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ORIGINAL RESEARCH

Thermal properties of rhinoceros ovum pick-up probes: Possible effects of temperature shock on oocyte maturation potential

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Background: The implementation of assisted reproductive techniques and use of *in vitro* embryo production has highlighted their potential in attempts to prevent the extinction of the northern white rhinoceros (*Ceratotherium simum cottoni*). However, *in vitro* embryo production has proved challenging. Transrectal ovum pick-up (OPU), while done successfully with custom-made aspiration probes (AP), may expose oocytes to detrimental temperature shock.

Objective: This study compares the temperature regulation properties between two AP, made from different materials and used during OPU in southern white rhinoceros (*C. s. simum*).

Methods: The initial PVC plastic AP, produced using a computer numerical control machine, was compared to a modified aluminum AP. A rhinoceros model was constructed using a 210 L water container fitted with heating elements and a water pump. Water was warmed to 37.5–38.5 °C to simulate rhinoceros body temperature. The AP was equilibrated to room temperature (22 °C; simulating summer) or in a walk-in fridge (4 °C; simulating winter) overnight. Temperature loggers were placed inside and on the outside of the AP.

Results: The aluminium AP equilibrated to the model water temperature within 15 minutes, of submersion in the model, while the temperature of the PVC AP remained significantly lower during the three-hour period. The inside of the PVC AP remained significantly colder compared to outside during both winter and summer (33.23 ± 4.28 °C vs. 36.51 ± 2.13 °C and 36.04 ± 1.69 °C vs. 37.22 ± 0.92 °C, respectively). There was no significant difference between the inside temperature of the aluminium AP compared to the outside temperature during both the winter and summer simulations (36.75 ± 0.49 °C vs. 37.83 ± 0.40 °C and 37.86 ± 0.16 °C vs. 38.04 ± 0.11 °C, respectively).

Conclusion: This research highlights the importance of using material with good thermal properties during assisted reproductive procedures. It also contributes to further advancement during the establishment of a successful *in vitro* embryo production protocol for rhinoceros in South Africa.

Keywords: aspiration probe, *in vitro*, oocytes, Rhinocerotidae, white rhinoceros, temperature

Introduction

The Rhinocerotidae are considered one of the most threatened large mammal families world wide (IUCN 2023). The northern white rhinoceros (*Ceratotherium simum cottoni*) is functionally extinct as only two females remain, both in captivity (Emslie 2020; International Rhino Foundation 2023). The southern white rhinoceros (*C. s. simum*) is the most abundant of the rhinoceros with the greater one-horned rhinoceros (*Rhinoceros unicornis*) considered vulnerable and the black (*Diceros bicornis*), Javan (*R. sondaicus*) and Sumatran rhinoceros (*Dicerorhinus sumatrensis*) classified as critically endangered (International Rhino Foundation 2023; IUCN 2023). The current state of the northern white rhinoceros along with the rest of the family led to the investigation of alternative methods of reproduction in attempts to save these keystone species from extinction (Hildebrandt et al. 2018; Hildebrandt et al. 2023).

Assisted reproductive technology (ART) and the *in vitro* production of embryos are well documented in both domestic and wild mammals (Herrick 2019; Sjunnesson 2020). These techniques have been studied and modified to produce species-specific protocols for implementation in rhinoceros conservation; however this has proved challenging, with limited success (Hermes et al. 2009; Stoops et al. 2011a,b; Hildebrandt et al. 2018). The anatomy of the female reproductive tract required adaptation of the commonly used transvaginal oocyte aspiration to a transrectal oocyte pick-up (OPU) due to the cranial position of the ovaries (Ververs et al. 2015; Meuffels-Barkas et al. 2023). The change in protocol also required modification of the aspiration probe (AP) used during the OPU (Hermes et al. 2009; Hildebrandt et al. 2023). These modifications have been primarily focussed on the dynamic structure of the AP with limited information on the temperature regulation properties of the AP material.

Temperature is one of the many factors known to impact on the successful outcome of ART procedures. Temperature fluctuations during OPU can affect the developmental potential of oocytes (Wang et al. 2001; Foss et al. 2013). Therefore, it is important to maintain a constant temperature when follicular fluid is aspirated from the ovarian follicle. Preventing a decrease in rectal temperature through the administration of a warmed enema prior to OPU in southern white rhinoceros resulted in

higher *in vitro* oocyte maturation compared to rhinoceros that received an unwarmed enema (Vorster et al. 2024). Oocytes may experience a decrease in temperature of up to 7 °C as the follicular fluid travels through the aspiration system into the collection tube (Redding et al. 2006). This drop in temperature affects oxygen tension (pO2) in the follicular fluid and also results in a higher *pH* which negatively impacts oocyte health (Redding et al. 2006).

The overall aim of this study was to investigate the temperature regulation properties of the PVC AP used during rhinoceros OPU in South Africa. We predict that the temperature inside the AP (the temperature oocytes will be exposed to) and the outside temperature of the AP (the rhinoceros rectal temperature) will be similar and not differ significantly following a three-hour equilibration period. The findings from this study were used to investigate the thermal properties of a newly designed AP used during southern white rhinoceros OPU in South Africa. The final conclusions are useful for: 1) modifying future APs to improve *in vitro* success for all rhinoceros species and 2) designing APs for OPU in other species.

Materials and methods

Study design

Rhinoceros simulation (RS)

A plastic container (210 L, 560 m x 975 mm; Jojo Tanks, South Africa) was filled with tap water and heated using portable water heating elements (2000W, Krazeprice, South Africa) inserted through the top of the container. The heating elements increased the water temperature to 37.5 °C to 38.5 °C, simulating the core body temperature of a rhinoceros (Citino & Bush 2007). A digital thermostat (STC 1000, 2KW, RA Smart Technologies, South Africa) ensured that the appropriate temperature was reached and maintained. Three temperature loggers (iButtons, ColdChain ThermoDynamics, South Africa) were placed inside the container: just below the water's surface (high), approximately 48 cm from the top (middle) and at the bottom (low). A water pump (Dragonfly 4P0006, 28W 2 m high pump; Chamberlains, South Africa) was positioned at the bottom of the container to evenly circulate the water throughout.

Polyvinyl chloride aspiration probe (PAP)

The AP used during rhinoceros OPU was made of PVC (143 x 11 x 10 cm, 11.3 kg) using a computer numerical control machine (Figure 1a). To measure the temperature of the PAP, two temperature loggers were placed on the inside of the instrument (next to the aspiration line; Figure 2a) with another two placed on the outside (Figure 2b). The rhinoceros rectal wall was simulated by covering the PAP with an elastic stocking embedded in silicone. To simulate seasonal differences, the PAP was left at room temperature (22 °C; summer simulation) or in a 4 °C walk-in fridge (winter simulation) overnight, before being submerged in the RS for three hours. This process was repeated three times during both seasonal simulations. Ambient temperature was measured by placing temperature loggers within 2 m from the plastic RS.

Figure 1: The aspiration probes used for rhinoceros oocyte pick-up in South Africa made from (A) polyvinyl chloride (14 3x 11 x 10 cm, 11.3 kg) and (B) Aluminium (144 x 9,5 x 7.5 cm, 10.8 kg).

Aluminium aspiration probe (AAP)

Following the outcome of the initial experiment, a modified AP was designed and constructed of aluminum (Figure 1b; 144 x 9,5 x 7.5 cm, 10.8 kg), then submerged in the RS for similar data collection as described above. Temperature measurements were recorded once every minute to an accuracy of 0.06 °C and stored on the iButtons for later download.

Statistical analyses

Temperature data were downloaded from each iButton using the ColdChain ThermoDynamics programme (version 4.9.2013.12.06.100) before being exported into a Microsoft Excel® spreadsheet. Datapoints before each AP was submerged into, and after it was removed from, the water tank were excluded from the analyses. All temperature measurements were averaged for each simulation (winter, summer, inside the

Figure 2: The iButtons were placed inside the PAP next to the aspiration line (A), and on the outside (B) of the PAP in order to record the temperature the oocytes will be exposed to and the rhinoceros' rectal temperature, respectively.

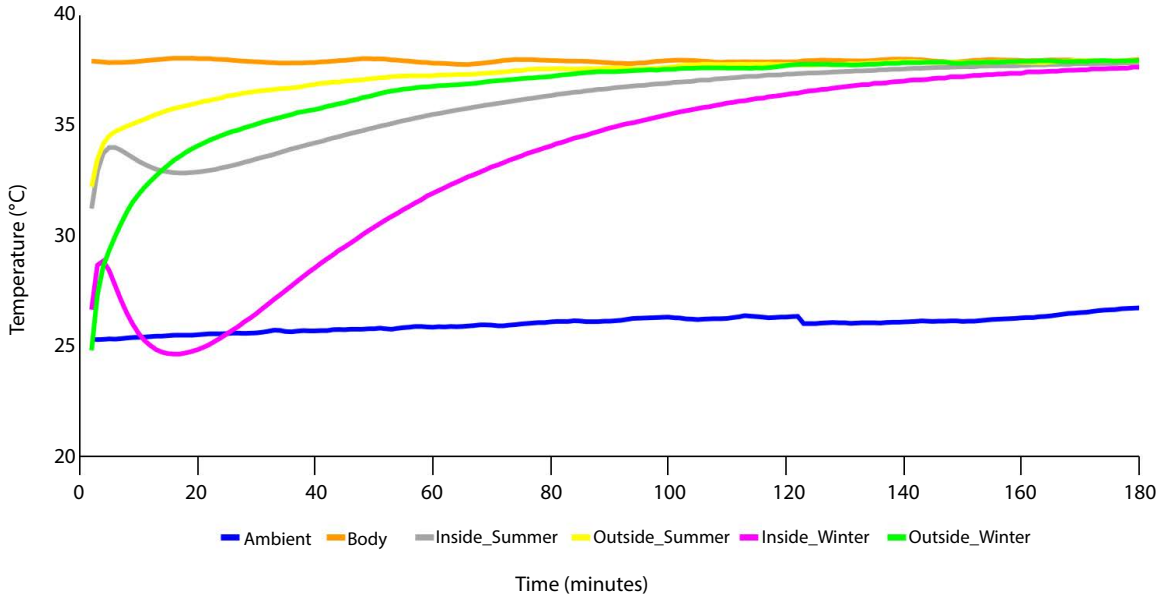

Figure 3: A graphic representation of the temperature associated with the PVC aspiration probe (PAP) in relation to ambient temperature and rhinoceros body temperature (RS) over a 180 minute calibration time.

AP and outside the AP) before being separated into one-hour intervals. Temperature data of each of the APs and the water bath were compared using Dunn's post-hoc test in the statistical program PAST (version 4.03). Because the AAP experiment was done after the initial experiment on the PAP, the temperature data of the two APs were compared to the RS and not to each other. However, the time taken for each AP to reach equilibrium with the RS temperature (i.e. no statistically significant difference in temperature between the AP and RS) was compared as an indication of the difference in thermic properties of the two APs. Data are presented as mean ± standard deviation (SD).

Results

PAP

Ambient temperature (blue, n = 181 [observations]; 26.04 ± 0.34 °C) and body temperature (RS; orange, n = 181; 37.89 ± 0.06 °C) remained stable throughout the experiment (Figure 3). During the summer simulation, the temperature inside the PAP (grey, n = 181; 36.04 ± 1.69 °C) was significantly lower than the outside PAP temperature (yellow, n = 181; 37.22 ± 0.92 °C; $p < 0.05$). Both the inside and outside temperatures of the PAP were significantly lower than the RS temperature during the summer simulation ($p < 0.05$; Figure 3). Neither inside nor outside

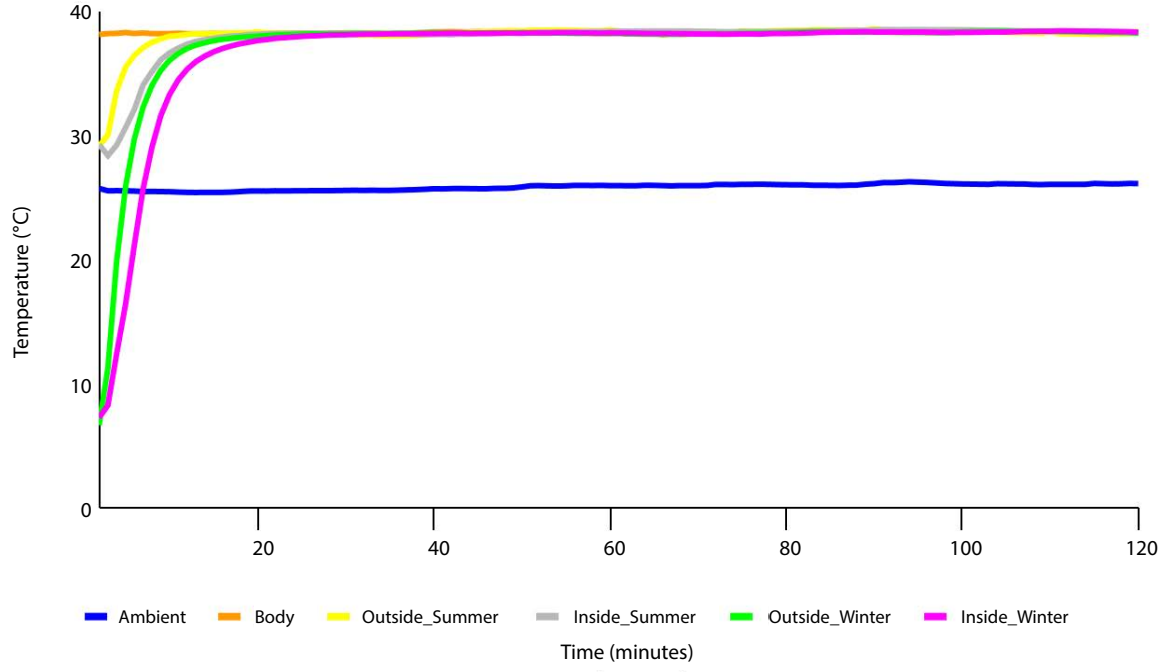

Figure 4: A graphic representation of the temperature associated with the aluminium aspiration probe (AAP) in relation to ambient temperature and rhinoceros body temperature (RS) over a 120 minute calibration time.

the PAP equilibrated to the rhinoceros body temperature during the three-hour recording period in summer (Figure 3).

During the winter simulation, the inside temperature of the PAP (purple, $n = 181$; 33.23 ± 4.28 °C) was significantly lower than the outside temperature (green, $n = 181$; 36.51 ± 2.13 °C; $p < 0.05$). The temperature inside and outside of the PAP was significantly lower compared to the RS temperature during the winter simulation ($p < 0.05$). During the winter simulations, neither the inside nor outside temperature of the AP reached the rhinoceros body temperature during the three-hour period (Figure 3).

AAP

Ambient temperature (blue; $n = 120$; 25.85 ± 0.02 °C) and water temperature inside the RS (orange; $n = 120$; 38.26 ± 0.01 °C) remained stable throughout the experiment (Figure 4). During the summer simulation, there was no significant difference between the inside (grey; $n = 120$; 37.86 ± 0.16 °C) and outside temperature of the AAP (yellow; $n = 120$; 38.04 ± 0.11 °C; $p = 0.80$), respectively. During the same simulation (summer), there was also no significant difference between the temperature inside the AAP ($p = 0.60$) and temperature outside the AAP ($p = 0.79$) compared to the RS temperature (Figure 4) respectively. The temperature of the outside and inside of the AAP equilibrated to the RS temperature after approximately 6 and 10 minutes, respectively (Figure 4).

During the winter simulation, there was no significant difference between the inside temperature of the AAP (purple; $n = 120$; 36.75 ± 0.49 °C) and the outside temperature of the AAP (green; $n = 120$; 37.83 ± 0.40 °C; $p = 0.65$). The inside ($p < 0.05$) and outside temperatures ($p < 0.05$) of the AAP were significantly lower than the RS temperature; however, the AAP equilibrated to the rhinoceros body temperature after approximately 12 and 15 minutes, respectively (Figure 4).

Discussion

The material used to manufacture the AP had a significant effect on temperature regulation during the two simulations. The inner and outer temperatures of the PAP and the RS temperature were significantly different from each other. The temperature of the PAP did not stabilise after a three-hour equilibration period and approached, but did not reach the RS temperature. Inner and outer temperatures of the AAP did not differ from each other, and the temperatures stabilised after approximately 15 minutes of equilibration time.

Because the PAP failed to reach the simulated rhinoceros body temperature, even after the extended equilibration time, oocytes would have been exposed to an approximately 5 °C lower temperature than rhinoceros body temperature. This implies that oocytes would experience temperature shock which potentially would have a detrimental impact on *in vitro* maturation and fertilisation success rates. In order to stabilise the rectal temperature and increase the temperature of the AP, a warmed enema can be used prior to OPU to prevent a temperature drop as a result of an unwarmed enema (Vorster et al. 2024). Even though the effect of the temperature drop was not directly investigated, the effect has been reported in cattle where the change in temperature had a cascading effect on dissolved oxygen (pO_2) and *pH* (Redding et al. 2006).

An extended period for temperature equilibration is also not plausible during rhinoceros OPU in the field. In order to limit any potential animal welfare issues, anaesthesia needs to be administered and reversed as quickly as possible with the procedure performed within the shortest possible time (de Lange et al. 2017; Meuffels et al. 2022). The modified AAP was significantly better at reaching the simulated rhinoceros body temperature with limited difference in temperature after approximately 15 minutes. This means the time required to prepare the rhinoceros cow for OPU can easily be utilised to heat the AAP using a device specifically designated for this purpose. During summer months, inserting and positioning the AP, visualising the ovaries with ultrasound and counting follicles could allow enough time for the AP to equilibrate to rhinoceros body temperature before the start of the OPU; eliminating the need to heat the AP. The AAP makes this a feasible option while the PAP poses significant challenges as it might be impractical to heat the equipment for more than three hours prior to the OPU.

The silicone stocking might have acted as an insulator preventing the AP from reaching the appropriate temperature during this simulation, which may have exaggerated the time needed for the AP temperature to equilibrate in this experiment. On the other hand, during the OPU the presence of air in the rectum and restriction of blood supply to the perirectal space are factors that could potentially increase equilibration time in the field.

The findings of this study are based on a rhinoceros model under controlled conditions. The next step should be to investigate temperature characteristics of the PAP and AAP during an actual rhinoceros OPU. Based on our findings, we estimate that oocytes will experience significantly less detrimental temperature fluctuations when using the AAP compared to the PAP under field conditions, in particular if the AP is heated for at least 15 minutes prior to the procedure. It is important to consider how the AP connects to the system and the overall impact on ART success. Future research should consider all of these aspects including the flushing media used during rhinoceros OPU. The flushing media should be maintained at an appropriate temperature to avoid oocyte heat or cold shock. Methods must be developed and refined to regulate the temperature of the flushing media and the collection bottle in which oocytes are collected before being moved to the laboratory. This is of particular importance during rhinoceros OPU procedures due to the length of the AP necessitating a longer time for oocytes to pass through it. Rhinoceros OPUs, even if performed in a zoo-setting, are also subject to the external environmental conditions. This is in contrast to the controlled circumstances possible for humans and some domestic species. Even though evaluation of the individual stages of the OPU process is valuable to refine and improve, it is important to remember the entire system should function in unison in order to have a successful outcome.

Our initial predictions about the temperature of the PAP were incorrect; the findings from this study were used to modify and improve the AP that has since been used for rhinoceros OPUs in South Africa. When using a PAP, the probe should be preheated

to 37.5–38.5 °C prior to the start of the OPU. This may prove challenging under field conditions as our results indicate a required three-hours of heating prior to OPU. When using an AAP, a short pre-warming period would be beneficial; however, the AP would likely equilibrate to the rectal temperature in the time frame needed before the first aspiration. Alternatively, placing the AP into the rectum following a warmed enema could be sufficient to allow the AP to reach the appropriate temperature to prevent the oocytes from experiencing excessive temperature shock (Vorster et al. 2024). This needs to be verified under field conditions.

Our findings and recommendations may not only improve the OPU procedure and *in vitro* success of southern white rhinoceros ART, but additionally, may be incorporated into OPU in different rhinoceros and other species where OPU procedures are not yet well defined and improvements are needed.

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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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