COMPARATIVE STUDY OF FRESH AND FROZEN THAWED SEMINAL CHARACTERISTICS OF THREE INDIGENOUS BREEDS OF STALLIONS[#]

Yogesh Soni[1], Ashok Kumar, J. S. Mehta, T. R. Talluri[2], S. K. Ravi[2], R. A. Legha[3], B. N. Tripathi[4] and Tejpal[5]
Department of Veterinary Obstetrics and Gynaecology, College of Veterinary and Animal Science
Rajasthan University of Veterinary and Animal Sciences, Bikaner-334 001, Rajasthan, India

Received on: 05.08.2016
Accepted on: 11.01.2017

ABSTRACT

The present study was undertaken with the objective to study and compare the seminal parameters of fresh and frozen thawed semen of stallions of Indian breeds viz., Marwari, Manipuri and Zanskari. Semen was collected from nine apparently healthy stallions (three stallions from each breed) aged between 4 to 6 years. A total of 54 ejaculates (6 ejaculates from each stallion) were evaluated on the basis of colour, consistency, total volume, gel free volume, pH, progressive sperm motility, sperm concentration, live sperm count, sperm abnormality, hypoosmotic swelling test, acrosomal integrity test and DNA integrity test. Post thaw evaluation of each semen sample was done on the basis of post thaw motility, live sperm count, hypo osmotic swelling test, acrosomal integrity test and DNA integrity test. It was observed that for the fresh semen evaluation parameters the stallions, but not breeds were found to be significant source of variation, for total semen volume ($P \leq 0.05$); gel free volume ($P \leq 0.05$); progressive sperm motility ($P \leq 0.05$); total sperm concentration ($P \leq 0.01$); live sperms percentage ($P \leq 0.05$), HOST ($P \leq 0.05$); acrosomal integrity test ($P \leq 0.05$). For the post thaw seminal parameters the stallions and breeds both were found to be significant source of variation.

Key words: Stallion, pony, semen, Marwari, Manipuri, Zanskari, frozen

Introduction

India harbours two horses (Kathiawari and Marwari) and four pony (Manipuri, Spiti, Zanskari and Bhutia) breeds inhabiting in different agro-climatic regions (Gupta *et al.*, 2014). Over the time, these breeds have adapted with certain unique traits like endurance, relative disease tolerance, sturdiness, sure-footedness to sustain and work in harsh environmental conditions. All the four pony breeds are endangered breeds because of their shrinking number in their respective breeding tracts (Gupta *et al.*, 2014). The population of purebred animals of these breeds has reduced considerably low, which has brought this breed in the category of threatened equine breed in the country. This necessitates immediate measures to be taken for conservation and propagation of indigenous breeds of horses. In the horse breeding industry, there is a growing demand for semen that can be used for artificial insemination independent of location and availability of stallions. The use of cryopreserved sperm is one way to face this dilemma, but sperm from some stallions exhibits unsatisfactory post-thaw survival and fertility rates (Vidament *et al.*, 1997; Loomis and Graham 2008). In order to improve cryosurvival, fundamental research on the seminal characteristics and events that induce and prevent cryodamage is essential. Keeping in view of the above cited factors, as a measure to conserve the Indigenous equine germplasm, ICAR-NRC on Equines, Bikaner, Rajasthan is making valiant efforts for efficient breeding and their *ex-situ* conservation and propagation by semen collection and cryopreservation of these breeds and recording the fundamental breeding related data of these breeds. The present study was conducted with an objective to study and compare the seminal parameters of fresh and frozen thawed semen of stallions of these Indian breeds.

Materials and Methods

The experiment was conducted on 4 to 6 years aged stallions (n=9) of three indigenous breeds (Marwari, Zanskari and Manipuri) (three stallions from each breed), maintained at the Equine Production Campus, Bikaner, Rajasthan under uniform management and housing conditions, during the months of breeding season from April to July. All the animals were fed uniformly and no source of artificial light and no special feed were provided. All the animals were provided with water and feed *ad lib* during all the time.

A total of 54 ejaculates from 9 stallions (6 ejaculates from each) were collected. Semen was collected from stallions in the morning hours before feeding, twice a week, using artificial vagina (AV) (Colorado model) and a mare in oestrus as dummy as per the standard method described previously (Talluri *et al.*, 2012a). Semen collection, evaluation and processing for freezing were done according to Talluri *et al.* (2012a). In brief, soon after semen collection, seminal parameters like colour, consistency, and total volume were recorded by visual observation and seminal pH by pH strips. Then, the gel portion of the semen was filtered through sterilised gauze filter and the volume of gel free semen was calculated visually in another sterile graduated semen collection bottle. Gel free semen was used for evaluation of other semen parameters viz., progressive sperm motility, sperm concentration, livability, plasma membrane integrity, acrosome integrity via Giemsa staining and chromatin integrity via Acridine orange stains to study the chromatin stability and DNA intactness of the spermatozoa.

A Computer Assisted Semen Analyzer (HTB CEROS II, Version 1.3, Hamilton Thorne Research, Beverly, MA, USA) equipped with a thermo stage was used to analyze the progressive sperm motility. Sperm concentration was

[#1]Part of M.V.Sc. Thesis of the first author, present and corresponding address, Teaching Associate, Teaching Veterinary Complex, College of Veterinary and Animal Science, RAJUVAS, Bikaner, Rajasthan-334 001,email:dr.ysoni89@gmail.com,[2]Scientist (Senior Scale), Equine Production Campus, NRC on Equines, Bikaner, Rajasthan-334001, [3]Principal Scientist and Incharge, NRC on Equines, Bikaner, Rajasthan-334 001,[4]Director, ICAR-NRC on Equines, Hisar, Haryana-125001, [5]Veterinary Officer, Incharge Govt. Veteterinary Hospital, Leensar, Distt. Barmer, Rajasthan.

determined using haemocytometer. The sperm morphology and live/dead status were determined according to Bloom's method (eosin/nigrosine) (Samardzija *et al*., 2008). Plasma membrane integrity/functional integrity of sperm membrane was determined by hypoosmotic swelling test (HOST) as described by Talluri *et al*. (2012b).The number of spermatozoa (%) with tail coiling (HOS +ve) was recorded for each sample. The experiment was repeated six times in order to obtain a consistent result. For the detection of acrosome integrity of the sperms, Giemsa method as described by Brito *et al*. (2011) was followed. For detecting the chromatin integrity of spermatozoa the method described by Irma Virant-Klun *et al*. (2002) was adopted. The percentage of spermatozoa with single-stranded DNA was calculated from the ratio of spermatozoa with red, orange, or yellow fluorescence to all spermatozoa counted per sample.

Semen was cryopreserved by traditional method of freezing as per the Talluri *et al*. (2016). Briefly, Semen was extended up to 150-200 million sperm/ml with freezing extender (glucose-EDTA-lactose extender containing 5% Dimethyl-formamide as cryoprotectant), and filled and sealed into 0.5 ml straws with the help of automatic filling and sealing machine (IMV, France). Filled straws were kept for equilibration in an automated cooling chamber at 4˚C for 2 h. Pre-freeze motility was recorded after equilibration and before exposing straws to liquid nitrogen (LN_2) vapour. Straws were spreaded over freezing racks, 4 cm above the LN_2 level in a styrofoam box for exposure to LN_2 vapour for 10-12 min and then dipped into LN_2 and stored. Microscopic evaluation of frozen semen was done at least after 24 hrs of its storage. For this, frozen semen straws were dipped into water bath at 37˚C for 30 sec to thaw the semen and post-thaw motility (PTM), live sperm count, hypo osmotic swelling, acrosomal integrity and DNA Integrity were recorded. Semen samples having PTM $\geq$30% were kept stored into separate canisters dipped in LN_2 containers for future use in artificial insemination (AI).

Results and Discussion

It is well established fact that freezing and thawing process produces various stresses to spermatozoa that leads to morphological or biochemical damages to sperm (Tariq *et al*., 2015). Spermatozoa are terminal and highly differentiated cells, which have many characteristics required for fertilization. A combination of tests, measuring one or more characteristics, gives a better estimation of semen quality than using only single test (Colenbrander *et al*., 2003; Rodriguez-Martinez, 2003; Estrada and Samper, 2007). In this context, the present study was conducted to evaluate fresh and post thaw quality of the stallions' semen of Marwari, Manipuri and Zanskari Indian breeds. The observed basic semen characteristics of all three breeds are discussed below:

In this study colour of the semen of all three breeds was graded as milky white. Similar observation was made by Arifiantini *et al*. (2013) and Tejpal (2016). In other studies, Pal *et al*. (2009) and Ravi *et al*. (2013) reported colour of Indian breed stallions as milky white to creamy. Consistency of the semen in present study was graded as variably thin in all three Indian breeds similar to the reports of Pal *et al*. (2011a), Arifiantini *et al*.(2013) and Ravi *et al*. (2013). Normal stallion semen colour should be white, greyish-white to slightly creamy and it provides a rough estimate of sperm concentration. Usually, larger volume semen are more watery instead of creamy and it reflects low sperm concentrations. Colour and consistency of semen are important indicative of quality of semen (Samper, 2009).

In the present study, the total semen volume of Marwari stallion ranged from 24.00±1.78 to 59.20±5.66 ml with an overall mean of 45.06 ±4.75 ml, and of Manipuri stallion 29.40 ± 1.96 to 76.60 ± 11.88 ml with an overall mean of 54.53 ± 7.09 ml, and of Zanskari stallion 31.20 ± 4.30 to 51.80 ± 5.63 ml with an overall mean of 41.06 ± 3.59 ml. Non-significant difference was found in total semen volume among these three breeds but significant difference was seen among individuals within breeds. The mean total volume of all three breeds observed in this study was within the range of observation made by Dowset and Pattie (1982), Pickett *et al*. (1988), Rickets (1993) and Arangasamy *et al*. (2013) as they found range of normal semen volume 30-300 ml. The individual variation in semen volume may be due to various factors viz., breed, age, season, teasing time, frequency of semen collection, work load etc. (Pickett *et al*., 1988; Gunnarsson, 1997; Estrada and Samper, 2007). Variation in the semen volume could be due to difference in individual semen production (Picket *et al*., 1976; Picket and Shiner, 1994) and the teasing time (Ionata *et al*., 1991). Total ejaculate volume is increased through accessory gland secretion by excessive sexual stimulation of the stallion prior to collection (Pickett *et al.,* 1987).

The seminal pH of Marwari stallions ranged from 7.28 ± 0.02 to 7.34 ± 0.04 with an overall mean of 7.30 ± 0.02, and of Manipuri stallions ranged from 7.25 ± 0.04 to 7.32 ± 0.02 with an overall mean of 7.29 ± .036, and of Zanskari stallions ranged from 7.31 ± 0.04 to 7.34 ± 0.07 ml with an overall mean of 7.32 ± 0.04. No significant difference was found among these three breeds as well as among stallions within breeds (P>0.05). The observations in present study were in accordance with the previous observations made by Arifiantini *et al*. (2013) (pH was 7.0 ± 0.20 of stallion semen), Pal *et al*. (2011b) (semen pH in Marwari stallions as 7.2 ± 0.02).

In the present study, mean progressive sperm motility of all 9 stallions was more than 70%. Progressive sperm motility of fresh semen more than 60% might be considered appropriate for cryopreservation of stallion semen (Davies Morel, 1993). In the present study, progressive motility observed in fresh semen of Marwari stallion ranged from 79.40 ± 1.16 to 84.40 ± 1.69% with an overall mean of 81.46 ± 0.87%, and of Manipuri stallion from 68.00 ± 6.04 to 80.00 ± 4.18% with an overall mean of 75.00 ± 2.84%, and of Zanskari stallion from 70.00 ± 2.73 to 84.00 ± 1.87% with an overall mean of 77.00 ± 2.22%. The results are consistent with the previous observations made by Sieme *et al*. (2002) and Talluri *et al*. (2012) who found progressive sperm motility in gel free stallion semen 75.2 ±2.70% and 77.00 ±1.51%, respectively. The post thaw motility percentage observed for the three breeds viz., Marwari, Manipuri and Zanskari were 39.55± 2.67, 39.72±4.80 and 33.88±3.37. A significant difference ($P\leq0.05$) was observed between the post thaw motility of Zanskari breed stallions and to that of stallions of Marwari and Manipuri breeds. A significant difference ($P\leq0.01$) was observed between the individual stallions of each breed. The post thaw motility observed in the

present study are in accordance with the results of Alvarez *et al*. (2014) who also reported the post thaw motility of the stallions in the same range.

Overall range of sperm concentration recorded in the present study was 164.00±12.08 to 293.20±16.31 million/ml with an overall mean of 260.55±5.86 million/ml. Talluri *et al*. (2012) and Talluri *et al*. (2016) in their study found the values ranged from 115 to 275 million/ml for Marwari and Zanskari stallions, while Pickett *et al.* (1988) observed the values from 100 to 200 million/ml sperm concentration in horse semen samples.

Viability of spermatozoa is important criterion while selecting ejaculates for freezing to get more number of post thaw live spermatozoa. The mean live sperm count recorded for three breeds viz. Marwari, Manipuri and Zanskari was 85.2±0.78%, 80.9±0.9% and 82.67±0.73**%,** respectively with an overall mean of 82.92±0.59%. Non-significant difference was found among breeds for mean live sperm count (P>0.05). Significant difference (P<0.05) was found among stallions for live sperm count for stallions of different breeds. The post thaw livability percentage of the spermatozoa of the three breeds were calculated and observed a significant difference ($P\leq0.05$) between the stallions of Zanskari to that of the stallions of Marwari and Manipuri breeds. The livability percentage of spermatozoa of all the stallions varied from 50.17±0.79 to 58.67±0.49. These results are in concurrence with the earlier findings of Talluri *et al.* (2012) and Tejpal *et al.* (2016) who recorded the same range of values for indigenous stallions in their study.

The sperm morphology evaluation is an essential component of any semen analysis and provides the clinical information about the potential fertility of semen sample. The morphological defects observed in this study were within the range of 2.07±0.27 to 3.27±0.85%. It was observed that highest abnormality in Marwari breed was associated with sperm tail, while in Manipuri and Zanskari breeds mid piece abnormality was more common. It has generally been accepted that certain morphological structural deviations correlate with male sub fertility and infertility (Pesch and Bergmann, 2006). Stallions with good fertility will often have >60% normal sperm and <5% abnormalities occurring in the acrosome and midpiece (Samper *et al*., 2007).

The HOS test seems more appropriate for predicting the fertilizing capacity of frozen-thawed than fresh semen, because membrane damage is here a more important limiting factor than in the former (Colenbrander *et al*., 2003). The mean HOST positive sperms recorded for three breeds viz. Marwari, Manipuri and Zanskari was 56.43±0.64%, 57.00±2.27% and 59.36±2.65%, respectively with an overall mean of 57.59±1.29. Significant difference (P<0.05) was found among stallions for HOST positive sperm, while non-significant difference was found among breeds. The mean HOST positive sperms of frozen thawed semen of Marwari stallions was recorded as 45.17±1.43, for Manipuri stallions it was 44.05±1.40 and for Zanskari stallions it was observed to be 46.39±0.68 and a significant difference was observed between the stallions of Zanskari breed, to that of Marwari and Manipuri breed stallions (Fig.1b). This test highlights the permeability and functionality of sperm membrane up on exposure to hypo-osmotic solution and the observation of higher value is a valid indication of intact membrane and sample with higher value is re-garded as potent for establishing pregnancy. The HOST values observed in the present study were found to be low as recorded by Talluri *et al.* (2012) in indigenous stallions but found to be in accordance with that of Tejpal *et al.* (2016).

The mean intact acrosomal count recorded for each of the Marwari stallions was 94.8 ± 1.15, 86.8 ± 0.81 and 92.00 ± 0.92 %, respectively with an overall mean of 92.2 ± 0.56% for the Manipuri stallions 93.60±0.37, 89.20±0.31, 94.80±0.67 % with an overall mean of 92.46±0.37%, and of the Zanskari stallion 94.80±0.74, 89.20±0.31 and 95.60±1.88 % with an overall mean of 93.20±0.73% in fresh semen samples. The determination of the acrosome status in cryopreserved sperm is of the fundamental importance as cryopreservation directly damages sperm membrane, which could be followed by a loss of the acrosomal matrix contents. Pukazhenthi *et al.* (2014) measured lower values i.e. 84% and 76% acrosome intact spermatozoa in fresh semen of Przewalski's wild horses and domestic horses, respectively by using Coomassie stain method. In the present experiment, there was significant difference among stallions within breed but no significant difference (P>0.05) among three breeds. The observed mean percentage of acrosome intact sperms of frozen thawed semen of Marwari stallions 89.67±0.58, and of the Manipuri stallions was 87.00±0.75 and of the Zanskari stallions was 87.72±0.65, respectively. There is a significant difference ($P\leq0.05$) was observed between the acrosome integrity of the Marwari stallions to that of Manipuri and Zanskari stallions in the post thaw acrosome integrity percentage of the spermatozoa (Fig. 1c). There was also a non-significant difference ($P\leq0.01$) between the stallions of different breeds. The values observed in the present study are well in concurrence with the earlier reports of Talluri *et al*. (2012).

While the conventional parameters influence the capability of the spermatozoa to fertilize the oocyte, chromatin integrity is essential for the further development of the embryo. It has been postulated that poor chromatin packaging and/or damaged DNA may contribute to failure of sperm decondensation and consequently in fertilization failure (Sakkas *et al.,* 1996). Damaged DNA might thereby be a factor of increasing importance in stallion sub fertility (Silva and Gadella, 2005). The mean DNA intact sperm count recorded for each of the Marwari stallions was 93.2±0.23, 93.5±0.53 and 94.2±0.36% with an overall mean of 93.63±0.37%, and for Manipuri stallions 95.20 ± 0.88, 96.80 ± 0.72 and 95.40 ± 0.59% with an overall mean of 95.80 ± 0.73%, and of Zanskari stallions 94.6±0.43, 93.8±0.86 and 94.8±0.52% with an overall mean of 94.4±0.60%. No significant difference (P>0.05) was found among stallions within breed or among three breeds i.e. Marwari, Manipuri and Zanskari for DNA integrity values for fresh semen. Significant differences (P<0.05) were found for the DNA intact sperm percentage in frozen thawed semen among stallions of each breed and also between the Marwari stallions to that of Manipuri and Zanskari breed Stallions (Fig. 1d).

Stallions were the principal source of variation in fresh semen parameters in the present experiment and for the post thaw seminal parameters the stallions and breeds both were found to be significant source of variation.

Fig.1. Comparative post thaw seminal parameters of stallions of three indigenous breeds viz., Marwari, Manipuri and Zansakari.

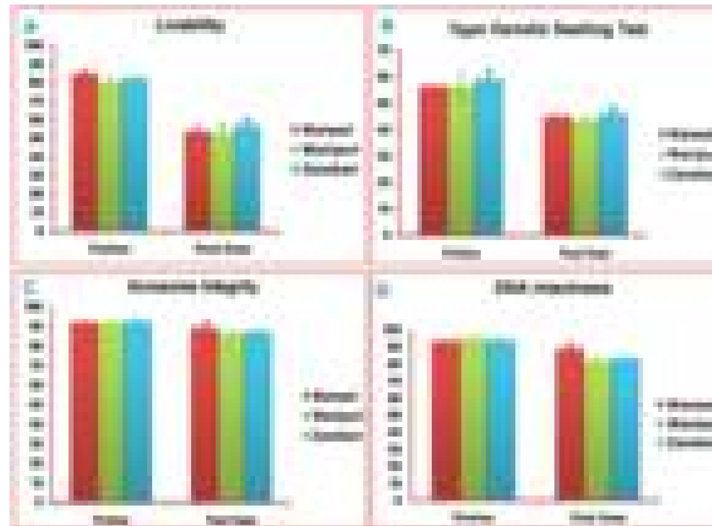

Table 1: Fresh semen parameters

Seminal Attribute	Fresh Semen (Mean±SE)		
	Marwari	Manipuri	Zanskari
Total Volume	45.06±4.75^A	54.53±7.09^A	41.06±3.59^A
Colour	White to Milky white	Creamy white to white	white
Consistency	Thin to thick	Thick	thick
pH	7.30±0.026^A	7.29±0.03^A	7.32±0.04^A
Gel free volume	35.73±4.22^A	34.93±4.29^A	30.86±2.85^A
Pro. motility	81.46±0.87^A	75.00±2.84^A	77.00±2.22^A
Sperm conc.	253.33±8.52^A	262.33±15.89^A	266.00±6.12^A
Live %	85.2±0.78^A	80.9±0.99^A	82.67±0.73^A
Host	56.43±0.64^A	56.80±5.37^A	59.36±1.61^A
Acrosome integrity	92.2±0.56^A	92.46±0.37^A	93.20±0.73^A
Chromatin integrity	93.63±0.37^A	95.8±0.73^A	94.4±0.60^A

Means with different superscripts differ significantly ($P\leq0.05$* or $P\leq0.01$**), letters in uppercase denote significance among breeds.

Acknowledgements

The authors are highly thankful to the Dean CVAS, Bikaner, Director of ICAR-NRCE and to the Incharge Equine Production campus for providing excellent facilities for smooth conducting of the present study.

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