The Indian Journal of Animal Reproduction, 24(1): 32-35, June 2003

Enzyme leakage from spermatozoa of Black Bengal and beetal bucks during freezing with various cryoprotectants

M.P. SINGH[1†], A.K. SINHA[2], BALRAJ SINGH[3] AND D.K. SINGH[4]

Department of Gynaecology and Obstetrics
Ranchi Veterinary College, Ranchi (Jharkhand)-834 006

Received: May 11, 2001
Accepted: July 1, 2002

ABSTRACT

The present experiment was conducted to study the effect of cryoprotectants (glycerol, DMSO and lactose) containing extenders on leakage of acrosin and hyaluronidase enzymes from spermatozoa of Black Bengal and Beetal bucks. Activities of acrosin and hyaluronidase were assayed in neat, equiliberated (pre-freezing) and frozen thawed (post-freeze) semen samples. The variations in acrosin activity at pre- and post-freeze stages due to extender were significant ($P<0.01$). Mean intracellular acrosin activities in the neat semen of Black Bengal and Beetal bucks were 0.0275± 0.00084 and 0.0301± 0.00105 units/10^9 sperms, respectively. Acrosin activity decreased in equiliberated and frozen thawed semen as compared to neat semen, indicating the cryoprotective ability of extenders. Extracellular mean activities of hyaluronidase in the neat semen of Black Bengal and Beetal bucks were 233.78 ±12.688 and 220.58±7.261 units/10^9 sperms. Hyaluronidase activity increased in equiliberated and frozen thawed semen as compared to neat semen. It also indicate cryoprotective properties of extenders.

Key words: Acrosin, Hyaluronidase, Buck, spermatozoa

Acrosin and hyaluronidase play an important role in the process of fertilization. In absence of these enzymes, the sperms are not capable of penetrating the cumulus/zona (Allison and Hartree, 1970 and McRorie and Williams, 1974). Freezing procedures have been reported to alter the cell permeability and damage the acrosome causing release of enzymes present therein (Zaneveld *et al.*, 1971, McRorie and Williams, 1974). The estimate of these enzymes at pre- and post-freeze stages is indicator of quality/ extent of sperm damage. The present study was planned to assess the cryoprotective ability of glycerol, DMSO and lactose in various combinations and concentrations by estimating leakage of these enzymes from the spermatozoa of Black Bengal and Beetal bucks during the process of freezing.

MATERIALS AND METHODS

The present study was conducted on 180 semen samples (90 from each breed) collected twice weekly from 3 Black Bengal and 3 Beetal bucks aged about 2-4 years maintained under goat breeding project at Ranchi Veterinary Colleg Ranchi, Jharkhand. Immediately after routin evaluation, the semen samples were diluted (1:5 with tris buffer and centrifuged ar 3000 rpm for 1 minutes. Ten extenders were prepared containin different cryoprotectants (Table 1). The ratio of eg yolk to buffer with cryoprotectants in extender wa 1:5 and antibiotics were added at suitabl concentrations (Pencillin G sodium 1000 I.U. an Streptomycin sulphate 1000 mg/ml of extende Semen samples were extended in such a way tha each straw contains approximately 100 millio spermatozoa. Subsequent to filling and sealing, th semen filled mini straws (0.25 ml) wer equiliberated at 5.00±1°C for 5 hours (Sinha, 198 and frozen by an instant freezing techniq (Bhandari *et al.*, 1982).

Acrosin activity (intact with the sperm cell was estimated as intracellular level (Amann *et* 1986) after activation of total proacrosin (Mann a Mann, 1981), which was released by incubating sperm pack (10^9spermatozoa over night at 4°C the medium (pH 2.8) containing 10% glyce (Polakoski *et al.*, 1977). Subsequent to incubatio the samples were centrifuged at 3000 rpm for

[1,3]Assoc. Prof., [2]Dean, [4]Associate Professor, Animal Genetics & Breeding

†Corresponding author

Table 1: Composition of Semen extenders

Ingredients	EXTENDERS									
	TYG	TYD			TYGD			TYGL		
		TYD_3	TYD_6	TYD_8	TYG_3D_5	TYG_5D_3	TYG_7D_1	$TYGL_{60}$	$TYGL_{120}$	$TYGL_{180}$
Tris (hydroxymethyl) amino methane (g)	12.10	12.10	12.10	12.10	12.10	12.10	12.10	12.10	12.10	12.10
Fructose (g)	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Citric acid (g)	6.80	6.80	6.80	6.80	6.80	6.80	6.80	6.80	6.80	6.80
Glycerol (ml)	32.00	-	-	-	12.00	20.00	28.00	20.00	20.00	20.00
DMSO (ml)	-	12.00	24.00	32.00	20.00	12.00	4.00	-	-	-
Lactose (g)	-	-	-	-	-	-	-	8.64	17.28	25.92
Double glass Distilled water (ml)	368.00	388.00	376.00	368.00	368.00	368.00	368.00	380.00	380.00	380.00
Total volume (ml)	400.00	400.00	400.00	400.00	400.00	400.00	400.00	400.00	400.00	400.00

TYG= Tris Yolk Glycerol (glycerol 8%), TYD= Tris Yolk DMSO (DMSO 3, 6 and 8%), TYGD= Tris Yolk Glycerol DMSO (glycerol 3 + DMSO 5%,Glycerol 5 + DMSO 3%, glycerol 7 + DMSO 1%), TYGL= Tris Yolk Glycerol Lactose (Lactose 60, 120 and 180 mM).

minutes and supernatant was utilized for the estimation of acrosin activity, which was determined following the hydrolysis of Benzoyle arginine-p-nitroanilide (BAPNA) and was expressed as $D^{OD}{}_{420}$/minute/10^9 spermatozoa and considered as one unit of enzyme activity (Sinha *et al.*, 1996). Extracellular hyaluronidase activity was assayed by the method of Linker (1956) and expressed as mM-N-acetyle glucosamine liberated /minute/10^9 spermatozoa as unit of enzyme (Sinha *et al.*, 1996). Data were analysed to study the effect of extender on enzyme activities at pre- and post-freeze stages as per Snedecor and Cochran, 1967.

RESULTS AND DISCUSSION

Acrosin

Mean intracellular acrosin activity in the neat semen of Black Bengal bucks was 0.0275±0.00084 units/10^9 sperms. The mean activity of acrosin ranged between 0.0130±0.00035 (TYD_8) and 0.0201 ±0.00042 ($TYGL_{180}$) at pre-freeze stage. Significant variation in acrosin activity was observed due to extender. Acrosin activity was significantly ($P<0.01$) higher in $TYGL_{180}$ and $TYGL_{120}$ than other extenders (Table 2). Mean intracellular acrosin activity in the neat semen of Beetal bucks was 0.0301±0.00105 units/10^9 sperms. The mean activity of acrosin ranged between 0.0134± 0.00028 (TYD_8) and 0.0211±0.00040 ($TYGL_{180}$) at pre-freeze stage. Significant variation in acrosin activity was observed due to extender. Acrosin activity was significantly ($P<0.01$) higher in $TYGL_{180}$ than other extenders but the difference with $TYGL_{120}$ was not significant (Table 2). Decline in acrosin activity was observed in each extender from pre-freeze to post-freeze stage. The findings pertaining to decrease in acrosin activity from pre- freeze to frozen thawed stage are in accordance with the findings of Buruiana *et al.*, 1980 in cattle, Chinnaiya and Ganguli, 1980, Kakar and Anand, 1984 in buffalo and Nehring, 1988 in ram semen. However at this stage significantly higher ($P<0.01$) activity was observed in lactose containing extenders as compared to DMSO alone or in combination with glycerol extenders. Maximum intracellular acrosin was observed in lactose containing extenders at pre- and post-freeze stages of freezing in the spermatozoa of bucks. Whereas minimum acrosin activity was recorded in extender TYD_8 with highest concentration of DMSO. It was further observed that with decreasing concentration of DMSO either alone or in combination with glycerol, the enzyme activity increases. In lactose containing extenders, the activity increased with the increasing concentration of lactose. These findings indicate better cryoprotective properties of lactose and glycerol in comparison to other combinations of cryoprotectants. The lactose extender were most effective with respect to viability and motility of spermatozoa also. Acrosomal damage was also lower in lactose extenders as compared with DMSO extenders (Singh *et al.*, 1995).

Hyaluronidase

Extracellular mean activities of hyaluronidase in the neat semen of Black Bengal bucks was 233.78±12.688 units/10^9 sperms. The mean activity at pre-freeze stage ranged between 277.16±16.735 ($TYGL_{180}$) and 324.30±11.819(TYD_8) units/10^9 sperms (Table 2). The hyaluronidase activity at post-freeze stage was significantly ($P<0.01$) lower in $TYGL_{180}$ than TYG, TYD and TYGD extenders

Table 2: Pre- and post freeze mean activities of acrosin and hyaluronidase enzymes of Black Bengal and Beetal buck's spermatozoa in different extenders with various cryoprotectants (units/10^9 spermatozoa)

Extenders (n= 9 in each extender)	Enzyme activity			
	Acrosin		Hyaluronidase	
	Pre-freeze	Post-freeze	Pre-freeze	Post-freeze
Black Bengal				
TYG	0.0170±0.00112^{c}	0.0134±0.00078^{a}	288.69±15.372	438.97±15.332^{bcd}
TYD_3	0.0143±0.00080^{d}	0.0102±0.00045^{bc}	297.90±17.000	454.37±13.192^{abc}
TYD_6	0.0137±0.00056^{d}	0.0095±0.00039^{cd}	309.21±15.695	465.63±10.640^{ab}
TYD_8	0.0130±0.00035^{d}	0.0086±0.00028^{d}	324.30±11.819	480.75±8.010^{a}
TYG_3D_5	0.0137±0.00039^{d}	0.0100±0.00043^{cd}	305.44±17.666	460.01±8.964^{ab}
TYG_5D_3	0.0144±0.00048^{d}	0.0108±0.00043^{bc}	294013±18.551	450.58±10.235^{abc}
TYG_7D_1	0.0173±0.00078^{c}	0.0116±0.00041^{b}	290.35±14.547	418.58±10.166^{d}
$TYGL_{60}$	0.0181±0.00078^{bc}	0.0136±0.00079^{a}	286.58±14.820	435.53±13.251^{bcd}
$TYGL_{120}$	0.0192±0.00054^{a}	0.0142±0.00071^{a}	284.70±15.175	424.18±12.303^{cd}
$TYGL_{180}$	0.0201±0.00042^{a}	0.0148±0.00058^{a}	277.16±16.735	412.89±9.775^{d}
Pooled	0.0161±0.00033	0.0117±0.00027	295.62±5.096	443.95±4.075
Beetal				
TYG	0.0179±0.00066^{cd}	0.0133±0.00085^{de}	269.61±13.982^{bcd}	414.81±12.346^{de}
TYD_3	0.0162±0.00061^{ef}	0.0110±0.00043^{e}	282.80±14.419^{abc}	431.77±9.414^{bcd}
TYD_6	0.0147±0.00041^{gh}	0.0091±0.00023^{b}	296.00±13.599^{ab}	448.70±7.535^{ab}
TYD_8	0.0134±0.00028^{h}	0.0079±0.00012^{a}	311.10±16.005^{a}	461.90±6.806^{a}
TYG_3D_5	0.0149±0.00043^{fg}	0.0097±0.00026^{b}	288.69±15.372^{abc}	441.17±10.564^{abc}
TYG_5D_3	0.0167±0.00057^{de}	0.0112±0.00052^{c}	282.80±14.419^{abc}	417.45±9.658^{cde}
TYG_7D_1	0.0178±0.00062^{cd}	0.0126±0.00043^{d}	269.61±13.982^{bcd}	409.15±11.083^{def}
$TYGL_{60}$	0.0190±0.00059^{bc}	0.0139±0.00049^{ef}	273.38±16.836^{abcd}	403.55±8.835^{ef}
$TYGL_{120}$	0.0204±0.00053^{ab}	0.0146±0.00049^{fg}	254.52±11.995^{cd}	399.74±7.520^{ef}
$TYGL_{180}$	0.0211±0.00040^{a}	0.0153±0.00045^{g}	239.44±9.976^{d}	386.59±4.713^{f}
Pooled	0.0160±0.00076	0.0114±0.00070	276.80±4.723	421.48±3.629

Values with the same superscript in a column for each breed separately did not differ significantly.

except TYG_7D_1. The overall mean hyaluronidase activities at pre- and post freeze stages were 295.62±5.049 and 443.95±4.075 units/10^9 spermatozoa, respectively. At pre-freeze stage , the hyaluronidase activity was higher in DMSO extenders as compared to lactose extenders, though the difference was not significant. It is also evident that the activity increased with the incresing concentration of DMSO alone or in combination with glycerol. Extracellular mean activities of hyaluronidase in the neat semen of Beetal bucks was 220.58±7.261 units/10^9 sperms. The mean activity at pre-freeze stage ranged between 239.44± 9.976 ($TYGL_{180}$) and 311.10 ±16.005(TYD_8) units / 10^9 sperms (Table 2).The hyaluronidase activity at pre-freeze stage was significantly ($P<0.01$) lower in $TYGL_{180}$ than TYG, TYD and TYGD extenders except TYG_7D_1. The overall mean hyaluronidase activities at pre- and post-freeze stages were 276.80±4.723 and 421.48±3.629 units/10^9 spermatozoa, respectively. At post–freeze stage, the hyaluronidase activity was significantly higher in TYD_8 extender as compared to lactose extenders. It is also evident that the activity increased with the increase in concentration of DMSO alone or in combination with glycerol.

The activity was maximum in TYD_8 extender in both breeds. The hyaluronidase activity decreased with the increasing concentration of lactose in extenders containing glycerol with lactose. The activity was lowest in $TYGL_{180}$ extender with highest concentration of lactose. At post-freeze stage there was an overall increase in hyaluronidase activities was observed in all extenders. The trend observed at pre-freeze stage continued in the frozen thawed semen also. The variation in hyaluronidase

activity at post-freeze stage was significant (P<0.01) as reported by Kakar and Anand (1984) in buffalo semen. However Patil *et al.* (1981) could not observe significant difference in hyaluronidase release among extenders in buffalo semen.

REFERENCES

Allison, A.C. and Hartree, E.F.1970. Lysosomal enzymes in the acrosome and their possible role in fertilization. *J. Reprod. Fertil.*, **21** :501-505.

Amann, R.P., Froman, D.P. and Schenk, J.L.1986. A microassay for bovine acrosin. *J. Androl.*, **7**:27.

Bhandari, N., Chauhan, R.A.S. and Mathew, A. 1982. Note on the effect of rate of freezing on the freezability of buffalo spermatozoa. *Indian J. Anim. Sci.*, **52**:1237-1238.

Buruiana, L.M., Khalil, M., Seiciu, F. and Niculescu, S. 1980. Effect of freeze preservation on acrosin and hyaluronidase activity of bull spermatozoa. *Anim. Breed. Abstract.*, 50:3711.

Chinnaiya, G.P. and Ganguli, N.C.1980. Acrosomal damage of buffalo spermatozoa during freezing in extenders. *Anim. Breed. Abstract.*, **52**:331.

Kakar, S.S. and Anand, S.R. 1984. Acrosomal damage and enzyme leakage during freeze preservation of buffalo spermatozoa. *Indian J. Expt. Biol.*, 22:5-16.

Mann, T. and Mann, C.L.1981. ***Male reproductive function and semen***. Springer – Verlag. Berlin, Heidelberg, New York.

McRorie, R.A. and Williams, W.L.1974. Biochemistry of mammalian fertilization. *Annual Rev. Biochem.*, **43**:777.

Nehring, H. 1988. Evaluation of acrosin integrity during semen storage. Zeitschrift der Humboildt Universitat Zu Berlin, *R. Med., 37*,1:109-116.

Patil, D.S., Hukeri, V.B. and Seshpande, B.R.1981. Acrosomal damage in fresh and frozen thawed buffalo spermatozoa. *Indian J. Anim. Reprod.*, 1: 90-93.

Polakoshi, K.L., Zahler, W.L. and Paulson, J.D.1977. Demonstration of proacrosin and quantitation of acrosin in ejaculated human spermatozoa. *Fert.and Sterl.*, **28**:668-670.

Singh, M.P., Sinha, A.K., Singh, B. K.1995. effect of cryoprotectants on certain seminal attributes and fertility of buck spermatozoa. *Theriogenology*,**43**:1047-1053.

Singh, M.P., sinha, A.K., Singh, B.K. and Prasad, R. L. 1996. Effect of cryoprotectants on release of various enzymes from buck spermatozoa during freezing. *Theriogenology*,**42**:405-416.

Sinha, S. N. 1986. Studies on freezability, certain characteristics and fertility of buck spermatozoa. Ph. D. Thesis, Birsa Agricultural University, Ranchi, Jharkhand, India.

Snedecor, G. W. and Cochran, W.G.1967. ***Statistical Methods***. 6th Edition, Oxford & IBH publication Co., Calcutta, India.

Tsekova, E., Spasov, K.H., Angelova, M. and Georgiev, G.K.H.1986. The effect of cryopreservation on the activity of acrosomal enzymes in ram spermatozoa. *Anim. Breed. Abstract.*, **55**:5600.

Zaneveld, L.J.D., Robertson, R.T., Kessler, M. and Williams, W.L. 1971. Inhibition of fertilization *in vitro* by pancreatic and seminal plasma trypsin inhibitors. *J. Reprod. Fertil.*, **25**:287.

Zlatarev, S.T., Georgiev, G.H., Tzekova, E.I., Petrova, K.G. and Mincheva, M.B.1988. Influence of pH of dilutors on some acrosomal enzymes of ram spermatozoa stored at 0-4°C up to 48 hours. *Anim. Breed. Abstract.*, **57**:5618.