

Insemination doses: How low can we go?

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Abstract

This manuscript presents a brief historical review of investigations related to equine artificial insemination. The origin of recommended insemination doses for use fresh, cooled and frozen semen will be reviewed. Over 30 years ago, an insemination dose of 500×10^6 progressively motile sperm (PMS) was recommended to maximize pregnancy rates when mares were bred with fresh semen under less than ideal conditions. Since that time, 500×10^6 progressively motile sperm has been almost universally accepted as a standard insemination dose, regardless of a stallion's fertility or the refinements that have been made in mare management and semen extenders. Insemination doses for cooled-transported and frozen-thawed semen have also been extrapolated from this dose. Data from a number of studies will be presented which demonstrate the feasibility and rationale of reducing sperm numbers used to breed mares with fresh, cooled and frozen-thawed semen, including the use of deep-horn insemination techniques.

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1. Introduction

Recent advances in semen processing and insemination techniques have permitted successful breeding with semen doses several orders of magnitude lower than those in conventional use. While these techniques have stimulated a large amount of interest, they are primarily employed in research settings or special clinical circumstances. In general, they are more time consuming and labor intensive, often requiring meticulous attention to detail, and as such are not yet practical for the average breeder. However, it appears that even using conventional techniques, breeding mares with insemination doses lower than those commonly recommended can result in acceptable pregnancy rates.

2. Conventional insemination techniques

2.1. Fresh semen

Time-honored insemination doses recommended to achieve satisfactory pregnancy rates in mares have been in the range of $250\text{--}500 \times 10^6$ progressively motile sperm (PMS), with 500×10^6 sperm being the dose most commonly recommended. The origin of these recommendations however is somewhat nebulous. Most of the early work regarding equine artificial insemination, published by Russian and Eastern European workers in the 1930s–1950s, focused on extender composition, with little if any regard given to sperm numbers. The studies of Pickett and Voss [1], Pickett et al. [2] and Householder et al. [3] are most often cited to endorse the use of 500×10^6 progressively motile sperm. Yet, when one reads these manuscripts, it is apparent that sperm number was only one of several facets examined and that the determination of 500×10^6 PMS as being the optimal insemination

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dose was often confounded by multiple inseminations and the use of inferior extenders. The methods of the time also resulted in less than optimal hCG timing, based on day of estrus rather than size of dominant follicle, and pregnancy determinations were not performed until 50 d post insemination. Other work from this laboratory, conducted during this same time period, demonstrated that while an insemination dose of 50×10^6 PMS significantly reduced fertility, when using semen from fertile stallions under ideal conditions, insemination doses of 100×10^6 PMS would not reduce fertility [4–6]. Kenney et al. [7] also reported that they consistently established pregnancy when 100×10^6 PMS was used as an insemination dose from certain stallions. In fact, more than 10 years earlier, the Chinese reported that they had established a very successful equine artificial insemination program involving 40 stallions and thousands of mares, and they found that the insemination dose could be reduced from 400×10^6 to 100×10^6 PMS when diluted in powdered milk-based extenders [8]. The other essential finding in this report was that pregnancy rates were optimized when mares were inseminated close to ovulation. Unfortunately, it appears that western nations were slow to recognize and accept these findings and despite these results, a dose of 500×10^6 progressively motile sperm continues to be most commonly recommended and employed in the equine breeding industry, even under ideal conditions.

In 1975, Kenney et al. [7] published the formulation of a non-fat dried milk solids-glucose extender (NFDMS-Gluc). This ‘Kenney extender’ as it is known, revolutionized equine artificial insemination in the western world. Once a convenient, reliable semen extender became available, the use of artificial insemination in horses increased worldwide. The effect of insemination dose could now be compared without the confounding effects of inferior extender composition. However, while ample anecdotal information has been generated from breeding farms using artificial insemination doses ranging from 200 to 400 million progressively motile sperm, controlled studies designed to determine a minimum effective insemination dose are lacking. Surprisingly, it was not until 1997 that such a study was published in a major English language journal [9]. In that study, 75% (21/28) of mares became pregnant when bred with 300×10^6 progressively motile sperm compared with 64% (23/36) mares becoming pregnant when bred with 500×10^6 progressively motile sperm ($P = 0.34$). Unfortunately, the authors did not investigate lower insemination doses, probably because the study was performed at a

commercial breeding operation. However, it is clear from this study and data from more recent studies where insemination doses of 20×10^6 PMS [10], 50×10^6 PMS [11] or 100×10^6 PMS [12] from fertile stallions did not reduce fertility, that an insemination dose of 500×10^6 progressively motile sperm is not necessary to achieve acceptable pregnancy rates. So where did this recommendation originate and why does it persist? In all likelihood, summation of the cumulative efforts of Colorado workers is the main source [13]. Based on numerous studies from their laboratory, Pickett et al. created a graph representing the theoretical relationship between sperm number and pregnancy rate [13]. This graph presents a dramatic increase in pregnancy rate from 100×10^6 sperm per inseminate to 500×10^6 sperm per inseminate, after which the slope of the pregnancy rate curve slightly declines relative to 1×10^9 sperm per inseminate. The authors suggested that these pregnancy rates were what would be expected on average breeding farms at the time. The wide-ranging interpretation of this theoretical graph was that for the average breeding farm, pregnancy rates are maximized by using insemination doses of 500×10^6 progressively motile sperm and that increasing the dose does not improve fertility. Hence, an insemination dose of 500×10^6 progressively motile sperm became dogma and even into the present day is rarely challenged. Pickett et al. pointed out that when conditions are less than optimal, factors such as inferior extenders and poor management, are additive in depressing pregnancy rates when insemination doses lower than 500×10^6 progressively motile sperm are used [13]. One would expect that, since that time, improvements in semen extenders and mare management would allow for lower insemination doses to be used successfully on the average breeding farm. This is evidenced by acceptable pregnancy rates being obtained with the widespread use of cooled-transported and frozen-thawed semen where insemination doses are commonly much lower than 500×10^6 progressively motile sperm.

2.2. Cooled-transported semen

The use of cooled-transported equine semen continues to gain popularity among breeders. Coincident with this widespread use is a tremendous disparity in success rates. Much of this variation can be attributed to differences in the inherent fertility of the mares and stallions or in the ability of the semen from certain stallions to survive the rigors of cooling, storage and transport. In many practice situations, the ability of the

inseminator to critically evaluate cooled-transported semen before or after insemination is precluded by the lack of convenient laboratory facilities. Therefore, the quality of the inseminate, particularly the number of progressively motile sperm inseminated, is often unknown. However, under favorable conditions when fertile mares are bred with good quality semen, pregnancy rates using cooled-transported semen are equivalent to those obtained with fresh semen. As such, the most commonly recommended insemination dose for cooled-transported semen mimics those that of fresh semen, i.e., 500×10^6 progressively motile sperm.

When packaging semen for cooling and transport, a dose of 1×10^9 PMS placed in the transport container is almost universally recommended. The origin of this recommendation goes back to Douglas-Hamilton et al.'s original study in which they used an early prototype of the EquitainerTM [14]. In that study, citing Pickett and Voss's report [1] that 500×10^6 was the optimum sperm number required for insemination, they assumed "a conservative 50% mortality in transit" and hence packaged $1.0\text{--}1.5 \times 10^9$ sperm per shipment. Since that time, advances in transport container design and function, semen extenders, semen dilution ratios and sperm concentrations have greatly improved sperm survival after cooling and storage; yet 1×10^9 PMS remains the most commonly recommended dose to package for shipment. The current industry standard for semen stored >12 h is to dilute semen containing 1×10^9 PMS at least 1:3 in a NFDMS-Gluc extender containing antibiotics, so that the final sperm concentration is $25\text{--}50 \times 10^6$ sperm/mL and to store semen at $4\text{--}6^\circ\text{C}$. Even with optimal processing and storage conditions, semen from some stallions demonstrates $\leq 30\%$ progressive sperm motility after cooling and pregnancies do occur. However, as with fresh semen, controlled studies have not been performed and the minimum insemination dose of cooled equine semen that will result in good pregnancy rates is unknown.

To investigate whether the total number of progressively motile sperm inseminated after being cooled and transported affects pregnancy rate, medical records were retrospectively examined from a group of mares presented for breeding with transported cooled semen at the Texas Veterinary Medical Center. Mares that were inseminated with <500 million (range 70–481 million) PMS had a lower per-cycle pregnancy rate (2/10; 20%) than mares inseminated with ≥ 500 million (range 500–2670 million) PMS (15/22; 68% per-cycle pregnancy rate). When progressive sperm motility was $<60\%$, pregnancy rate per cycle was only 48% (11/23). Pregnancy rate per cycle was 67% (15/22) when the

percentage of progressively motile sperm in the cooled semen was $\geq 60\%$ [Varner and Blanchard, unpublished data]. While this was not a controlled experiment (many different stallions and mares, breeding to ovulation time was not controlled, and insemination dose was not controlled), it indicates that the number and quality of sperm present in an insemination dose of cooled equine semen are critical factors impacting fertility. Interpretation of these data suggests that when breeding with transported cooled semen, prudence dictates a minimum of 500 million progressively motile sperm be inseminated as a starting point, unless successful testing has been conducted with lower doses. Perhaps if the data could have been further stratified, lower doses of PMS may have been found to result in acceptable pregnancy rates. Data such as this should also provide impetus for evaluating the quality of cooled semen used to inseminate each mare.

Before cooled semen is to be offered on a commercial basis, it is important to perform a test cool on the semen to ensure that it is suitable for this purpose. For some stallions, even when optimal semen handling techniques are employed (reviewed by [15]), insufficient numbers of their sperm cells survive the cooling and storage process to provide an adequate number of motile sperm for insemination. Since sperm viability decreases with storage time and the effect varies with stallion, the minimum dose of progressively motile sperm necessary to achieve satisfactory pregnancy rates for a given stallion should be tested. This would allow more efficient use of semen for stallions whose sperm motility is only slightly ($<30\%$) reduced after cooling and storage, as well as indicate the need to increase the packaged dose for stallions whose semen has poor tolerance ($>50\%$ reduction in motility) to cooling and storage.

Semen shipments often arrive containing two insemination doses. When the question of whether breeding with a single dose of cooled semen after 24 h of storage, a double dose of semen after 24 h of storage or a dose after 24 h and a dose after 48 h of storage affected pregnancy rates, fertility trials done at two different laboratories resulted in disparate results. One study found no difference in pregnancy rate [16], whereas the other found an improvement in pregnancy rate with the two-dose, 2-d method [17]. The study that showed an advantage to breeding with a full dose after 24 h and a full dose after 48 h of storage, packaged 1×10^9 PMS/dose and used three stallions of varying fertility [17]. However, the number of mares bred among stallions and treatments was not balanced, which resulted in one stallion being responsible for the overall

difference in pregnancy rate. Unfortunately, sperm motility after cooling was not evaluated in that study so the actual insemination dose of PMS among treatments and stallions cannot be determined. The other study [16] used one fertile stallion and packaged 500×10^6 PMS to breed 18 mares with the whole dose 24 h later and 18 mares with half the dose at 24 h followed by half the dose at 48 h. Post-cooling sperm motility was evaluated in this study and pregnancy rates were identical (12/18; 67%) when mares were bred with 298×10^6 PMS after 24 h of cooling or with 150×10^6 PMS after 24 h of cooling followed by 125×10^6 PMS after 48 h of cooling. A noticeable stallion effect was apparent with these studies, emphasizing that it is important to consider the quality of semen after cooling and the inherent fertility of the stallion when deciding how to best utilize the semen when two doses are sent. It is also clear that an insemination dose of 500×10^6 PMS is not necessary to achieve acceptable pregnancy rates when mares are bred with good quality cooled semen. If the semen quality is good, a single insemination dose on the day of arrival should be sufficient. If sperm motility is poor on arrival, motility will be even lower the following day. In such cases, putting both doses in the mare at the same time will help maximize the number of motile sperm in the insemination dose. This may provide the best chance for obtaining a pregnancy on that cycle, especially if a second semen shipment cannot be expedited to arrive prior to ovulation.

2.3. Frozen-thawed semen

The processes of cryopreservation and thawing exposes sperm to a number of potentially injurious stresses which are most obviously manifested as a dramatic reduction in post-thaw motility (reviewed by [18]). However, even sperm that remain motile after the freeze-thaw process may have suffered sublethal cryodamage, which could adversely affect fertility. In general, pregnancy rates obtained with frozen-thawed equine semen are lower than those obtained with fresh or cooled semen, even when similar numbers of motile sperm are inseminated. Despite this, recommended insemination doses for breeding with frozen-thawed semen usually contain lower numbers of motile sperm than those recommended for fresh or cooled semen.

When examining early studies utilizing frozen-thawed semen, comparison of pregnancy rates based on insemination dose is problematic. Numbers of total sperm inseminated rather than the number of motile sperm inseminated were commonly reported. As with early studies involving fresh and cooled semen, fertility

results were also confounded by numerous factors including freeze-thaw methods, insemination timing and frequency, as well as the use of inferior extenders, especially those containing contraceptive levels of glycerol. For example, Pace and Sullivan [19] reported that increasing the insemination dose from 40×10^6 motile sperm to 80×10^6 motile sperm improved foaling rate but increasing the number to 160×10^6 motile sperm did not result in further improvement. The extender used contained 7% glycerol and the authors stated that the foaling rate for all treatment groups ($\leq 35\%$) was suboptimal. Using an improved cryopreservation procedures, Volkmann and vanZyl [20] were able to achieve a 44% per-cycle pregnancy rate with $137\text{--}210 \times 10^6$ PMS after thawing, but the pregnancy rate increased to 73% per cycle when mares were inseminated with $>220 \times 10^6$ PMS post-thaw (range, $222\text{--}333 \times 10^6$ PMS). Morris et al. [21] were able to impregnate 8 of 12 mares inseminated in the uterine body with 14×10^6 motile, frozen-thawed sperm from a highly fertile stallion.

Post-thaw sperm motility is quite variable among stallions and even among different ejaculates from the same stallion. Generally, only ejaculates demonstrating $\geq 30\%$ post-thaw sperm motility are selected for insemination. While 0.5 mL straws are most commonly used, the number of sperm packaged per straw as well as the recommended number of straws per insemination varies among laboratories. As such, there is no standard insemination dose based on numbers of progressively motile sperm for frozen-thawed equine semen and many recent reports describe insemination doses based on total numbers of sperm. In an effort to address this, standards are being recommended in Europe that can be found on The World Breeding Federation of Sport Horses website (<http://www.wbfs.org>).

The 4th International Symposium on Stallion Reproduction provided insights on breeding with frozen semen from several commercial breeding operations. Although data generated from commercial breeding operations may lack the stringent controls that are desired in academic settings, they may, in fact, provide more useful information to the practitioner because of the large number and variety of mares and stallions involved. For example, based on data obtained from breeding records of 46 stallions and 90 mares bred over 312 cycles, Metcalf [22] compared pregnancy rates of mares grouped by the total number of progressively motile sperm inseminated per cycle. Pregnancy rates for mares inseminated with $<200 \times 10^6$, $200\text{--}400 \times 10^6$, and $400\text{--}600 \times 10^6$ PMS ranged from 54.5 to 60% and were lower than those for mares inseminated with 600--

800×10^6 PMS (88.2%; $P < 0.02$). Barbacini et al. [23] found no difference in per-cycle pregnancy rate for mares that were inseminated 24 and 40 h after hCG with 400×10^6 total sperm (46%; 129/280) compared with those inseminated once postovulation with 800×10^6 total sperm (47%; 120/255). The most extensive data set was presented by Vidament [24] who scrutinized records from 20 years of breeding with frozen semen from the French National Stud encompassing hundreds of stallions and thousands of mares bred at numerous artificial insemination centers. She reported that when post-thaw motility was $<45\%$, pregnancy rate per cycle was 43%, whereas when post-thaw motility was $>45\%$, the per-cycle pregnancy rate was 52%. Data analyses resulted in recommendations that included selecting ejaculates that exhibit $>30\text{--}35\%$ post-thaw motility and inseminating mares twice with 400×10^6 sperm, 24 h apart before ovulation. Loomis and Squires [25] also recently summarized data on frozen semen in a commercial setting for the 2003 breeding season. Insemination doses generally contained $800 \times 10^6\text{--}1 \times 10^9$ total sperm exhibiting at least 30% progressive motility and doses reportedly contained from 240 to 600×10^6 PMS. First cycle pregnancy rate was 58.1% (126/217) with an overall per-cycle pregnancy rate of 52.7%. Pregnancy rate did not differ for mares bred once postovulation or multiple times pre- and post-ovulation. Another commercial operation in Germany [26] reported that when post-thaw motility was $>35\%$, pregnancy rates did not differ when mares were inseminated once prior to ovulation, 30 h after hCG with either 100×10^6 or 800×10^6 total frozen-thawed sperm. In the study by Morris et al., 9/14 mares became pregnant with approximately 14×10^6 PMS inseminated hysteroscopically at the uterotubal junction (UTJ), 30–32 after hCG administration [21]. Based on results such as these, some semen freezing centers in Europe are advocating deep-horn insemination and the use of a single 0.5 mL straw per insemination dose.

2.4. Deep-horn insemination

In 1998, two reports describing hysteroscopic insemination with low numbers of sperm at the uterotubal junction stimulated resurgence in the use of deep-horn insemination techniques for the mare [27,28]. This subsequently resulted in numerous publications from several laboratories. The term ‘resurgence’ is used because while this technique may seem to be novel approach, deep-horn insemination for routine equine artificial insemination was reported by Russian workers in the 1930s [29]. The

difference is that current methods are aimed at inseminating ultra-low numbers of sperm in small volumes and have also incorporated newer technology, i.e., hysteroscopy for fresh, cooled and frozen-thawed semen. Comprehensive summaries of these studies have recently been published [30–32].

Development of these techniques was primarily motivated by the desire to increase insemination efficiency with low numbers of sperm when using frozen semen, sex-sorted semen, semen from subfertile stallions, or epididymal sperm. It is theorized that by depositing semen higher in the reproductive tract, a greater proportion of sperm would survive to colonize the oviduct and therefore fewer sperm would be necessary to achieve the same probability of fertility than with conventional artificial insemination [18]. This is most evident in sheep where in order to achieve similar fertility, cervical insemination requires an insemination dose 10 times higher than that used for uterine horn inseminations [33]. While the cervix of the mare does not impose the same anatomical and functional restrictions to artificial insemination as does that of the ewe, deposition of semen on or near the UTJ ipsilateral to the ovulatory follicle should allow for much lower insemination doses to be used successfully. Rigby et al. [34] demonstrated that depositing semen in the tip of the uterine horn, ipsilateral to the dominant follicle, resulted in a greater proportion of sperm being recovered from the ipsilateral oviduct (77% of recovered sperm), than when sperm were deposited in the uterine body (54% of recovered sperm). That study also demonstrated that following insemination of 500×10^6 sperm, an average of only 0.0007% of the inseminated sperm appeared to inhabit the oviducts. Morris et al. [35] were able to achieve pregnancy rates of 60–75% with hysteroscopic insemination of fresh semen when 1×10^6 , 5×10^6 or 10×10^6 motile sperm were deposited at the UTJ ipsilateral to ovulation. Pregnancy rates declined to $<30\%$ when 0.5 or 0.1×10^6 motile sperm were inseminated. However, 1 of 10 mares did get pregnant when only 1000 motile sperm were inseminated with this technique.

Insemination of frozen-thawed equine semen would appear to readily lend itself to these techniques since sperm are typically packaged at $100\text{--}200 \times 10^6$ sperm/mL in 0.5 mL straws. Semen from a single 0.5 mL straw could be deposited on or near the uterotubal junction. However, several studies have provided mixed results with uterine body inseminations resulting in pregnancy rates as good if not better than those obtained by using equal or even higher sperm numbers deposited at the UTJ (reviewed by [30–32]). In a study involving a large

number of commercial mares, Alvarenga et al. [36] inseminated $50\text{--}75 \times 10^6$ frozen-thawed sperm hysteroscopically at the UTJ; 57.2% (95/166) of mares became pregnant. In another study, hysteroscopic insemination of 14×10^6 motile, frozen-thawed sperm at the UTJ resulted in a pregnancy rate of 64.3% (9/14) which did not differ from results with conventional insemination (66.7%; 8/12) using the same number of frozen-thawed sperm [21]. However, in that same study, when the hysteroscopically inseminated dose was reduced to 3×10^6 motile, frozen-thawed sperm, deposition of semen at the UTJ showed a distinct advantage over uterine body deposition (16/34 versus 2/14 pregnant, respectively).

Initial studies involving low-dose, deep-horn insemination techniques were performed hysteroscopically. Use of a transrectally guided technique however, greatly reduces the time and expense required for low-dose insemination compared to using hysteroscopy. As with the hysteroscopic technique, results of different studies have varied widely and pregnancy rates have ranged from 0 to 64% (reviewed by [31]). Lindsey et al. [37] used transrectal ultrasonography to verify placement of the pipette near the tip of the uterine horn; 0 of 10 mares became pregnant following deposition of 5 million sperm from a fertile stallion, compared with a 50% pregnancy rate using hysteroscopic insemination. Based on these results and those of others, investigators from that laboratory have been adamant that hysteroscopy should be used when insemination doses contain fewer than $5\text{--}20 \times 10^6$ progressively motile sperm [38]. However, Brinsko et al. [39] reported that when placement of the insemination pipette was assisted and confirmed by transrectal palpation, deposition of 200 μL of cooled semen containing $3.3\text{--}3.6 \times 10^6$ PMS resulted in pregnancy rates (10/18; 56%) that were similar to hysteroscopic insemination (12/18; 67%). The disparity in pregnancy rates obtained between these two studies is difficult to explain, but could be related to differences in semen quality, mare populations, or the skill of the inseminators. Although some practice is required, smooth passage of the pipette up the uterine horn to the UTJ can readily be mastered. This technique also has applications with frozen-thawed semen as evidenced by the study of Petersen et al. [40] where embryos were recovered from 7 of 11 mares (63.6%) bred with 50×10^6 frozen-thawed sperm deposited at the tip of the uterine horn with the pipette being directed per rectum.

Vazquez et al. stated that the purpose of their low-dose hysteroscopic insemination study was to develop a management tool for subfertile stallions [27]. Since that

time, others have reported that deep-horn insemination techniques have failed to improve pregnancy rates of sub fertile stallions, particularly those with concomitant oligospermia and poor sperm morphology ([30,31], Blanchard unpublished data). However, application of these techniques to several subfertile stallions presented to our laboratory with low numbers of normal sperm has yielded very promising results [Varner unpublished data]. Varner conducted breeding trials with two stallions having per-cycle pregnancy rates of $<20\%$ and showed improvement in pregnancy rate after their semen was centrifuged through a discontinuous density gradient (EquiPureTM) to increase the percentage of progressively motile, morphologically normal sperm. Hysteroscopic insemination with 100 μL of extended semen containing 20×10^6 PMS increased the pregnancy rate from 20 to 35% (7/20) for one stallion and transrectally guided deep-horn insemination of $60\text{--}150 \times 10^6$ PMS in 250 μL resulted in seven of eight mares (87.5%) becoming pregnant for the other horse. Varner recently instituted these techniques on a breeding farm for two stallions that historically had per-cycle pregnancy rates of $<40\%$, one of which had recently declined to $<20\%$. Both stallions produced dilute ($<100 \times 10^6$ sperm/mL) ejaculates containing $<1 \times 10^9$ progressively motile, morphologically normal sperm. Ejaculates were centrifuged to concentrate sperm, but a density gradient was not used. Mares were inseminated at the tip of the uterine horn using the transrectally guided technique with 1 mL of fresh extended semen containing an average of $231\text{--}310 \times 10^6$ PMS. Using these methods on the farm, pregnancy rates increased to 52% per cycle (58/112) for one stallion and to 62.4% (78/125) for the other stallion.

3. Conclusions

Although the time-tested insemination dose of 500×10^6 PMS has served us well over the years for both fresh and cooled semen, improvements in extender composition and mare management should allow conventional doses to be reduced to at least 100×10^6 PMS for fertile stallions bred to fertile mares under good management. However, when conditions are less than ideal, e.g. subfertile stallions, subfertile mares or poor management, it would seem prudent to follow the earlier recommendations for using higher sperm numbers [13]. Low-dose deep-horn insemination techniques can and are being used by practitioners for fresh, cooled and frozen semen and it appears that the threshold dose to achieve acceptable pregnancy rates is at least 1×10^6 progressively motile

sperm even for highly fertile stallions. Employment of these methods in the management of subfertile stallions has been met with mixed success but appears to show promise in selected cases. Reducing the insemination dose can improve the efficient use of semen but the level of reduction that still results in acceptable fertility varies among stallions. Only by breeding sufficient numbers of mares with reduced sperm numbers and/or alternative insemination techniques will we know how low we can go with regards to an insemination dose for any particular stallion.

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