Original Research Article

Fresh Seminal Characteristics of Marwari Stallionsin Breeding Season

ABSTRACT

Preserving the sperm of stallions with significant genetic worth is a widely accepted procedure. Thus, the species' typical reproductive behaviour is altered to facilitate the retrieval of sperm for the use of assisted reproductive biotechnologies, like artificial insemination and cryopreservation of sperm. As the Marwari breed of horse is a native breed of our country, the present experiment was designed to define the basic semen parameters of Marwari stallions. The experiment was conducted on six healthy Marwari horses aged between 50 and 140 months with a semen collection frequency of twice a week. A total of thirty six semen samples (six from each horse) were collected using artificial vagina. Following a macroscopic examination, fresh semen samples underwent a microscopic evaluation. The appearance of fresh semen was milky white or creamy white and the consistency was thin to thick. Fresh seminal pH was 7.54±0.03. Total ejaculated semen volume, gel volume and gel-free semen volume were 67.22±5.77 ml, 14.08±1.98 ml and 53.14±4.16 ml, respectively. Progressive sperm motility of sperm was 78.99±0.65%. Sperm concentration was 195.50±5.67 million/ml. Viability and total morphological abnormalities of sperm were 85.82±0.48% and 4.42±0.23%, respectively. Plasma membrane integrity (HOST), acrosome integrity and DNA integrity of sperm were noted as 53.65±0.85%, 87.36±0.36% and 95.83±0.24%. Significant difference was observed among Marwari stallions for semen volume (total ejaculated, gel and gel-free) ($P<0.05$), progressive motility ($P<0.05$), viability ($P<0.05$), concentration ($P<0.05$), plasma membrane integrity (HOST) ($P<0.05$), acrosome integrity ($P<0.05$), and DNA integrity ($P<0.05$) of sperm. Non-significant difference was observed for seminal pH ($P>0.05$) and total sperm morphological abnormalities ($P>0.05$).

Keywords:acrosome integrity, DNA integrity,fresh semen, HOST, Marwari horse, semen parameters.

Comment [D1]: Upper case letter

INTRODUCTION

A valuable diagnostic approach for determining the reproductive status of a person is through the examination of his or her sperm (Lodhi *et al*., 2008). It is necessary to use specialised processes in order to identify some types of damage, particularly in sperm membranes, as common methods are not able to detect certain types of damage (Celeghini*et al*., 2010a). The plasma membrane is responsible for the maintenance of cellular homeostasis (Oura and Toshimori, 1990). As a result, the integrity of the plasma membrane plays a significant role in ensuring the survival of sperm in the female reproductive tract and in maintaining the fertile capacity of the sperm. Since the acrosome response is crucial for sperm penetration into the zona pellucida and for oocyte plasma membrane fusion, acrosome integrity is required for the event of oocyte fertilisation (Flesch and Gadella, 2000). Oocytes must be protected in order to complete fertilisation. Sperm motility, morphology, and viability are the routine criteria that are used to evaluate the status of the sperm. Aside from

that, the sperm concentration, the volume, and the colour of the ejaculate are also important factors to be examined. The motility of sperm is the measure that is used most commonly in order to evaluate the quality of stallion semen (Tejerina *et al*., 2009). Computer Assisted Semen Analysis (CASA) lets you objectively evaluate many aspects of sperm motility. It is a more reliable, impartial, and repeatable way to measure sperm motility than looking at them with your own eyes (Colenbrander*et al*., 2003). CASA allows for the objective measurement of various aspects of sperm function. The measurement of an individual's membrane integrity (HOST) and motility was able to accurately predict the individual's positive fertility rate (Henkel *et al*., 1993). Studies have demonstrated that there is a significant correlation between fertility and the proportion of intact acrosomes (Saackle*et al*., 1980; Berndtson *et al*., 1981). On the other hand, the cold shock resistance test and the lifespan assay are equally useful techniques for determining whether or not stallion sperm exhibit fertility. It is common practice to preserve the sperm collected from stallions that have a high genetic value. Therefore, the normal breeding behaviour of the species is modified in order to enable the collection of sperm for the purpose of utilising reproductive biotechnologies such as artificial insemination (AI) and cryopreservation of sperm. Therefore, the purpose of this experiment is to study and define certain fresh seminal characteristics of Marwari stallions in arid region during the breeding season and also to compare the values of seminal parameters among the stallions via statistical tools.

MATERIALS AND METHODS

Experimental Conditions

The experiment was carried out on six healthy Marwari stallions well maintained at the Equine Production Campus, ICAR-National Research Centre on Equines, Bikaner, Rajasthan, India. Animals used in the present study were aged between 50 and 140 months and reared under uniform conditions of feeding and management (Table 1). The study was carried out during the breeding season with a frequency of semen collection twice a week and a total of 36 semen samples were collected (six samples per animal). Semen collection was done early in the morning before feeding via Colorado model artificial vagina (Figure 2) with a female in the oestrus as a dummy (Figure 1).

Table 1: Identification of horses with their age

S. No.	Marwari Stallion (identification)	Age (months)
1.	Mohit	140
2.	139	86
3.	Dogger	78
4.	167	52
5.	170	51
6.	175	50

Figure 1: Semen collection from Marwari stallion using artificial vagina

Figure 2: Assembled artificial vagina (Colorado model)

Evaluation of Semen Sample

Following collection, each freshly obtained semen sample was examined macroscopically to evaluate its colour, consistency, semen volume and seminal pH via bare eyes without the aid of any equipment and to identify any variations in the quality of the semen. As soon as the semen was collected, seminal parameters, such as seminal pH and the total volume of semen, were recorded. A digital pH metre was used to measure the seminal pH by dipping its probe into semen and recording the reading that appeared on the screen. The total volume of ejaculated semen was recorded from the semen collection bottle. After gross evaluation, each ejaculate was filtered through a sterile gauge into a graduated vial that had been prewarmed to 37°C in order to extract gel-free semen. Gel-free and gel volumes of

semen were also recorded. Each ejaculate was filtered through a sterile gauge to get gel-free semen following macroscopic examination. Each gel-free semen sample underwent a microscopic analysis after the macroscopic evaluation, which involved the evaluation of progressive motility, viability, concentration, total morphological abnormalities, plasma membrane integrity (HOST), acrosome integrity and DNA integrity of the sperm. A Computer Assisted Semen Analyzer (CASA) was used to evaluate the progressive motility of each sample. Following the procedure outlined by Kumar (2018), 20 µl of gel-free semen mixed with 980 µl of diluting fluid was used to determine the sperm concentration in each sample. The evaluation was carried out in an improved Neubauer's counting chamber at 37°C.

The final, sperm concentration was calculated by the following mathematical formula:

Sperm concentration (million/ml) = diluting factor (50) x sperm count in 5 square chambers x 0.05 x 10^6

Following the methodology of Treulen*et al*. (2018), the fluorescent dyes SYBR-14 and propidium iodide were employed to assess the viability of sperm. Kumar (2019) outlined a procedure for determining sperm abnormalities using 100X oil immersion. The Nie and Wenzel (2001) method was used to determine the integrity of the plasma membrane of the sperm (HOST). As per Soni *et al*. (2018), the fluorescent dye FITC-PNA was used to assess the sperm acrosome integrity. The DNA integrity was assessed following the method outlined by Liu and Baker (1994) using Acridine Orange fluorescent dye.

Data Analysis

The data of seminal parameters of fresh semen from Marwari stallions were gathered, arranged, and summarised. Statistical evaluations were conducted for mean, standard error (SE), analysis of variance (ANOVA), and Duncan's new multiple range test (DNMRT) using IBM-SPSS Statistics Version 26.

RESULTS AND DISCUSSION

Fresh Semen Evaluation

A total of 36 ejaculates, including six from each of the six stallions, were collected and analysed in this study to evaluate various characteristics of the fresh semen quality of Marwari horses.

Semen Colour andConsistency

Semen colour was classified as milky white or creamy white. The colour of stallion semen was observed as white-cloudy by Arifiantini *et al*. (2013). Legha*et al*. (2013) graded jack semen as milky white to creamy in their study and similar colour observations were made for Poitou jack semen by Pal *et al*. (2009) and Legha and Pal (2012). Ejaculate colour is normally opaque white and any deviation from this may indicate contaminants or abnormalities (Jasko, 1992). Common contaminants that may change the colour of an ejaculate are blood (red colour) and/or urine (yellow colour), which may have damaging effects on spermatozoa. In the present study, the seminal consistency was evaluated as thin to thick, more or less similar to the results obtained by Gupta *et al*. (2003) and Pal *et al*. (2009)

for Poitou Jacks semen, although Arifiantini *et al*. (2013) observed the thin consistency of stallion semen. A good stallion's semen should be milky white, but it can also be watery or creamy; a creamy ejaculate indicates a higher number of sperm, whereas a watery ejaculate frequently indicates a lower number (Kuklin, 1983).

Seminal pH

Seminal pH in freshly ejaculated semen of Marwari stallions was recorded as 7.48±0.08 to 7.58±0.09 with an average of 7.54±0.03. A non-significant difference (P>0.05) was observed among horses for value of seminal pH. A wider range for seminal pH of 7.2 to 7.7 and 6.9 to 7.8 was recorded by McKinnon *et al*. (2011) and Davies-Morel (2015), respectively. Seminal pH in the present study was consistent with findings ofHeckenbichler*et al*. (2011), who reported stallion semen pH range of 6.0-7.5, with a mean value of 6.6±0.0, and also similar to the findings observed by Arifiantini *et al*. (2013), who reported a pH of 7.0±0.2. Ravi *et al*. (2013) found that the seminal pH of Kathiawaristallions ranged from 6.7 to 7.4, with a mean value of 7.05±0.04. Seminal pH is affectedmainly by difference in secretions from the accessory sex glands. The pH of the secretion of these glands is alkaline and with an increase in ejaculation frequenciestends towards further alkalinity. (Tejpal *et al*., 2017).

Semen Volume (total ejaculated, gel and gel-free volume)

The total ejaculated volume of freshly ejaculated semen from Marwari stallions was recorded as 48.33±3.57 to 120.83±17.10 ml, with an average of 67.22±5.77 ml. A significant difference (P<0.05) was observed among the horses for total ejaculated semen volume. Tejpal *et al*. (2017) also observed a significant difference (P<0.05) amonghorses for total ejaculated volume with a lower average value of 48.69±8.01 ml (ranged between 31.33±3.12 to 75.83±15.35). Talluri *et al*. (2017) found the semen volume of 45.06±4.75 ml for Marwari stallions to be within the range of current values. The findings of this study were in line with those of Bustamante-Filho *et al*. (2009), who found that six fertile Criollo stallions had a typical semen volume range of 45.7-7.8 ml. Pal and Legha (2009) investigated a mean total ejaculated semen volume of 81.46±7.14 ml for Marwari stallion, which was within the range of the present study. Sieme*et al*. (2002) found 42.15±2.5 ml of stallion semen in ejaculates collected with the initial mount, which is lower than the current study. However, differences in individual semen production and teasing time have been linked to variations in semen volume (Pickett and Shiner, 1994; Ionata*et al*., 1991). Gamal *et al*. (2016) measured the semen volume in young, mediumaged, and old Arabian stallions. The average mean values were 38.33±8.53 ml, 40.0±10.37 ml, and 67.78±6.016 ml, respectively. They found that ejaculated volume was significantly greater in old stallions (P<0.05) than in young and moderately aged stallions. The gel volume of freshly ejaculated semen from Marwari stallions ranged from 8.33±1.67 to 32.50±6.29 ml, with an average of 14.08±1.98 ml. A significant difference (P<0.05) was observed among the horses for gel volume. A lower average gel volume of 3.75±7.63 ml for Marwari horses was recorded by Pal and Legha(2009). Gel-free volume of freshly ejaculated semen from Marwari stallions was recorded as 37.50±3.10 to 88.33±12.29 ml, with an average of 53.14±4.16 ml. A significant difference (P<0.05) was observed among the horses for gel-free volume. Gel-free volume was discovered by Kavak *et al*. (2003) to be between 61.9±35.1 and 42.9±28.1 ml, which were within the range of the present results. Similarly, in studies of Bozkurt *et al*. (2007) and

Arifiantini *et al*. (2013), the gel-free volume of horse semen was 51.1±6.4 and 33.6 ±12.9 ml, respectively. Contrary to the present results, Tejpal *et al*. (2017) noted a lower value for gel-free volume (17.5±2.5 to 34.16±5.54 ml) with an average of 25.99±3.88 ml, showing a non-significant difference (P>0.01) among the horses. Excessive sexual stimulation of the stallion before collection increases the volume of the total ejaculate by secreting accessory glands (Pickett *et al*., 1987).

Progressive Sperm Motility

Progressive sperm motility in freshly semen samples of Marwari horses was recorded as 75.48±1.21 to 82.08±1.48% with an average of 78.99±0.65%. A significant difference (P<0.05) was found among the stallions for progressivesperm motility. Tejpal *et al*. (2017) recorded a non-significant difference against the present observations. Values similar to those in the currentinvestigation were recorded by Pal *et al*. (2009) with an average sperm motility of 73.33% in the Marwari breed of horse. Although Ravi *et al*. (2013) reported the progressive sperm motility in gel-free semen to be 77.0±1.51% in Kathiawari horses andSieme*et al*. (2002) observed progressive motility of 75.2±2.7% in stallion semen. In contrast to the current investigation, Arifiantini *et al*. (2013) found 70.0±5.4% motility in fresh semen from stallions. Kavak *et al*. (2003) studied Tori and Estonian breed stallions and reported a lower sperm motility of 67.8±6.4% and 67.5±7.5%, respectively, than the present study in freshly ejaculated semen. A similar value was reported by Khalifa *et al*. (2006) at El-Zahra Arab Horse. Bustamante-Filho *et al*. (2009) in Criollo stallions reported progressive motility values of 71.3±20.0 % and 57.0±21.9 %, respectively. Talluri *et al*. (2012) investigated the parameters of fresh semen of Zanskari stallions andprogressive sperm motility in fresh semen was recorded 77.24±11.5%, which was in favour of the present study.

Sperm Concentration

Sperm concentration in freshly ejaculated semen of Marwari horses was recorded as 131.17±6.34 to 226.67±6.96 million/ml, with an average of 195.50±5.67 million/ml. A significant difference (P<0.05) was found among the horses for sperm concentration. Sieme*et al*. (2002) observed the sperm concentration with different numbers of mounts and recorded a similar value in the first mount (200±0.2 million/ml) and the second mount (190±0.2 million/ml), followed by decreased values of sperm concentration in succcessive mounts as compared to the present study. On the other hand, a higher sperm concentration of 378.79±0.15 million/ml was observed by Talluri *et al*. (2012) for Zanskari Stallions. However, Pal and Legha (2009) recorded a lower value of 154.81±12.56 million/ml for sperm concentration of Marwari stallions. Ravi *et al*. (2013), in their study, noted the values varried from 115-275 million/ml, which was within the range of the present study. Likewise, in another study, Kumar *et al*. (2014) and Arifiantini *et al*. (2013) found similar sperm concentrations of 217.43±11.76 and 213.0±21.9 million/ml of stallion semen, respectively.

Sperm Viability

Sperm viability in fresh semen of Marwari stallions was recorded with a range of 82.50±0.63 to 88.42±0.87% with an average of 85.82±0.48%. A significant difference (P<0.05) was found among the stallions for sperm viability. Lower values of sperm viability were recorded by Talluri *et al*. (2012) and Tejpal *et al*. (2017) in fresh semen of Zanskari stallions and Marwari stallions in the arid region, with mean values of 76.78±0.08% and 78.36±2.16%, respectively. In contrast to the present study, Tejpal *et al*. (2017) observed a

non-significant (P>0.01) among stallions for mean values of sperm viability. Sperm viability of horses reduces in the summer and differs significantly (P<0.05) from other seasons (Janett *et al*., 2003). Arifiantini *et al*. (2013) found a higher livability of fresh horse semen than the present study. A lower viability of 65.69±235.18% was observed by Gil *et al*. (2013) in fresh stallion semen. The live and dead sperm count gives a reliable measure of spermatozoa viability and a live sperm ratio >50% is consideredgood for AI (Davis-Morel, 1990; Rickets, 1993).

Sperm Total Morphological Abnormalities

Total morphological sperm abnormalities in freshly ejaculated semen of Marwari stallions were observed as 3.33±0.61 to 5.33±0.33%, with an average of 4.42±0.23%. A non-significant (P>0.05) difference was observed among the stallions for total morphological sperm abnormalities. Similar to the present study, a non-significant difference was observed among stallions for total sperm morphological abnormalities by Soni (2016), Kumar (2017), Kumar (2018), Kumar (2019) and Jhamb (2021). Soni (2016) recorded a lower mean value of 2.07±0.27% as compared to the present study. A higher mean value of 6.78±0.52% was reported by Kumar (2017). Kumar (2018) observed a very high mean value of 15.16±0.97%. Kumar (2019) and Jhamb (2021) recorded a high mean value of 7.19±0.15% and 8.8±0.14%, respectively in contrast to the present values. Morrell *et al.* (2008) recorded the morphological abnormalities were proximal cytoplasmic droplets (7–27%), pear-shaped heads (3.6-7%), heads with narrow bases (1.5-5%) and narrow heads (1-3%).

Sperm Plasma Membrane Integrity (HOST)

Sperm plasma membrane integrity (HOST) in freshly semen samples of Marwari stallions ranged from 46.83±1.43 to 58.17±2.32%, with an average of 53.65±0.85%. A significant difference (P<0.05) was found among the stallions for sperm plasma membrane integrity (HOST). Similar findings were noted by Tejpal *et al*. (2017), with HOST-positive sperm from six stallions ranging between 48.5±1.23 and 59±1.73% with an overall mean of 54.11±2.08%. The current findings were very similar to those of Henry *et al*. (2002) and Gil *et al*. (2013), who obsereved that in a 100 mM sucrose solution, fresh stallion semen had a mean value of 58.22±168.92% and 58.3±10.95%. Bustamante-Filho *et al*. (2009) reported very low average membrane functionality of 24.2±15.9% as compared to the present study on Criollo stallions. Talluri *et al*. (2012) recorded 79.41±1.67% of HOST-positive sperm in fresh semen from Zanskari stallions, which is very high in comparison to current experimental values. HOST is a basic but authentic sperm membrane integrity measurement test (Chowdhury *et al*., 2013).

Acrosome Integrity

Acrosome integrity in freshly ejaculated semen of Marwari stallions ranged from 84.92±0.51 to 89.00±0.86%, with an average of 87.36±0.36%. A significant difference (P<0.05) was found among the stallions for acrosome inegrity. Tejpal *et al*. (2017) noted a very high significant difference (P<0.01) among the stallions with an average acrosome integrity of 87.33±1.53%, which is similar to the current study. Pukazhenthi*et al.* (2014) evaluated the acrosome-intact sperm percentage in the fresh semen of Przewalski'sdomestic and wildstallions. The values of intact acrosomes reported in fresh semen of wild and domestic horses were 84.80±3.10% and 76.30±4.20%, respectively, which were very high as compared to Marwari stallion. Compared to the current study, Celeghini*et al*. (2010b) and Gil

et al. (2013) reported 75±6% and 62.36±85.15% of intact spermatozoa in fresh stallion semen, respectively. If a spermatozoon's acrosome is destroyed, it will not capable to fertilise mature female gamete, even if it is alive and exhibiting increasing motility (Graham, 2001).

DNA Integrity

DNA integrity in freshly ejaculated semen from Marwari horses ranged from 94.17±0.48 to 97.33±0.33%, with an average of 95.83±0.24%. A significant difference ($P<0.05$) was found among the stallions for DNA integrity values. Similar to this study, Kumar (2019) and Jhamb (2021) found a significant difference ($P<0.05$) in DNA integrity values between stallions. They found an average value of 94.1±0.36% in the fresh semen of a Marwari stallion. Kumar (2018) recorded an average DNA integrity value of 93.56±0.29% in freshly ejaculated semen of Marwari stallions, which was in favour of the present observations. In contrast to the present study, a non-significant difference among stallions for DNA integrity was reported by Soni (2016) and Kumar (2017), with an average value similar to the current investigation.

CONCLUSION

The study clearly defines the basic seminal characteristics of Marwari stallions in the arid region during breeding season and also provides a comparative evaluation of basic seminal parameters among Marwari stallions. Macroscopic evaluation of fresh seminal parameters revealed a non-significant difference ($P>0.05$) for seminal pH, while semen volume (total ejaculated, gel and gel-free) was significantly different ($P<0.05$) among stallions. A significant difference ($P<0.05$) was observed among stallions for all microscopic parameters, including progressive motility, viability, concentration, plasma membrane integrity (HOST), acrosome integrity and DNA integrity of the sperm, except total sperm morphological abnormalities, which was non-significantly ($P>0.05$) different among stallions. The effect of age and body weight on fresh seminal parameters should be studied to know the individual horse pattern.

REFERENCES

1. Arifiantini, R. I., Purwantara, B., Yusuf, T. L. and Sajuthi, D. (2013). The quality of stallion semen in skim milk and dimitropoulos extenders preserved at 50°C and ambient temperature supplemented with different sugar. Journal of Animal Science and Technology, 36(1):45-55.
2. Berndtson, W. E., Olar, T. and Pickett, B. W. (1981). Correlations between post-thaw motility and acrosomal integrity of bovine sperm. Journal of Dairy Science, 64:346-349.
3. Bozkurt, T., Turk, G. and Gur, S. (2007). The time-dependent motility and longevity of stallion spermatozoa diluted in different spermatozoal concentrations and extenders during cool storage. Revue de MedecineVeterinaire, 158:67-72.
4. Bustamante-Filho, I. C., Pederzolli, C. D., Sgaravatti, A. M., Mattos, R. C., Dutra-Filho, C. S. and Jobim, M. I. M. (2009). Activity of glutathione peroxidase and

catalase in stallion semen during cryopreservation. Animal Reproduction Science, 94(1-4):70-73.
5. Celeghini, E. C. C., Andrade, A. F. C., Raphael, C. F., Nascimento, J., Ticianelli, J. S. and Arruda, R. P. (2010a). Damage assessment of the equine sperm membranes by fluorimetric technique. The Brazilian Archives of Biology and Technology, 53(6):1285-1292.
6. Celeghini, E. C. C., Andrade, A. F. C., Raphael, C. F., Nascimento, J., Ticianelli, J. S. and Arruda, R. P. (2010b). Damage assessment of the equine sperm membranes by fluorimetric technique. The Brazilian Archives of Biology and Technology, 53(6):1285-1292.
7. Chowdhury, S., Das, S., Gupta, T., Sana, D. and Bose, S. (2013). A comparative study with Murrah buffalo and indigenous Gir spermatozoa to hypo-osmotic swelling test. Exploratory Animal and Medical Research, 3(2):159-164.
8. Colenbrander, B., Gadella, B. M. and Stout, T. A. E. (2003). The predictive value of semen analysis in the evaluation of the stallion fertility. Reproduction in Domestic Animals, 38:305-311.
9. Davies-Morel, M. C. G. (2015). Equine Reproductive Physiology, Breeding and Stud Management (Fourth Edition). CABI Publishing.
10. Davies-Morel, M. C. G. (1993). Equine Reproductive Physiology, Breeding and Stud Management,1st ed. Farming Press, Ipswich, UK.
11. Flesch, F. M. and Gadella, B. M. (2000). Dynamics of the mammalian sperm membrane in the process of fertilization. Biochimica et Biophysica Acta, 1469:197-235.
12. Gamal, A., El-Maaty, A. M. A. and Rawash, Z. M. (2016). Comparative blood and seminal plasma oxidant/antioxidant status of Arab stallions with different ages and their relation to semen quality. Asian Pacific Journal of Reproduction, 5(5):428-433.
13. Gil, L., Galindo-Cardiel, I., Malo, C., Gonzalez, N. and Alvarez, C. (2013). Effect of cholesterol and equex-STM addition to an egg yolk extender on pure Spanish stallion cryopreserved sperm. International Scholarly Research Notices, 2013.
14. Graham, J. K. (2001). Assessment of sperm quality: a flow cytometric approach. Animal Reproduction Science, 68(3-4):239-247.
15. Gupta, A. K., Singh, R., Mamta, Yash, P., Singh, M. K. and Yadav, M. P. (2003). Influence of season on characteristics of jack semen. Indian Journal of Animal Science, 73:986-991.
16. Heckenbichler, S., Deichsel, K., Peters, P. and Aurich (2011). Quality and fertility of cooled-shipped stallion semen at the time of insemination. Theriogenology, 75:849-856.
17. Henkel, R., Muller, C., Miska, W., Gips, H. and Schill, W. B. (1993). Determination of the acrosome reaction in human spermatozoa is predictive of fertilization in vitro. Human Reproduction, 8(12):2128-2132.
18. Henry, M., Snoeck, P. P. N. and Cottorello, A. C. P. (2002). Post thaw spermatozoa plasma membrane integrity and motility of stallion semen frozen with different cryoprotectants. Theriogenology, 58:245-248.

19. Ionata, L. M., Anderson, T. M., Pickett, B. W., Heird, J. C. and Squires, E. L. (1991). Effect of supplementary sexual preparation on semen characteristics of stallion. Theriogenology, 36(6):923-957.
20. Janett, F., Thun, R., Niederer, K., Burger, D. and Hassig, M. (2003). Seasonal changes in semen quality and freezability in the Warmblood stallion. Theriogenology, 60(3):453-461.
21. Jasko, D. (1992). In: Evaluation of stallion semen. Veterinary Clinics of North America; Equine Practice, Stallion Management, vol. 8. Philadelphia: WB Saunders Co., pp. 129-148.
22. Jhamb, D. K. (2021). Effect of L-arginine and trehalose supplementation to semen extender on quality and fertility of cryopreserved stallion semen. PhD Thesis, College of Veterinary and Animal Science, Navania, Rajasthan.
23. Kavak, A., Johannisson, A., Lundeheimd, N., Rodriguez-Martinez, H., Aidnik, M. and Einarsson, S. (2003). Evaluation of cryopreserved stallion semen from Tori and Estonian breeds using CASA and flow cytometry. Animal Reproduction Science, 76(3-4):205-216.
24. Khalifa, T. A. A., Waheed, M. M. and Lymberopoulos, A. G. (2006). An endeavor to improve longevity of cryopreserved equine sperm. Journal of Hellenic Veterinary Medicine Society, 57(3):195-204.
25. Kuklin, A. D. (1983). Artificial insemination of horses. Veterinaria, Moscow, USSR, 7:57-58.
26. Kumar, A. (2018). Evaluation of various parameters affecting semen quality in Marwari stallion. PhD Thesis, College of Veterinary and Animal Science, Bikaner, Rajasthan.
27. Kumar, D., Jhamb, D., Kumar, N. and Saxena, A. (2014). Effect of breeding and non-breeding seasons on post thaw quality of stallion semen. Indian Journal of Animal Reproduction, 35(1):33-38.
28. Kumar, P. (2019). Effect of addition of cholesterol-loaded cyclodextrin on quality of semen in Marwari horse and Poitou donkey. PhD Thesis, College of Veterinary and Animal Science, Bikaner, Rajasthan.
29. Kumar, P. (2017). Effect of ascorbic acid and glutathione on pre-freeze and post-thaw quality of equine semen. MVSc Thesis, College of Veterinary and Animal Science, Bikaner, Rajasthan.
30. Legha, R. A. and Pal, Y. (2012). Seminal characteristics of exotic jacks under arid conditions in Indian. Veterinary Practitioner, 13(2):229-231.
31. Legha, R. A., Pal, Y., Ravi, S. K. and Dedar, R. K. (2013) Physical and biochemical properties of indian jack semen. Animal Science Reporter, 7:4.
32. Liu, D. Y. and Baker, H. W. G. (1994). A new test for the assessment of sperm-zona pellucida penetration: relationship with results of other sperm tests and fertilization in vitro. Human Reproduction, 9(3):489-496.
33. Lodhi L. A., Zubair, M., Qureshi, Z. I., Ahmad, I. and Jamil, H. (2008). Correlation between hypo-osmotic swelling test and various conventional semen evaluation parameters in fresh Nili-Ravi buffalo and Sahiwal cow bull semen. Pakistan Veterinary Journal, 28(4):186-188.

34. McKinnon, A. O., Squires, E. L., Vaala, W. E. and Varner, D. D. (2011). Equine Reproduction, 2nd. John Wiley and Sons, UK.
35. Morrell, J. M., Johannisson, A., Dalin, A. M., Hammar, L., Sandebert, T. and Martinez, R. H. (2008). Sperm morphology and chromatin integrity in Swedish Warmblood stallions and their relationship to pregnancy rates. Acta Veterinaria Scandinavica, 50(1):1-7.
36. Nie, G. and Wenzel, J. (2001). Adaptation of the hypoosmotic swelling test to assess functional integrity of stallion spermatozoal plasma membranes. Theriogenology, 55(4):1005-1018.
37. Noue, P., Bernabe, J., Rampin, O., Vidament, M., Dumas, T., Palmer, E. and Magistrini, M. (2001). Sexual behavior of stallions during in-hand natural service and semen collection: an observation in French studs. Animal Reproduction Science, 68:161-169.
38. Oura, C. and Toshimori, K. (1990). Ultrastructural studies on the fertilization of mammalian gametes. International Review of Cytology, 122:105-151.
39. Pal, Y. and Legha, R. A. (2009). Seminal characteristics of Marwari stallions. Indian Veterinary Journal, 86(9):918-920.
40. Pal, Y., Legha, R. A. and Tendon, S. A. (2009). Comparative assessment of seminal characteristics of horse and donkey stallions. Indian Journal of Animal Science, 79(10):1028-1029.
41. Pickett, B. W. and Shiner, K. A. (1994). Recent developments in AI in horses. Livestock Production Science, 40:31-36.
42. Pickett, B. W., Squires, E. L. and McKinnon, A. O. (1987). Procedures for collection, evaluation and utilization of stallion semen for artificial insemination. Animal Reproduction Laboratory, Colorado State University, pp. 1-125.
43. Pukazhenthi, B. S., Johnson, A., Guthrie, H. D., Songsasen, N., Padilla, L. R., Wolfe, B. A., Silva, M. C. D., Alvarenga, M. A. and Wildt, D. E. (2014). Improved sperm cryosurvival in diluents containing amides versus glycerol in the Przewalski's horse (*Equus ferusprzewalskii*).Cryobiology, 68(2):205-214.
44. Ravi, S. K., Legha, R. A., Pal, Y. and Sharma, R. C. (2013). Characteristics and freezability of Kathiawari horse semen. Indian Journal of Animal Science, 83(11):1146-1148.
45. Rickets, S. W. (1993). Evaluation of stallion semen. Equine Veterinary Education, 5(5):232-237.
46. Sacckle, R. G., Marshall, C. E., Vinson, W. E., Oconner, M. L., Chandler, J. E., Mullins K. J., Amann, R. P., Wallace, R. A., Vincel, W. N. and Kellgree, H. C. (1980) Semen quality and heterospermic insemination in cattle. In Proceedings: 9th International Congress. Animal Reproduction and Artificial Insemination (Medrid), 5:75-78.
47. Sieme, H., Chte, A. and Klug, E. (2002). Effect of frequency and interval of semen collection on seminal parameters and fertility of stallion. Theriogenology, 58(2-4):313-316.
48. Soni, Y., Talluri, T. R., Kumar, A., Ravi, S. K., Mehta, J. S. and Tripathi, B. N. (2018). Effects of different concentration and combinations of cryoprotectants on

sperm quality, functional integrity in three Indian horse breeds. Cryobiology, 86:52-57.
49. Talluri, T. R., Arangasamy, Ravi, A. S. K., Pal, Y., Gupta, A. K. and Singh, R. K. (2012). Characterstics of fresh and frozen semen of Zanskari stallion. Indian Veterinary Journal, 89(7):62-64.
50. Talluri, T. R., Mal, G. and Ravi, S. K. (2017). Biochemical components of seminal plasma and their correlation to the fresh seminal characteristics in Marwari stallions and Poitou jacks. Veterinary World, 10(2):214-220.
51. Tejerina, F., Morrell, J., Petterson, J., Dalin, A. M. and Rodriguez-Martinez, H. (2009). Routine assessment of motility of ejaculated stallion spermatozoa using a novel computer-assisted motility analyzer (Qualisperm™). Animal Reproduction, 6(2):380-385.
52. Tejpal, Mehta, J. S., Ravi, S. K., Ruhil, S., Talluri, T. R., Kumar, A. and Singh, D. (2017). Study of certain physical parameters of fresh semen in Marwari horse. Veterinary Practitioner, 18(1):47-51.
53. Treulen, F., Aguila, L., Arias, M. E., Jofre, I. and Felmer, R. (2018). Impact of post-thaw supplementation of semen extender with antioxidants on the quality and function variables of stallion spermatozoa. Animal Reproduction Science, 201:71-83.