Insemination Results with Slow-Cooled Stallion Semen Stored for approximately 40 Hours

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Heiskanen, M.-L., M. Huhtinen, A. Pirhonen and P. H. Mäenpää: Insemination results with slow-cooled stallion semen stored for approximately 40 hours. Acta vet. scand. 1994, 35, 257-262. – Semen from 3 stallions was extended using 2 methods (Kenney extender and a modified Kenney extender), slowly cooled, and stored for 41 ± 6 (s.d.) h before insemination. An insemination dose (40 ml) contained 1.5 - 2 billion spermatozoa. In the experiment, 26 mares were inseminated in 30 cycles. The pregnancy rate per cycle obtained with sperm stored in the Kenney extender was 87% (n=15). When the semen was extended with the modified extender, centrifuged and stored, the pregnancy rate was 60% (n=15). Inseminations were done every other day until ovulation was detected. If a mare ovulated more than 24 h after the last insemination, she was inseminated also after ovulation. The single-cycle pregnancy rate was 58% when the mares were inseminated only before ovulation (n=19) but the rate was 100% when the inseminations were done both before and after ovulation (n=9) or only after ovulation (n=2). The difference in pregnancy rates was significant (p<0.05), indicating that postovulatory inseminations probably serve to ensure the pregnancies. The extending and handling methods used in this study resulted in a combined pregnancy rate of 73%, and appear thus to be useful for storing stallion semen for approximately 2 days.

artificial insemination; horse.

Introduction

Transport of slowly cooled stallion semen is becoming popular throughout the world because pregnancy rates with frozen semen are often rather low. The longer stallion semen can be stored and its fertility retained, the more flexibility it offers to the breeder for semen transport. However, the pregnancy rate of equine semen stored for 24 h or longer has been evaluated in only a few studies so far (*Douglas-Hamilton et al.* 1984, *van der Holst* 1984, *de Vries* 1987, *Heiskanen et al.* 1987, *Francl et al.* 1987, *Zidane et al.* 1991). The objective of this study was to investigate the pregnancy rate using sperm diluted with 2 different methods and stored for about 40 h.

Materials and methods

Three stallions, a Finnhorse, a Standardbred, and a warmblooded riding horse with no previous problems in fertility, were used in our breeding trial at the Equine Research Station in Ypäjä. Three sperm-rich fractions from each ejaculate were collected with an open-ended vagina[a] every other day in May. The semen was extended with the Kenney extender (*Kenney et al.* 1975) or with the Kenney ex-

Group 1. Inseminations before ovulation

Group 2. Inseminations before and after ovulation

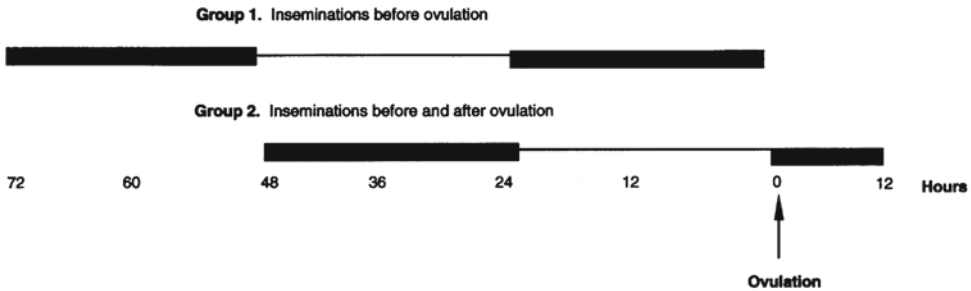| 72 | 60 | 48 | 36 | 24 | 12 | 0 | 12 | **Hours** |

Ovulation

Figure 1. Schematic presentation of the inseminations in relation to ovulation.

tender supplemented with 10 mM theophylline and 10 mM Hepes, pH 7.2 (modified extender) (*Heiskanen et al.* 1987). With this modified extender, the semen was first diluted, 1:1, thereafter concentrated by centrifugation for 3 min (700 x g), and resuspended with the extender. The total volume of the insemination dose with both methods was 40 ml, containing 1.5 to 2 billion spermatozoa. Sperm concentration of the ejaculate was estimated with a Spermfotometer[b] and sperm density in the final dilution with a Bürker haemocytometer. Motility was evaluated microscopically in a Makler chamber[c]. The semen was slowly cooled in syringes using a Retainer[d] for 24 h and then stored at 5 to 7°C in a refrigerator for about 2 days. Before insemination, the diluted semen was pre-warmed for 15 min at +37°C to prevent possible damage caused by cold semen (+ 5°C) in the uterus (Vanha-Perttula, personal communication). During the breeding season of 1991, 26 fertile mares of different breeds, aged 3-15 years, were inseminated during 30 estrous cycles. The mares' ovaries were palpated and monitored by ultrasonography every other day of estrus until a preovulatory follicle reached 35 mm in diameter, and thereafter every 12 h until ovulation was detected.

The mares were inseminated every other day until detected ovulation. The mares were divided into 2 groups according to the timing of the inseminations (Fig. 1). If the mare ovulated less than 24 h from the last insemination, she was not inseminated again (Group 1; 19 estrous cycles). If ovulation occurred more than 24 h from the last insemination (Group 2; 9+2), the insemination was repeated after ovulation (9 estrous cycles). In addition, 2 mares were inseminated only after ovulation due to their short estrus (2 estrous cycles). The mares were inseminated on the average 1.9 times per cycle. Both extenders were used in the two insemination groups.

The mares were scanned for pregnancy by ultrasound on Days 16, 23 and 43 after ovulation. Eight of the 22 pregnancies were terminated by a prostaglandin injection 16 days after the insemination, since the mares were to be used in other experiments.

Statistical analysis

The results are presented in tables as arithmetic means and standard deviations (s.d.). The differences between the pregnancy rates of

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[a]Model Krakow-72, [b]LeoDiagnostiks Ab, Helsingborg, Sweden, [c]Sefi Medical Instruments, Haifa, Israel, [d]The Hamilton System, MA, USA.

Table 1. Spermatozoal numbers and motility characteristics of the 2 different methods after extending and storage (40 h) (mean ± s.d.).

Insemination dose	Kenney extender	Modified extender
Total number (x 10^6) of spermatozoa	1925 ± 875	1474 ± 613
Percentage (%) of progressively motile spermatozoa	19 ± 10	33 ± 13[a]
Number (x 10^6) of progressively motile spermatozoa	401 ± 339	436 ± 207

a) Statistically significantly different from the Kenney extender ($p<0.001$).

the extending methods were compared using the chi-square test or Fisher's test. The differences in the mean number and percentage of spermatozoa in the 2 types of extenders and in the pregnant and nonpregnant groups were tested statistically by Student's t-test. The Mann-Whitney test was used to compare the semen storage times between Groups 1 and 2.

Results
Insemination doses
The initial percent of progressively motile spermatozoa after dilution was 45 ± 12%, the volume of the insemination dose was 40 ml and the mean concentration of spermatozoa 44 ± 19 million/ml. The dilution ratio (volume of semen to volume of semen + extender) was 38 ± 11% in the insemination doses extended with the Kenney extender. The volume of seminal plasma was minimal when the extended semen was centrifuged and the sperm pellet was resuspended with the modified Kenney extender.

The total number of spermatozoa was found to be higher in doses extended with the Kenney extender than in doses extended with the modified extender (Table 1). This was due to the centrifugation step included in the modified extender method, in which about 25 % of the sperm were lost. In contrast, the percentage of progressively motile spermatozoa after storage was significantly higher using the modified extender ($p<0.001$) than using the Kenney extender. Thus, the numbers of progressively motile spermatozoa in the insemination doses were almost equal in both methods (Table 1).

Pregnancy rates
Between the extending methods (Table 2), a difference in pregnancy rates was only seen in Group 1, but it was not significant (Fisher's

Table 2. Pregnancy results with the Kenney extender and the modified extender.

Insemination in relation to ovulation	Storage time of semen (h) (x ± s.d.)	Pregnancy rate on day 16 / inseminated cycle (%)		
		Kenney extender	Modified extender	Both extenders
24-0 h before (Group 1)	43 ± 4	6/8 (75)	5/11 (45)	11/19 (58)
48-24 h before and/or (Group 2) 0-12 h after	42 ± 5 38 ± 7	7/7 (100)	4/4 (100)	11/11 (100)
	41 ± 6	13/15 (87)	9/15 (60)	22/30 (73)

Table 3. Spermatozoal numbers (mean ± s.d.) and motility characteristics in the pregnant and nonpregnant groups of mares.

Insemination dose	Pregnant (n=22)	Nonpregnant (n=8)
Total number (x 10^6) of spermatozoa	1751 ± 832	1500 ± 555
Percentage (%) of progressively motile spermatozoa	25 ± 12	29 ± 13
Number (x 10^6) of progressively motile spermatozoa	416 ± 305	421 ± 184

exact test). Further, no statistically significant differences in semen characteristics of insemination doses were observed between the pregnant and nonpregnant groups (Table 3). The single-cycle pregnancy rates and the percentage of progressively motile spermatozoa of the individual stallions are shown in Table 4.

A statistically significant difference in pregnancy results was obtained between the timing of inseminations in Groups 1 and 2. The pregnancy rate was 58% (n=19) when the mares were inseminated only before ovulation, but 100% (n=11) when the insemination-

tions were done both before and after ovulation or only after ovulation ($p<0.05$, χ^2-test) (Table 2). The storage time of the semen was 43 ± 4 h in Group 1. In Group 2, it was slightly shorter when insemination was repeated after ovulation (38 ±7) but the difference is not statistically significant. The mean storage time for all doses before insemination was 41 ± 6 h and an overall pregnancy rate of 73% was achieved 16 days after ovulation (Table 2). One embryonic loss was detected between days 16 and 23 in Group 1.

Discussion

The timing of the insemination in relation to ovulation is of great importance when cooled and transported semen is used (*Jasko et al.* 1991). This view is consistent with our experiment, in which the relatively high pregnancy rates obtained were probably due to a careful timing of the inseminations close to ovulation. A 100% pregnancy rate was achieved regardless of the extending method by repeating the insemination after ovulation with those mares inseminated more than 24 h before ovulation. *Pickett et al.* (1975) and Jasko et al. (1992) have demonstrated that dilution ratios smaller than 1 : 2 to 1 : 4 (semen : extender) decrease the number of motile spermatozoa after 24 h of cooling. In this study, the content

Table 4. Percentage of progressively motile spermatozoa (PMS) (mesn ± s.d.) and the single-cycle pregnancy rates of the different stallions on day 16.

Stallion	PMS initial (%)	PMS after storage (%)	Single-cycle pregnancy rate			
			Kenney extender	Modified extender	Total	(%)
Finnhorse	45 ± 5	21 ± 12	8/10	4/6	12/16	(75)
Warmblooded	49 ± 7	28 ± 12	4/4	1/4	5/8	(63)
Standardbred	38 ± 7	32 ± 16	1/1[a]	4/5	5/6	(83)

[a]One embryonic death (between days 16 and 23) is included in the pregnancy rate on day 16.

of semen (sperm + seminal plasma) was relatively high (about 1 : 2 to 1 : 3) in doses with the Kenney extender. This may partly be the cause of lower percentages of progressively motile spermatozoa in this extender compared to the modified extender where the seminal plasma was removed. In previous experiments we have found that supplementation of the extender with theophylline and Hepes increases the motility after storage (*Heiskanen et al.* 1987). However, the pregnancy rates with the Kenney extender were not statistically significantly different compared to the rates with the modified extender and centrifuged semen. A difference was seen in Group 1, in which the mares were inseminated only before ovulation and the number of inseminations with sperm stored in the modified extender was slightly higher. In Group 2, no differences were found between these methods.

Pre-warming of semen has not been recommended by *Douglas-Hamilton & Viale* (1984). However, our results indicate that warming of semen for 15 min at 37°C is not harmful. The recommended number of spermatozoa in insemination doses is 500 million progressively motile spermatozoa (*Pickett et al.* 1975), which means about one billion spermatozoa for a 24 h storage. In this study, we used 1.5 to 2 billion spermatozoa in the insemination doses, because it was assumed that the percentage of progressively motile spermatozoa varies between 20 and 30 % after storage.

The fact that the sperm of the 3 stallions maintained its viability and fertility for about 40h, offers the possibility to transport or store the semen for similar periods of time. In practice, our recommendation is to collect the first sperm-rich fractions of the ejaculate. The semen should then be immediately diluted and centrifuged if necessary. It seems to be important to inseminate mares close to ovulation to ensure a high pregnancy rate. To ascertain pregnancy, mares can be inseminated twice or, under frequent ovarian control, the insemination can be timed to be done only after ovulation.

We will need more information in the future about how long the extended stallion semen can be stored before insemination without major loss of its fertility capacity. Further, it is important to know how long the chilled sperm remains viable in the reproductive tract of the mare in order to reduce the number of laborious ovarian examinations.

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Sammanfattning

Inseminationsresultat av långsamt nerkyld hingstsperma, som lagrats ca 40 timmar.

Hingstsperman späddes med 2 vätskor (Kenney extender eller modified Kenney extender). De tre första spermierika fraktionerna av ejakulatet samlades upp med öppen vagina. Sperman späddes antingen omedelbart med "Kenney extender" eller centrifugerades och tillfördes "modified extender" före och efter centrifugeringen. Sperman kyldes med sk. "slow-cool method" och lagrades ca 40 timmar innan inseminationen. Sammanlagt insemineredes 26 ston i 30 brunster. Dräktighetsresultatet med "Kenney extender" var 87% (n=15) och med "modified extender" kombinerad med centrifugering 60% (n=15). Sista inseminationerna gjordes 24-0 timmar före eller 48-24 timmar före och 12 timmar efter ovulationen. När ston insemineredes endast före ovulationen, var dräktighetsresultatet 58% (n=19). I de fall ston insemineredes både före och efter ovulationen var resultatet 100% (n=11). Skillnaden var statistiskt signifikant (p<0.05). Undersökningen visar att i båda metoderna bibehålles spermans fertilitet och kan utnyttjas på hingstationerna. Centrifugering rekommenderas när spermiekoncentrationen är låg. Enligt våra undersökningsresultat bör stona insemineras strax efter ovulationen för att uppnå bästa dräktighetsresultat.