EFFECT OF DILUTORS ON MOTILITY, MORPHOLOGY AND ACROSOME INTEGRITY OF RAM SPERMATOZOA DURING FREEZING

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ABSTRACT

The effects of dilutors, rams, stages of freezing process and their interactions were studied in 90 ejaculates in a 3x5x3 factorial experiment for biophysical characteristics of sperms during freezing of sheep semen. The diluents used was 20% egg yolk and 4% glycerol with tris fructose citric acid. (TFCEG), a synthetic phosphate medium (Phos) and sodium citrate glucose egg yolk glycerol (SCGEG). The influences of dilutors, rams, stages of freezing and interactions of dilutor with ram, and dilutor with stages of freezing were highly significant (P<0.01) on motility and abnormal sperm as well as acrosome score. Maximum post-thaw motility (36.67 ± 1.13%) was observed in TFCEG diluent followed by Phos (31.00 ± 1.43%) and the least in SCGEG (19.83 ± 0.85%) diluent. Rams having better sperm motility at dilution stage could express equally good post-thaw motility in Phos diluent. The percentages of morphologically abnormal sperms and acrosome scores were also significantly (P<0.05) influenced by the dilutors, being minimum in Phos and maximum in TFCEG diluent. Further, the observations indicated that TFCEG provided better protection to sperm motility whereas Phos diluent provided maximum protection to acrosme morphology while cryofreezing of ram semen.

Key Words: Dilutors, Freezability, Motility, Morphology, Acrosome integrity, Ram semen.

Composition of diluent is very important for optimum post-thaw survival of spermatozoa (Mathur et al., 1991). Several reports on different media used for extending ram spermatozoa for successful pellet and ampoule freezing have appeared in literature (Mathur, 2003), but reports on the comparative efficacy of different media for straw cryopreservation are meagre (Gil et al., 2000; 2003). Vulnerability of ram spermatozoa to dilution and cold shock, sensitivity to the alteration in pH and composition of medium, and less tolerance to glycerol pose practical problems in straw cryopreservation leading to lower post- thaw recovery rate (Mathur, 2003). Hence, the present study was attempted to evaluate the interaction of individual sire with different diluents for straw cryopreservation of Patanwadi ram semen in terms of motility, morphology and acrosome integrity.

The study was conducted on semen of five mature Patanwadi rams maintained in semi-intensive system at AICRP on sheep breeding, at University farm, Sardarkushinagar, North Gujarat. Semen was collected in separate AV at weekly intervals from each ram. Immediately after collection, the cups were kept at 37°C in water-bath. The samples were evaluated for routine macro-microscopic quality and split-diluted with three extender formulations with 20% egg yolk and 4% glycerol in a 3x5x3 factorial experiment. The extenders used were (i) Sodium citrate glucose egg yolk glycerol -SCGEG), (ii) Phosphate buffer with glucose fructose

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sodium citrate potassium chloride egg yolk glycerol (Synthetic phosphate medium) - Phos and (iii) Tris fructose citric acid egg yolk glycerol -TFCEG

Split-ejaculates of individual rams after first step dilution (1:3) at 37°C temperature were cooled to 5°C over 1.5 hr. In the second step of dilution, equal volume of first step diluted pre-cooled semen and glycerolated buffer (at 3-5°C) was mixed so as to maintain 150 million sperm per straw and then equilibrated for 4 hrs in the refrigerator. French medium straws of different colour markings were then filled and sealed with PVA powder. The straws were frozen in liquid nitrogen vapour in a thermocoal box using standard freezing protocol, as recommended by Sahni and Mohan (1988). The straws were thawed at 37°C for 15 seconds in a water-bath after 24 hr of freezing. Each sample was studied for sperm motility, total morphological abnormalities and acrosome score in freshly diluted, equilibrated (pre-freeze) and post-thawed semen. The data were analyzed statistically using 3 factors factorial randomized block design (Steel and Torrie, 1981).

The findings on motile and abnormal sperm per cent and acrosome score of individual rams at three stages of freezing process in three diluents are presented in Tables 1 and 2.

Maximum sperm motility was observed at all three stages of freezing process in TFCEG diluent followed by Phos and SCGEG diluents. The effects of all three variables, i.e. rams, dilutors and freezing steps, and the interaction of dilutor with the ram and the freezing step on sperm motility were highly significant ($P<0.01$). At post-thaw stage, all the rams had maximum sperm motility in TFCEG dilutor followed by Phos and SCGEG, but not at initial stage or after equilibration. Behaviour of spermatozoa of individual ram in different dilutors is suggestive of the fact that ram with better semen quality can sustain even a second or third grade dilutor though the best performance is enhanced in the superior dilutor, thus emphasizing the need of judicious selection of ram for freezing of semen. Further, rams with even a comparatively lower initial semen quality can desirably undergo dilution and freezing process with a better dilutor. However, the data in Table 2 suggests that though the sperm motility was maximum in phosphate dilutor initially, the effect of equilibration / glycerolisation and freezing thawing was much detrimental in SCGEG and Phos dilutors than the TFCEG. These observations are suggestive of the fact that probably Phos diluent could have better protective effect during chilling of ram semen. Findings of present study suggest that TFCEG is the best diluent for straw freezing of ram semen as far as post-thaw motility is concerned.

Significant effect of rams over the sperm motility corroborated with the findings of D'Alessandro et al. (1999) who found significantly higher motility, viability, acrosome integrity and even fertility of ram semen frozen in TFYG than in SMLYG diluent. Further, the post-thaw motility with tris diluent in the present study (36.67 ± 1.13%) is comparable to that reported by Lopez et al. (1988) and Kandasamy et al. (1989), but was lower than that reported by Saxena et al. (1979). Significant reduction in the motility of sperms in tris diluent after equilibration and freezing process is comparable with the reports of Mathur (2003).

The overall post-thaw motility in phos dilutor (31.00 ± 1.43%) is comparable with that of Varnavaskii et al. (1989). The post-thaw motility in SCGEG (19.83 ± 0.85 %) is comparable to the report of Zamfirescu et al. (1980) with a similar composition of diluent and freezing process. However, the post-thaw motility obtained by Londhe et al. (2005) with a different glycerol concentration and/or freezing process was better than the present findings. The trend of gradual loss in the post-thaw motility at the three stages of freezing process compared well with the report of Mathur (2003).

The percentage of morphologically abnormal spermatozoa in the three dilutors varied significantly ($P<0.05$), being higher in TFCEG and SCGEG dilutors than in the Phos diluent. The variation between rams and stages of freezing process was also highly significant ($P<0.01$) for this trait. The interaction of rams with the dilutors for the percent morphological abnormalities was significant ($P<0.05$). All the rams had minimum sperm abnormalities in Phos diluent at post-thaw stage. Effect of rams over the stages of freezing process was non-significant; however, dilutors had highly significant influence over the stages. Critical difference test revealed that at dilution stage the morphological abnormalities were minimum in TFCEG, and maximum in Phos and SCGEG diluents. After glycerolisation and equilibration, the gradation from minimum to maximum abnormalities turned over to Phos, TFCEG and SCGEG diluents, respectively, while after undergoing the freeze-thaw process, the gradation from minimum to maximum per cent sperm abnormalities was in Phos, SCGEG and TFCEG, suggesting the fact that dilutors highly significantly influence the morphological characteristics of

spermatozoa during cryopreservation. The phos diluent proved to be the most efficient to maintain normal sperm morphology.

Saxena and Tripathi (1984) reported non-significant variation between dilutors for morphological abnormalities of sperm head. Gokcen et al. (1992) found significant variation between dilutors for sperm abnormalities of ram semen, which is in agreement with the present findings. Variation between rams in morphological abnormalities of sperm was in accordance with the reports of Kakadia (1993) for chilled ram semen, while significant stage variation agreed with the findings of Mathur et al. (1991). The effect of dilutors over per cent acrosome score was highly significant ($P<0.01$), the overall per cent acrosomal score being lower in Phos than the SCGEG and TFCEG diluents. Further, the variation in per cent acrosome score due to rams and stages of freezing process was also significant ($P<0.01$). It was observed that though the per cent acrosomal score was minimum in freshly diluted semen in TFCEG, maximum in Phos, and intermediate in SCGEG diluent, while after freeze-thaw process the maximum score was in SCGEG and minimum in Phos, TFCEG being intermediate. The observations indicated that during glycerolisation and freeze-thaw process, Phos diluent could provide maximum protection to the morphology of acrosome and SCGEG the minimum, tris being intermediate.

The percentage of intact acrosome of present study corroborated with the findings of Lopez et al. (1988) for the tris diluent and Watson and Martin (1972) for Phos diluent. Further, comparatively higher per cent acrosome score after freeze-thaw process using tris lactose than milk lactose egg yolk diluent has been reported (Mathur, 2003). It was attributed to the relative osmotic pressure, which may be a probable factor of having significant influence between the diluents leading to variation in the per cent acrosome score in the present study also. The influence of stags of freezing process over the post-thaw acrosomal score was highly significant in this study, which was in accordance with the reports of Gil et al. (2003).

Thus, it can be concluded that both dilutors and freezing steps significantly influence the cryopreservability of ram semen, and that tris-fructose-citric acid-egg yolk-glycerol (TFCEG) diluent protected sperm motility better, while Phos diluent provided maximum protection to sperm and acrosme morphology during freezing of ram semen. However, these subjective observations need to be supported by actual fertility trials.

Table 2 : Effect of dilutor x stage of freezing interaction on per cent motile and abnormal spermatozoa and acrosome score of Patanwadi ram semen (Mean ± SE)

Trait	Stage of freezing	Dilutors		
		TFCEG	Phos	SCGEG
Sperm motility (%)	Dilution	$72.50^{a}\pm1.57$	$73.67^{a}\pm1.32$	$68.00^{b}\pm1.30$
	Equilibration	$60.83^{c}\pm1.75$	$57.83^{d}\pm1.90$	$47.50^{e}\pm1.35$
	Post-thaw	$36.67^{f}\pm1.13$	$31.00^{g}\pm1.43$	$19.83^{h}\pm0.85$
Abnormal Sperm (%)	Dilution	$8.79^{g}\pm0.36$	$9.91^{f}\pm0.25$	$9.58^{f}\pm0.25$
	Equilibration	$13.17^{e}\pm0.44$	$13.09^{e}\pm0.56$	$14.84^{d}\pm0.36$
	Post-thaw	$22.64^{a}\pm0.72$	$18.48^{c}\pm0.57$	$21.28^{b}\pm0.52$
Acrosome score (%)	Dilution	$7.09^{b}\pm0.34$	$8.23^{a}\pm0.18$	$7.86^{a}\pm0.24$
	Equilibration	$10.13^{b}\pm0.59$	$11.33^{ab}\pm0.42$	$12.39^{a}\pm0.43$
	Post-thaw	$17.22^{b}\pm0.89$	$15.59^{c}\pm0.55$	$18.09^{a}\pm0.53$

Means carrying similar superscript do not differ significantly among dilutors for a trait ($P>0.05$)

Table 1. Per cent sperm motility, abnormal sperm and acrosome score at different stages of freezing process of Patanwadi ram semen using three extenders (Mean ± SE)

Trait	Ram No.	Stages of freezing process in different extenders								
		Diluted			Equilibrated			Post-thawed		
		TFCEG	Phos	SCGEG	TFCEG	Phos	SCGEG	TFCEG	Phos	SCGEG
Sperm motility (%)	P1	75.00 ±2.45	75.83 ±4.54	68.33 ±2.79	65.00 ±2.24	62.50 ±2.81	47.50 ±2.50	39.17 ±2.71	35.83 ±1.54	19.17 ±1.54
	P2	80.00 ±1.29	76.67 ±1.67	75.00 ±1.83	65.83 ±2.01	62.50 ±3.35	51.67 ±4.77	40.83 ±2.39	34.17 ±2.39	21.67 ±2.71
	P3	68.23 ±2.79	70.00 ±2.89	64.17 ±2.01	55.00 ±4.28	55.00 ±3.65	45.00 ±2.24	32.50 ±2.14	25.83 ±2.39	16.67 ±1.05
	P4	77.50 ±2.14	79.17 ±2.01	70.00 ±2.24	68.33 ±2.11	64.17 ±3.75	49.17 ±2.71	39.17 ±1.54	37.50 ±2.50	22.50 ±2.14
	P5	61.67 ±2.79	64.17 ±2.01	62.50 ±3.09	50.00 ±2.58	45.00 ±2.58	44.17 ±2.01	31.67 ±1.67	21.67 ±1.05	19.17 ±2.01
Abnormal sperm (%)	P1	7.80 ±0.92	8.97 ±0.36	9.33 ±0.49	11.82 ±1.03	12.75 ±0.69	14.03 ±0.51	22.02 ±1.28	18.22 ±0.50	20.27 ±0.86
	P2	8.25 ±0.75	10.97 ±0.34	8.87 ±0.59	11.63 ±0.87	12.25 ±0.24	11.52 ±2.01	20.35 ±1.13	17.05 ±0.59	21.18 ±1.72
	P3	8.43 ±0.83	9.71 ±0.34	9.00 ±0.36	13.89 ±0.52	13.38 ±0.89	14.76 ±0.53	23.75 ±1.56	19.15 ±0.61	21.37 ±0.65
	P4	8.65 ±0.60	9.38 ±0.48	9.50 ±0.32	13.52 ±0.87	12.10 ±0.66	15.77 ±1.03	22.23 ±1.89	15.43 ±0.56	19.97 ±0.69
	P5	10.85 ±0.45	10.55 ±0.37	11.18 ±0.49	15.10 ±1.02	16.00 ±0.99	16.10 ±0.99	24.97 ±1.88	22.58 ±1.53	23.63 ±1.20
Acrosomal score (%)	P1	6.33 ±0.95	7.59 ±0.39	7.59 ±0.46	9.14 ±1.33	11.05 ±0.80	11.10 ±0.33	16.62 ±1.68	15.13 ±0.50	16.91 ±0.97
	P2	6.48 ±0.76	8.43 ±0.17	7.33 ±0.68	8.36 ±0.69	10.56 ±0.39	11.15 ±0.41	13.43 ±1.59	13.71 ±0.50	16.26 ±0.82
	P3	6.71 ±0.66	8.30 ±0.59	7.69 ±0.41	10.93 ±0.51	11.07 ±0.75	11.31 ±0.51	17.93 ±2.05	16.26 ±0.67	18.56 ±0.86
	P4	7.07 ±0.66	7.86 ±0.40	7.76 ±0.47	11.09 ±1.03	9.90 ±0.42	14.07 ±1.24	16.86 ±1.68	13.21 ±0.47	16.59 ±1.22
	P5	8.60 ±0.39	8.98 ±0.25	8.92 ±0.46	13.18 ±1.14	14.32 ±1.13	14.36 ±1.07	21.26 ±2.12	19.56 ±1.51	21.12 ±1.43

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