What do women and rhinos have in common?

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The concept that human reproductive physiology is unique, and little can be learned or benefits gained by studies in other model system or exotic species is woefully out of date. The cross-fertilization that can be gleaned by furthering our knowledge about the basic biology and molecular mechanisms that regulate everyday physiological processes in other species is vast. In this review, I share how studying rhinoceros ovarian physiology, exploring novel culture techniques, and overcoming unexpected challenges that are presented by working with such unique samples has benefitted my program of research that focuses on developing fertility preservation technologies for women — and rhinoceros. (F S Rep® 2025;6:19–23. ©2025 by American Society for Reproductive Medicine.)

Key Words: Rhino, ovary, culture, human, fertility preservation, rhinoceros, follicle

The title of this article could be perceived controversial by those less familiar with biology, indeed many have raised eyebrows when hearing it. The concept that women and rhino have things in common can be hard to fathom, and may be considered inflammatory at the extreme or are also words that form the beginning of a joke. However, for those with a more physiological background, the use of model systems such as drosophila (fruit files) and mice are well established to interrogate and decipher the fundamentals of biological mechanisms that lead to ground breaking changes in clinical treatment for humans.

Considerable research is conducted into the biological processes of numerous other non-model species with the aim of learning about the wonderful diversity of species that inhabit this planet away from clinical application. On occasion, such explorations into the unknown can lead us to species where fully understanding the molecular processes that drive their everyday biology or reproductive systems could have tremendous impacts on human health and human assisted reproductive technologies (ARTs). For this there are many examples, such as the Australian gastric brooding frog, *Rheobatrachus silus*, which became extinct in the mid-1980s. Understanding how these frogs were able to halt the process of digestion, altering their stomach from an acidic, hostile environment to a supportive "tadpole incubator," could have held the key to developing clinical treatments for gastric diseases in humans (1). Fully understanding the mechanisms by which embryonic diapause occurs, (where blastocysts exist for many months not proliferating in suspended animation, free floating in the female reproductive tract: best described in marsupials (2), but also known in many deer such as roe deer (3)), could reveal crucial mechanisms for utilization in human fertility clinics. In addition, these strategies may be the basis for novel cancer drug developments by halting cell proliferation. Such basic research studies in non-model species other than humans and other non-domestic animals, brings us to the rhinoceros and the Rhino Fertility Project.

ENDANGERED RHINOS

The Rhino Fertility Project was conceived when I learned about the extent of ovarian dysfunction and reproductive disorders in captive rhinos, akin to conditions found in humans (4–6). This, combined with my knowledge about the declining numbers of individuals of each rhino species on our planet — the majority on the brink of extinction (Table 1) — led me to take action. Most critical is the northern white rhinoceros where the population has dwindled and now consists of just two females, both of which are infertile. Monumental efforts have been made in a desperate attempt to save this iconic species from extinction. In 2009, four of the last remaining northern white rhinos were transferred to a single location, the Ol Pejeta Conservancy, in Kenya, but the lack of natural breeding with the few remaining individuals aging year by year meant that the possibility of rescuing this species became ever more challenging. Assisted reproductive technologies (ARTs) were therefore brought into play as an additional tool to prevent extinction

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TABLE 1

Population status of rhino species according to IUCN red list.

Species	Scientific name	IUCN red list status	Numbers remaining (assessment date)	Population trend
Southern White Rhino	*Ceratotherium simum simum*	Near threatened	10,080 (2020)	Decreasing
Northern White Rhino	*Ceratotherium simum cottoni*	Critically endangered	2 (2020)	Decreasing
Black rhino	*Diceros bicornis*	Critically endangered	3,412 (2020)	Increasing
Javan Rhino	*Rhinoceros sondaicus*	Critically endangered	18 (2019)	Stable
Sumatran rhino	*Dicerorhinus sumatrensis*	Critically endangered	30 (2019)	Decreasing
Greater one-horned rhino/Indian rhino	*Rhinoceros unicornis*	Vulnerable	2,100–2,200 (2018)	Increasing

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(7). In 2018, with the demise of the last male, Sudan, the northern white rhino is now listed as functionally extinct. However, because a number of semen samples have been cryopreserved from several northern white rhinos over the years, efforts to save this species have ramped up with the utilization of advanced ARTs (aARTs) (8, 9). These include stimulating the remaining female northern white rhinos with exogenous gonadotrophins to support the development of numerous eggs to be harvested using ultrasound-guided aspiration for subsequent in vitro fertilization using intracytoplasmic sperm injection (10). However, ovum pick up rates remain low, potentially due to follicle abnormalities (11), and as this technique is invasive and requires a general anaesthetic with associated risks, additional aARTS are being explored. A game-changing technology has been developed and this is the ability to produce functional oocytes in the laboratory using in vitro gametogenesis. A technology developed in mice, in vitro gametogenesis is an exciting, and complex, process whereby skin fibroblasts are de-differentiated into induced pluripotent stem cells, which are then directed to differentiate into primordial germ cells which then undergo gametogenesis to form functional oocytes in culture (12). Since samples of somatic tissue from numerous northern white rhinos have been frozen and biobanked around the world, this ground breaking technology paved the way for developing much needed eggs in vitro for use in aARTS; an endeavor being led by Prof. Katsuhiko Hyashi (13); this concept is discussed in depth in another article in this *Fertility and Sterility* series "Outside the box: comparative stem cell insights for fertility biotechnology and conservation" by Hutchinson et al. (14). This technique cannot only be used for mice or rhinos but in fact multiple species, including humans, which could benefit numerous infertile patient groups, the most obvious being cancer survivors where in many cases, treatment renders the patients devoid of gametes.

Learning about the presence of reproductive disorders in captive female rhinos was salient to my research program which focuses on ovarian function in health and disease, and fertility preservation in humans (15, 16) and various model species (17, 18). The existence of rhino ovarian dysfunction is particularly pertinent to our studies into a mouse model of premature ovarian insufficiency (19). In this mouse model, we have restored follicle development using the reaggregated ovary technique (20). To achieve this, we isolated germ cells from infertile females and combined them with wildtype ovarian somatic cells resulting in the generation of a so-called reaggregated ovary. This reaggregated ovary was then transplanted into a mouse host and resulted in follicle development (20). Using techniques established in our group, we set out to develop a methodology to culture rhino ovarian tissues with the aim of generating eggs in the laboratory from quiescent primordial follicle pools; a currently unexplored area of research. Thus, the notion of exploring ovarian function for rhino species was too big an opportunity to pass up, and our unique skillset in ovarian function/culture combined with my deep-rooted passion for species conservation made the study of rhino ovarian function one of my laboratory's priorities.

From its inception, the Rhino Fertility Project has used the southern white rhino as a model species for its more endangered northern white cousin, using ovaries collected at post mortem. However, there was a dearth of published literature about ovaries from any rhino species. Although papers have been published describing in situ ultrasonographic visualization of rhino ovaries (21–23), and there is a wonderful website (24) with links and images of the broader rhino reproductive tract, there were no publications detailing southern white rhino ovary morphology or histology. Intriguingly, this does not mean that such information is unknown – just unpublished. Herein lies the first challenges: availability of data, and how novel descriptive data about exotic species has, in the past, been hard to publish due to a lack of a hypothesis, which is usually the basis for publications focusing on common model organisms. Yet the lack of such information holds back true research. In more recent years, such crucial knowledge about fundamental biology has started being accepted again for publication.

CHALLENGES WITH SOURCING RELEVANT TISSUES

Obtaining relevant tissues for rhino research is challenging – as is the case for humans. It is clear that a healthy woman of reproductive age would have no reason to give up her ovaries for research, much less undergo surgery to make such a gift. Hence, human ovarian tissues are either obtained for research as a generous donation after surgical removal for clinical reasons (and thus are often abnormal), a portion is donated for research after an ovary has been collected for fertility

preservation (25), samples biopsied during caesareans (26), or samples from transgender patients (27). Thus, the supply is unpredictable and the quality of the samples is highly variable since very few will be from healthy patients. Ovarian samples collected for fertility preservation are commonly a result of a cancer diagnosis and thus these patients will often have been subjected to a first line chemotherapy treatment, and their overall health and wellbeing may be sub-optimal. Samples collected from transgender patients have often been treated with exogenous testosterone, which was initially presumed to have little impact, but more recent studies have revealed that such treatments do affect ovarian tissues and the follicles within (27). In contrast, the ovarian biopsies collected during caesareans are more likely to be from patients who have been taking care of their health and as such may well be in 'optimal' condition. Either way, each piece of human ovarian tissue donated for research is incredibly valuable.

Similar challenges exist with rhino ovaries; scarcity of samples, albeit for different reasons. Samples can only be obtained from animals that have been euthanized for humane reasons or have died, and as would be expected, these are usually older animals. Such scarcity leads to more considered decisions about how best to process and utilize the ovarian samples to maximize the information generated — akin to the precious human samples we use for our culture research. This scarcity of samples results in limited information, and constraints on what work can be carried out to fully understand ovarian morphology and function.

There can also be challenges with time delays during the attainment of tissues for both human samples and rhinos. For humans, there can clearly be logistical challenges and indeed, with the "Danish Model" the patient stays and the tissue is transported to the central facility for processing and cryopreservation. There were concerns that delays could prove deleterious to the tissue but using sheep ovaries to explore this, we revealed that a delay in cryopreservation of 24–48 hours actually led to improved follicle health after thawing and xenotransplantation (18). We are now exploring the mechanisms behind this finding as clearly incorporating a delay to cryopreservation could be of benefit when considering developing fertility preservation protocols for humans. Moreover, such a discovery will also be highly advantageous when considering freezing rhino ovaries or ovaries from other endangered species because it is unlikely that the cryopreservation and processing facilities are near the point of collection if outside of a zoo.

CHALLENGES WITH AVAILABILITY OF RELEVANT INFORMATION

Having been fortunate enough to obtain ovaries from a euthanized southern white rhino for the Rhino Fertility Project, the first challenge was to consider normality. There was no published information about what ovaries from southern white rhinos looked like. The rhino's closest domestic relative is the horse whose ovary is well known to be quite unusual. In older horses, the ovary is considered to be "inside out" with follicles growing from the inside as opposed to the external cortex. Also, unlike human ovaries, each equine ovary has a single site for ovulation, the ovulation fossa, where follicles migrate to ovulate. Therefore, it was not inappropriate, because of the close phylogenetic proximity, to assume that the rhino ovary would resemble the horse ovary. However, when we obtained our first ovary from the southern white rhino, it looked quite different to the horse. Therefore, we first had to carry out rudimentary dissection and basic histology to ascertain where the ovarian follicles were located (28). Our studies revealed that the follicles, albeit in older animals, were heterogeneously distributed, as is also observed in human ovarian tissues. This results in a significant investment of time to properly analyze the tissues.

CHALLENGES WITH CULTURE AND ANALYSIS OF OVARIAN FOLLICLES

The heterogeneous nature of follicle distribution in human and rhino ovaries compounds challenges associated with analyzing follicle development within tissues. When preparing ovarian tissues for culture, the pieces of ovarian tissue are cut into much smaller pieces to enable nutrient penetration in vitro. However, because of the heterogeneous follicle distribution in both human and rhino ovaries, pieces can exist that contain no follicles at all, resulting in considerable wasted effort in terms of culture and analysis. Interestingly, the dye Neutral Red has been shown to aid in locating small follicles in sheep (29) and human (15, 30) ovarian tissues. This dye accumulates in small growing follicles that are located near smaller clusters of primordial follicles. Indeed, when we selected pieces of human ovarian tissue to culture based on a positive Neutral Red stain, this resulted in more tissues with follicles at final analysis (15). Thus, we explored using Neutral Red to similarly identify rhino tissue pieces that contained ovarian follicles. Unfortunately, these efforts using the older rhino ovaries proved futile with analysis of Neutral Red positive tissues revealing neutral red accumulation in blood vessels only and not follicles (unpublished). There are of course transgenic mouse models with tissue-specific florescent markers and thus visualization of growing follicles is achievable; however, alternative strategies are required when considering human or rhino ovarian tissues. Such challenges with finding follicles in rhino tissues stimulated us to explore novel concepts to enable follicles to be visualized in growing tissues; such studies are in progress. The development of tools to enable follicles to be visualized while growing, rather than in fixed tissues that require laborious sectioning, will have a significant impact on developing protocols for culturing human ovarian tissues by dramatically speeding up wet laboratory experimentation. Such concepts will help the development of robust protocols for culturing human ovarian tissue.

For patients who have survived childhood blood cancer and have undergone fertility preservation (i.e., cryopreservation of ovarian tissues), it is imperative that we develop robust techniques to culture ovarian tissues and generate mature eggs that can be rolled out as a clinical technique. A repeatable multistep protocol that enabled the maturation of fertilizable eggs from primordial follicles in mouse ovarian tissues was established decades ago (31, 32). However, a

robust multistep protocol does not yet exist for human ovarian tissue despite considerable global efforts. To date, only two research groups have successfully generated mature eggs in vitro from human primordial follicles contained in fresh ovarian cortex (26, 33). However, these techniques have not been replicated. It is important to highlight that these studies were conducted on fresh ovarian tissue. As the majority of ovarian samples for human fertility preservation and conservation biobanking are cryopreserved, it is vital to develop similar techniques for these stored tissues and therefore cryopreserved ovarian tissue most need our focus. It is well known that cryopreservation has a negative effect on tissue viability and although reimplantation of cryopreserved ovarian tissue in women of reproductive age has resulted in hundreds of livebirths, little research is actually undertaken on these tissues. With the goal of generating mature human eggs in culture, many research groups are focusing on specific stages of follicle development and developing different culture protocols and techniques with the agenda that the optimization of each different step required will ultimately lead us to a clinical technique.

We have established protocols for culturing mouse (17) and human ovarian tissues (15, 16) that exhibit a number of similarities. However, when planning to culture rhino ovarian tissues, we need to bear in mind that the rhino ovary is considerably larger than the human ovary with follicles distributed over a larger area, with much more "dead space" that does not harbor follicles (11, 28). This was compounded by the heterogeneous nature of follicle distribution and we noted during analysis that many tissues no longer contained follicles. Thus, it would take vast amounts of tissue culture effort using standard techniques to culture equivalent amounts of rhino ovarian tissue. This has challenged us and pushed us to think outside of the box as to how we can culture larger amounts of tissue for the same culture effort. We are in the process of establishing a new system that looks promising for mouse ovarian tissues (unpublished) and hope to validate this for rhino ovarian tissues in the next year. It has also not escaped our notice that to optimize this system for human ovarian tissues would also be advantageous and thus studies are underway to explore and validate the methodology for human application.

One aspect that has huge impact on the development of protocols to optimize follicle development in the laboratory is the labor-intensive and time-consuming analyses that are required. These need to be completed to determine the effect of any culture treatment on follicle development in the tissues before further optimization can be explored. Follicle development is hard to view in real time in culture because of the dense extracellular matrix in the cortex because this precludes visualization of small follicles. Thus, tissues need to be fixed for histological analyses. For this, fixed tissues are paraffin embedded, sectioned, stained, imaged, and finally follicle development can be assessed and decisions taken about what to explore next. We have explored using tissue clearing to assess follicle development circumnavigating the need for sectioning; however, this does not ameliorate the need for fixation and does not enable assessment in real time (34). Analysis of follicle development in real time is crucial enabling protocol optimization to be swift with an immediate readout. Such a challenge is significantly hindering progress in developing a robust repeatable clinical treatment and thus we are exploring new ways to tackle this roadblock.

CONCLUSION

Extending our program of research deciphering ovarian function in health and disease to include rhino has resulted in multiple technological challenges that we have needed to overcome. In the process of overcoming these challenges, we have identified numerous aspects that will help in the development of fertility preservation protocols for the benefit of human patients showcasing how exploring non-model species can have beneficial effects on human health.

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Suzannah A. Williams: Conceptualization, Funding acquisition, Investigation, Writing – original draft, Writing – review & editing.

Declaration of Interests

S.A.W. has nothing to disclose.

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