-Smith 1973; Ververs et al. 2015). White rhinoceros are non-seasonal breeders with calves born throughout the year (Roth 2006; van der Groot et al. 2015). However, birth-peaks have been reported in captive populations with the majority of calves born between November and April in the southern hemisphere (Ververs et al. 2017). Although breeding can occur naturally, several reproductive pathologies (e.g. anoestrus, "silent oestrus") and disease susceptibility have led to a slow population growth across southern Africa (Pennington & Durrant 2018).

Assisted reproductive technology (ART) has been implemented across different genera in an attempt to overcome fertility issues (Hansen 2020). Applying ART to wildlife offers new possibilities for improved breeding programmes in endangered species (Herrick 2019; Pope 2019). Artificial insemination (AI) has successfully produced rhinoceros calves in several captive populations (Hildebrandt et al. 2007; Hermes et al. 2009a; Pennington et al. 2020). During AI, semen is deposited through the cervix and the sperm fertilises an *in vivo* ovulated oocyte (Stoops et al. 2016). This AI process becomes challenging when wild rhinoceros are not habituated to human presence and is also limited by semen quality and "silent oestrus" in captive populations (Stoops et al. 2016).

The implementation and development of processes like ovum pick-up (OPU) and other *in vitro* techniques (i.e. intracytoplasmic

sperm injection) have proved challenging in rhinoceros (Hermes et al. 2009b; Hildebrandt et al. 2023). Due to the anatomy of the reproductive system, OPUs are done transrectally rather than transvaginally on chemically immobilised cows (Ververs et al. 2015; Meuffels-Barkas et al. 2023). Given the logistical challenges associated with performing regular ultrasound scans on wild rhinoceros to monitor ovarian activity, determining follicular development and oocyte developmental stage becomes more complex. However, fresh OPUs still result in better quality oocytes compared to oocytes collected post-mortem (Stoops et al. 2011a, 2011b).

Oocytes are particularly sensitive to changes in temperature with both heat and cold stress affecting their function and development (Hansen 2009; Latham 2016). The result of any temperature fluctuations experienced by mature oocytes include, but are not limited to, meiotic spindle disruption, abnormal spindle formation and altered zona pellucida or nuclei (Parks & Ruffing 1992; Takahashi 2012; Wang et al. 2001). A drop in temperature for even two minutes can result in irreversible depolymerisation of the meiotic spindles inside the oocyte (Foss et al. 2013). Although temperature fluctuations can occur during the OPU, temperature control must remain a high priority to limit any developmental anomalies during the ART process (Wang et al. 2001).

The aim of this study was to investigate the effect of enema warming prior to OPU, on the maturation rate of oocytes recovered from Southern white rhinoceros (*C. s. simum*). This was done by comparing oocyte maturation between cows receiving a warmed versus an unwarmed enema. It is expected that oocytes retrieved during OPU following a warmed enema will mature at a higher rate compared to oocytes retrieved following an unwarmed enema. This study is the first to report on the effect of enema warming prior to OPU and its effect on oocyte maturation in Southern white rhinoceros. The conclusions from this research can be implemented in other ART protocols to maximise the potential *in vitro* success not only for rhinoceros but also other endangered wildlife species.

Materials and methods

Study site, species, and management of rhinoceros

Rhinoceros OPUs (n = 20) were conducted at three privately owned game farms in South Africa between February 2021 and November 2021. The exact location of the farms is not disclosed due to potential security risks. Farming systems ranged from an intensive system with animals housed in bomas and fed lucerne (*Medicago sativa*; Site 1) to completely free range where the animals grazed on natural veld (Site 3). Site 2 was a semi-intensive farming system where animals were provided with shelter and access to natural veld. For the purpose of this research, a primary breeding season was defined from November to April with a secondary breeding season ranging from May to October (Ververs et al. 2017).

Anaesthetic protocol

Cows were visually evaluated to determine general health and family status (if the cow had a calf and the age of the calf if applicable) to assess their suitability for the trial. Only healthy individuals were selected for the OPU procedure. Anaesthesia was induced through an intramuscular dart (Motsumi® 1.5 ml with 1.5″ needle) shot with a Pneudart® .22 blank 397 dart gun. Animals were immobilised using a combination of an opioid etorphine (9.8 mg/ml; Novartis, South Africa) and azaperone (100 mg/ml; VTech, South Africa) at a flow rate of 1.5–5 mg etorphine and 30–60 mg azaperone in the dart. A blindfold and ear muffs were used to lower external stimuli. Immobilisation was achieved within 4–15 minutes dependent on topography of the area, temperament, weight and dose given. Anaesthesia was maintained at acceptable levels for OPU procedures through a combination of ketamine (100 mg/ml; VTech, South Africa), medetomidine (20 mg/ml; VTech, South Africa) and butorphanol (50 mg/ml; Kyron, South Africa). Anaesthesia reversal was achieved through intravenous administration of antipamezole (5:1 dosage rate of medetomidine used) and naltrexone (20 mg/mg of opioid used). A portable aluminium structure (3 x 3 m) covered with shade canvas was erected to cover the hind quarters of the cow and aspiration lines. Ambient temperature was recorded using an iButton (ColdChain ThermoDynamics, South Africa) covered by a small sealable plastic bag attached to the aluminium structure (2 m above ground level). Anaesthesia administration and reversal times were recorded for each OPU.

Oocyte aspiration and maturation

The anaesthetised cows were placed in sternal recumbency before the rectum was cleaned and a trans-rectal reproductive ultrasound examination was performed. If a rhinoceros was found to be pregnant, the anaesthesia was reversed and the cow was released; cows suitable for OPU were placed in left lateral recumbency in preparation for the OPU procedure. Faecal matter was manually removed from the rectum before flushing with 25 L buffered double distilled salt solution (205.00 g sodium chloride, 18.10 g disodium phosphate and 5.25 g potassium dihydrogen phosphate; Modulab Technologies, USA). Enemas were either warmed (32.0 ± 3.79 °C; n = 7) before being used as flush for the rectum or used unwarmed (23.33 ± 2.08 °C; n = 13).

Immature oocytes were aspirated directly from the ovary using the aspiration probe (AP; 1.45 m x 38.50 cm). The AP was constructed from polyvinyl chloride plastic (PVC) using a computer numerical control machine. The AP consisted of three double lumen 17G needles (Marcus Medical, South Africa) and an ultrasound transducer (Shenzhen Mindray Bio-Medical electronics, China). The AP was guided through the rectum until the left ovary was visible. Each follicle was flushed three times using fresh ABT flushing media (31350 IU/2L Heparin; Sigma-Aldrich, USA) using a 20 ml syringe (Swavet, South Africa) attached to the AP. The flushing media was heated to and maintained at 37 °C using a water bath and a plastic container with heating pads (Shenzhen Anpan Health Industry, China) and heating blanket (Panamedic, South Africa). The media was then collected from the follicle into a sterile 250 ml collection bottle (Lasec, South Africa) using a Craft suction unit pump (Imperial Way Watford, United Kingdom).

The cow remained in left lateral recumbency while the procedure was repeated for the right ovary. Ovum pick-up starting and

completion times were recorded to determine the duration of the procedure.

The collected flushing fluid was filtered (Embryo Filter, Spitronics Micro Ignitions, South Africa) before identifying the oocytes under 20x magnification (Olympus SZX7, South Africa) at 38 °C. Oocytes were placed in 2 ml sterile cryogenic vials (Nalgene®, Merck, Germany) containing either holding media ($n = 4$; Hinrichs 2010) or maturation media ($n = 15$; Choi et al. 2006). Oocytes in holding media were transported to the *in vitro* fertilisation laboratory of the Veterinary Faculty of the University of Pretoria (OP). Oocytes placed in maturation media were transported to EmbryoPlus (EP), a privately owned veterinary IVF laboratory in Brits, North West Province, South Africa. Transportation time was dependent on the distance to the laboratory but ranged between 30 minutes and seven hours (256.0 ± 132.60 minutes) at 34.07 ± 5.64 °C.

Upon arrival at the respective laboratories, oocytes were placed in maturation media in 4-well culture dishes (AEC Amersham, South Africa) and covered with 400 µl mineral oil (Harrilabs, South Africa). At the OP laboratory oocytes were placed in CO_2 incubators (Thermo Forma Steri-cycle; Labotec, South Africa) and cultured at 38.5 °C in 5.6% CO_2, 20% O_2 and balanced N_2 atmosphere. At the EP laboratory oocytes were placed in benchtop incubators (Minc I, Marcus Medical, South Africa) and cultured at 37.5 °C in 7% CO_2, 7% O_2 and 86% N_2 atmosphere. At both laboratories, oocytes were matured for 40–44 hours. Cumulus cells were mechanically removed before morphologically classifying oocytes as mature (MII) based on presence of a polar body.

Data analysis

Temperature data were downloaded using the ColdChain ThermoDynamics program (version 4.9.2013.12.06.100) and exported to Microsoft Excel® spreadsheet. All temperature data points before the start and after the end time of each procedure were removed from the downloaded iButton data in Excel. A shapiro-Wilk normality test was used to investigate the distribution of the data. Univariate models were fit to evaluate each variable individually. Mixed effects multivariable models were fitted using location (Site 1; Site 2; Site 3), enema temperature (warmed vs. unwarmed), ambient temperature (°C), AP temperature (°C), OPU duration (minutes), season (primary vs. secondary breeding season), transportation media (holding vs. maturation media) and laboratory (EP vs. OP) as predictor variables. Animal identification was included as random effect. The predictor variable with the largest Wald *p*-value was stepwise removed and the analysis rerun until all remaining variables had a Wald $p < 0.1$. To investigate confounding factors, all variables were inserted back into the final model one by one; if the risk ratio (RR) of other remaining variables changed with more than 15% the factors were considered confounding and kept in the model. The effect size was estimated using the RR and corresponding 95% confidence interval (CI). Where collinearity existed between two variables (as in the case of laboratory and transport media), only one of the two covariates was included in the model.

All statistical analyses were done using the statistical program STATA® (Version 14; California, USA). Data is presented as mean ± standard deviation.

Results

There were 78 oocytes harvested in total, resulting in approximately four oocytes recovered per OPU (3.90 ± 4.94; Table I). Three cows were excluded in total from the analysis due to technical issues ($n = 1$); pregnancy ($n = 1$) and no follicular development ($n = 1$). All linear variables were distributed normally.

During the univariate analysis, enema temperature was the only predictor that had a significant effect on oocyte maturation rate (Table II). Site 3 was omitted from the analysis due to no oocytes retrieved from this site reaching the MII stage.

Using multivariate modelling, enema temperature was, again, the only significant predictor of oocyte maturation with more oocytes reaching the MII stage when a warm enema was used prior to OPU (Table III).

No significant animal welfare incidents were recorded during this study.

Table I: A summary of the general field conditions and ovum pick-ups performed per study site between February 2021 and November 2021

	Site 1	Site 2	Site 3
Ovum pick-up (OPU) performed*	8	10	2
Warmed enemas used*	3	4	0
Follicles aspirated per OPU	10.00 ± 4.00	9.00 ± 8.00	7.00 ± 0.00
Oocytes harvested per OPU	4.75 ± 4.83	3.80 ± 5.44	1.00 ± 0.00
Aspiration rate (%) per follicle aspirated	45.42 ± 25.75	29.11 ± 27.99	14.29 ± 0.00
Maturation rate (%) per oocyte cultured	58.91 ± 36.01	38.78 ± 36.99	–
Ambient temperature (°C) per OPU	32.41 ± 7.45	25.38 ± 7.19	26.45 ± 0.73
Aspiration probe temperature (°C) per OPU	35.70 ± 2.64	33.51 ± 2.11	36.02 ± 0.05
Aspiration duration (minutes) per OPU	61.13 ± 39.56	64.60 ± 52.66	41.50 ± 9.19

Data is presented as mean ± SD. *denotes the absolute number of OPUs performed and number of warmed enemas used prior to OPU.

Table II: The univariate associations between potential predictors of oocyte maturation success for white rhinoceros oocyte pick-ups from South Africa

Variable	Level	Coefficient (°C; 95% CI)	Risk ratio (95% CI)	*p*-value
Location	Site 1 Site 2* Site 3	-0.45 (-1.34, 0.44) – Omitted	0.64 (0.26, 1.55) – Omitted	0.32 –
Enema	Warmed Unwarmed*	0.81 (-0.10, 1.73) –	2.26 (0.91, 5.63) –	0.08 –
Ambient temperature (°C)	–	-0.02 (-0.08, 0.03)	0.98 (0.92, 1.03)	0.40
Aspiration probe temperature (°C)	–	-0.04 (-0.30, 0.21)	0.96 (0.74, 1.23)	0.73
Aspiration duration (minutes)	–	-0.00 (-0.01, 0.01)	1.00 (0.99, 1.01)	0.70
Season	Primary breeding Secondary breeding*	0.06 (-0.84, 0.97) –	1.07 (0.43, 2.63) –	0.89 –
Media type	Holding media Maturation media*	0.92 (-0.82, 2.69) –	2.55 (0.44, 14.76) –	0.30 –
Lab	EP OP*	-0.58 (-1.98, 0.82) –	0.56 (0.14, 2.27) –	0.42 –

CI = Confidence interval; *Reference variable

Table III: The multivariate associations between potential predictors of oocyte maturation success during oocyte pick-ups in white rhinoceros cows from South Africa

Variable	Level	Risk ratio (95% CI)	*p*-value
Enema temperature	Warmed Unwarmed*	2.26 (0.91, 5.63) –	0.08 –

CI = Confidence interval; *Reference variable

Discussion

Twenty oocyte pick-ups were performed on white rhinoceros cows over a 10-month period at three different locations across South Africa. Seventy-eight oocytes were aspirated in total and the potential factors affecting oocyte maturation were investigated. Enema warming was the only factor affecting oocyte maturation with oocyte maturation higher in cows receiving a warmed enema prior to the onset of the OPU compared to cows where the enema was unwarmed.

On average, four oocytes were recovered during each OPU procedure for this study. Although the number of oocytes harvested is relatively low, the numbers are consistent with other reports where OPU procedures in white and black rhinoceros resulted between two and nine oocytes retrieved per procedure (Hermes et al. 2009b). Similarly, Hildebrandt et al. 2018, reported an average of six oocytes retrieved per OPU in white rhinoceros resulting in a retrieval rate of 34% (Hildebrandt et al. 2023). Rhinoceros anatomy presents specific challenges during OPU; these can include locating the ovaries using ultrasound and distance to the ovaries (Ververs et al. 2015; Meuffels-Barkas et al. 2023). Specific modifications have been made to the AP used during OPU (Hildebrandt et al. 2018) which can increase the successful retrieval of oocytes; however, the low recovery rate still leaves room for improvement. The specific skills and experience of the person performing the OPU is another aspect that needs to be considered. The more experienced a person is, the higher the oocyte retrieval rate could be. These factors highlight the importance of using the appropriate tools and the experience of the team performing the OPUs.

Heating the enema used to flush the rectum before starting with the OPU increased the chances of oocytes maturing more than two-fold. Oocytes retrieved from rhinoceros cows that received the warmed enema probably experienced less temperature fluctuations than the oocytes from cows receiving a cold enema. This is likely due to the warmed solution increasing the warming rate of the AP by not decreasing rectal temperatures. The PVC material used to construct the AP is a poor heat conductor and oocytes retrieved from cows that received a cold enema probably experienced greater temperature fluctuations that impaired the maturation outcome. No other reports have been made on the importance of enema temperature during rhinoceros OPU although luke-warm water has been used to clean the rectum prior to the procedure (Hildebrandt et al. 2023). Due to different teams and techniques it is possible that other groups do not use an enema before an OPU procedure; however, these results highlight the importance of warming when it is employed. During field conditions when warming of enemas is not possible, oocyte processing and transport should be conducted at lower temperatures to limit temperature shock. Reducing temperature fluctuations during oocyte retrieval and transport is crucial in successful IVF programmes (Parks & Ruffing 1992; Zubor et al. 2020) where even a slight drop in temperature could result in detrimental *in vitro* outcomes (Takahashi 2012; Zubor et al. 2020).

Although this study highlights an important aspect of rhinoceros OPU, there are limitations that need to be addressed during future work. The conclusions are based on a relatively small sample size; however, the logistics surrounding rhinoceros

anaesthesia and OPU needs to be taken into consideration and is a general limiting factor during wildlife research. The AP is constructed from PVC, which is generally believed not to be a good heat conductor. The impact from the AP was not directly investigated during this study and further research is needed to limit the potential impact of the temperature characteristics of the AP on oocyte maturation and development. Using material with better heat conducting properties such as metal could potentially decrease the temperature fluctuations during OPU, and improve oocyte maturation rate.

Developing successful species-specific *in vitro* fertilisation protocols for rhinoceros has proved challenging. To the best of the author's knowledge, this is the first study investigating a specific aspect of the OPU procedure in attempts to increase oocyte maturation and embryo development in Southern white rhinoceros. These findings represent one of the puzzle pieces that can improve the overall aspiration process and be implemented in other OPU protocols and IVF in general. Enhancements and improvements to the current ART protocol will ultimately aid the conservation of one of the world's most threatened, keystone species.

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Conflict of interest statement

The authors declare they have no conflicts of interest that are directly or indirectly related to the research.

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Compliance with ethical guidelines

A Section 20 permit was obtained for this study from the Department of Nature Conservation in South Africa with ethical approval from the animal ethics committee (REC053-19) of the Faculty of Veterinary Science, University of Pretoria. An addendum application (REC176-21) to the original agreement was approved by both the animal ethics committee and animal research committee of the Faculty of Veterinary Science, University of Pretoria.

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