

POST-THAW MOTILITY AND LONGEVITY OF MOTILITY OF IMIPRAMINE-INDUCED EJACULATES OF PONY STALLIONS

S. M. McDonnell and R. M. Oristaglio Turner

University of Pennsylvania School of Veterinary Medicine
Mcconnett Square, PA 19348

Received for publication: *September 20, 1993*

Accepted: *July 6, 1994*

ABSTRACT

Imipramine-induced ex copula ejaculates (11) and fractionated in copula ejaculates were collected from each of 5 pony stallions for freezing in 5-ml straws (6), using a modified Kenney glucose skim-milk extender (2). Initial post-thaw total and progressive motilities and daily post-thaw total and progressive motilities, as well as the number of days to reach 0 progressively motile spermatozoa, were also similar for the 2 methods of collection. The percentage of morphologically normal spermatozoa both before freezing and after thawing were also similar for in copula and ex copula ejaculates. Consistent with previous work (1,1), imipramine-induced ejaculates were of extremely high sperm concentration and low volume compared with those of in copula ejaculates. In this study, imipramine-induced ejaculates were of significantly higher concentration of sperm and lower volume than fractionated (first 2-3 jets) in copula ejaculates. These results suggest that imipramine-induced ejaculates may be suitable for cryopreservation. Breeding trials are necessary to evaluate actual fertility of semen.

Key words: semen, cryopreservation, imipramine, xylazine, induced-ejaculation

INTRODUCTION

This study is part of ongoing work toward developing pharmacologic methods of enhancing and inducing ejaculation in stallions. Our principal applied objective is to enable collection of semen from stallions that, as a result of physical impairment or psychological sexual behavior dysfunction, are unable to ejaculate in copula. Thus far, we have begun to develop three methods for inducing ex copula ejaculation in stallions, using a variety of agents that affect genital smooth muscle activity (8,10,11). All of the protocols reported or presently under study have relatively low overall reliability of success (20 to 70%) with considerable variability among individual stallions. Nonetheless, there are stallions for which pharmacologically-induced ejaculation has been a satisfactory aid to breeding, either temporarily or permanently. Examples include stallions with limb weakness or pain that precludes safe mounting or ground collection, inadequate libido, injury of the penis that interferes with erection, and consistent or intermittent ejaculation failure of unknown etiology.

Acknowledgments

This was a NIH NS01537 and Dorothy Russell Havemeyer Foundation project conducted at the Havemeyer Barn and Georgia and Philip Hofmann Research Center for Animal Reproduction at New Bolton Center. We thank Dr. Charles Love for helpful review of this report.

Of recently reported methods for pharmacologic induction of ex copula ejaculation in stallions, protocols employing imipramine consistently result in an ejaculate of superphysiologic concentration of spermatozoa ($400-1200 \times 10^6$ spermatozoa/ml), very low volume and, usually, higher total numbers of spermatozoa per ejaculate compared with that of in copula methods of collection. Such characteristics suggest emission of principally the sperm rich component with little accessory gland fluid, and suggest that imipramine-induced ejaculates might be well-suited for cryopreservation.

For stallions with ejaculatory dysfunction, particularly for those with deteriorating physical conditions, cryopreservation of ex copula ejaculates would be a useful management tool. If reliability of ejaculation can be improved with further research, cryopreservation of pharmacologically-induced ex copula ejaculates or pharmacologically-enhanced in copula ejaculates might be useful for other stallions as well. An example would be stallions whose in copula ejaculates are of very low concentration of spermatozoa or whose spermatozoa are especially adversely affected by exposure to seminal plasma or centrifugation.

The main objective of the work reported here was to compare post-thaw motility and longevity of motility of imipramine-induced ex copula ejaculates with those of fractionated in copula ejaculates obtained from the same stallions.

METHODS

General Design

Over a period of 4 wk during November and December of 1992, four ejaculates were obtained for freezing from each of 5 pony stallions. For each animal, 2 ejaculates were obtained in copula and 2 ex copula. (For one stallion, only 1 ex copula ejaculate met minimum concentration criteria for freezing; see results.) For each animal, the order of method was alternated, such that for each freezing session, there were 1 or more samples for each of the 2 methods. The ejaculation interval was standardized to 2 to 3 d. One experimenter performed all collections and another did the freezing and semen measures, remaining blind to the method of collection of ejaculates.

Subjects

Five sexually experienced pony stallions (6 to 18 yr old; 175 to 200 kg) were used. These animals had been used as subjects of semen and behavior studies for the previous one to 5 yr. During this study, they were kept together at pasture, with supplemental hay provided once daily and free access to water. On 8 semen collection days, stallions were kept from 0800 to 1600 h in individual tie-stalls in a stable. They were provided with hay and water ad libitum when stabled.

Semen Collection

Ex copula induced ejaculates. Ejaculations were induced using imipramine hydrochloride (2 mg/kg, iv) followed by xylazine hydrochloride (0.3 mg/kg iv) as described by McDonnell and Odian (11), with the following exceptions. In the present study, xylazine was not administered until 1 h after imipramine with no ejaculation. Instead of after 10 min in the previous study. Animals were kept undisturbed in their tie-stalls during the induced-ejaculation procedure. They were remotely monitored by video surveillance to detect ejaculation, and videotaped for subsequent review, rather than observed by a person

nearby as in the initial study. As in our previous study, ejaculates were collected using a plastic bag positioned over the prepuce by a 5-inch plastic embroider-v hoop attached to a girth. The samples were removed from the collection bag within 1 min of ejaculation.

In copula fractionated ejaculates. Fractionated **semen was** obtained using manual stimulation with the stallion mounted on a tethered mare or dummy mount, as described by McDonnell and Love (9). The method employs a plastic semen collection bag that is secured with tape to the erect penis **just** before the stallion mounts the dummy. In the present study, one person handled the stallion, washed the stallion's penis and attached the collection bag, performed the manual stimulation, and fractionated the ejaculate. As the first 2 jets were palpated and visualized, the plastic collection bag was pinched proximally such that subsequent jets were separated from the initial jets.

Freezing and Thawing Processes

Semen was frozen as described by Love et al. (6). This centrifugation-free method uses only ejaculates exceeding 200 million spermatozoa per ml. After the raw measures described below were obtained, semen was slowly diluted at room temperature (22 C) to a concentration of 100×10^6 spermatozoa per ml using a modified Kenney extender containing glucose, dry skim milk, egg yolk, and glycerol (2). The extender had been prepared earlier on the day of freezing and allowed to equilibrate to 37°C in a water bath. The extended semen was packaged at room temperature in 5-ml plastic straws such that each straw contained 450×10^6 total spermatozoa. The straws were then sealed with metal balls and labeled. In most cases, packaging was complete within 15 min after collection. Straws were placed for 90 min into a cabinet that was pre-cooled to 4°C. After this initial cooling, straws were frozen on a metal screen at 3 cm above liquid nitrogen for 15 min, and subsequently submersed into liquid nitrogen for storage.

After a minimum of 72 h in liquid nitrogen, straws were thawed for 45 sec in a water bath of 50°C. After initial post-thaw semen measures (described below) were obtained, the remaining thawed semen was extended to a concentration of 50×10^6 spermatozoa per ml with standard Kenney skim milk glucose extender (5) and stored for 5 d at 4°C in a storage container. ^b

Semen Measures and Analysis

Semen was evaluated before freezing and after thawing using methods described by Kenney et al (5). Measures of raw semen immediately after collection included: percentage total motile spermatozoa (visually estimated), percentage progressively motile spermatozoa (visually estimated), gel-free semen volume, pH, concentration of spermatozoa (densimeter^c), total number of spermatozoa, and percentage morphologically normal spermatozoa (visually estimated using phase contrast at $\times 1000$). Raw and extended total and progressive motilities were estimated for all samples. In some cases, motilities of raw semen were difficult to evaluate due to high concentrations of spermatozoa. For this reason, only motilities of extended semen were used for analyses. Immediate post-thaw measures analyzed included spermatozoa total and progressive motilities and percentage morphologically normal spermatozoa. Total and progressive motilities of extended semen maintained at 4°C were estimated daily for the first 5 d following thawing.

a Minitube of America, RR 1, Box 3, Cambridge, IA 50046.

b Equitainer, Hamilton-Thorn Research, 30A Cherry Hill Dr., Danvers, MA 01923.

c "A" Model Densimeter, Animal Reproduction Systems, Chino, CA 91710.

Table 1 Prefreeze and post-thaw semen characteristics

Semen Characteristics	Fractionated in copula ^a		Imipramine-induced CX copula ^b		P ^c
	X	SEM	X	SEM	
A. Pre-freeze	(n=10)		(n=10)		
Sperm concentration (10 ⁶)	300.0	(27.3)	811	(76.6)	0.001
Semen volume (ml)	10.0	(1.20)	6.2	(1.41)	0.05
PLB:	6.9	(0.51)	6.8	(0.06)	
Morphologically normal (%) ⁹	64.0	(4.85)	59.4	(5.02)	
Total number spermatozoa (10 ⁹)	2.92	(0.40)	4.56	(0.82)	
Total motile spermatozoa ^d (%)	79.4	(1.76)	79.4	(1.30)	
Progressively motile ^d (%)	71.7	(3.12)	73.9	(1.39)	
B. Post-thaw ^d	(n=9)		(n=9)		
Morphologically normal (%)	61.4	(2.94)	61.7	(3.54)	
Initial total motile (%)	41.7	(5.59)	47.2	(2.65)	
Initial progressively motile (%)	36.7	(5.59)	41.7	(2.76)	
Days to 0 progressively motile ^e	4.3	(1.50)	4.1	(0.41)	
Day 1 Total motile spermatozoa (%)	18.9	(3.61)	22.2	(2.65)	
Day 1 Progressively motile (%)	10.2	(3.03)	16.7	(2.36)	
Day 2 Total motile (%)	13.4	(2.12)	19.6	(3.60)	
Day 2 Progressively motile (%)	7.8	(1.69)	10.0	(2.63)	
Day 3 Total motile (%)	6.3	(0.97)	8.9	(2.28)	
Day 3 Progressively motile (%)	1.9	(0.79)	4.1	(1.77)	
Day 4 Total motile (%)	4.7	(1.17)	6.1	(1.76)	
Day 4 Progressively motile (%)	7.6	(0.73)	1.3	(0.71)	
Day 5 Total motile (%)	3.8	(1.29)	2.4	(1.12)	
Day 5 Progressively motile (%)	0.8	(0.32)	0.1	(0.11)	

a Manual stimulation fractionation, first 2 to 3 jets only (9)

b Imipramine induced (2 mg/kg iv: 11).

c Paired t-tests.

d Extended to 100 x 10⁶ spermatozoa/ml using modified Kenney extender.

e Stored at 4°C.

For 1 stallion, repeated in copula fractionation attempts yielded samples with concentrations less than 200×10^6 spermatozoa per ml. For this same stallion, repeated attempts to obtain a second ex copula induced ejaculate failed. Accordingly, for this animal only 1 ejaculate from each method was frozen.

For each semen characteristic listed above, as well as number of days post thawing to reach 0% progressively motile spermatozoa, ex copula (n=9) and in copula ejaculates (n=9) were compared using paired t-test procedures.

RESULTS

Semen

Semen characteristics at the time of collection for each method are summarized in Table 1A. Imipramine-induced ex copula ejaculates were of significantly higher concentration and lower volume than in copula fractionated ejaculates. The mean total number of spermatozoa was 50% higher for imipramine-induced ex copula ejaculates, however this difference was not statistically significant. However, it is worth noting that for 4 of the 5 stallions, both ex copula ejaculates contained higher total numbers than either of the in copula ejaculates. The mean percentage of morphologically normal spermatozoa as well as the mean pH of raw semen and the mean percentage of total and progressively motile spermatozoa (extended semen) were similar for the 2 collection methods.

Post-thaw semen morphology and motilities are summarized in Table 1B. Initial post-thaw motilities as well as daily motilities and the mean number of days to reach 0% progressively motile were all similar for the two methods of collection. Mean percentage of morphologically normal spermatozoa was also similar for in copula and ex copula ejaculates.

Efficiency of Methods

For the in copula fractionated semen collection method, a total of 12 trials were required to obtain 9 ejaculates for freezing. 3 failures were due to insufficient concentration of spermatozoa (less than 200×10^6 spermatozoa/ml). For the induced ex copula collection method, a total of 17 attempts were required to obtain 9 ejaculates. All induced ejaculates were sufficiently concentrated for freezing; failures were due to no ejaculation. For 10 trials, xylazine was administered when ejaculation had not occurred within 60 min after imipramine treatment. Ejaculation occurred after administration of xylazine in 2 of these trials.

DISCUSSION

These findings suggest that post-thaw motility is similar for imipramine-induced ex copula ejaculates and fractionated in copula ejaculates of stallions. In our laboratory, initial post-thaw progressive motility of at least 35% is considered acceptable for insemination of mares. This criteria was met for all except 1 in copula sample, which had an initial post-thaw progressive motility of 25%. Actual breeding trials are necessary to evaluate the fertility of semen, however, these results suggest that imipramine-induced ejaculates may be suitable for cryopreservation.

There are several characteristics of imipramine-induced ejaculates that theoretically might render them especially well-suited for cryopreservation. First, if a higher total number of spermatozoa per ejaculate could be obtained, as was the case in our previous study (11) and for 4 of the 5 stallions in this study, a higher number of insemination doses

could be frozen per collection. Second, in order to package sufficient numbers of appropriately extended spermatozoa a single straw, current methods of freezing stallion semen require a sample of higher concentration than is typically ejaculated in copula (13) or, with certain stallions, that can be obtained using fractionation methods (14). Imipramine-induced ejaculates are consistently of very high concentration. Third, although the mechanism is not known, seminal plasma has been found to have adverse effects on sperm motility (4,15). It has been suggested that differences in seminal plasma composition may, at least partially, account for differences in post-thaw motility or fertility among individual stallions, between ejaculates from the same stallion, or in association with season (1,7). Highly concentrated semen samples relatively free of seminal plasma are thus considered desirable to minimize such effects. Centrifugation of complete ejaculates collected by traditional in copula methods is the most common method used for increasing concentration of spermatozoa and removing seminal plasma from samples for freezing. Centrifugation has been shown to adversely affect sperm motility (3,13). Both centrifugation and fractionation result in exposure of spermatozoa to seminal plasma for variable periods of time. In boars, it has been shown that some seminal plasma proteins bind to spermatozoa after only a few minutes of exposure (12). Thus, theoretically, it may be beneficial to reduce spermatozoa contact with seminal plasma, as appears to occur when ejaculation is induced with imipramine. Spermatozoa have been shown to be capable of surviving normally after removal of seminal plasma and resuspension in semen extender (4).

The rate of ejaculation using this articular imipramine treatment protocol was greater (53%) than for our initial study (3%; 11). Several factors may account for this difference. In the present study, we waited longer (60 min) to administer xylazine after imipramine treatment than we had in the initial study (10 min). Our rationale for the longer interval was that findings from the initial study suggested that imipramine alone may be relatively effective. In that earlier study, 6 of the 16 (37.5%) ejaculations during a total of 24 trials (25%) occurred with imipramine alone (within the 10 min before administration of xylazine). The overall ejaculation rate was 33%. In the present study, 7 of 9 (78%) ejaculations out of a total of 17 (41%) trials occurred within 60 min using imipramine alone. In the previous study, one or more experimenters remained within sight of the stallion to observe ejaculation whereas in the present study, animals were left undisturbed after treatment. It is our experience that induced ejaculation rate is higher in undisturbed animals. Another difference between the 2 drug-induced ejaculation protocols was the length of the ejaculation interval, which was shorter (2 to 3 d) in the present study than in the previous one (4 d). Though not systematically tested, it is our impression that, for all the induced-ejaculation regimens we have tested, the longer the interval between ejaculations, the greater the likelihood that ejaculation will occur.

REFERENCES

1. Amann, R.P., Cristanelli, M.J. and Squires, E.L. Proteins in stallion seminal plasma. *J. Reprod. Fertil.* 1987; 35 (Suppl):113-120.
2. Burns, P. J. Modification of Kenney's extender for cryopreservation of equine spermatozoa. Proceedings 12th International Congress on Animal Reproduction, The Hague, The Netherlands, 1992; 1849-1851.
3. Cochran, J.D., Amann, R.P., Froman D.P., and Pickett, B.W. Effects of centrifugation, glycerol level, cooling to 5°C, freezing rate and thawing rate on post thaw motility of equine sperm. *Theriogenology* 1984; 22:25-38.
4. Jasko, D.J., Moran, D.M., Farlin, M.E. and Saires, E.L. Effect of seminal plasma dilution or removal on spermatozoal motion characteristics of cooled stallion semen. *Theriogenology* 1991; 35: 1059-1067.

5. Kenney, R.M., Hurtgen, J.P., Pierson, R.H., Witherspoon, D. and Simons, J. Clinical fertility evaluation of the stallion. *Society for Theriogenology*, Hastings, NE, 1983; 19-25.
6. Love, C.C., Loch, W.L., Bristol, F., Garcia, M.C. and Kenney, R.M. Comparison of pregnancy rates achieved with frozen semen using two packaging methods. *Theriogenology* 1989; 31:613-622.
7. Magistrini, M., Chanteloube, Ph. and Palmer, E. Influence of season and frequency of ejaculation on production of stallion semen for freezing. *J. Reprod. Fertil. Suppl.* 1987; 35:127-133.
8. McDonnell, S.M. Pharmacologically induced ex copula ejaculation in stallions. *Proceedings 12th International Congress on Animal Reproduction*, The Hague, The Netherlands, 1991; 1894-1896.
9. McDonnell, S.M. and Love, C.C. Manual stimulation collection of semen from stallions: Training time, sexual behavior and semen. *Theriogenology* 1990; 33:1195-1210.
10. McDonnell, S.M. and Love, C.C. Xylazine-induced ex copula ejaculation in stallions. *Theriogenology* 1991; 36:73-76.
11. McDonnell, S.M. and Odian, M.J. Imipramine and xylazine-induced ex copula ejaculation in stallions. *Theriogenology* (in press).
12. Metz, K.W., Berger, T. and Clegg, E.D. Adsorption of seminal plasma proteins by boar spermatozoa. *Theriogenology* 1990; 34:691-700.
13. Pickett, B.W. and Amann, R.P. Cryopreservation of semen. *In: McKinnon, A.O. and Voss, J.L. (eds), Equine Reproduction*. Lea and Febiger, Philadelphia, 1993; 769-789.
14. Tischner, M., Kosiniak, K. and Bielanski, W. Analysis of the pattern of ejaculation in stallions. *J. Reprod. Fertil.* 1974; 41:329-335.
15. Varner, D.D., Blanchard, T.L., Love, C.L., Garcia, M.C. and Kenney, R.M. Effects of semen fractionation and dilution ratio on equine spermatozoal motility parameters. *Theriogenology* 1987; 28:709-723.