Research update from Newcastle's Centre for Reproductive Science: Ambient temperature sperm storage and shipment

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For over a decade, our team at the Centre for Reproductive Science (University of Newcastle, Australia) have collaborated closely with the equine breeding industry and clinicians across the country to work on solving reproductive challenges faced by breeders and vets. Our goal has been to exploit the latest knowledge and the most advanced techniques, to get to the bottom of fundamental reproductive biology that can then allow us to deliver practical outcomes. One of the challenges we were tasked with early on was that of improving chilled storage and shipping of stallion semen. This was a particular concern for the standardbred industry, where a majority of foals are produced by artificial breeding, but breeders were concerned with the short lifespan of sperm in chilled storage and the logistical limitations that this brought.

While we have all become used to the routines of collecting semen from a stallion, packaging it in a box with some ice-bricks and rushing it off with a courier to get to the mare before she (hopefully) ovulates, the process is far from ideal, both in terms of practicality and biology. From logistical limitations to severe biological compromise of the sperm cell, there were good reasons to work on a new alternative: ambient temperature storage. What followed was a scientific journey that touched on everything from molecular biology and bat oviducts to comparing the shapes of plastic pots and testing wine fridges. Here I will attempt to concisely outline the research journey and more pertinently, the current state of affairs for ambient temperature sperm storage in practice, which is now ready for clinical use.

The history: an accident in the snow

The idea of using cold temperatures to preserve sperm is credited to Lazzaro Spallanzani, who in 1776 accidentally dropped a sample of sperm in the snow. It is said he looked at the sperm after they thawed and found that some were still alive. This paved the way for cryopreservation of sperm, which relies on using cold temperatures to slow down cell metabolism, and cryoprotectants to simultaneously protect the sperm from the damage of extreme temperature changes. In the early 1900s, while working on refining cryopreservation, scientists noticed that bull sperm survived reasonably well – and retained good fertility – after being refrigerated at 5°C for 24 hours, with milk and glycerol added to the storage media. This laid the foundations for chilled storage and shipment, which became commonplace in livestock breeding by the 1950s, and is now widely used in equine practice.

The science: chilled storage

For many years now chilled storage has allowed stallions to breed mares that are geographically removed, with comparatively lower labour input (versus, for example, frozen semen, or relocating the mare to the stallion farm), and less risk of injury to horses and humans. Chilling the sperm cells to around 5°C degrees is required in order to slow down their metabolism, conserve energy and thus prolong their lifespan outside the body, long enough that the semen can be shipped overnight to arrive in time for the mare's impending

ovulation. Sperm under these conditions can typically survive at most around 2-3 days. The semen can only be cooled once it has been mixed with a specialised extender, usually containing milk or milk-derived products that stabilise the cell membrane to help tolerate cooling and adsorb toxic waste products. While there have been some refinements to these extenders and their exact composition, the concept has remained largely the same as that developed in the 1950s. Furthermore, these extenders were developed based on what is known about better-studied species such as cattle, and then typically used on horses with little to no modification. Yet tweaking and refining the extenders can only improve things so far – the negative effects of cooling a cell cannot be alleviated completely. This is mostly because of the 'phase transition' and how it affects the lipids in the cell membrane. Think of it like butter that melts at room temperature, but becomes a solid in the fridge: when the lipids in the cell's plasma membrane cool down and become rigid, the sperm cell can no longer effectively control what goes in and out across the membrane, wreaking havoc on the sperm's ability to survive and function. The only way to avoid this is to avoid cooling the sperm past the phase transition temperature (around 10 - 15°C), and this means finding alternative ways to support – rather than to slow down – their metabolism during their time in storage.

Stallion sperm in the spotlight: Live fast and die young

The first step in developing a tailored extender for stallions was to go back to basics and understand the metabolism used by stallion sperm cells. In a seminal study by Gibb et al,[1] we found that, compared to most other well studied species, stallion sperm rely more heavily on a metabolic pathway called oxidative phosphorylation (rather than glycolysis), which generates energy quickly, allows them to swim faster, but also means the sperm become exhausted quickly and produce high levels of toxic byproducts in the form of reactive oxygen species (ROS): they 'live fast, and die young'. This meant we needed to find new ways to support their metabolic needs during storage, and to more effectively remove the toxic byproducts they generate. We also wanted to reduce the energy used up by the cells during storage, but without dropping the temperature. So we got to work doing experiments to test out hundreds of different components that might address these issues. This even included components found in bat oviducts, where sperm are known to survive for several months, and a compound that shifts the sperm metabolism to behave more like human sperm – burning energy more slowly and steadily.

The solution: SpermSafe

We eventually gathered up all the components that these experiments showed were beneficial for the sperm (i.e., those that supported the stallion sperms' metabolism, protected them from toxic reactive oxygen species, and reduced their energy wastage) and combined these into an extender, now called 'SpermSafe'. The end result was that by two weeks in storage, motility had only dropped by about a third from the start of the experiment, with more than 50% of the cells still alive and motile for most stallions. At this point we were excited but sceptical – good motility in the lab is a good start, but are the sperm actually able to fertilise an egg? We tested the stored sperms' ability to capacitate (i.e., go through the molecular activation steps required to fertilise an egg), and to bind to oocytes *in vitro*. The one-week-old sperm performed better than the traditionally chilled sperm on these assays.[2] The first SpermSafe foal, 'Tinsel', was born just before Christmas

2018, with many more foals on the ground since then as we've continued to work with a small number of vets in Australia to trial the extender.

No-chill storage in action

SpermSafe is a new extender but it is worth noting it brings with it an entirely new way of storing and shipping stallion sperm. Being able to collect a stallion once, and have usable sperm for 1-2 weeks can radically change the way that stallion owners organise their schedules and will reduce the stress (and costs) of time-critical logistics. It also involves doing things a little differently and requires procedural change that may take time to adopt widely.

Storing sperm for a prolonged period requires careful processing and some extra steps to remove bacteria and fungal contaminants from the sample, as these would also thrive on the nutrients and metabolism-supporting components in the extender. Dead and dieing sperm release toxic waste molecules that can trigger a domino effect in the living cells and compromise long-term storage, so these also need to be removed as much as possible at the beginning of the storage period. A simple and effective way to do this is to pre-process semen by centrifuging through a colloid such as EquiPure; this both isolates the high-quality cells from debris and low-quality sperm whilst removing much of the microbial contamination. Other exciting new techniques are in development including electrophoretic separation and membrane based approaches. Latest developments on this front are discussed in a recent review paper.[3] We are continuing to refine prototypes for new, simpler and more effective strategies in our lab.

Of course, the shipping and storage temperature is different to traditional chilled AI. After testing a range of storage temperatures, we found that 17°C is optimal for SpermSafe-stored sperm as it allows for some of the protective effects of low temperatures without crossing into the 'danger zone' below the phase transition temperature of ~15°C. Fortunately this is the standard temperature of a wine fridge, and there are a few available options for 17°C shipping containers. Our team is currently working on a bespoke shipping container that will use phase transition gels to optimally maintain a stable temperature between 15-17°C.

Case study: Poor chillers

While all stallions' sperm will suffer to some degree with chilling, some stallions are particularly susceptible to these ill-effects and their sperm will not tolerate chilled storage at all – in some cases, to the point that they are unable to achieve pregnancies with chilled semen. For these horses, breeding mares on-site with fresh semen is considered the only option. A significant benefit of ambient temperature storage is the ability to facilitate shipped semen breeding for these poor chilling stallions. We had the opportunity to investigate whether such stallions could benefit from using Sperm Safe when our collaborator, Dr Lisa Maclellan, was presented with such a stallion – he had achieved zero pregnancies after multiple attempts using chilled semen, despite having very high fertility on-farm. After extending with SpermSafe and storing at 17°C, he had a fertility rate of 67%, vs a rate of 0% with chilled semen[4]. The stallion is now able to service mares off-farm, which was previously not an option. It has been reported that up to 30% of all stallions may be "poor chillers". [5] We hope that SpermSafe can help these stallions reach their maximum reproductive potential and give breeders access to a greater range of stallion options.

Further applications: Frozen semen, IVF and epididymal sperm

Dr Lee Morris (EquiBreed ART, NZ) has pioneered the use of SpermSafe as a new way to thaw and process cryopreserved sperm, in combination with microfluidic isolation and fixed-time, low-dose AI. Sperm were thawed and could then be stored (or shipped) in SpermSafe for up to 24 hours, achieving embryo recovery rates of 52%.[6]

Conventional IVF in horses is making a research comeback since promising results were published by Felix et al in 2022.[7] The success of the IVF procedure relies heavily on whether high sperm viability and motility can be maintained in vitro. We are now testing the ability of SpermSafe to facilitate more flexible IVF protocols as well as developing custom media that will enhance physiological capacitation of sperm, as these are two of the major challenges preventing the wider uptake of IVF in clinical practice.

Other clinical applications we hope to test in coming months is the use of SpermSafe as a medium for recovering and processing epididymal sperm and its use as an intrauterine treatment to alleviate persistent breeding-induced endometritis (PBIE) in susceptible mares. We always welcome expressions of interest from potential collaborating clinicians who wish to be involved in these studies.

Final points

SpermSafe can be used on most stallions, and, just like any extender, should be trialled for compatibility. For the majority of stallions, sperm can be stored at 17-22°C for up to 1 or 2 weeks, depending on the initial sperm quality at collection - which of course varies widely. We know it can help poor chiller stallions, while or stallions with low motility, short term storage in SpermSafe is beneficial as it minimises the rapid loss of sperm quality typically seen with chilled storage. Careful processing between collection and storage is needed, because removing seminal plasma, dead or dieing sperm cells, as well as microbes, is crucial for extended storage.

Extended liquid storage means that a stallion might only need to be collected once or twice a week and sperm can be stored for use throughout the week, while the stallion can continue with his training and competition routine with minimal interruption. Semen can also be shipped to the mare in advance of ovulation and thus reduce the risk of courier delays or early/late ovulations messing up the cycle and resulting in wasted costs. Excitingly, SpermSafe can prolong the lifespan of thawed cryopreserved sperm, potentially removing the need for unpleasantly timed mare scans during frozen semen breeding cycles.

The Newcastle team is excited to get our research out into clinical practice and to develop tailored protocols for challenging or unusual cases. We are always open to new collaborative relationships to continue trialling our innovations and exploring new research questions.

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