



The efficient use of equine cryopreserved semen

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Abstract

In order to optimize the efficient use of cryopreserved stallion semen, recent research has focused on the minimum insemination dose of frozen–thawed spermatozoa required for maximum fertility rate. The results appear to be highly stallion-dependent. Factors such as the timing of AI with respect to ovulation, as well as the site of insemination within the mare's reproductive tract, also affect success in breeding with frozen–thawed semen. Since acceptable pregnancy rates can be achieved from insemination of mares with very low numbers of spermatozoa, increasing the number of insemination doses processed from a single ejaculate may prove more cost-effective to stallion owners.

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1. Introduction

In the USA, insemination of mares with frozen–thawed stallion semen has become widely accepted and grown in popularity over the past 15 years, as experience in both the cryopreservation of sperm and optimal management of the mare has developed. Methods of freezing sperm, composition of extenders necessary to preserve the integrity and fertility of sperm, timing of insemination, site of insemination, and optimal dose of insemination, all appear to contribute to variability in pregnancy rates [1]. In lieu of this variability, researchers and clinicians strive to develop optimal standardized protocols both for freezing spermatozoa and insemination. Future research is also directed toward the ability to predict the fertility of frozen–thawed semen, particularly with the identification of specific molecular “markers” that may be highly correlated with fertility. And, in further attempt to

enhance the “freezability” and fertility of stallion semen, several laboratories have recently found improvement of certain semen parameters in some stallions through specific feeds and nutraceuticals; this effect is suspected to take place by inhibiting the detrimental events of oxidative stress concomitant with the freezing and thawing of semen.

2. Expected pregnancy rates using frozen–thawed semen

Pregnancy rates of mares bred with frozen–thawed semen are highly variable (range, 0–100% per-cycle [2]). Because of this variability, studies reported from insemination of large numbers of mares may provide the most accurate assessment of pregnancy rates with frozen–thawed semen. Loomis [3] reported that 876 mares were bred with frozen–thawed semen from 106 stallions; the per-cycle and per-season pregnancy rates were 53.5 and 81.9%, respectively. Similar results were reported by Vidament [4] in a retrospective analysis of 20 years of breeding records within the French National Stud; based on post-thaw motility of frozen–thawed

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semen, per-cycle pregnancy rates ranged from 43 to 52%. These aforementioned studies are the results of many inseminations performed by several individuals, thereby lacking in intensive quality control. It is possible in highly managed mare herds to achieve higher per-cycle pregnancy rates. In a study where mares were managed by a single veterinarian, Metcalf reported a per-cycle pregnancy rate of 73% when 45 mares were bred over 63 cycles with frozen–thawed semen from 22 stallions [5]. Although these per-cycle pregnancy rates appear to be affected by mare management, other specific factors, such as the number and concentration of progressively motile spermatozoa within an insemination dose, the timing of insemination, and site of semen deposition, affect success rates using frozen semen.

3. Optimal insemination dose for maximizing pregnancy rates

When breeding mares with fresh semen, it was determined long ago that a dose of 500×10^6 progressively motile spermatozoa (PMS) inseminated every 48 h while a mare was in estrus yielded maximum pregnancy rates [6]. Although setting a standard for the equine breeding industry, these results have been challenged, as many stallions have excellent fertility results with insemination doses $<500 \times 10^6$ PMS [7]. Hence, fertility of cooled stallion semen appears highly stallion-dependent. The same variability and individuality is suspected to be true for frozen–thawed semen, and thus, it becomes difficult to set a rigid standard insemination dose of frozen–thawed semen that applies to all stallions. Regardless, several published studies with results from breeding many mares offer useful guidelines.

In the retrospective examination of breeding records from >30,000 mare inseminations within the French National Stud, Vidament [4] concluded that mares should be inseminated with a minimum of 750×10^6 total frozen–thawed sperm per-cycle; doses lower than this critical value were likely to result in lower per-cycle pregnancy rates. Based on this study, this figure has been adopted and recommended by the World Breeding Federation of Sport Horses. However, determining the optimal dose of *motile* frozen–thawed spermatozoa (as opposed to total number) that will maximize pregnancy rates is perhaps more important in the USA, since stallions are not selected as sires on the basis of post-thaw motility of frozen–thawed spermatozoa. Many years ago, Volkmann and van Zyl [8] reported that per-cycle pregnancy rates were significantly higher when

the insemination dose of frozen–thawed semen was increased from 137 to 210×10^6 to 222 to 333×10^6 PMS (44% versus 73%, respectively). In a more recent study by Metcalf [9], breeding records of 90 mares bred over 312 cycles with frozen–thawed semen from 46 stallions were analyzed. The per-cycle pregnancy rates for mares inseminated with $<200 \times 10^6$, 200 to 400×10^6 , 400 to 600×10^6 , 600 to 800×10^6 , and $>800 \times 10^6$ PMS were 54% (12/22), 70.2% (66/94), 60% (30/50), 88.2% (60/68), and 56.4% (44/78), respectively. These very high pregnancy rates represent a small population and possibly, both mares and stallions with high fertility. Notwithstanding, there were significant differences in per-cycle pregnancy rates based on the insemination dose of frozen–thawed PMS; mares bred with 600 to 800×10^6 PMS had significantly higher per-cycle pregnancy rates than mares bred with other doses. Squires et al. [10] reported similar high per-cycle pregnancy rates (83–86%) in mares that were bred with a *minimum* of 250×10^6 frozen–thawed PMS from seven fertile stallions. In a larger, commercial study, Loomis and Squires [11] reported a per-cycle pregnancy rate of 52.7% (126/217) when mares were bred with doses equal to 240 to 600×10^6 PMS. Based on these studies, it appears that insemination doses exceeding 250×10^6 PMS are likely to optimize fertility.

The optimal concentration of PMS within an insemination dose has also been investigated. In an early study that examined 63 mares bred with frozen–thawed semen from five stallions, Leipold et al. [12] reported significantly higher per-cycle pregnancy rates (40% versus 15%) when mares were bred with 320×10^6 PMS at high concentration (1.6×10^9 spermatozoa/mL) versus low concentration (400×10^6 spermatozoa/mL). However, in a large study conducted over a number of years, Vidament [4] cited that the present French system recommends freezing equine semen at a lower concentration (100×10^6 /mL), since it appears to result in fewer cycles per pregnancy.

It is important to realize that, at least in the USA, frozen semen is not selected for use by any standardized criteria; therefore, it is difficult to recommend an optimal concentration or optimal number of sperm for an insemination dose. In addition, comparison of per-cycle pregnancy rates and insemination dose is confounded by many variables, which include, but are not limited to, individual stallion variation, timing of insemination, limited numbers of mares in studies that actually determined the exact number of motile sperm (and not just a minimum motility or total sperm) in the inseminate, and post-insemination treatments of mares.

4. Timing of insemination

Insemination with frozen–thawed semen should be timed to occur close to ovulation; specific protocols designed to coordinate the timing of insemination with induction of ovulation have yielded encouraging results for “appointment breeding”. Samper demonstrated that the majority of mares (94%) ovulate 36–42 h following induction of ovulation with either hCG or deslorelin if the mare is in estrus, and a dominant preovulatory follicle and endometrial edema are present at the time of drug administration [13]. Based on these data, “appointment breeding” has grown in popularity and has proven successful utilizing several protocols. Using very low numbers of motile, frozen–thawed spermatozoa (14×10^6), Morris et al. [14] inseminated mares once at 32 h post hCG administration and obtained a pregnancy rate of 67%. There are, however, conflicting theories regarding the optimal timing of insemination with respect to ovulation itself. Many research laboratories have examined pregnancy rates after mares were inseminated pre-ovulation, post-ovulation, or on either side of ovulation. Some researchers advocate breeding mares both pre- and post-ovulation. They recommend inseminating mares 24 and 40 h after induction of ovulation, whereas others advise breeding at 36 h and again at 42–44 h after an ovulation-inducing agent is given. With both regimens, it is assumed that ovulation occurs between the two inseminations. Other researchers argue that only a single insemination is needed for acceptable pregnancy rates. Barbacini et al. [15] evaluated insemination protocols and found no difference in pregnancy rates between mares bred 24 and 40 h after hCG administration (46%), (when ovulation was likely to occur between 36 and 42 h), and pregnancy rates of mares that were bred only once post-ovulation with the same total insemination dose (47%). Following a single post-ovulation breeding, similar per-cycle pregnancy rates have been reported by Hemberg et al. (45.4%) [16] and by Metcalf (47%) [9]. In the latter study, if mares were inseminated both pre- and post-ovulation, per-cycle pregnancy rates were increased to 70% (164/234), suggesting that, although more labor intensive, pregnancy rates may be higher when mares are bred on either side of ovulation. However, if frozen–thawed semen is selected from only highly fertile stallions, pregnancy rates after a single post-ovulation breeding may be as high as 80% [10].

Disparity in pregnancy rates following a single insemination after ovulation versus insemination on either side of ovulation likely reflects the variation in inherent fertility of the stallions. In addition, it is often

challenging to predict ovulation even when “appointment breeding” is anticipated. In studies where examiners bred a diverse population of mares with frozen–thawed semen from many stallions, highest pregnancy rates were attained with pre- and post-ovulation insemination [4,9]. However, if the mare gets pregnant, post-ovulation insemination represents the most economical means of breeding a mare. In this case, breeding management of the mare is more cost-effective in that the management needed for predicting ovulation is eliminated. The mare requires examinations only twice daily and, unlike regimens that call for a pre-ovulation breeding whereby multiple doses may be used if the mare fails to ovulate as predicted, only a single dose of semen is used following identification of a corpus hemorrhagicum. Conversely, if the mare fails to get pregnant with a single post-ovulation insemination, then it becomes ultimately more expensive for both the mare and stallion owners.

5. Site of insemination

Acceptable pregnancy rates can be achieved with very low doses of frozen–thawed semen when insemination occurs close to or upon the oviductal papilla. Both deep-horn and hysteroscopic insemination of mares using low doses of sperm have been recently studied. Although hysteroscopic insemination was originally designed to improve the pregnancy rates of subfertile stallions [17], the technique has not appeared to significantly improve fertility of many subfertile stallions [18]. Regardless, both methods of deep-horn insemination are gaining popularity in stallions that breed many mares.

Pregnancy rates after hysteroscopic or deep-horn insemination with low numbers of frozen–thawed semen have varied widely. Lindsey et al. [19] reported a 37.5% pregnancy rate in mares bred with 15×10^6 motile frozen–thawed sperm inseminated via hysteroscopy at the UTJ, whereas Morris et al. [14] reported a higher pregnancy rate of 64.3% (9/14) following insemination of a dose of 14×10^6 motile, frozen–thawed spermatozoa. She obtained the same pregnancy rate when mares were bred with 14×10^6 motile, frozen–thawed sperm in the uterine body (66.7%; 8/12). Petersen et al. [20] reported a 64% (7/11) pregnancy rate in mares bred with 50×10^6 frozen–thawed progressively motile spermatozoa at 12 and 24 h post hCG by the deep-horn insemination technique. When the same mares were bred with 500×10^6 cooled progressively motile spermatozoa in the uterine body, only 4/11 mares became pregnant (37%). These results

suggest that pregnancy rates may be higher with low-dose insemination when the semen is placed in the uterine horn or at least, closer to the UTJ. In contrast, Sieme et al. [21] did not find a significant difference between insemination techniques (uterine body versus deep intracornual versus hysteroscopic) when reproductively normal mares were bred with 100×10^6 frozen–thawed total sperm (48.6, 48.0, and 48.4% per-cycle pregnancy rates, respectively). It is noteworthy that apparent differences among studies may have been due to inherent fertility of the relatively few stallions used.

6. Future direction of study

Current research is focused on both stallion management and molecular reproduction. The ability to predict, assess, and enhance fertility of frozen semen is challenging because there are no reliable and repeatable means of predicting fertility of frozen–thawed stallion semen. Assessing fertility of frozen semen from pregnancy data can be misleading because of the multitude of confounding variables when a small sample size is studied [22]. Although post-thaw motility appears to have little correlation with fertility [23–25], it is commonly measured, as it is a rapid “stall-side” test. A battery of tests performed on the frozen–thawed semen appears to provide the best data for predicting fertility. Graham [26] reported that motility, membrane integrity, morphology and energy metabolism were the most important attributes of fertilizing capacity of sperm. Hence evaluation of fertility status of a frozen semen sample includes evaluation of motility, morphology, plasma membrane integrity, response to osmotic stress, and sperm chromatin denaturation. Often, however, results of studies that test some of these parameters in the laboratory are not only contradictory with respect to a single parameter and its relationship to fertility, but they also show conflicting results when attempting to establish correlation between the parameters themselves. As an example of such disparity, Jasko et al., in two separate studies [27,28], reported a positive correlation between morphology of fresh spermatozoa and fertility. In contrast, after examining frozen–thawed sperm from 27 stallions, Kuusma et al. [23] failed to find a correlation between morphology and foaling rate. Results from these studies maybe somewhat difficult to compare, for data from the latter study was derived from stallions that had already been selected for commercial use in Sweden, and the study examined frozen–thawed sperm. Regardless, it is important to consider that a high percentage of

morphologically normal sperm, at least those that have undergone cryopreservation, may not be as highly correlated with fertility as once thought. Brinsko et al. [29] reported that there was no correlation between morphology and percentage of membrane-damaged sperm in sexually rested stallions. Although this study did not perform fertility trials, the lack of correlation may further offer further explanation for the difference in results between Jasko et al. [27,28] and Kuusma et al. [23].

Results of other *in vitro* tests suggest that there is a relationship between fertility and specific semen parameters. Certain parameters measured by fluorescent labeling and flow cytometry, such as DNA fragmentation [30], and plasma membrane integrity [23], have been shown to be correlated with stallion fertility. The sperm chromatin structure assay has been reported to be a particularly valuable test in assessing and predicting stallion fertility, for there appears to be a correlation between fertility and the intrinsic susceptibility of stallion sperm to chromatin denaturation [30]. Additionally, the use of specific molecular probes designed to test for “fertility factors” both within the spermatozoa themselves and the seminal plasma, also have promise in evaluating predicting stallion fertility. For example, Gradil et al. [31] identified a specific sperm phospholipase believed to be associated with initial embryo development; expression of this molecule was reduced in subfertile stallions. Finally, since the damage to sperm function measured by these *in vitro* tests is likely a result of oxidative reactions, investigation into measurements of, as well as protection from, oxidative stress associated with freezing and thawing semen, represents another promising area of research [32].

With regards to management of stallions, fertility of frozen semen may be affected by diet, season and exercise. Fertility of stallion semen may be enhanced through diet. Several preliminary studies [33,34] reported that feeding a nutraceutical compound rich in omega-3 fatty acids increased sperm production, motility characteristics, morphology, and most importantly, fertility of some stallions. These studies suggested that ingestion of omega-3 fatty acids may alter the cholesterol:phospholipid ratio of the sperm plasma membrane [35]. Manipulation of this ratio may render greater fluidity of the membrane and more resilience to oxidative stress. *In vitro* research supports this hypothesis; loading spermatozoa with cholesterol-dextrin appeared to increase motility and viability of both jack [36] and stallion cryopreserved semen [37]. Other feed supplements suspected to enhance the antioxidant characteristics of sperm cells are under

investigation, particularly feeding compounds rich in antioxidants, such as Vitamin C, selenium, and ergothionine.

Seasonal variability and the effect of training may affect fertility of frozen–thawed semen. Recent publications have demonstrated that there are seasonal differences in quality of frozen stallion semen, suggesting that the highest number of progressively motile sperm can be obtained from stallions in the autumn [38–40]. Certain training techniques and exercise levels appear to be detrimental to the freezability of stallion semen [41], perhaps as a result of hyperthermic injury to the spermatogenic cells [42].

7. Conclusion

The primary goal in a frozen semen program is to determine the most efficient use of semen without compromising pregnancy rates. Techniques used must be cost-effective. Stallion owners continue to seek noninvasive and practical treatment of the horse, perhaps through feed additives, that will enhance sperm parameters and ultimately, fertility and output. Current research in the equine frozen semen industry is aimed at determining the minimal insemination dose needed for maximizing pregnancy rates, for both the individual stallion and for the industry as a whole. Maximizing fertility of spermatozoa, including epididymal sperm [43], through manipulation of preservation techniques and changes in diet and exercise, will continue to be evaluated. Finally, the ongoing quest to reliably predict fertility remains at the forefront of future study.

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