LIVESTOCK RESEARCH INTERNATIONAL
Journal homepage: www.jakraya.com/journa/lri

ORIGINAL ARTICLE

Freezability and Fertility of Marwari Horse Semen at Farmer's Doorstep

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Received: 09/01/2015

Revised: 04/03/2015

Accepted: 05/03/2015

Abstract

The present study was aimed to assess the success of Marwari horse semen freezing at owner's doorstep (field level) besides to record conception rate and fertility using frozen semen for artificial insemination (AI). A total of 108 ejaculates out of twenty stallions were collected, evaluated and subjected for freezing. Ejaculates that had progressive sperm post-thaw motility (PTM) ≥30% were kept stored for use in AI as this is an internationally accepted minimum standard. AI was performed in Marwari mares at farm using frozen-thawed semen. In present study, sperm PTM in ejaculates subjected for freezing ranged between 7.5±1.44 to 46.5±1.44%. Based on sperm PTM% range <20, 20 to 40 and >40%, horses were categorized as poor, moderate and good freezers, respectively. Conception at the end of breeding season was 37.5% (36/96), while the average number of AI per conception was 2.66**.** Fertility of stallion's using frozen semen AI was 37.5% and 36.45%, based on pregnancy at the end of breeding season and foal born, respectively. There were individual variations in ejaculates success to freezing, however, semen can be frozen with satisfactory results in field that resulted in an acceptable conception and fertility using frozen semen in horses.

Keywords: Conservation, Conception, Freezing, Marwari horse, Semen.

1. Introduction

Marwari horses are native of Marwar (Barmer, Jalore, Jodhpur, Lakshman Nagar, Nagaur, Pali) and Mewar (Bhilwara, Chittorgarh, Rajsamand, Udaipur) regions of Rajasthan, India. This breed of horse is known for compact conformation and endurance which has good export potential (Gupta *et al.*, 2014). This population of Indian horses has declined rapidly to an endangered category (Gupta and Pal, 2010; Gupta *et al.*, 2012) as a result of indiscriminate breeding with exotic or nondescript animals and heavy mechanization. This necessitates steps to be taken to conserve and multiply these animals as Purebred. Availability of Marwari stallions is insufficient and scattered at distance which put limitations to breed a mare. Cryopreservation of semen is a way to conserve and disseminate superior germplasm at a desired time and place. The use of frozen semen in AI increases the spectrum of an elite stallion for breeding. However, the use of frozen semen in general is limited by the variability between stallions and between ejaculates within stallions; ability of the spermatozoa to tolerate the freezing-thawing process and thus subsequent variable reduction in fertility (Amann and Pickett, 1987). Variability in post-thaw spermatozoa motility has been reported between ejaculates within (Arangasamy *et al.*, 2008) and between (Pal *et al.*, 2011) Marwari horses.

Classification of stallions as 'good' moderate or 'poor' freezers is based on post-thaw motility characteristics, including percentage of progressively motile sperm and velocity rate. According to Tischner (1979), about 20% of the stallions show good semen freezability with more than 40% progressively motile sperm after freeze–thaw, 60% has acceptable or fair freezability (20–40% post-thaw progressive sperm motility), and 20% has poor survival showing less than 20% progressively motile sperm cells after freeze–thaw. Loomis and Graham (2008) accepted stallions for their freezing program when post-thaw progressive sperm motility was greater than 30% following 30 min incubation at 37^0C for more than 25% of the ejaculates. On farm semen collection from Marwari horses, its cryopreservation (Pal and Legha, 2009; Pal *et al.*, 2009) and use for AI in mares (Arangasamy *et al.*, 2008) was initiated in the past decade at Equine

Production Campus, Bikaner but the reports on seminal characteristics (Ravi *et al.*, 2013) and fertility rates of stallion's semen frozen under field conditions is scanty. Therefore, present study was undertaken with an aim to assess success of semen freezing at farmer's doorstep; to evaluate their seminal characteristics and also to assess the conception rate and fertility by using frozen semen for AI.

2. Materials and Methods

2.1 Semen Collection

Twenty apparently healthy Marwari stallions aged between 6 to 12 years were used for semen collection at owner's doorstep from places including Udaipur, Ladnu, Dundlodh (Jhunjhunu) in Rajasthan and Gumjal in Punjab. Semen was collected by artificial vagina (Colorado model) equipped with a disposable liner as per the standard method. Semen was processed for freezing as per the method described by Pal *et al.* (2011) after recording the fresh seminal characteristics. The semen samples having progressive sperm motility more than 60% were further processed for cryopreservation. Semen freezing laboratory was set up at space provided in field. Both primary (containing Glucose 0.15g, Sodium citrate dehydrate 2.6g, Disodium EDTA 0.37g, Sodium bicarbonate 0.12g, Streptomycin 0.10g, Benzyl penicillin 0.10g, made up to 100ml with double distilled water) for initial sperm washing and secondary media (mixture of two solutions i.e. 25 ml from Solution 1 + 50 ml from Solution 2 with 20 ml egg yolk) for final dilution were prepared in fresh within an hour of semen collection, respectively. Gel free semen was mixed with modified glucose EDTA primary extender in the ratio of 1:1 and centrifuged at 2000 RPM at 10º C for 3 min. Supernatant was discarded and the modified secondary extender was added to sperm pellet as per desired dilution rate. Secondary extender was prepared by mixing of solution 1 (contains Glucose EDTA: Glucose 6g, Sodium citrate dehydrate 0.37g, Disodium EDTA 0.37g, Sodium bicarbonate 0.12g, Streptomycin 0.10g, Benzyl penicillin 0.10g, made up to 100ml with double distilled water) and solution 2 (contains Lactose 11 %: lactose 11g, Streptomycin 0.08g, Benzyl penicillin 0.08g, made up to 100ml with double distilled water). This mixture was then filled in to plastic centrifuge tubes (50 ml capacity), centrifuged at 3000 RPM at 10º C for 30 min, collected clear supernatant fluid and added with glycerol at 2% of the total volume.

2.2 Evaluation of Fresh Semen Quality

Soon after semen collection, seminal characteristics were recorded by direct visual observation (color, consistency) and examined under microscope (total and progressive sperm motility, sperm count). Parameters like total seminal volume, gel volume, gel free volume and pH were also recorded. Gel free semen was used to evaluate seminal pH, total and progressive sperm motility percent. Sperm concentration was determined using hemocytometer. Semen was extended to contain approximately 150 to 200 x 10^6 sperm ml^{-1} before subjected to freezing protocol.

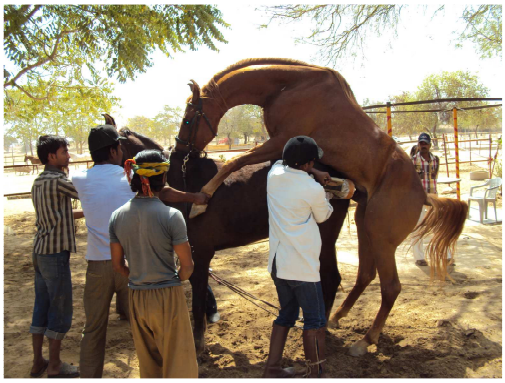

Fig 1: Semen collection from an elite Marwari stallion at owner's doorstep

2.3 Semen Cryopreservation

Straws filled with extended semen (150 to 200 x 10^6 sperm ml^{-1}) were kept for equilibration in refrigerator at 4 to 5°C for 2 hour. These pre equilibrated straws were placed inside chamber of programmable planner when temperature in chamber reached to 4 to 5°C. The cooling rate of chamber was at 0.3°C/min from 18°C to 5°C, and frozen at 10°C/min from +5°C to –15°C, then at 19°C/min from –15°C to –100°C. After reaching –100°C, the straws were taken out and plunged into canisters dipped in to liquid nitrogen (-196°C) till further use for AI.

Fig 2: Semen evaluation and processing laboratory set up at owner's doorstep

2.4 Evaluation of Post Thaw Sperm Motility

Two frozen semen straws of each ejaculate were thawed at 37^0 C for 30 sec, placed on a pre warmed slide, covered with a cover slip and evaluated visually for motility under the microscope. Semen samples with sperm PTM ≥30% were kept stored for further use in AI.

2.5 Artificial Insemination in Mares

Farm mares (>3.5 years of age) at Equine Production Campus, Bikaner were inseminated towards the end of the estrus once, twice or thrice per cycle before ovulation and up to 6 hours post ovulation which was carefully monitored through per-rectal ultrasonography. AI was done once the follicle diameter reached ≥40 mm and at every 24 hr till ovulation was confirmed. Based on sperm PTM% range <20, 20 to 40 and >40%, horses were categorized as poor, moderate and good freezers, respectively. The frozen semen from stallions categorized as moderate to good freezers were used for AI. Eight frozen thawed straws of 0.5 ml capacity (75 to 100 x 10^6 sperm per 0.5 ml straw) were used (approx. a volume of 4 ml) for each insemination into the uterine body when the AI catheter was just crossed the cervix. Number of inseminations per cycle was counted as single AI.

2.6 Statistical Analysis

All the experimental data were analyzed using following calculations

2.6.1 Freezing Success/Freezability

The percent of freezing success for the ejaculates was calculated as per the given formula:

$$\textit{Ejaculate freezability \%} = \frac{\textit{Number of ejaculates selected (PTM} \geq 30\%)}{\textit{Total Number of ejaculates frozen}} \times 100$$

2.6.1 Conception Rate

The conception percent was calculated as per the given formula:

$$\textit{Conception \%} = \frac{\textit{Number of mares conceived at the end of season}}{\textit{Number of mares inseminated during the season}} \times 100$$

2.6.3 Foaling Rate

The foaling percent was calculated as the percentage of inseminated mares which delivered a foal.

$$\textit{Foaling \%} = \frac{\textit{Number of inseminated mares delivered a live foal}}{\textit{Number of mares inseminated during the season}} \times 100$$

2.6.4 Fertility Evaluation

Individual and overall fertility of stallions using frozen semen AI was calculated as percentage of mares that are pregnant at the end of each breeding season after a single or multiple inseminations during a single or several estrous cycles.

$$\textit{Fertility \%} = \frac{\textit{Pregnant mares at the end of breeding season after a single or multiple inseminations during a single or several estrous cycles during the one/all breeding season}}{\textit{Mares inseminated during the one/all breeding season}} \times 100$$

Similarly, fertility based on foaling was calculated as percentage of mares that deliver a live offspring after single or multiple inseminations over one or many estrous cycles.

3. Results and Discussion

3.1 Seminal Characteristics

Milky white to creamy appearance with variably thin consistency of gel free semen observed in present study was similar to observation of Pal *et al*. (2009) in Marwari horses. Total, gel free and gel volume of semen were recorded (mean±SEM) as 84.61±6.14, 46.20±2.46 and 38.34±5.26 ml, respectively. Variations in semen volume may be caused by age, season, teasing time, frequency of semen collection and workload which had also been described by Pickett *et al*. (1988). Previous records of semen volume reported to vary from 20 to 160 ml (Pal and Legha, 2009) and 30 to 225 ml (Pal *et al.*, 2009*)* in Marwari horses. Previous observations of mean±SEM of total, gel and gel free volume of semen reported as 81.46±7.14 (Pal and Legha, 2009), 23.21±6.48 and 53.69±2.64 ml (Pal *et al.*, 2009), respectively in Marwari horses.

Mean±SEM of total and progressive sperm motility percent in gel free semen was 77.61±0.77 and 70.98±0.79 which is comparable to recent study of Ravi *et al*. (2014) in Marwari horses. Pre-freeze motility observed was 64.85±0.94 percent after 2 hour of incubation at 4 to 5^0C in refrigerator. Mean±SEM of sperm concentration in fresh semen was 192.74±4.26 x 10^6 ml^{-1} (ranged 100 to 280 x 10^6 ml^{-1}) which is

comparable 182.0±11.89 x 10^6 ml^{-1} as reported by Ravi *et al*. (2014) in Marwari horses.

3.2 Semen Freezability

Mean±SEM sperm PTM in ejaculates subjected for freezing ranged between 7.5±1.44 to 46.5±1.44%, with wide individual variation in the semen freezing success. Stallions which had post-thaw motility <20, 20–40 and >40% were categorized as poor, moderate and good freezers, respectively as per previous study (Pal *et al.*, 2011). In present study, only 30% stallions were graded as good (6/20), majority (65%) were moderate (13/20) and 5% as poor (1/20) freezers. Mean±SEM sperm PTM in selected ejaculates of moderate to good freezer stallions was 36.41±0.74 percent. Freezing success of ejaculates varied widely between stallions (Table 1).

Table 1: Stallion-wise freezing success of ejaculates

Name of stallion	Ejaculates subjected for freezing (n)	Ejaculates cryopreserved successfully (n)	Freezing success (%)
Ashwini	3	3	100
Badal	3	1	33.33
Bahadursah	1	1	100
Chandravansh	6	4	66.66
Deewana	10	4	40
Dilbag	6	5	83.33
Dilsher	12	5	41
Gajraj	4	1	40
Ganesh	4	4	100
Hansraj	15	9	60
Kohinoor	3	2	66.66
Kuber	7	6	85.71
Kuldeepak	9	9	100
Rajrajeshwar	5	3	60
Rajratan	4	3	75
Rajtilak	4	None	Nil
Samrat	4	3	75
Sanjay	2	1	50
Shekha	2	2	100
Sikander	4	4	100
Overall	Total=108	Total=71	Average =65.13

3.3 Conception Rate and Foaling

A total of 96 inseminations with frozen semen were performed since year 2006 to 2012 in 23 mares repetitively in different breeding seasons. Overall conception observed based on pregnancy at the end of breeding season year (2006 to 2012) was 37.5% (36/96). Average number of AI per conception calculated was 2.66**.** In general, pregnancy rates from frozen semen are not as high as those expected from fresh semen in horses and a difference of 5 to 30% is often quoted (Wockener and Colenbrander, 1993). Gary (2005) reported conception rate between 20 to 55% in Thoroughbred horses. Jhamb *et al.* (2006) reported pregnancy rate as 61 and 36%, using fresh semen *vs.* frozen semen, respectively, in Thoroughbred horses. In a pilot study, Arangasamy (2008) observed a conception rate of 72.72% using frozen semen of stallions maintained at farm and the number of mares inseminated was very less (8/11). Conception rate of 37.5% achieved in the present study is appreciable due to the fact that the longevity of frozen semen in female reproductive tract is reduced (Watson, 2000) as well as mare exhibits estrus for approximately one week. A total of 35 foals were born out of 36 pregnancies observed at the end of respective breeding season during 2006 to 2012, with a foaling percent 36.45%. One still birth occurred in a mare in the year 2009. Following AI with frozen semen, foaling rate reported to vary from 30% (Wockener and Colenbrander, 1993) to 60% (Carnevale and Ginther 1995). Average pregnancy rates per cycle in mares bred with frozen semen was reported between 30 and 40% with a wide range between sires (Samper, 2001). The quality of the semen, the breeding status of the mare and the management of the mare during the estrus period are the three factors that had greatest impact on pregnancy rates inseminated with frozen semen (Vidament *et al*., 1997). More than one insemination with frozen semen in the same cycle can results in slight but consistently higher pregnancy rates compared to a single insemination (Vidament *et al*., 1997). Mares with histories of reproductive problems in general had lower pregnancy rates than the average (Samper *et al*., 1994).

3.4 Fertility of Stallion

Overall fertility of stallion's using frozen semen AI was 37.5% and 36.45%, based on pregnancy at the end of breeding season and foal born, respectively. To study stallion-wise fertility, frozen semen of 9 stallions (graded as moderate to good freezers) was used for AI. Conception rate was observed more than 50% in 3 stallions (good freezers), between 30 and 49% in 3 stallions and less than 30% in 3 stallions graded as moderate freezers (Table 2). Fertility per cycle with frozen semen reported to vary between 30 and 55% (Palmer and Magistrini, 1992). Our findings of fertility rate are well supported by these workers. Number of mares allotted to each stallion for fertility trial was less during the study and stallion wise fertility which correspond to conception and foaling are presented in Table 2. Breeding closer to ovulation and deposition of semen close to the utero-tubal junction can increase the fertility for most stallions-

Table 2: Stallion-wise inseminations with frozen semen (2006-2012)

Name of stallion	Number of mares inseminated	Mares pregnant at the end of breeding season	Conception (%)	Foaling (%)
Bahadursah	8	6	75	62.5
Dilsher	13	7	53.84	53.84
Gajraj	6	1	16.66	16.66
Ganesh	9	5	55.55	55.55
Hansraj	25	8	32	32
Kohinoor	8	2	25	25
Rajrajeshwar	9	3	33.33	33.33
Rajratan	10	3	30	30
Samrat	7	1	14.28	14.28
Overall	Total=96	Total=36	Average=37.5	Average=36.45

with frozen semen (Samper *et al*., 1994).

Amann (2005) emphasized that fertility studies in horse are notoriously difficult to interpret and sample sizes of statistical relevance are almost impossible to achieve. Also, mares could not be evenly distributed to each stallion due to colour preference of particular stallion by the owner of the mare. It was reported that in order to determine a true fertility percentage with a variation of approximately 10%, nearly 75 females per treatment must be inseminated, whereas at least 100 females must be inseminated to reduce the variation to 7% (Amann, 2005). Lower fertility occurs when mares are inseminated with frozen semen (Samper, 2001). Variables affecting the fertility include the number of spermatozoa per dose, the number of inseminations per cycle and the timing of the insemination with respect to ovulation (Amann, 2005; Samper *et al.,* 2002). Finally, fertility is also dependent upon the number of spermatozoa inseminated (den Daas *et al.*, 1998), the age of the females being inseminated (Carnevale and Ginther, 1995); Samper *et al*., 2002) and timing of insemination to ovulation (Amann, 2005; Samper *et al.,* 2002). These variables are not detected in semen analysis. Despite difficulties correlating laboratory data with fertility, laboratory evaluation of semen quality remains important. Although laboratory semen analyses may not predict actual fertilizing potential of a semen sample, the analysis may predict samples of low fertility and allow exclusion of those samples from an artificial insemination program (Amann, 2005).

4. Conclusions

From the present study, it is clearly evident and can be concluded that ejaculates from all the stallions do not freeze alike and semen from some stallions those categorized as poor freezers cannot be cryopreserved. In general, semen from stallions that freezes had less frozen-thawed sperm motility per cent as compared to bovines. Good results of AI with frozen semen can be obtained by selection of good freezer stallions, monitoring of estrous cycle to predict time of ovulation and timely insemination of mares.

Acknowledgements

The authors are thankful to Director, National Research Centre on Equines for providing facilities and funds to carry out this study in field.

References

Amann RP (2005). Weaknesses in reports of ''fertility'' for horses and other species. *Theriogenology*, 63: 698-715.

Amann RP and Pickett BW (1987). Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa. *Journal of Equine Veterinary Science*, 7: 145-173.

Arangasamy A, Legha RA, Pal Y, Bansal RS, Singh J and Sharma RC (2008). Conception rate of Marwari stallion's semen cryopreserved by custom freezing. *Indian Veterinary Journal*, 85: 1001-1002.

Carnevale EM and Ginther OJ (1995). Defective oocytes as a cause of subfertility in old mares. *Biology of Reproduction (Monograph),* 1: 209-214.

den Daas JHG, de Jong G, Lansbergen LMTE and van Wagtendonk-de Leeuw AM (1998). The relationship between the number of spermatozoa inseminated and the reproductive efficiency of individual dairy bulls. *Journal of Dairy Science*, 81: 1714-1723.

Gary E (2005). Fertility and Obstetrics in the Horse. 3rd edn. *Blackwell Publishing Ltd UK.*

Gupta AK and Pal Y (2010). Genetic resource of equines in India and their status. In the compendium: Equine

Health and Production Management, NRCE Hisar, India, 103-106.

Gupta AK, Chauhan M, Bhardwaj A, Gupta Neelam, Gupta SC, Pal Y, Tandon SN and Vijh RK (2014). Comparative genetic diversity analysis among six Indian breeds and English Thoroughbred horses. *Livestock Science*, 163: 1-11.

Gupta AK, Tandon SN, Pal Y, Bhardwaj A and Chauhan M (2012). Phenotypic characterization of Indian horse breeds-a comparative study. *Animal Genetic Resources*, 50: 49.

Jhamb D, Kumar D, Kumar N, Tiwari D, Sharma G, Rathore GS and Prasad DM (2006). Artificial insemination in mares: fresh vs. frozen semen. In the National symposium on application of recent biotechnological advances in equine reproduction. Remount Veterinary Corps, Equine Breeding Stud, Babugarh, Uttar Pradesh.

Loomis PR and Graham JK (2008). Commercial semen freezing: individual male variation in cryosurvival and the response of stallion sperm to customized freezingprotocols. *Animal Reproduction Science*, 105: 119-128.

Pal Y and Legha RA (2009). Seminal characteristics of Marwari stallions. *Indian Veterinary Journal*, 86: 918-920.

Pal Y, Arangasamy A, Legha RA, Singh J, Bansal RS, Khurana SK and Tandon SN (2011). Freezability and fertility of Marwari stallion semen. *Indian Journal of Animal Science*, 81(5): 445-447

Pal Y, Legha RA and Tandon SN (2009). Comparative assessment of seminal characteristics of horse and donkey stallions. *Indian Journal of Animal Science*, 79(10): 1028-1029.

Palmer E and Magistrini M (1992). Automated analysis of stallion semen post-thaw motility. *Acta Veterinaria Scandinavica*, 88(Suppl): 137-152.

Pickett BW, Voss JL, Bowen RA, Squires EL and McKinnon AO (1988). Comparison of seminal characteristics of stallion that passed or failed seminal evaluation. In the 11th International Congress on Animal Production and Artificial Insemination. University College Dublin, Dublin Republic of Ireland, 380.

Ravi SK (2014). Effect of dietary n-3 PUFA on ovarian function, embryonic development and semen quality in horses. PhD Thesis, Indian Veterinary Research Institute (Deemed University), Izatnagar (UP), India.

Ravi SK, Legha RA, Pal Y and Sharma RC (2013). Characteristics and freezability of Kathiawari horse semen. *Indian Journal of Animal Science*, 83(11): 1146-1148.

Samper JC (2001). Management and fertility of mares bred with frozen semen. *Animal Reproduction Science*, 58: 219-228.

Samper JC, Hearn PH and Ganheim A (1994). Pregnancy rates and effect of extender and motility and acrosome status of frozen–thawed stallion spermatozoa. In the Proceedings of the 40th Annual Convention of American Association of Equine Practitioners, 41-43.

Samper JC, Vidament M, Katilia T, Newcombe J, Estada A and Sargent J (2002). Analysis of some factors associated with pregnancy rates of frozen semen: a multi-center study. *Theriogenology*, 58: 647-650.

Tischner M (1979). Evaluation of deep-frozen semen in stallions. *Journal of Reproduction and Fertility*, 27: 53-59

Vidament M, Dupere AM, Julienne P, Evain A, Noue P and Palmer E (1997). Equine frozen semen freezability and fertility results. *Theriogenology*, 48: 907-917.

Watson PF (2000). The causes of reduced fertility with frozen semen. In the Proceedings of the 14th International Congress on Animal Reproduction Stockholm, Sweden, 481-492.

Wockener A and Colenbrander B (1993). Liquid storage and freezing of semen from New Forest and Welsh pony stallions. *Deutsche Tierarztliche Wochenscbrift*, 100: 125-126.