# Practical experience with artificial insemination (AI) using fresh chilled and frozen semen in mares

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## ORIGINAL ARTICLE

### ABSTRACT

The objective of this study was to compare the efficiency of artificial insemination (AI) carried out with frozen and fresh, diluted and chilled semen under field conditions. One hundred and twenty-nine mares of different breeds were included in the study. Eighty-one out of the 107 mares inseminated with fresh, chilled semen got pregnant. Seven pregnant mares aborted and 74 foals were born. Out of the 22 mares inseminated with frozen semen, 17 mares got pregnant. Two mares out of the 17 pregnant mares aborted and finally 15 healthy foals were born. No difference was found between the two groups in the ratio of the foals born ($P > 0.05$). The comparison of medians for the number of insemination cycles did not show significant differences. However, a significant difference (Kruskal–Wallis test, $P = 0.014$) was found in the number of the inseminations per conception in favour of frozen semen (2.5 vs. 1.8 with fresh chilled and frozen semen, respectively). The Cox regression revealed that the type of semen has a significant impact ($P < 0.001$) on the service period (duration of the insemination period): the use of frozen semen prolonged the insemination period. This could be due to management issues, since re-insemination with frozen semen took place after only one/a few missed oestrous cycles not used for AI.

## INTRODUCTION

One of the main goals of horse breeding is to get as many foals as possible from valuable parents within a short time. Research data indicate that only a few spermatozoa get into the ampulla of the oviduct where fertilisation takes place. Moreover, only a few spermatozoa take part in the fertilisation process, but only one of them enters and fertilises the oocytes. Based on this observation, between 200 and $500 \times 10^6$ spermatozoa are used for artificial insemination (AI) in the practice. Morris (2004) reported that pregnancies were obtained after AI with a much lower number of spermatozoa ($5 \times 10^6$). However, if the semen was deposited deep into the uterine horn, close to the opening of the oviduct, pregnancies were achieved with as few as $1 \times 10^6$ fresh spermatozoa. However, when frozen semen is used, the recommendation is to provide minimum $3 \times 10^6$ spermatozoa. Unfortunately, the spermatozoa of some stallions cannot be cryopreserved with a good survival rate. Minimum 35% of the spermatozoa must show progressive motility after thawing. Based on clinical observations, the semen of 30–50% of stallions has the appropriate number of motile spermatozoa after thawing (Scherzer et al., 2009; Aurich et al., 2016).

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The preparation of semen for AI is very important and may greatly increase or lower the success of fertilisation. According to Jasko et al. (1992), a 5–20% volume of seminal plasma is the best for the viability and motility of spermatozoa. The best method for seminal plasma removal is low speed centrifugation (Roach et al., 2016). Single layer centrifugation (SLC) has a positive effect on the fertilisation rate, because only spermatozoa with appropriate motility, morphology and an integrated chromatin stock can get through the special colloids. In an experiment, 69% of mares inseminated with semen prepared with SLC for AI became pregnant, compared to the control group in which only 45% pregnancy rate was obtained ($P < 0.05$; Morrell et al., 2014).

Cryopreservation greatly supports the application of AI in horse breeding, as in the case of other farm animal species (Fodor et al., 2018). However, during semen freezing the spermatozoa are exposed to oxidative stress which may decrease their fertilising capability. Numerous oxidative stress markers have been detected in spermatozoa, and their increase clearly proves the harmful effect of oxidative processes induced by cooling/freezing. Certain data of investigations indicate that these markers might be used to predict the cryopreservability/freezability of semen (Muñoz et al., 2016). The decreased fertilising ability of spermatozoa induced by oxidative stress is related to methylation activity, assuming that the otherwise low concentration will increase after thawing. Although the methylation level is significantly higher in frozen/thawed semen, the role of DNA methylation in decreased fertility is not entirely known (Aurich et al., 2016).

For the protection of spermatozoa against cryo-damage, cryoprotective substances are added to the spermatozoa (to the freezing solution) prior to cooling/freezing. For semen freezing, the most widely used cryoprotectant is glycerol. The concentration of glycerol is very important because the spermatozoa are very sensitive to it (glycerol may be toxic to them). Glycerol concentrations higher than 3.5% are toxic to spermatozoa (Fuller, 2004; Vidament, 2005; Carleton, 2011), but lower concentrations support the survival of sperm cells (Santiani et al., 2017). Because of their very intense motility, spermatozoa have a very intense metabolic activity which produces energy for the motility. Since spermatozoa rapidly use up the fructose (energy carrier) content of the seminal plasma, the collected semen must be diluted (extender must be added to the collected semen as soon as possible) in order to provide energy carriers (nutrients) for the spermatozoa. Many different extenders/diluents are used in the practice of chilled and frozen semen production in various species. Nowadays, in the horse, the most popular extender/diluent is Kenney's diluent, which contains skimmed milk and glucose. According to some research results, a diluent (Equi Pro) containing whey protein, sugars, casein and glycine better supports sperm motility than does skimmed milk. Phosphocasein also protects the morphology of spermatozoa and increases their longevity (Lagares et al., 2012).

The objective of this study was to compare the efficiency of AI carried out with frozen as well as with fresh, diluted and chilled semen in mares under field conditions.

## MATERIALS AND METHODS

### Animals

One hundred and twenty-nine mares of different breeds were included in the study. They were artificially inseminated in the breeding season. The youngest animal was 3 years old and the oldest was 26 years old (average age: 9.5 years). The mares have private owners and they were kept in different farms under different keeping and feeding conditions which represents the random environment background well.

The mares were subjected to a thorough clinical examination (vaginal and rectal examination, palpation, and ultrasound) before entering the AI study programme.

### Artificial insemination (AI)

One hundred and seven and 22 mares were inseminated with chilled and frozen semen, respectively. The fresh, diluted and chilled semen was stored at 4–5 °C prior to AI. In the case of AI with frozen semen the cryopreserved semen was thawed in a 38 °C water bath (10 sec). In order to avoid the damage of frozen and thawed spermatozoa, the catheter used for AI was kept at the same temperature on a heated/warming plate. The number of spermatozoa in the insemination dose in the case of both chilled and frozen-thawed AI was approx. $500 \times 10^6$/mL.

### Statistical analysis

Data on reproduction were analysed according to the following criteria:

(A) First we calculated the frequency of reproduction features (pregnant/non-pregnant ratio, ratio of fetal absorption and abortions, foal birth ratio), and then we made statistical comparisons according to the type of the insemination dose (cooled or frozen) with two-sided difference test of the ratios (simultaneously taking into account the ratio and the number of observations). Then, we compared the number of insemination cycles (in which the insemination was carried out) as well as the number of inseminations per conception by type of the insemination dose using Kruskal–Wallis median test. In order to determine the possible influence of stallions on the success of fertilisation, their distribution according to semen type was checked also by the chi-squared test. Exclusively chilled or only frozen semen was used from 54 and 7 stallions, respectively, and from a single stallion both types of semen were used.

(B) After that we tested how long it took to get a successful insemination (duration of insemination period, service period, SP). The length of the insemination period was evaluated by Cox regression, taking into account the following effect: type of semen (2, 1 = cooled and 2 = frozen), mare age group (2, 1 = up to 10 years and 2 = above 10 years), breeding season (2, 1 = edge of the breeding season [3–4 and 8–9 months] and 2 = high

season [5–7 months]), hormone treatment (2, 1 = with hormone treatment and 2 = without treatment) and number of inseminations (continuous variable from 1 to 8). In 65 mares, hCG (Chorulon[R], MSD Animal Health; dose 2000–3000 IU IM) was used for ovulation induction (58 of those mares became pregnant).

During the evaluation of the SP, we determined the day when half of the mares were fertilised with the two types of semen and the ratio of the fertilised mares by day 42 (end of the third oestrous cycle).

(C) We also studied the changes of the insemination timespan during the breeding season. We also show the results on a diagram, not just the correlation between insemination timespan and breeding season but also taking into consideration the two types of semen (cooled or frozen).

The statistical analysis of the data was performed using the Dell Statistic Program (Dell Inc., 2015).

# RESULTS

(A) Out of the 107 mares inseminated with fresh chilled semen, 81 mares became pregnant (81/107; 75.7%; Table 1). Seven pregnant mares aborted and 74 foals were born (74/81; 91.4%).

Out of the 22 mares inseminated with frozen semen, 17 mares became pregnant (17/22; 77.3%). Two mares out of the 17 pregnant mares aborted, and finally 15 healthy foals were born (15/17; 88.2%). In the ratio of the foals born no significant difference was found between the two groups (two-sided difference test, $P > 0.05$).

Figure 1 shows the median, the lower and the upper quartiles and the minimum-maximum values of the number of oestrous cycles and AIs per conception. We did not find significant differences by the comparison of medians for the number of insemination cycles (Kruskal–Wallis median test, $P = 0.155$; averages: 1.48 with cooled semen vs. 1.53 with

frozen semen). However, a statistically proven difference was found between medians for the number of inseminations per conception (Kruskal–Wallis median test, $P = 0.014$).

For information purposes, the mean values of inseminations are presented. Considering the pregnant mares only, the mean values of AI numbers per conception in the two groups were as follow: with fresh chilled semen $2.49 \pm 1.56$ ($n = 81$) and with frozen semen $1.82 \pm 0.81$ ($n = 17$). The mean of AI numbers per mare in the two whole groups were as follows: using fresh chilled semen it was $2.54 \pm 1.62$ ($n = 107$), while using cryopreserved semen it was $1.81 \pm 0.80$ ($n = 22$).

Table 2 presents the number and proportion of stallions according to their prolificacy and semen type. The distribution of stallions used with fresh chilled semen versus frozen semen appeared to be significantly different ($P = 0.003$). Stallions having fully successful pregnancy results with fresh semen constituted the most populous efficacy category (67%).

(B) Analysing the data presented in Table 3, we found no significant effect of the mares' age category, breeding season or hormone treatment on the length of the service period. However, both the type of semen and the number of inseminations have a significant impact ($P < 0.001$) on the time of conception.

Generally, negative beta values for an explanatory effect indicate that the hazard is higher and thus the prognosis is better for a lower code value of effect (Fatma and Eman, 2018). In our study, the negative beta value (regression coefficient, $-1.1156$) for semen type means that the use of frozen semen prolongs the insemination period. The negative beta value for number of insemination ($-0.6425$) indicates that the higher the number of inseminations, the longer the inseminations period. In our case, code 1 was given for fresh chilled semen, and the numbering of the inseminations is obvious.

Data of Fig. 2 show that half of the mares inseminated with cooled semen got pregnant by the 5th day, while half of the mares inseminated with frozen semen became pregnant by the 35th day based on the functions of survival analysis

Table 1. Reproductive data of mares inseminated with fresh chilled and frozen semen

| Reproductive features | Mares inseminated with fresh chilled semen ($n = 107$) | Mares inseminated with frozen semen ($n = 22$) | Difference test of the ratios ($P$) | Main average |
|---|---|---|---|---|
| Number and proportion of mares that became pregnant, $n$ (%) | 81 (75.5) | 17 (77.3) | 0.888 | 98 (76.0) |
| Number and proportion of non-pregnant mares, $n$ (%) | 26 (24.3) | 5 (22.7) | 0.939 | 31 (24.0) |
| Number and proportion of absorbed embryos and abortions[a], $n$ (%) | 7 (8.6) | 2 (11.8) | 0.891 | 9 (9.2) |
| Number and proportion of foals born, $n$ (%) | 74 (91.4) | 15 (88.2) | 0.695 | 89 (90.8) |

[a] In this group the mares were pregnant on the 18th day based on ultrasound investigation, then later we could not confirm the pregnancy, indicating that the mare had probably aborted.
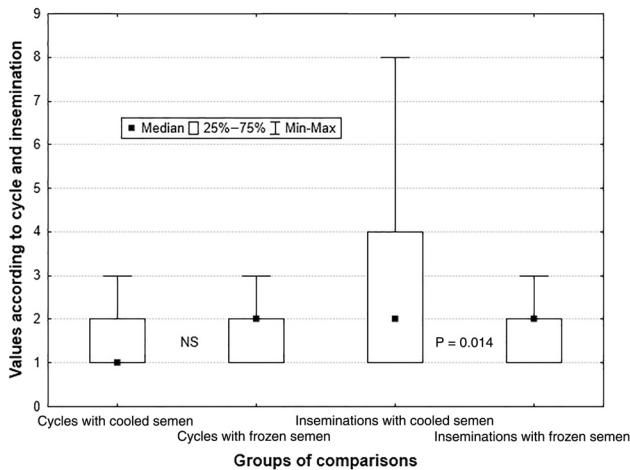
*Figure 1.* Box and whisker plot of the number of cycles and inseminations by semen type

(lowest curve fitting). Furthermore, until the 42nd day of the breeding season, more than twice as many mares were fertilised in the group of mares inseminated with chilled semen than in the frozen semen group (92% vs. 53%).

*Table 2.* The distribution of stallions used for insemination with fresh chilled and frozen semen

| Efficacy categories of pregnancy | Inseminations with fresh chilled semen | Inseminations with frozen semen |
|---|---|---|
| In total: | | |
| No. and proportion of stallions | 55 (100%) | 8 (100%) |
| No. of mares inseminated | 107 | 22 |
| No. of mares per stallion | 1.95 | 2.75 |
| In successful pregnancies: | | |
| No. and proportion of stallions | 37 (67%[a]) | 4 (50%[a]) |
| No. of mares | 61 | 4 |
| No. of mares per stallion | 1.65 | 1.00 |
| In partially successful pregnancies: | | |
| No. and proportion of stallions | 10 (18%[a]) | 2 (25%[a]) |
| No. of mares | 35 | 16 |
| No. of mares per stallion | 3.50 | 8.00 |
| In unsuccessful pregnancies: | | |
| No. and proportion of stallions | 8 (15%[a]) | 2 (25%[a]) |
| No. of mares | 11 | 2 |
| No. of mares per stallion | 1.38 | 1.00 |

[a]Chi$^2$ test for stallion distribution among pregnancy categories by semen type. $P = 0.003$.

(C) Figure 3 shows the relation of insemination timespan to the breeding season. It is obvious that the longer the time until a mare becomes pregnant (the later she becomes pregnant in the breeding season), the longer is the duration of the breeding season. Mares inseminated with chilled semen become pregnant sooner compared to those inseminated with frozen semen. Their short SP is almost independent of the day of the breeding season. AI performed with frozen semen led to a prolonged SP.

## DISCUSSION

Our data confirmed the observations of other authors that an acceptably high pregnancy rate can be obtained after AI with frozen stallion semen. The prerequisites for success are the careful preparation of the semen (dilution, cooling, cryopreservation, etc.) and the mare for the AI (timing of AI), as well as the correct execution of the insemination technique. Based on a literature review by Horváth and Szenci (2018), there is no significant difference between the results of AI with fresh chilled and frozen semen. The effectiveness of both AI types depends on the interaction of three major factors: the quality of semen, the reproductive performance of the mare, and the insemination management, including the insemination technique applied.

In the present study carried out under Hungarian field conditions, no difference was found between the semen types in the number of insemination cycles by median test. This result confirms the above observations, and it suggests that all conditions were satisfactorily met. The number of inseminations shows that more chilled semen is required and less frozen semen is sufficient for achieving conception. Re-insemination with chilled semen is more common than the use of frozen semen for this purpose. Frozen semen was used more prudently, perhaps because of its higher price, especially when it originated from abroad.

The large proportion of stallions whose use resulted in a certain pregnancy provides an indirect evidence that the higher conception rate achieved with frozen semen could not be due to better males.

With Cox regression we could prove that the SP is substantially extended when AI with frozen semen is practised. This finding could also be explained as a result of the management challenges associated with the use of frozen semen.

To discuss this phenomenon, it is necessary to distinguish the number of insemination cycles (which we dealt with) from the number of oestrous cycles of the mare. Often, the first insemination carried out in an oestrous (breeding season) cycle is not successful, and must be repeated. From the shorter service period found when using chilled semen, we conclude that if the insemination was unsuccessful in a given oestrous cycle of the mare, a re-insemination took place already in the subsequent cycle. In contrast, in the case of insemination with frozen semen, the mare was not rebred at the subsequent oestrus. So for price or other reasons many owners will be hesitant to immediately rebreed a mare who has failed to become pregnant. The next insemination cycle

*Table 3.* Presentation of the effects on the length of the insemination period in the Cox regression model (Chi$^2$ = 80.4813, df = 5, $P < 0.001$)

| Effects ($n$ = number of mares) | Beta[a] | Standard error | Wald statistics | Hazard ratio (95% CI for HR) | $P$ value |
|---|---|---|---|---|---|
| Types of semen:<br>  1 = fresh chilled ($n$ = 81)<br>  2 = frozen ($n$ = 17) | −1.1156 | 0.2803 | 15.841 | 0.328<br>0.189–0.568 | < 0.001 |
| Age group of mares:<br>  1 = up to 10 years ($n$ = 54)<br>  2 = above 10 years ($n$ = 44) | 0.2308 | 0.1881 | 1.506 | 1.260<br>0.871–1.821 | 0.220 |
| Breeding season:<br>  1 = out of season ($n$ = 41)<br>  2 = high season ($n$ = 57) | 0.3684 | 0.2105 | 3.064 | 1.445<br>0.957–2.184 | 0.080 |
| Hormone treatment:<br>  1 = with treatment ($n$ = 58)<br>  2 = without treatment ($n$ = 40) | 0.1550 | 0.1894 | 0.670 | 1.168<br>0.806–1.693 | 0.413 |
| Number of inseminations ($n$ = 1–8, all 233) | −0.6425 | 0.1016 | 39.974 | 0.526<br>0.431–0.642 | < 0.001 |

[a]Regression coefficient; CI = confidence interval; HR = hazard ratio.
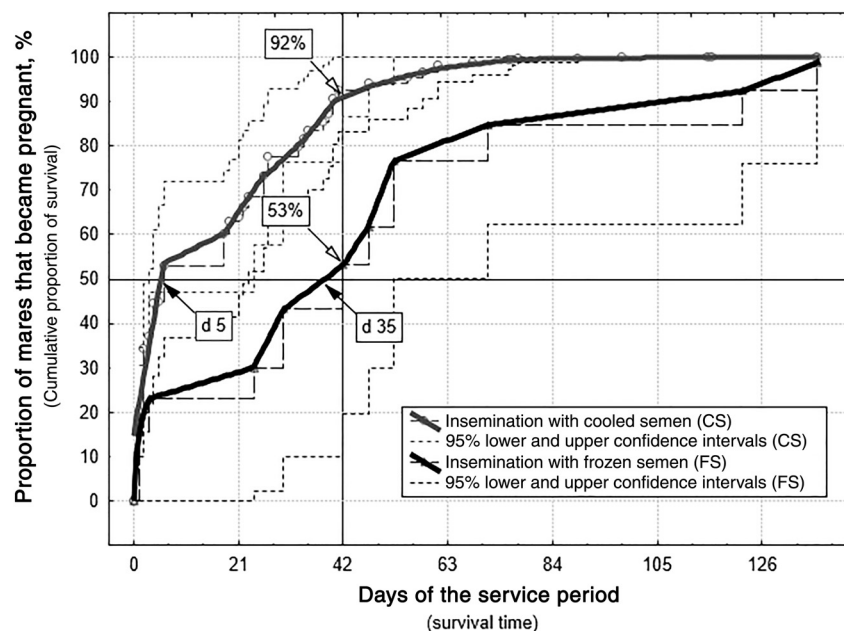


*Figure 2.* Pregnancy rate of mares by time according to semen type

with frozen semen will only take place after one (or few) missed oestrous cycles. The mare's cycle must be closely monitored by frequent ultrasound examinations when using AI with frozen semen, which is done only when the signs of ovulation are accurately detected. This seems a plausible reason for the lengthening of the SP. We would like to note that this investigation also deals with the characterisation of the use of frozen semen in the practice. For some humane reason, there is no insemination at the next oestrus after the first AI. This leads to an 'inexplicable' prolongation of the SP. We believe that the exact 'biological comparison' of semen types with regard to the number of inseminations per conception (Fig. 1; Kruskal–Wallis median test, $P = 0.014$) clearly shows the professional side of this issue. Although there was no statistically proven difference in the number of cycles,

Fig. 1 gives the impression that the number of AI cycles would be higher with frozen semen than with chilled semen. It is a usual practice in Hungary to carry out inseminations with frozen semen in the second part of the breeding season.

Our data were collected from AI programmes under field conditions, and they demonstrate a very good success rate. AIs with frozen semen also showed very good pregnancy rates. One of the most important factors determining the good outcome of AI in mares is that the females must be inseminated in the right phase of the oestrous cycle, very close to the time of ovulation. This is especially important when frozen semen is used for AI.

The importance of the precise preparation of mares for AI is also underlined by the fact that only 65 mares were treated with hCG in order to support the maturation
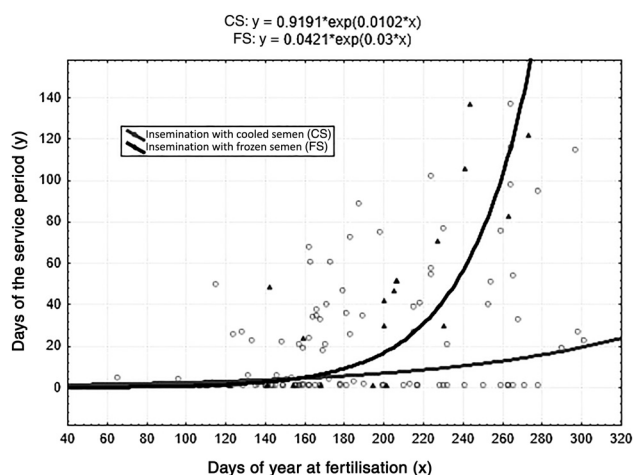
CS: y = 0.9191*exp(0.0102*x)
FS: y = 0.0421*exp(0.03*x)

*Figure 3.* Connection of the service period to the breeding season according to semen type

of the oocyte and induce ovulation of the follicle. Fifty-eight out of the 65 mares treated with hCG became pregnant. However, in this case the treated cycle must be monitored more closely by more frequent ultrasound examinations.

## ACKNOWLEDGEMENT

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