



Semen cryopreservation - challenges and perspectives

Cryopreservação de semen: desafios e perspectivas

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Abstract

Frozen semen has many advantages over fresh or cooled semen. Management of the stallion before and during the freezing process is extremely important for obtaining good quality frozen/thawed semen. Frequency of ejaculation will affect the quality of frozen semen as well as age of the stallion and breed. Furthermore the initial concentration prior to freezing also affects the post-thaw motility. Other factors affecting freezability include type of cryoprotectant and level of glycerol. Cooling rate from minus 10 to minus 50 degree centigrade do not appear to affect post thaw motility but the concentration within a 0.5 ml straw does affect motility. Proper handling and storage of frozen semen is extremely important and stallion semen can last indefinitely if maintained in liquid nitrogen.

Keywords: freezing, semen, semen quality, stallion.

Resumo

Sêmen congelado tem muitas vantagens sobre o sêmen fresco ou descongelado. O gerenciamento do garanhão antes e depois do processo de congelamento é extremamente importante para obter boa qualidade de sêmen congelado/descongelado. A frequência da ejaculação afetará a qualidade do sêmen congelado, além da idade e raça do garanhão. Além disto, a concentração inicial antes do congelamento também afeta a mobilidade pós descongelamento. Outros fatores que afetam a capacidade de congelamento incluem tipo de crioprotetor e nível de glicerol. A taxa de descongelamento de 10 graus centígrados negativos para 50 graus centígrados negativos não parece afetar a mobilidade pós descongelamento, mas a concentração do conteúdo de 0.5 mL na palheta afeta a mobilidade. O manuseio correto e armazenamento do sêmen congelado é extremamente importante e o sêmen do garanhão pode ter validade indefinida se mantido em nitrogênio líquido.

Palavras-chave: criopreservação, desafios, semen.

Introduction

There has been an increased use of frozen semen in the past decade. This is because of the many advantages of utilizing frozen-thawed equine semen. Some of those advantages include preserving genetic material for the future and the ability to utilize the stallion's semen to produce pregnancies even after the death of the stallion. Properly stored frozen semen can remain viable in the nitrogen tank for many decades (Amann and Pickett, 1987). Cryopreservation can become an insurance policy against the untimely loss of the stallion due to death. Other advantages include the ability to ship semen internationally and thus have access to a greater population of mares. Frozen semen can be more convenient and economical for breeding mares in that the semen can be available at the farm and once the mare reaches the appropriate stage of the cycle she can be inseminated (Graham, 1996b; Loomis and Squires, 2005). Thus, the semen can be shipped early in the breeding season and be available for insemination at the most appropriate time for fertility. The majority of semen is collected during the non-breeding season and frozen. This allows stallion's to be used in a fresh or cool-semen program during the breeding season, and frozen semen can be obtained during the non-breeding season. There are, however, disadvantages to frozen semen. One major disadvantage is that there is no good test for fertility of frozen semen except insemination of mares, which is quite costly (Graham, 1996a; Kirk et al., 2005). There is also the perception by the breeder and veterinarian that the mare must be examined with ultrasound and palpation multiple times a day in order to achieve excellent fertility with frozen semen. However, this is only true if a single dose of semen is provided per cycle. If multiple doses of semen are provided for each cycle, then mares can be bred at a fixed time after injection of an ovulatory agent, thus allowing the mare to be managed much like those that are inseminated with cool semen (Barbacini et al., 2005)



Management of the stallion in a frozen semen program

The ejaculation frequency of the stallion prior to semen collection for freezing can have a dramatic effect on the freezability of semen (Loomis and Squires, 2005). It is best that the stallion be collected several times within a few days of being placed into a frozen semen program. This could mean collecting twice on one day, once on the following day, and then on the third day begin the collection for freezing purposes. Other approaches would include collecting stallion every other day for several days prior to placing them into a frozen semen program. The most efficient collection schedule for most stallions in a frozen semen program is in every other day collection schedule. However, if the stallion produces low numbers of sperm then perhaps collections twice a week may be more appropriate. Alternatively, if the semen quality varies dramatically between ejaculates then it may be necessary to collect the stallion daily during the freezing process. Based on a recent study (Kalmar et al., 2013) involving over 800 stallions and nearly 14,000 ejaculates the best posts-thaw motility was obtained in the fall and winter as opposed to spring and summer. In addition, if one examined the change from initial motility to post-thaw motility (freezability), the best time to freeze semen was also in the fall or winter. This may be due to the chances of heat stress that occurs during the spring and summer in some areas within the United States. The same data set was also examined for the effect of age on post-thaw motility. Stallions were divided into age categories of: 2 to 5, 6 to 10, 11 to 15, 16 to 20 and greater than 20 years. Post-thaw motility was decreased in stallions 11 to 15 yr compared to younger stallions and post-thaw motility continued to decrease as the stallion got older than 15 years. Thus, it would seem that the best time to freeze semen from a stallion would be after he is sexually mature i.e. 5 to 6 years of age, and up until approximately 11 years of age. That is not to say that semen cannot be frozen successfully from older stallions but generally the sperm numbers may be decreased and the posts-thaw quality decreased in these older stallions. This data set was also examined for the effect of breed on freezability of stallion. Post-thaw semen quality was best in our stock horse breeds (Appaloosa, Paints and Quarter horses), whereas the poorest post-thaw semen quality was obtained from Friesian, Andalusian and Saddlebreds.

Cryopreservation procedures

There have been a few subtle changes in the cryopreservation procedures over the last decade that have helped improve the quality of frozen thawed stallion spermatozoa (Loomis and Squires, 2005). Once the semen is collected the first step after initial evaluation is centrifugation to concentrate sperm and to remove seminal plasma. The use of centrifugation cushions have increased the recovery of sperm during the centrifugation process by allowing one to use higher G forces and longer centrifugation times. This increases the number of sperm available for processing and freezing.

There are numerous commercially available extenders for freezing equine spermatozoa. Generally, one would test freeze stallion in several extenders and then determining which extender provides the highest posts-thaw motility. That extender would then be used to freeze all the semen for that particular stallion. There are certainly preferences stallions have for particular extenders. The most common extenders are the skim milk, egg yolk glycerol extenders and the lactose EDTA extender containing glycerol (Graham, 1996b).

More recently an extender from Brazil has been developed that contains a combination of dimethyl formimide and glycerol (Alvarenga et al., 2005). The level of glycerol in extenders has been shown to affect posts-thaw motility (Ecot et al., 2000). Levels higher than 2.5% have been shown to be detrimental to post-thaw motility.

Cooling rate does not appear to be a major factor affecting the freezeability of stallion semen. Cooling rates from -10 to -50°C provided similar post-thaw motility (Graham, 1996b). It has been suggested that the thawing rate and the dilution of the cryoprotectant after thawing may be more important than the freezing rate (Vidament, 2005). Equine sperm appear to be quite sensitive to rehydration and it may be that serial dilution after thawing may improve fertility. However, these same studies have not shown an effect on post-thaw motility.

Frozen equine sperm can be stored in either five ml 2.5 ml or 0.5 ml straws but most of the equine frozen semen is stored in 0.5 ml straws. Each straw generally contains 100 million total sperm and eight straws are generally considered a dose of semen (total 800 million). However, there are some occasions where 4 straws are considered a dose of semen or even one straw containing 800 million total sperm is considered a dose of semen. The advantage of the eight straws dose is that if fertility is extremely good with a full dose then fewer numbers of straws can be used per insemination. Studies have shown that mares inseminated with a half dose of semen (4 straws) resulted in similar fertility to those inseminated with a full dose (Barbacini et al., 2005). It is advisable however, to use a full dose of semen from a given stallion until his fertility has been established then one can decrease number of straws per breeding depending upon the stallions fertility. More recently, veterinarians have utilized only one or two straws of semen per breeding by placing semen at the tip of the uterine horn at the entrance of the utero-tubal junction using rectally-guided deep horn insemination approach. Others have utilized centrifugation through density gradient as a means of enhancing the quality of semen prior to freezing or after freezing and thawing. Few studies have evaluated fertility of frozen semen that has been



layered on a density gradient and centrifuged. In a study conducted in our laboratory (Cerny et al., 2011), there was no difference in fertility of mares inseminated with four straws of frozen-thawed semen that have been centrifuged through a density gradient compared non-centrifuged frozen thawed sperm.

Storage and shipment of frozen /thawed semen

Frozen equine sperm can either be stored in liquid nitrogen tanks at the farm or veterinarian's office or at a commercial semen freezing facility. Regardless of where the semen is stored is important to maintain adequate levels of liquid nitrogen in the tank in order to properly maintain the viability of sperm (Loomis and Squires, 2005). It has been estimated that sperm maintained in liquid nitrogen will not change in their composition for as much as 50,000 years. However, there are many occasions where both equine and bovine sperm were damaged by improper handling during the storage and distribution. It is important to pre-freeze the forceps involved in taking the semen out of a liquid nitrogen tank and also to work below the frost line. Anytime the sperm are exposed to temperatures above -100°C there is a chance of damage. One of the most common ways of damaging sperm is during the process of removing samples from a liquid nitrogen tank. The safest way is to work below the frost line of the tank and also make sure that the goblets are full of liquid nitrogen when the sperm are brought up into the neck of the tank. Most semen that is shipped to the mare for breeding is transported in dry vapor shippers. These shippers are designed to maintain the temperature -196°C for up to 21 days. However, it is essential that the dry shippers be properly charged prior to being used. Semen freezing facilities are able to provide semen to the breeder within 24 hours of making a request. However it is more efficient if the breeder or veterinarian order semen several days prior to the mare having a pre-ovulatory follicles. The other option is to order the semen several months ahead of the breeding season and transfer the semen into a liquid nitrogen tank at the breeding farm or veterinary clinic.

Insemination of mares with frozen equine sperm

There are two general approaches to insemination of mares with frozen-thawed equine sperm. One is to breed the mare post-ovulation within 6 hours of ovulation. The other is to breed the mare within 12 hours pre-ovulation or breed the mare both pre-and post-ovulation (within 12 hr before and up to 6 hr after ovulation). The scheme that is chosen depends upon how much semen is made available per cycle. If multiple doses of semen are made available, then mares can be managed much like those in a cool semen program. In this case, the mare would be palpated and ultrasound only once a day during the estrus. Once a pre-ovulatory follicle is identified then an ovulatory agent is given and the mare is bred 24 to 30 later (Barbacini et al., 2005). She is then checked with ultrasound the following day and if she has not ovulated she is inseminated a second time. Studies have shown similar fertility for mares inseminated once or twice in the cycle with frozen semen (Barbacini et al., 2005). A fixed time insemination after giving an ovulatory agent has also been published (Barbacini et al., 2005). The first insemination is performed 24 hours after hCG or 30 hours after GnRH and the mare is automatically inseminated the second time 16 hr post the first insemination. If only one dose of semen is made available, then mares are typically palpated and ultrasound 4 to 6 times a day and then upon detecting an ovulation the mare is bred within 6 hours after ovulation. The decision as to which insemination schemes to use is based upon the amount of semen made available. One of the criticism of the fixed time insemination or two dose insemination scheme is a possibility of the second dose of semen being inseminated into an inflamed uterus. However, studies have shown that there is no greater incidence of retained uterine fluid in mares inseminated once versus twice per Cycle (Barbacini et al., 2003). That is not to say that some problem mares, that are susceptible to post breeding endometritis, may have better fertility if they are inseminated only once the cycle. If one anticipates the mare will have an exaggerated post-breeding endometritis after insemination with frozen semen then it would be best to administer oxytocin six hours after insemination in order to eliminate uterine fluid.

Certainly one of the major trends in insemination of mares with frozen equine sperm is the use of deep horn insemination as a means of utilizing fewer sperm per insemination. Studies are needed to confirm the advantage of deep horn insemination over body insemination, particularly when relatively high sperm numbers are used i.e. greater than 100 million total sperm.

Conclusion

More than likely if the fertility of frozen-thawed equine sperm was equal to that of cool semen then there would be an even greater trend in the industry towards use of frozen semen. There are studies (Loomis and Squires, 2005) that have shown similar fertility between cooled and frozen semen, although there is great stallion variation in the fertility with frozen semen. Certainly the advantage of using frozen semen is greater than those for cooled semen and it is likely that in the future fertility of frozen semen will improve for the majority of stallions. Thus, there will be an increase in the use of frozen semen and decrease in the use of cooled semen in the equine industry.



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