An update on cooling and freezing extenders for stallion sperm
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Introduction
Methods for preserving stallion sperm, by cooling the sperm to 5°C or by cryopreservation, have been developing for >20 years.1,2 Preserving sperm at 5°C, is usually accomplished by diluting sperm in a milk-based extender at room temperature and slowly cooling the sperm to 5°C using a passive cooling system.2-4 The original milk-based extender was very simple and consisted of only dry skim milk, glucose, sodium bicarbonate and gentamicin sulphate.5 Modifications off this main theme, including adding salts and proteins, have improved the survival of stallion sperm cooled to 5°C.

Most extenders originally developed to cryopreserve stallion sperm consisted of milk and/or egg yolk, with sugars, salts and glycerol.6 Stallion sperm cryopreservation generally requires centrifuging the sperm, to concentrate the sperm and remove most of the seminal plasma; diluting the sperm in the cryo-diluent, packaging the sperm into straws and freezing the straws in liquid nitrogen vapor, either directly from room temperature or after cooling the sperm slowly to 5°C. Recent developments in both the freezing procedure and diluent composition have improved sperm cryo-survival rates. This review will update the practitioner on the recent changes made in diluents for cooling and freezing stallion sperm, as well as changes in techniques that improve the survival rates of sperm after cooling or after cryopreservation.

Cooled stallion sperm
Incubating sperm with high levels of seminal plasma, for extended lengths of time, is detrimental to stallion sperm. Therefore, sperm stored at 5°C, survive much better if the seminal plasma concentration is reduced to < 25% of the fluid volume. This can be achieved by diluting the sperm at least 1:3 with the cooling extender prior to cooling, or by centrifuging the sperm prior to mixing the sperm with the cooling extender.3 Sperm from some stallions, do not cool very well, and removing the majority of seminal plasma (the final solution should contain 5% seminal plasma), from these samples, can sometimes benefit these sperm.3 If the semen is centrifuged, care must be taken not to centrifuge the sperm with too much force or for too long, as this can damage the sperm. The use of a 'cushion' in the bottom of the centrifuge tube allows the sperm to be centrifuged with greater force and/or for longer times, which allows more sperm to be collected during the process, while limiting damage to the sperm.3

Modifications to the original skim-milk formula have produced a number of cooling extenders that exhibit improved sperm survival after cooling. A number of researchers have investigated adding different antioxidants to the media,4 but for the most part these have not benefitted sperm significantly. However, some of the most promising additives to cooling extenders are additional milk casein proteins, including phosphocasienate.2 Casein components are the main additional ingredients in the INRA96 and ARSBlue extenders, and sperm cooled to and held at 5°C in these extenders maintain higher sperm motility for 24-96 hours, than sperm diluted in the classic Kenney extender.2 Cooling extenders with added casein products maintain higher sperm motility for sperm from most stallions, but seem to be especially effective for sperm that normally do not cool very well.

Cryopreserved stallion sperm
Cryopreserving stallion sperm typically involves centrifuging the sperm (to concentrate the cells and reduce the seminal plasma to ~5%), diluting the sperm into the cryo-diluent containing glycerol (2.5-5%) and packaging the sperm into straws. Depending upon the cryo-diluent used, sperm are often frozen in liquid nitrogen vapor directly from room temperature (lactose-EDTA extender; LEDTA) or first cooled to 5°C and then frozen (skim milk-egg yolk extenders; SMEY). Sperm are usually packaged into 0.5 mL straws, but straws up to 5 mL are sometimes used. The smaller, 0.5 mL straws are usually thawed in a 37°C water bath for 30 seconds prior to insemination, while the larger straws are normally thawed in a...
50°C for 45 sec before being plunged into a 37°C water bath to stop the warming process, prior to insemination.

Again, many changes have been made in both the procedures used to cryopreserve stallion sperm as well as the diluents used to cryopreserve them. Both LEDTA and SMEY extenders contain various sugars in them, which act as non-permeating cryoprotectants. Studies have investigated whether sugars, other than lactose, can cryopreserve sperm better than lactose. However, these alternative sugars do not have major beneficial effects for cryopreserving stallion sperm.7,8

Although the most commonly used cryoprotectant, glycerol, permeates stallion sperm membranes; it does so rather slowly.9 This means, when sperm are initially added to the freezing extender, that contains glycerol; and when frozen-thawed sperm are diluted from the freezing extender at insemination, water rushes out of the cell or into the cell, respectively; before the glycerol can slowly cross the membrane.9 This causes the sperm to shrink or swell, respectively; and these cell volume changes can damage the cell (especially sperm swelling when the cryoprotectant is removed). These volume excursions can be lessened if cryoprotectants that are more permeable to the stallion sperm membranes are used, instead of glycerol. Experiments utilizing cryoprotectants that have lower molecular weight and that are more membrane permeable, than glycerol, such as methyl formamide and dimethyl formamide either alone or replacing part of the glycerol normally used in the cryo-diluent, result in higher post-thaw sperm survival than similar diluents containing only glycerol.1,10

Current dogma dictates that sperm frozen in LEDTA can be frozen in liquid nitrogen vapor directly from room temperature, while sperm frozen in SMEY should be first cooled to 5°C before being frozen. This suggests that sperm do not 'cold shock' in LEDTA, but are susceptible to cold shock in the SMEY extender. Recent experiments have shown that sperm cryosurvival rates are higher in both LEDTA and SMEY if the sperm are cooled slowly to 5°C, to prevent cold shock, prior to freezing.8 This simple procedure can easily be implemented into a cryopreservation protocol, to increase stallion sperm cryosurvival rates.

Finally, there is sometimes a desire to cryopreserve sperm collected from the epididymides of stallions that have recently died or that have been castrated. Currently, seminal plasma (collected from other stallions) is added to the epididymal sperm prior to cryopreserving the cells. In a recent study, we diluted epididymal sperm with either seminal plasma or a basic salt solution containing added sugars and protein, and found that sperm diluted in the salt solution exhibited higher post-thaw motility than sperm diluted in seminal plasma prior to cryopreservation (Graham; unpublished). This indicates that seminal plasma is not necessary for sperm cryopreservation.

Conclusions

Recent progress has been made in both the methodology and the extenders for cooling and cryopreserving stallion sperm. In particular a 'cushion' at the bottom of centrifuge tubes protects sperm from damage, while allowing greater centrifugal force and time to be used during centrifugation. This permits more sperm to be collected during the centrifugation process. New cooling extenders, which contain additional casein products, help stallion sperm survive the cooling process and storage at 5°C, better than extenders that do not contain added casein. Alternative cryoprotectants, which are of lower molecular weight than glycerol, create less osmotic damage to sperm, during their removal and result in higher cryosurvival rates, than glycerol alone. Epididymal sperm can be cryopreserved using the same methods and cryo-diluents as ejaculated sperm, but do not need to have seminal plasma added to them after collection. Diluting the epididymal sperm first in a basic salt solution containing sugar and protein is sufficiently effective to activate sperm motility and permit sperm concentration to be determined prior to diluting the sperm in a cryo-diluent. Finally, sperm should be cooled to 5°C before being frozen.

References
