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Cryosurvival of Marwari stallion sperm in different extenders

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ABSTRACT

Extenders (3) were compared for frozen-thawed semen quality of Marwari horses in the present study. Ejaculates (36) including 6 ejaculates from each of 6 stallions were collected using artificial vagina (AV) method. The ejaculates were cryopreserved either with glucose-EDTA-lactose, HF-20 or egg yolk-skim milk extender using custom freezing technique. Sperm characteristics, viz. motility, viability, acrosome intactness and plasma membrane integrity were studied in frozen-thawed semen besides record of fresh semen quality as a prerequisite. Present study indicated a non-significant difference in frozen-thawed semen parameters with glucose-EDTA-lactose and HF-20 extenders, however, both the extenders resulted in a higher percentage of post-thaw motility (PTM), viability, intact acrosome and plasma membrane integrity of sperm than the egg yolk-skim milk extender. It was concluded that glucose-EDTA-lactose and HF-20 are better semen extenders as compared to egg yolk-skim milk and they can be used effectively for semen cryopreservation in horses.

Key words: Cryopreservation, Extender, Marwari horse, Semen

The acceptance of cryopreserved stallion's semen is hindered by great variability in post-thaw sperm viability as indicated also in Marwari horses (Pal *et al.* 2011). Several factors are involved in the poor freezability of equine sperm, including the composition of extender, type of cryoprotectant (Squires *et al*. 2004) and the freezing technique (Metcalf 2007). During cryopreservation, both physical and chemical factors, including thermal stress, oxidative stress and osmotic stress may damage sperm plasma membrane (Medeiros *et al*. 2002).

Several freezing extenders were found satisfactory for maintaining sperm viability and pregnancy rates in a high percentage of horses (Vidament 2005). The composition of extenders varies enormously, but they are usually based upon milk or egg yolk products with addition of antibiotics (Arifiantini 2013). Braun *et al.* (1995) observed that macromolecules such as egg yolk and skim milk can influence frozen-thawed horse spermatozoa. Likewise, different types of sugars (lactose, fructose, glucose, raffinose and sorbitol) supplemented in semen extenders can affect the sperm characteristics of frozen-thawed spermatozoa in horses (Pojprasath *et al.* 2011, Arifiantini 2013). Therefore, the present study was conducted to evaluate effect of different extenders on frozen-thawed semen quality of Marwari horses.

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MATERIALS AND METHODS

Experimental animals: Apparently healthy Marwari stallions (6), 4–6 year-old, maintained at NRCE, Bikaner, Rajasthan were used in present study. Experimental horses were kept in well ventilated boxes with feeding and water trough and attached to an open paddock. The horses were daily fed a standard diet that included 3 kg of concentrate with mineral mixture, salt and 9 kg fodder (green:dry in 3:1 ratio). Fresh drinking water was allowed *ad lib*. and the experiment was conducted during breeding season.

Preparation of primary extender: Addition of primary extender in equal volume of gel free semen and centrifugation is an essential step to remove supernatant (semen plasma) and to get sperm pellet. The sperm pellet in 3 aliquots from each ejaculate was in turn mixed with either of 3 secondary extenders used in this study. Primary extender containing glucose 0.15 g, sodium citrate dehydrate 2.6 g, disodium EDTA 0.37 g, sodium bicarbonate 0.12 g, streptomycin 0.10 g, benzyl penicillin 0.10 g and added double distilled water upto 100 ml, was prepared as per Pal *et al.* (2011).

Preparation of secondary extender: Secondary extenders (extenders A, B and C) were prepared as per composition mentioned in Table 1.

Semen collection and processing: Ejaculates (36; 6 ejaculates/stallion) were collected weekly from 6 stallions using Colorado model AV over a mare in estrus. Total

Table 1. Composition and preparation of secondary extenders

Constituents	Pal *et al.* (2011)		Nishikawa (1975)	Samper (1995)
	Extender A (Modified glucose-EDTA-lactose)		Extender B (HF-20)	Extender C (Skim milk egg yolk)
	Solution I (25 ml)	Solution II (50 ml)		
Glucose	6.0 g	-	5.0 g	-
Sodium citrate dehydrate	0.37 g	-	0.15 g	-
Disodium EDTA	0.37 g	-	-	-
Sodium bicarbonate	0.12 g	-	-	-
Lactose	-	11 g	0.3 g	-
Raffinose	-	-	0.3 g	-
Sucrose	-	-	-	9.3 g
Sodium phosphate	-	-	0.05 g	
Potassium sodium tartrate	-	-	0.05 g	-
Skim milk powder	-	-	-	2.4 g
Streptomycin	0.08g	0.08g	0.025 g	0.025 g
Benzyl penicillin	0.08 g	0.08 g		
Penicillin	-	-	25000 IU	25000 IU
Double distilled water up to	100 ml	100 ml	100 ml	100 ml
Egg yolk	20 ml		0.5 -2.0 g	8 ml
Glycerol	5%		10%	3.5%

ejaculate volume was noted as visible from graduated collection bottle, filtered through sterilized gauze and gel free semen volume was noted in another graduated bottle. Gel free semen was mixed with primary extender in equal and divided in 3 aliquots to centrifuge at 1,800 rpm for 3 min. The supernatant was discarded leaving sperm pellet which in turn extended with either extender A, B or C (as mentioned above) to get a final sperm concentration of approximately 150×10^6 ml^{-1}. Packaging of extended semen was done using automatic straw filling and sealing machine pre-cooled in a cooling cabinet maintained at 4–5°C for 2 h. The filled straws were laid horizontally onto a wired net and lowered into a styrofoam box 3 cm above the surface of liquid nitrogen for 12 min. The straws were turned around once or twice over wired net held in position to expose with liquid nitrogen vapour and then dipped into liquid nitrogen (–196°C). Two frozen semen straws were dipped in water bath maintained at 37°C for 30 sec for thawing and post-thaw evaluation.

Evaluation of semen quality: Progressive sperm motility was assessed by computer assisted semen analyzer equipped with a thermo stage. Hypoosmotic swelling (HOS) test was done as per Rao *et al.* (2012). Sperm acrosomal integrity was performed with fluorescein isothocynate-*Pisum Sativum* as per Cross *et al.* (1989). Live sperm % was determined using a drop of frozen-thawed semen sample (Khan and Ijaz 2008).

Statistical Analysis: Data were collected, arranged, summarized and then statistically analyzed using conventional statistical procedures. Analysis included mean values, standard error and analysis of variance (ANOVA) using F-test.

RESULTS AND DISCUSSION

Assessment of fresh semen is an integral part and is a prerequisite for semen cryopreservation. Fresh semen samples with 60% or above progressive sperm motility were considered appropriate for cryopreservation of semen. The seminal characteristics as observed in present study are presented in Table 2.

PTM % of sperm in frozen-thawed semen samples with extender A, B and C is presented in Table 3. Highest PTM % of sperm was observed with extender B whereas lowest with extender C. The difference was non-significant ($P>0.01$) between extender A and B, however, the difference of extender C with extender A and B was highly significant ($P<0.01$). The results indicated that monosaccharide based semen extenders (extender A and B) were better than extender C containing disaccharides (sucrose). Salazar *et al.* (2011) observed that egg yolk-based extender yielded higher values of sperm PTM % than milk egg yolk based extender ($P<0.05$) which was similar to the present study. This may be due to better availability of glucose as energy substrate to sperms, supplementation of EDTA and sodium bicarbonate as buffer compared to milk buffer (Martin and Klug Gunzel 1979) or increasing the glycerol concentration in extender (Ecot *et al.* 2000). In contrary, Torres-Boggino *et al.* (1995) found high PTM % of sperm with clarified egg yolk-skim milk than glucose-EDTA-lactose extender. Braun *et al.* (1995) observed 24.7% sperm PTM with skim milk-egg yolk extender which is similar to findings of this

Table 2. Some of the parameters (%) in fresh semen of Marwari horses (mean ±SE)

Progressive sperm motility (%)	Total semen volume (ml)	Live sperm (%)	Sperm with intact acrosome %	HOS positive sperm (%)
73.02 ± 2.47	48.69± 8.01	78.36 ± 2.16	87.33 ± 1.53	54.11 ± 2.08
		Range		
69.16 ± 1.68 to 76.16 ± 2.00	31.33 ± 3.12 to 75.83 ± 15.35	74.66 ± 1.66 to 81.16 ± 1.49	83.66± 1.11 to 91± 1.21	48.5 ± 1.23 to 59 ± 1.73

Table 3. Frozen thawed semen parameters of Marwari horses (mean ±SE)

Secondary extender	Sperm PTM (%)	Live sperm (%)	Sperm with intact acrosome (%)	HOS (%) positive sperm (%)
Extender A (modified glucose-EDTA-lactose)	37.61^{b} ± 1.11	47.16^{b}± 1.02	75.66^{b}± 0.96	37.36^{b} ± 1.33
Extender B (HF-20)	39.52^{b} ± 1.02	49.16^{b}± 1.09	76.83^{b} ± 0.79	38.97^{b} ± 1.00
Extender C (skim milk egg yolk)	29.88^{a} ± 1.29	40.30^{a}± 1.22	70.94^{a} ± 0.92	28.30^{a} ± 1.18

Values bearing different superscripts within column differ significantly (P<0.01).

study. Further, they stated that amount of egg yolk did not significantly influence the post thaw results that supports our findings as no significant difference between the results of glucose-EDTA-lactose and HF 20 extenders where egg yolk concentration was different. Moreover, Vidament *et al*. (2001) reported that varying glycerol concentration did not have any significant effect on sperm PTM %.

The live sperm count in frozen-thawed semen samples are mentioned in Table 3. Highest and lowest post thaw live sperm count was found with extender B and C, respectively. A non-significant difference (P>0.01) was found between extender A and B but it was highly significant (P<0.01) between extender C compared to extenders A and B. This may be due to glucose as substrate in these extenders that provide greater energy to support sperm survival. As per Jafaroghli *et al.* (2011), raffinose was better than sucrose in terms of sperm viability in frozen-thawed semen of ram which supports results of this study that extender B was better than extender C. Kumar *et al*. (2011) observed sperm viability of 30.34 ±0.85% in frozen-thawed stallion semen with glucose, lactose and raffinose based extender. Pugliesi *et al.* (2012) observed similar result that semen stored in Foote extender (glycine-egg yolk based extender) gave more sperm viability than Kenney extender (skimmed milk based extender). The factor that may be correlated to the survival of sperm cells during storage is the amount of energy reserves contained in extender and seminal plasma (Januaskauskas and Rodriguez-Martinez 1995). Milk contains natural buffer (McKinnon 1999) which may have less effect than citrate buffer on survivability of stallions' sperm which also supports our findings that extender A and B had better post thaw sperm viability than extender C. Casey *et al.* (1993) observed that the viability exceeded (P<0.04) motility when assessed by CASA, also correlates the present study. In the present study, extender that resulted in good post-thaw sperm motility also showed a good post-thaw viability of sperm as in agreement with the reports of Samardzija *et al.* (2008).

Disruption of the acrosome prior to sperm bounding to the zona pellucida renders a spermatozoa incapable of penetrating the zona pellucida and subsequently fertilizing the oocyte. It was observed that a high proportion of sperm that have lost their acrosomal contents are in fact dead. For these sperm, acrosomal loss may be due to degeneration of the acrosome associated with cell death (Casey *et al.* 1993). The sperm acrosomal intactness in frozen-thawed semen samples are reflected in Table 3. Highest value of sperm with intact acrosome (76.83 ±0.7923%) was found with extender-B and lowest (70.94 ±0.9223%) with extender-C in frozen-thawed semen. Non-significant difference (P>0.01) was found between extender A and B, however, the difference of extender C with extender A and B was highly significant (P<0.01). Kavak *et al.* (2003) observed similar result of 79.3 and 84.5% acrosome intact sperm in frozen-thawed semen in Tori and Estonian breeds of stallions, respectively using PI and FITC-PSA staining. Gil *et al.* (2013) observed post thaw acrosomal intact sperm of 36.60 ±13.64% (mean ±SD) which was lower than result of the present study. Teodora *et al.* (2008) observed 52.5 to 82.3% sperm with intact acrosome in frozen-thawed stallion semen using different semen extenders. Raffinose was reported better than sucrose in terms of sperm acrosomal integrity in frozen-thawed semen of ram (Jafaroghli *et al.* 2011) that support our findings that extender B had higher number of sperm with intact acrosome than extender C. Graham (2001) observed live acrosome intact spermatozoa from 28 to 67% with significant difference between stallions in frozen-thawed semen measured using flow cytometry, also supports our findings.

The HOS positive sperm in frozen-thawed semen samples are presented in Table 3. Highest post thaw HOS positive sperm was found with extender B and lowest with extender C. Non-significant difference (P>0.01) was observed between extender-A and B but the difference was highly-significant (P<0.01) between extender-C with extender A and B. Henry *et al.* (2002) observed that most frequent damage to sperm cells is the loss of membrane integrity which directly reflects the freezing ability. Similar observation was made by Kuisma *et al.* (2006) who found 30.1±1.6% post thaw HOS positive sperms in equine semen. Gil *et al.* (2013) also observed post thaw HOS positive sperm of 35.62±12.30% (mean ±SD) in lactose-EDTA-egg yolk extender of stallion semen similar to this study. Kumar *et al*. (2011) found 28.48±00.80% of HOS positive sperm of frozen-thawed equine semen with INRA–82 extender (glucose, lactose and raffinose based). Henry *et al*. (2002) found HOS positive sperm in frozen-thawed horse semen to be 18.9±13.6% which was lower than present study. Similarly, Pugliesi *et al.* (2012) reported superiority of egg yolk based extender over the milk based extender that gave better protection to the plasma membrane during cooling. Samardzija *et al.* (2008) and Mansour *et al.* (2009) found positive correlation between the percentage of motile spermatozoa and HOS positive sperm, supporting results of present study that extenders which had high PTM % also showed high HOS positive sperm.

The present study concluded that glucose-EDTA-lactose and HF-20 were better than egg yolk-skim milk as semen extenders and they could be used effectively for semen cryopreservation in horses.

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REFERENCES

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.

Braun J, Hochi S, Oguri N, Sato K and Torres-Boggino F. 1995. Effect of different protein supplements on motility and plasma membrane integrity of frozen-thawed stallion spermatozoa. *Cryobiology* **32**(5): 487–92.

Casey P J, Hillman R B, Robertson K R, Yudin A I, Liu I K M and Drobins E Z. 1993. Validation of an acrosomal stain for equine sperm that differentiates between living and dead sperm. *Journal of Andrology* **14**(4): 289–97.

Cross N L and Meizel S. 1989. Methods for evaluating the acrosomal status of mammalian sperm. *Biology of Reproduction* **41**: 635–41.

Ecot P, Vidament M, de Mornac A, Perigault K, Clement F and Palmer E. 2000. Freezing of stallion semen: interactions among cooling treatments, semen extenders and stallions. *Journal of Reproduction and Fertility* **56**: 141–50.

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* doi:10.1155/2013/280143.

Graham J K. 2001. Assessment of sperm quality. Proceedings of the Annual Convention of the American Association of Equine Practitioners. **47**: 302–05. 24–28 November. San Diego, California.

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**: 245–48.

Jafaroghli M, Khalili B, Farshadc A and Zamiri M J. 2011. The effect of supplementation of cryopreservation diluents with sugars on the post-thawing fertility of ram semen. *Small Ruminant Research* **96**: 58–63.

Januaskauskas A and Rodriguez-Martinez H. 1995. Assessment of sperm viability by measurement of ATP, membrane integrity and motility in frozen/thawed bull semen. *Acta Veterinaria Scandinavica* **36**: 571–74.

Kavak A, Johannisson A, Lundeheim 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): 205–16.

Khan M I R and Ijaz A. 2008. Effects of osmotic pressure on motility, plasma membrane integrity and viability in fresh and frozen-thawed buffalo spermatozoa. *Animal Reproduction Science* **2**: 548–53.

Kuisma P, Andersson M, Koskinen E and Katila T. 2006. Fertility of frozen-thawed stallion semen cannot be predicted by the currently used laboratory methods. *Acta Veterinaria Scandinavica* **48**: 14.

Kumar D, Jhamb D and Saxena A. 2011. Effect of different seasons and thawing protocols on certain seminal attributes of Indian standard bred stallion semen preserved using 1% glycerol and 1% dimethyl formamide as cryoprotectants. *Indian Journal of Animal Research* **45**(4): 247–55.

Mansour M M. 2009. Modification of hypo-osmotic swelling test to evaluate the integrity of stallion sperm plasma membrane. *Global Veterinaria* **3**(4): 302–07.

Martin J C and Klug Gunzel A R. 1979. Centrifugation of stallion semen and its storage in large volume straws. *Journal of Reproduction and Fertility* **27**: 47–51.

McKinnon A O. 1999. Breeding and its technology-now and the future. World trotting conference. November 25. Sydney.

Medeiros C M, Forell F, Oliveira A T and Rodrigues J L. 2002. Current status of sperm cryopreservation: why isn't it better? *Theriogenology* **57**: 327–44.

Metcalf E S. 2007. The efficient use of equine cryopreserved semen. *Theriogenology* **68**: 423–28.

Nishikawa Y. 1975. Studies on the preservation of raw and frozen horse semen. *Journal of Reproduction and Fertility* **23**: 99–104.

Pal Y, Arangasamy A, Legha R A, Singh J, Bansal R S, Khurana S K and Tandon S N. 2011. Freezability and fertility of Marwari stallion semen. *Indian Journal of Animal Sciences* **81**(5): 445–47.

Pojprasath T, Lohachit C, Techakumph M, Stout T and Tharasanit T. 2011. Improved cryopreservability of stallion sperm using a sorbitol-based freezing extender. *Theriogenology* **75**: 1742–49.

Pugliesi G, Carvalho G R D, Rates D M, Ker P G, Matta M P D, Oliveira R R D and Filho J M D S. 2012. Viability and fertility of cooled equine semen diluted with skimmed milk or glycine egg yolk-based extenders. *Revista Brasileira de Zootecnia* **41**(12): 2411–17.

Salazar J L Jr, Teague S R, Love C C, Brinsko S P, Blanchard T L and Varner D. 2011. Effect of cryopreservation protocol on post thaw characteristics of stallion sperm. *Theriogenology* **76**: 409–18.

Samardzija M, Dobranic T, Kruslin S, Cergolj M, Karadjole M, Prvanovic N, and Grizelj J. 2008. The use of the hypoosmotic swelling test and supravital staining in evaluation of sperm quality in boars. *Veterinarski Archiv* **78**(4): 279–87.

Samper J C. 1995. Stallion semen cryopreservation male factors affecting pregnancy rates. *Proceedings of the Society for Theriogenology.* pp. 160–165. 13–15 September. San Antonio Texas.

Squires E L, Keith S L and Graham J K. 2004. Evaluation of alternative cryoprotectants for preserving stallion spermatozoa. *Theriogenology* **62**: 1056–65.

Teodora V, Groza I, Morar I and Catana R. 2008. The effect of different freezing procedures on sperm head morphometry in stallions. *Veterinary Medicine* **65**(2): 1843–78.

Thirumala Rao Talluri, Arangasamy A, Sanjay Kumar Ravi. and Yash Pal. 2012. Hypo-Osmotic Swelling test for quality evaluation of fresh and frozen semen quality in horses. *Indian Veterinary Journal* **89** (11): 68–70.

Torres-Boggino F, Sato K, Oka A, Kanno Y, Hochi S, Oguri N

and Braun J. 1995. Relationship among seminal characteristics, fertility and suitability for semen preservation in draft stallions. *Journal of Veterinary Medicine* and *Science* **57**(2): 225–9.

Vidament M, Yvon J M, Couty I, Arnaud G, Nguekam-Feugang J, Noue P, Cottron S, Le Tellier A, Noel F, Palmer E and Magistrini M. 2001. Advances in cryopreservation of stallion semen in modified INRA82. *Animal Reproduction Science* **68**: 201–18.

Vidament M. 2005. French field results (1985–2005) on factors affecting fertility of frozen stallion semen. *Animal Reproduction Science* **89**: 115–36.