Ankara Üniv Vet Fak Derg, **63**, 359-363, 2016

The influence of insemination time, age and semen dose on fertility of mares inseminated with frozen semen

Mustafa AVCI[1], Mehmet Borga TIRPAN[2], Koray TEKİN[2], Ergun AKÇAY[2]

[1] General Directorate of Agricultural Enterprises, Ankara; [2] Ankara University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Ankara, Turkey.

Summary: In this study the influence of three different factors (semen dose, age, insemination time) on fertility were investigated. 48 mares were inseminated with 400×10^6 and 800×10^6 and 1200×10^6 (0,5 ml, French straw) frozen-thawed semen, in breeding season. Each group were randomly divided into two sub-groups, inseminated before (when follicle diameter was 40-45 mm) and after ovulation. The average number of pregnancy rates were 12.5 ± 9.4; 37 ± 11.7 and 37.5 ± 11.4 % at dose groups (400×10^6, 800×10^6 and 1200×10^6), 37.5 ± 9.6 and 20.8 ± 9.7 % at insemination time groups (before and after ovulation), 28.1 ± 8.2 and 31.3 ± 11.7 % in age groups (4-11, 12-19), respectively ($p>0.05$). As a result, for the optimization of fertility related issues such as; insemination time, dose, age have to be identified and solutions need to be revealed for the development of artificial insemination programs in Arabian horse breeding systems. Fertility related studies with frozen-thawed semen are quite limited in Arabian horse artificial programs. Current study is expected to provide important contribution to the artificial insemination programs with frozen thawed semen for Arabian horse breeds as a pioneer study in Turkey. This research will lead to future investigations on local Arabian horse breeding programs.

Keywords: Arabian horse, artificial insemination, equine reproduction, insemination dose, pregnancy rates.

Dondurulmuş sperma ile tohumlanan kısraklarda tohumlama zamanı, yaş ve sperma dozunun döl verimine etkileri

Özet: Bu araştırma, Safkan Arap atı kısraklarında, dondurulmuş-çözdürülmüş spermayla farklı dozlarda ve farklı zamanlarda yapılan tohumlamaların ve kısrak yaşının döl verimi üzerine etkisinin araştırılması amacıyla yapılmıştır. Toplam 48 Arap atı kısrağı 50×10^6 motil spermatozoa içeren 0.5 ml'lik (400×10^6), (800×10^6) ve (1200×10^6) dondurulmuş–çözdürülmüş sperma ile suni olarak tohumlanmıştır. Tohumlamalar yapılırken her bir grup kendi içerisinde rasgele iki alt gruba ayrılmış; yarısı ovulasyon öncesi (follikül çapı 40-45 mm iken), yarısı da ovulasyon sonrası dondurulmuş–çözdürülmüş sperma ile tohumlanmışlardır. Gebelik oranları, doz gruplarında (400×10^6, 800×10^6 ve 1200×10^6) sırasıyla % 12.5 ± 9.4; 37 ± 11.7 ve 37.5 ± 11.4; tohumlama zamanı gruplarında (ovulasyondan önce ve sonra) % 37.5 ± 9.6 ve 20.8 ± 9.7; yaş gruplarında (4-11, 12-19) sırasıyla % 28.1 ± 8.2 ve 31.3 ± 11.7 olarak kaydedilmiştir. Gebelik oranına sperma dozu, tohumlama zamanı ve yaşın etkisi istatistik olarak önemsiz ($p>0,05$) bulunmuştur. Sonuç olarak, Arap atı yetiştiriciliğinin gelişmesi için sorunların belirlenmesi, çözümlerin araştırılması ve çeşitli suni tohumlama uygulamalarının fertilite sonuçlarının ortaya konması gerekmektedir. Fertilitenin arttırılması için de tohumlama dozları ve zamanlarının belirlenmesi önem taşımaktadır. Arap atlarında dondurulmuş spermayla yapılan tohumlamaların fertilite ile ilişkilisini ortaya koyan araştırmalar sınırlıdır. Yapılan çalışma Türkiye arap atı yetiştiriciliğinde dondurulmuş sperma ile gerçekleştirilmiş olması ve fertilite oranlarını ortaya koyması açısından orijinal bir çalışmadır. Bu çalışma, ileride yapılabilecek arap atı yetiştirme programı çalışmaları için öncü olacağı düşünülmektedir.

Anahtar sözcükler: Arap atı, gebelik oranı, reprodüksiyon, suni tohumlama, tohumlama dozu.

Introduction

Artificial insemination (AI) with fresh, chilled transported or frozen semen have given the mare owner substantially greater possibilities to choose the most suitable stallion for a mare, even over great distances. There are several other advantages as well, with the decreased risk of spreading venereal diseases being one of the most important. In addition, several studies have shown that pregnancy rates are higher when using AI with fresh semen than when using natural service (2).

It is essential to maintain an acceptable pregnancy rate regardless of the type of AI used, i.e. fresh, cooled stored or frozen semen. Several factors are grown to affect the pregnancy rate.

The age, when the mare fertility starts to decrease, varies from 10 to 15 years in different publications (3, 4, 7, 11) along with the risk of pregnancy loss increase at a similar age. The decreasing pregnancy rates and increasing pregnancy loss rates lead to low foaling rates. Conversely, other researchers found that age has no

significant effect on fertility (15, 18). In order to achieve satisfactory pregnancy rates per cycle in mares, the minimum recommended number of spermatozoa included within a conventional insemination dose is generally $>300\times10^6$ progressively motile spermatozoa for fresh (2, 5, 12, 14, 20) and $>500\times10^6$ progressively motile spermatozoa for frozen semen (2). It has been shown that conventional insemination of mares with $\leq100\times10^6$ spermatozoa results in unsatisfactory pregnancy rates per cycle (13, 21). In certain studies small groups of mares were used, while in others semen from only a few stallions were used for the insemination. Thus, due to the variety of insemination regimens and evaluation schemes used, it is difficult to compare the results and draw conclusions from different studies. When comparing insemination doses it is important that an acceptable pregnancy rate (>60 % per cycle) is achieved in the insemination centers. To minimize the effect of individual mares and stallions, the mares should be divided into well-defined groups and the stallions must be fertile proven (12). All the mares and stallions were examined before the selection and fertility proven based on acceptable pregnancy rates according to breeding records.

The success of Arabian horse breeding activities related to the AI is not revealed yet. Therefore, effective improvement of various applications on fertility is important. Arabian stallions used for breeding purposes, have a great deal of economic value. Dissemination of genetic material is economically important. In this study, it was investigated for further benefit from mares and stallions by using frozen-thawed semen in different doses and insemination times. To increase fertility, information was revealed by determining insemination doses and time. On the other hand, this research has been planned to be the basis of other scientific studies.

The aim of this research is investigation of insemination time (before and after the ovulation), frozen-thawed semen doses (400×10^6, 800×10^6 and 1200×10^6) and age on pregnancy of purebred Arabian mares, which is expected to provide a basis for further investigations.

Materials and Methods

Study was performed at Sultansuyu, Karacabey and Anatolian Stud farms, subsidiary of General Directorate of Agricultural Enterprises (TIGEM). A total of 48 Arabian mares, 16 from each farm (4 to 19 years of age) were used in this study. Insemination programs were performed with frozen-thawed semen at Karacabey Agriculture Services (Ethic No: 2010-59-300).

Semen collection and examination: Fresh semen was collected with the aid of Missouri type artificial vagina while the stallion was mounting a teaser mare in estrus. After collection, quantity of semen was measured and filtrated with gauze to separate the gel. Spermatozoa motility was evaluated by Computer Assisted Semen Analysis (CASA- Hamilton Thorn-USA). The numbers of dead and abnormal spermatozoa were determined. The ejaculates were evaluated and accepted for evaluation if the following criteria were met: motility percentage higher than 70%, dead spermatozoa less than 25% and less than 20% abnormal spermatozoa in total concentration and pH of semen was evaluated with photometry (IMV-France) and pH indicator paper.

Semen dilution and freezing: For freezing concentration, 10 µl semen sample was diluted with 190 µl Inra 96 (IMV-France). 40 µl of sample were loaded into CASA chamber slide, quantity of semen and final dilution data were entered in to CASA and dilution processes were considered according to CASA result system. Sperm dosages were arranged as $100x10^6$ motile spermatozoa in each 0.5 ml straws before freezing.

EQCELLSIRE A (IMV-France) extender was used for the dilution process with a ratio of 1/1 according to CASA results and transferred into 2-4 falcon tubes for centrifugation (600×g/min). Mixtures were pipetted and gently transferred into uniform falcon tubes and 3-4 ml of EQCELLSIRE B (IMV-France). As soon as the mixtures were centrifuged, supernatant fluid were removed. Pellets were diluted and homogenized with Inra 96 extender. Then all tubes were placed in refrigerator at +4 C° for equilibration. Freezing was performed with a programmable freezer (nitrogen freezer, automatic Mini-Digitcool, IMV-Technologies, L' Aigle, France) (–60 C° min−1 until –140 C°). The straws were kept in liquid nitrogen and then thawed in a water bath (37 C° for 30 s) immediately before analyses or AI's. Minimum 50% motility rate was considered acceptable for performing AI protocol.

Reproductive examination: Mares, which exhibit external signs of estrus (mare squats, raises her tail, urinates, averts the clitoris and stands still) or vague were exposed to a stallion to detect their reproductive performance. Reproductive examination was performed with the use of speculum to observe both vaginal and cervical walls. Basically, dark pinkish and bright colors of vagina with secretion, and spread of cervical orifice were recorded. Rectal examination was performed to determinate ovarian status (graff follicle) and uterine edema. Uterus and ovarian status were examined by ultrasonography. Mares whose follicles were rough shaped, fluctuant and bigger than 35 mm; uterus was edematous and hypertonic, which is also expressed as "sliced orange shaped appearance" and, finally if there was hypertonic substance inside intralaminar liquid; it was considered as estrus.

Artificial insemination: 48 mares which were in estrus were separated into 3 groups and inseminated with 3 different frozen-thawed semen (400×10^6, 800×10^6 and 1200×10^6) doses with 2 different modules (before and after ovulation). First group was inseminated with 8 straws (400×10^6), second with 16 straws (800×10^6) and third with 24 straws (1200×10^6). From each ejaculate one straw were thawed and evaluated for motile spermatozoa concentration. Averagely 50×10^6 motile spermatozoa containing samples were used for AI. Ovulation surveillance was performed with ultrasonography. Ultrasonographic K-1 pregnancy examinations were done on the day 15th.

Statistical analysis: Effect of sperm doses, timing of insemination and age groups on pregnancy rates were calculated by Pearson Chi-square test when all the expected cell counts of the contingency table are bigger than five and fisher exact test when at least one of the expected counts of the contingency table is smaller than five. The results obtained were assessed by the use of variance analysis through calculating their efficiency. SPSS version 14.01 (Chicago, IL, USA) was used for data analyses. A minimum of 5 % significance level was used for all comparisons ($p<0.05$).

Results

Average values of sperm dose, time of insemination and age were shown in (Table 1). The differences in all groups regarding sperm dose (400×10^6, 800×10^6 and 1200×10^6) and pregnancy rate (12.5 ± 9.4; 37.5 ± 11.7 and 37.5 ± 11.4 % respectively) did not vary significantly ($p>0.05$). Differences in all groups regarding effect of insemination time (before and after ovulation) on pregnancy rate (respectively 37.5 ± 9.6 and 20.8 ± 9.7 %) were not statistically significant ($p>0.05$). In mares, age effect (4-11 and 12-19) on pregnancy rate (28.1 ± 8.2 and 31.3 ± 11.7 %) was not significant ($p>0.05$). The dose effect did not alter the pregnancy rates as well in mares along with at least 50% motile spermatozoa in each straw. Even so, there is not a standard motile spermatozoa dose in this matter. Studies focus on decreased doses in insemination processes due to optimal usage of old stallions, using low quality and sexed semen. This situation prevents determining a general standard dose for AI (Table 1).

Discussion and Conclusion

In this study, the lowest and highest pregnancy rates were obtained for 400×10^6 and 1200×10^6 doses respectively. The pregnancy rates were substantially similar in 800×10^6 and 1200×10^6 doses. On the other hand, the pregnancy rates in mares inseminated before ovulation were found to be higher than those inseminated after ovulation. Regarding the pregnancy rates, 4-11 years old mares were found to be higher than 12-19 years old mares. Even though these indicated differences were observed, semen dose, insemination time and age effect on pregnancy rates were found to be significant.

Semen dose: Post-thawed semen motility effects pregnancy rates in mares. Studies about optimal usage of poor quality, dead stallion semen or sexed semen can reduce the insemination dose and can cause an absence of a standard dose. On the other hand, researchers have investigated minimum insemination doses. Studies indicated that frozen - thawed semen, which is used for insemination, should have at least 240×10^6 and ≥30%

Table 1. Average pregnancy rates of mares.
Tablo 1. Kısraklarda elde edilen ortalama gebelik oranı.

Variants	n	Pregnancy rate (%) X ± Sx	P
Semen Dose		-	
400×10^6	16	12.5 ± 9.4	0.199
800×10^6	16	37.5 ± 11.7	
1200×10^6	16	37.5 ± 11.4	
Insemination time		-	
Before ovulation	24	37.5 ± 9.6	0.204
After ovulation	24	20.8 ± 9.7	
Age		-	
4 – 11	32	28.1 ± 8.2	0.998
12 – 19	16	31.3 ± 11.7	
Average value	48	29.1	

- : Not significant ($p>0.05$).
- : Önemsiz ($p>0.05$).
X ± Sx: Mean ± Standard error of mean.
X ± Sx: Ortalama ± Ortalama standart hata.

motility (9, 10). However according to Vidament et al. (20) insemination dose of frozen-thawed semen should be higher than 150×10^6. Morris (12) reported that insemination dose of $>300\times10^6$ motile spermatozoa for fresh semen, and $>200\times10^6$ motile spermatozoa for frozen semen is sufficient but $\leq100\times10^6$ motile spermatozoa is insufficient for pregnancy in mares.

In this study Arabian mares were inseminated with doses of; 400×10^6, 800×10^6 and 1200×10^6 frozen-thawed semen respectively, and pregnancy rates were as follows: 12.5 ± 9.4; 37.5 ± 11.7 and 37.5 ± 11.4 %, but no significant difference were seen between groups. During the insemination program, motile spermatozoa concentrations were relatively high compared to the literature (2, 8, 12). This is because insemination was performed on valuable racehorses, therefore freezing success of stallion semen is lower than other species and time of insemination was immediately before and after the ovulation. The reasons for no significant differences may be use of small number of mares in groups and large standard errors.

Acceptable pregnancy rate is regarded as at least 60% in mares (12). Pregnancy rates of this study are relatively low compared with this requirement. However, pregnancy rates of inseminations with frozen-thawed semen show wide variations in the literature. Variations at semen dose, insemination time, age, insemination method, maintenance and feeding schedules are very effective factors. Pregnancy rates with frozen - thawed semen are reported between 32–73% in commercial applications in the United States (9). In this study, pregnancy rates of insemination dose groups (800×10^6 and 1200×10^6) were within limits but closer to the lower limits of commercial applications in the United States. After inseminations of warm-blooded horse breeds done with stallions' frozen-thawed semen at privately owned, private companies and natural breeding public institutions in France, pregnancy rates were reported as follows; 64, 62, 57, 64% (19). Our pregnancy rates of insemination dose groups (800×10^6 and 1200×10^6) were lower.

Similarly, in German Hannover horse breeds pregnancy rates in one cycle after single or double inseminations with frozen-thawed semen were 42.2 and 50% (average 44%) respectively (18). In this study, pregnancy rates of insemination dose groups (800×10^6 and 1200×10^6) were similar to the present study. When compared with present study, (17) pregnancy rates were higher with frozen - thawed semen which were collected from Karacabey origin Haflinger stallions. Leipold et al. (8) reported that insemination doses of 1.600×10^6 and 400×10^6 had pregnancy rates of 37 and 22% respectively. When compared to the pregnancy rates, high concentration of insemination doses show similarity with the present study.

Insemination time: Insemination time has big impacts on pregnancy rates and insemination must be performed with ovulation detection. The importance of the ovulation identification rises, especially when it comes to frozen-thawed semen usage. Moreover, frozen-thawed spermatozoa are reaching the oviduct later when compared with the fresh semen. Intervals both between insemination–ovulation and ovulation–insemination decrease possibility of pregnancies (15, 18).

The pregnancy rates with frozen-thawed semen before and after ovulation were found 37.5 ± 9.6 and 20.8 ± 9.7 % respectively; also, differences between groups were negligible. Despite being negligible, insemination before ovulation was 16.7% higher than after ovulation. Low number of mares and high value of standard error may cause the differences between groups to be removed.

Although several literatures about insemination time (8, 9, 10, 15, 18), reports dissimilar results, it is commonly determined by ovulation time and chosen methods. For instance, in the USA, insemination with frozen-thawed semen was performed 6 hours either before or after ovulation (16). According to Miller (10), inseminations should be performed immediately after ovulation with one dose or double dose; one immediately and 18 hours after ovulation. Furthermore, Sieme et al. (18) reported that insemination with frozen-thawed semen must be performed 12 hours apart before and after ovulation. It is recommended to match the oocyte and sperm up during the insemination, second must be performed 24 hours after.

In our study single insemination dose was performed. But, second insemination dose should have been considered to increase fertility. According to our study, different results were recorded before and after ovulation. These results were found to be similar to Sieme et al. (18), whose research divided Hanoverian mares into 3 groups and inseminated all four groups in different time intervals (before and after ovulation) after hCG treatment with frozen-thawed semen.

Age groups: The age has great affects when frozen-thawed semen is used at artificial inseminations. Being too old (24-30) or too young (3-6) may have a negative effect on pregnancy. The pregnancy rates on several different ages (4–11, 12–19) with frozen-thawed semen were found 28.1 ± 8.2 and 31.3 ± 11.7 % respectively; also, differences between groups were negligible. Pregnancy rates were found 2.5% higher in 4-11 age groups than 12-19 age groups, which can be predictable.

There have been various reports about age effects on pregnancy rate in mares. For instance, Sieme et al. (18) grouped Hanoverian mares by age (2-4, 5-8, 9-12,

13-16 and 17-21) and inseminated them with frozen-thawed semen. Consequently, pregnancy rates were found similar in each group. Furthermore, research conducted in Tunisia (1) suggested that age effect might be disconsidered until 20 years old Arabian mares when inseminated by hand-mate method. Those results show similarity with our results.

In contrast, Vidament et al. (20) reported that pregnancy rates were found to be higher in <16 years old mares than ≥16 years old mares with frozen-thawed semen. Similarly, Miller (10) took 8 years of age as a limit and reported that mares which are more than 8 years old inseminated with frozen-thawed semen, are found to have a lower rate of pregnancy than mares above 8 years old. Those differences from our research may associate with racial and environmental factors.

In this study, effects of dose, ovulation time and age parameters were found to be negligible. On the other hand, pregnancy rates of 400×10^6 dose group were the lowest while 1200×10^6 was the highest. Also results of 800×10^6 dose group were close to 1200×10^6. In addition, pregnancy rates obtained from mares inseminated before ovulation were higher than mares inseminated after ovulation. Nevertheless, pregnancy rates of mares between 4-11 years of age were higher than 12-19 years of age.

In conclusion, this research was designed to obtain pregnancy rates of purebred Arabian mares inseminated with various doses of frozen-thawed semen (400×10^6, 800×10^6, 1200×10^6) and adjusted at different times (before and after ovulation). Purebred Arabian mares inseminated with three doses of frozen-thawed semen is practically quite low, pregnancy rates of 800×10^6 and 1200×10^6 sperm doses are relatively similar. Insemination before ovulation has a positive effect on pregnancy and fertility is prone to decrease with ageing though this is less effective.

References

1. **Benhajali H, Richard–Yris MA, Ezzaouia M, et al.** (2010): *Factors influencing conception rates of Arab mares in Tunisia.* Anim Rep Sci, **117**, 106 -110.
2. **Gahne S, Ganheim A, Malmgren L** (1997): *Effect of insemination dose on pregnancy rate in mares*. Theriogenology, **49**, 1071-1074.
3. **Hearn P, Bonnett B, Samper J** (1993): *Factors influencing pregnancy and pregnancy loss on one thoroughbred.* Farm. Proc. 39th Ann. Conv. Am. Ass. Equine Pract, 161-163.
4. **Hemberg E, Lundeheim N, Einarsson S** (2004): *Reproductive performance of thoroughbred mares in Sweden.* Reprod Dom Anim, **39**, 81-85.
5. **Householder DD, Pickett BW, Voss JL, et al.** (1981): *Effect of extender, numbers of spermatozoa and hCG on equine fertility*. Equine Vet Sci, **1**, 9-13.
6. **Katila T, Nivola K, Reilas T, et al.** (2010): *Factors affecting reproductive performance of horses*. Pferdeheilkunde, **26**, 6-9.
7. **Langlois B, Blouin C** (2004): *Statistical analysis of some factors affecting the number of horse births in France*. Reprod Nutr Dev, **44**, 583-595.
8. **Leipold SD, Graham JK, Squires PM, et al.** (1998): *Effect of spermatozoal concentration and number on fertility of frozen equine semen.* Theriogenology, **49**, 1537-1543.
9. **Loomis PR** (2001): *The equine frozen semen industry*. Anim Rep Sci, **68**, 191-200.
10. **Miller CD** (2008): *Optimizing the use of frozen–thawed equine semen*. Theriogenology, **70**, 463-468.
11. **Morris L, Allen WR** (2002): *Reproductive efficiency of intensively managed thoroughbred mares in new market*. Equine Vet J, **34**, 51-60.
12. **Morris LHA** (2004): *Low dose insemination in the mare: An update*. Anim Rep Sci, **82-83**, 625-632.
13. **Pace MM, Sullivan JJ** (1975): *Effect of timing of insemination, numbers of spermatozoa and extender components on the pregnancy rate in mares inseminated with frozen stallion semen.* J Reprod Fertil Suppl, **23**, 115-121.
14. **Pickett BW, Voss JL** (1975): *The effect of semen extenders and sperm number on mare fertility.* J Reprod Fertil Suppl, **23**, 95-98.
15. **Rossi R, Silva JM, Palhares MS, et al.** (2014): *Effect of age on fertility and embryonic loss of mares inseminated with jackass semen diluted and cooled at 5 degrees C for 12 hours*. Arq Bras Med Vet Zootec, **66**, 1442-1448.
16. **Samper JC** (2001): *Management and fertility of mares bred with frozen semen.* Anim Rep Sci, **68**, 219-228.
17. **Sevinç A, Yurdaydın N, Tekin N** (1984): *Karacabey harası safkan Arap ve Haflinger aygırlarından alınan spermaların dondurulması ve Haflinger kısraklarından elde edilen döl verimi*. Ankara Üniv Vet Fak Derg, **31**, 304-315.
18. **Sieme H, Schafer T, Stout TAE, et al.** (2003): *The effects of different insemination regimes on fertility in mares*. Theriogenology, **60**, 1153-1164.
19. **Vidament M** (2005): *French field results (1985-2005) on factors affecting fertility of frozen stallion semen*. Anim Rep Sci, **89**, 115-136.
20. **Vidament M, Dupere AM, Julienne P, et al.** (1997): *Equine frozen semen freezability and fertility fields results*. Theriogenology, **48**, 907-917.
21. **Voss JL, Wallace RA, Squires EL, et al.** (1979): *Effects of synchronisation and frequency of insemination on fertility*. J Reprod Fertil Suppl, **27**, 257-261.

Geliş tarihi: 04.02.2015 / Kabul tarihi: 03.12.2015

Address for correspondence:
Prof. Dr. Ergun AKÇAY
Ankara University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Ankara, Turkey.
e-mail: Ergun.Akcay@ankara.edu.tr