



## Efficacy of egg yolk based and egg yolk free soya bean milk based extenders for cryopreservation of Zebu cattle and buffalo semen

P.J. Chaudhary<sup>1</sup>, A.J. Dhama<sup>\*</sup>, D.V. Chaudhari, K.K. Hadiya and J.A. Patel

Department of Animal Reproduction Gynaecology and Obstetrics,  
College of Veterinary Science and Animal Husbandry, AAU, Anand-388 001, Gujrat, India.

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### ABSTRACT

This study was undertaken on three mature bulls each of Gir cattle and Surti buffalo breeds to evaluate the comparative efficacy of egg yolk based standard TFYG (Tris-citrate-fructose-yolk-glycerol) extender and egg yolk free soybean based commercial extenders Optixcell® (IMV, France) and Andromed® (Minitube, Germany) under split-sample technique. The ejaculates (9/bull) were extended @  $100 \times 10^6$  sperm  $\text{ml}^{-1}$  with three extenders and frozen using biofreezer following 4 hr of equilibration. The pooled means of progressively motile sperm observed (irrespective of extenders) at initial, pre-freeze and post-thaw stage in Gir bulls semen were  $76.53 \pm 0.53$ ,  $71.11 \pm 0.53$  and  $39.86 \pm 0.90\%$  and in Surti buffalo  $80.76 \pm 0.39$ ,  $74.65 \pm 0.45$  and  $40.35 \pm 1.07\%$ , respectively. The corresponding values for live sperm were  $75.64 \pm 0.76$ ,  $69.01 \pm 0.97$  and  $47.99 \pm 1.11\%$  for Gir and  $80.90 \pm 0.45$ ,  $75.76 \pm 0.48$  and  $52.33 \pm 0.86\%$  for Surti buffalo; and those of intact acrosome  $94.29 \pm 0.25$ ,  $90.29 \pm 0.27$  and  $79.29 \pm 0.33\%$  for Gir bulls, and  $93.94 \pm 0.21$ ,  $89.94 \pm 0.23$  and  $78.95 \pm 0.26\%$  for Surti buffalo semen, respectively. The HOS reactive sperm at initial, pre-freeze and post-thaw stage were  $76.18 \pm 0.74$ ,  $71.04 \pm 0.76$  and  $27.90 \pm 0.70\%$  for Gir, and  $81.83 \pm 0.35$ ,  $76.47 \pm 0.39$  and  $27.83 \pm 0.68\%$  for Surti bulls, respectively. The overall mean post-thaw incubation ( $37^\circ\text{C}$ ) survival of spermatozoa observed at 60, 120 and 180 min were  $28.40 \pm 0.91$ ,  $17.78 \pm 0.86$  and  $9.44 \pm 0.72\%$  for Gir bulls semen, and  $28.01 \pm 0.99$ ,  $18.40 \pm 1.01$  and  $10.51 \pm 0.93\%$  for Surti buffalo semen, respectively. Optixcell was proved superior, and at par with TFYG, than the Andromed in maintaining greater motility, viability, morphology, acrosomal/plasma membrane integrity including post-thaw sperm longevity of cattle and buffalo spermatozoa with significant differences only in sperm motility and post-thaw longevity. The motile, live and HOST reactive sperm were significantly higher in buffalo semen than cattle at initial and pre-freeze stage, but not at post-thaw stage. The results showed that egg yolk free commercial Optixcell extender and egg yolk based TFYG extender were at par in terms of most of the sperm quality traits, hence any one of them can be preferred over Andromed for successful routine cryopreservation of cattle and buffalo semen.

**Key words:** Relative efficacy, Egg yolk, Soybean milk, Bovine semen, Cryopreservation, Post-thaw longevity.

### INTRODUCTION

The two main concerns of bovine production systems regarding artificial insemination are the control of pathogens spread by semen contamination, and the total quality control of the batches produced (Leeuw *et al.*, 2000). Biological security of semen production is prejudiced by several factors such as the efficacy of the antibiotics used in the extenders, the quality control of reagents, hygiene during semen processing, and the quality of the extenders. Recent studies are in progress targeting to develop chemically defined extenders, free from compounds of animal origin. The semen-extenders generally used comprise of animal origin egg yolk, skim milk powder, or the combination of both, as primary source of lipoproteins, essential to membrane equilibrium during the freeze-thawing process (Bousseau *et al.*, 1998). Despite the significant benefits of egg yolk and milk on semen cryopreservation, such

components of animal origin may denote a latent microbiological threat, compromising the quality and standardization of cryopreserved semen, and are also responsible for immuno-infertility issues in inseminated females. As a consequence, the OIE recommended that animal origin products used in semen processing should be free of any biological hazard or processed so as to assure the safety of such compounds (Jimenez *et al.*, 2004).

Presently an alternative to substitute the components of animal origin in semen extenders is the soy lecithin, a natural mixture of phosphatidylcholine and several fatty acids. Studies targeting to assess the efficacy of soy lecithin in egg yolk free semen extenders were performed in bovine (Aires *et al.*, 2003; Muino *et al.* 2007; Veerabramhaiah *et al.*, 2011; Singh *et al.*, 2013; Beura *et al.*, 2014; Layek *et al.*, 2016) and bubaline (Akhter *et al.*,

\*Corresponding author's e-mail: ajdhami@aaui.in

<sup>1</sup>District Panchayat, Himmatnagar, Gujarat, India.

2010; Meena *et al.*, 2010; Ansari *et al.*, 2016; Chaudhari *et al.*, 2015, 2017). However, results obtained using lecithin as substitute to egg yolk are still a matter of debate (Leite *et al.*, 2010). Furthermore, due to the reduced technological inventions on semen cryopreservation over the last few years (Celeghini *et al.*, 2008; Chaudhari *et al.*, 2015), the tris-egg yolk-fructose-glycerol extender is still the most frequently employed semen extender worldwide (Chaudhari *et al.*, 2015). The aim of this study was therefore to assess the relative efficacy of egg yolk based and egg yolk free soybean based commercially available extenders for cryopreservation of Gir cattle and Surti buffalo bull semen.

## MATERIALS AND METHODS

This investigation was carried out during the favourable breeding season from September to February (2016-17) on three healthy mature Gir cattle and three Surti buffalo bulls maintained at Sperm Station of the College in Anand. The bulls were maintained under identical nutritional and managerial conditions. The semen was collected twice a week from all the bulls in the morning between 7.30 and 8.30 hr using artificial vagina. For this study, alternate ejaculates were used under split-sample technique to evaluate comparative efficacy of egg yolk based standard TFYG (Tris-citric acid-fructose-egg yolk-glycerol) extender and soybean based commercially available extenders Optixcell® (IMV, France) and Andromed® (Minitube, Germany) through various morphological and functional attributes of spermatozoa. The ejaculates (9/bull, total 54) with >70 % initial motility were divided in to three equal aliquots, and extended at the concentration of  $100 \times 10^6$  spermatozoa  $\text{ml}^{-1}$  at 34°C with three different extenders. The TFYG extender was prepared fresh daily using hen's egg, while commercial egg yolk free extenders were diluted with Mili-Q water @ 1:2 for Optixcell and 1:4 for Andromed as per the instructions of the manufacturers.

The extended semen samples were soon evaluated through standard procedures for sperm quality parameters, viz., motility, viability, morphology (eosin-nigrosin stain), acrosomal integrity (Watson, 1975) and plasma membrane integrity (HOST, Jayendran *et al.*, 1984), and were filled in French mini straws on IS4 system (IMV, France). After gradual cooling over 60-90 minutes and equilibration for 4 hrs in cold handling cabinet, the straws were frozen in liquid nitrogen vapour using a programmable bio-freezer (IMV, France). The straws of all three extenders were also evaluated at pre-freezing (after equilibration) and after 24 hrs of freezing (post-freeze stage) for the above five quality parameters. The straws were thawed in water bath at 37°C for 30 seconds. For post-thaw incubation test, the contents of three straws each were transferred to sugar tubes arranged in steel racks kept in a water bath and sperm progressive motility/longevity was assessed at 0, 30, 60, 120 and 180 min of incubation at 37°C. The data generated were analyzed

statistically using CRD, DMRT and 't' test by employing IBM software version 20.00 (Snedecor and Cochran, 1994).

## RESULTS AND DISCUSSION

**Sperm Quality Attributes during Cryopreservation:** The mean percentages of progressively motile, live and abnormal and HOS reactive sperm and those with intact acrosome observed at different stages of cryopreservation of Gir cattle and Surti buffalo bulls semen extended in conventional TFYG and commercial egg yolk free Optixcell and Andromed are presented in Tables 1-3. Statistical analysis revealed that there were significant differences ( $P < 0.05$ ) in the levels of these parameters between semen extenders and between stages of cryopreservation, but no individual bull variation was seen in any of the two species studied.

The mean percentages of progressively motile spermatozoa observed, irrespective of dilutors, at initial, pre-freeze and post-thaw stages in Gir bull semen were  $76.53 \pm 0.53$ ,  $71.11 \pm 0.53$  and  $39.86 \pm 0.90$ , and for Surti buffalo semen  $80.76 \pm 0.39$ ,  $74.65 \pm 0.45$  and  $40.35 \pm 1.07$ , respectively. The differences in sperm motility ratings were significant ( $P < 0.05$ ) between extenders at all three stages, where Optixcell showed superior results followed by TFYG and Andromed for cattle semen. Buffalo semen also revealed significant ( $P < 0.05$ ) variation for this trait at initial and pre-freeze stage, but had no significant ( $P > 0.05$ ) difference at post-thaw stage between extenders (Table 1). The pooled mean percentages of live spermatozoa observed, irrespective of extenders, at initial, pre-freeze and post-thaw stage in Gir bull semen were  $75.64 \pm 0.76$ ,  $69.01 \pm 0.97$  and  $47.99 \pm 1.11$ , whereas for Surti buffalo semen the values were  $80.90 \pm 0.45$ ,  $75.76 \pm 0.48$  and  $52.33 \pm 0.86$ , respectively. There were no significant differences between three extenders at any of the stages, except at pre-freeze stage in buffalo semen, where TFYG was found superior ( $P < 0.05$ ) in maintaining greater viability of sperms compared to soybean based commercial extenders (Table 1).

The pooled mean percentages of spermatozoa with intact acrosomes observed, irrespective of dilutors, at initial, pre-freeze and post-thaw stage were  $94.29 \pm 0.25$ ,  $90.29 \pm 0.27$  and  $79.29 \pm 0.33$  for Gir bulls, and  $93.94 \pm 0.21$ ,  $89.94 \pm 0.23$  and  $78.95 \pm 0.26$  for Surti buffalo semen, respectively. The corresponding values for sperms with intact plasma membrane (HOST reactive) were  $76.18 \pm 0.74$ ,  $71.04 \pm 0.76$  and  $27.90 \pm 0.70$  % for Gir, and  $81.83 \pm 0.35$ ,  $76.47 \pm 0.39$  and  $27.83 \pm 0.68$  % for Surti bulls, respectively (Table 2). The overall mean percentages of total sperms abnormalities recorded, irrespective of extenders, initially on dilution; at pre-freeze and post-thaw stage were  $5.20 \pm 0.22$ ,  $6.83 \pm 0.20$  and  $11.12 \pm 0.21$  in Gir bull semen, while  $4.76 \pm 0.22$ ,  $5.91 \pm 0.20$  and  $10.11 \pm 0.18$  for Surti buffalo semen, respectively. Statistically, there were no significant differences between three extenders at any of the stage of

**Table 1:** Mean ( $\pm$  SE) percentage of progressively motile and live spermatozoa in Gir cattle and Surti buffalo bulls' semen at different stages of cryopreservation in 3 extenders.

Freezing stage	Extender	Sperm motility (%)		Live sperm (%)	
		Gir	Surti	Gir	Surti
Initial	TFYG	75.00 $\pm$ 0.95 <sup>a</sup>	80.42 $\pm$ 0.73 <sup>ab</sup>	76.50 $\pm$ 1.30	81.75 $\pm$ 0.70
	Optixcell	78.33 $\pm$ 0.78 <sup>b</sup>	82.08 $\pm$ 0.60 <sup>b</sup>	75.63 $\pm$ 1.32	80.79 $\pm$ 0.79
	Andromed	76.25 $\pm$ 0.92 <sup>ab</sup>	79.79 $\pm$ 0.64 <sup>a</sup>	74.79 $\pm$ 1.38	80.17 $\pm$ 0.84
	Average	<b>76.53<math>\pm</math>0.53<sup>x</sup></b>	<b>80.76<math>\pm</math>0.39<sup>x</sup></b>	<b>75.64<math>\pm</math>0.76<sup>x</sup></b>	<b>80.90<math>\pm</math>0.45<sup>x</sup></b>
Pre-freeze	TFYG	69.38 $\pm$ 0.92 <sup>a</sup>	74.38 $\pm$ 0.81 <sup>ab</sup>	69.92 $\pm$ 1.74	77.04 $\pm$ 0.63 <sup>b</sup>
	Optixcell	73.13 $\pm$ 0.73 <sup>b</sup>	76.04 $\pm$ 0.74 <sup>b</sup>	69.42 $\pm$ 1.61	75.96 $\pm$ 0.79 <sup>ab</sup>
	Andromed	70.83 $\pm$ 0.94 <sup>ab</sup>	73.54 $\pm$ 0.71 <sup>a</sup>	67.71 $\pm$ 1.72	74.29 $\pm$ 0.95 <sup>a</sup>
	Average	<b>71.11<math>\pm</math>0.53<sup>y</sup></b>	<b>74.65<math>\pm</math>0.45<sup>y</sup></b>	<b>69.01<math>\pm</math>0.97<sup>y</sup></b>	<b>75.76<math>\pm</math>0.48<sup>y</sup></b>
Post-thaw	TFYG	40.42 $\pm$ 1.50 <sup>ab</sup>	39.58 $\pm$ 1.85	48.92 $\pm$ 1.94	52.38 $\pm$ 1.49
	Optixcell	42.08 $\pm$ 1.62 <sup>b</sup>	42.92 $\pm$ 1.80	49.42 $\pm$ 1.78	53.46 $\pm$ 1.47
	Andromed	37.08 $\pm$ 1.41 <sup>a</sup>	38.54 $\pm$ 1.87	45.63 $\pm$ 2.03	51.17 $\pm$ 1.51
	Average	<b>39.86<math>\pm</math>0.90<sup>z</sup></b>	<b>40.35<math>\pm</math>1.07<sup>z</sup></b>	<b>47.99<math>\pm</math>1.11<sup>z</sup></b>	<b>52.33<math>\pm</math>0.86<sup>z</sup></b>

Means bearing different superscripts between extenders (a,b,c) at each stage and between stages (x,y,z) differ significantly ( $P < 0.05$ ).

**Table 2:** Mean ( $\pm$ SE) percentage of sperms with intact acrosome and intact plasma membrane (HOST reactive) in Gir cattle and Surti buffalo bulls' semen at different stages of cryopreservation in three extenders.

Freezing stage	Extender	Intact acrosomes (%)		HOST (%)	
		Gir	Surti	Gir	Surti
Initial	TFYG	94.50 $\pm$ 0.40	94.04 $\pm$ 0.36	76.92 $\pm$ 1.27	82.25 $\pm$ 0.62 <sup>ab</sup>
	Optixcell	94.42 $\pm$ 0.46	94.00 $\pm$ 0.40	76.88 $\pm$ 1.28	82.63 $\pm$ 0.5 <sup>b</sup>
	Andromed	93.96 $\pm$ 0.43	93.79 $\pm$ 0.36	74.75 $\pm$ 1.27	80.63 $\pm$ 0.58 <sup>a</sup>
	Average	<b>94.29<math>\pm</math>0.25<sup>x</sup></b>	<b>93.94<math>\pm</math>0.21<sup>x</sup></b>	<b>76.18<math>\pm</math>0.74<sup>x</sup></b>	<b>81.83<math>\pm</math>0.35<sup>x</sup></b>
Pre-freeze	TFYG	90.50 $\pm$ 0.45	90.04 $\pm$ 0.39	71.79 $\pm$ 1.44	77.04 $\pm$ 0.56
	Optixcell	90.42 $\pm$ 0.47	90.00 $\pm$ 0.40	71.42 $\pm$ 1.24	76.92 $\pm$ 0.85
	Andromed	89.96 $\pm$ 0.50	89.79 $\pm$ 0.39	69.92 $\pm$ 1.26	75.46 $\pm$ 0.56
	Average	<b>90.29<math>\pm</math>0.27<sup>y</sup></b>	<b>89.94<math>\pm</math>0.23<sup>y</sup></b>	<b>71.04<math>\pm</math>0.76<sup>y</sup></b>	<b>76.47<math>\pm</math>0.39<sup>y</sup></b>
Post-thaw	TFYG	79.50 $\pm$ 0.59	79.04 $\pm$ 0.44	28.67 $\pm$ 1.29	28.04 $\pm$ 1.31
	Optixcell	79.42 $\pm$ 0.54	79.01 $\pm$ 0.46	28.63 $\pm$ 1.21	29.38 $\pm$ 1.27
	Andromed	78.96 $\pm$ 0.59	78.79 $\pm$ 0.46	26.42 $\pm$ 1.15	26.08 $\pm$ 0.85
	Average	<b>79.29<math>\pm</math>0.33<sup>z</sup></b>	<b>78.95<math>\pm</math>0.26<sup>z</sup></b>	<b>27.90<math>\pm</math>0.70<sup>z</sup></b>	<b>27.83<math>\pm</math>0.68<sup>z</sup></b>

Means bearing different superscripts between freezing stages (x,y,z) differ significantly ( $P < 0.05$ ), but not between dilutors.

cryopreservation process for total or even segment-wise sperm abnormalities (Table 3).

The findings regarding the performance of TFYG extender and Andromed extender were supported by Beran *et al.* (2012), who found better post-thaw motility, viability and post-thaw longevity in semen extended with egg yolk based extender than Andromed. Our findings are also in accordance with Kumar *et al.* (2015) that egg yolk based extender was more effective at preserving total and progressive sperm motility and viability than Andromed. The sperm cryopreserved in egg yolk free soybean based extenders (Andromed, Biociphos-Plus etc) showed higher straightness and linearity when compared to the Tris-egg yolk (TFYG) extender, but a decrease on post-thaw sperm survival was observed in Andromed cryopreserved samples when compared to TFYG extender in some studies (Gil *et al.*, 2000; Muino *et al.*, 2007). These varying findings could have been due to differences in extender density, viscosity or even the presence of large particles in different extenders, as previously suggested by Celeghini *et al.* (2008). The results of the study, however, were contrary to those reported by

Rastegarnia *et al.* (2013), who found higher post-thaw motility and viability of buffalo semen in Andromed than in standard TFYG extender.

In the current study, there were non-significant differences in values of acrosomal integrity of spermatozoa among three extenders at any of the stages of cryopreservation of cattle and buffalo semen, however significantly ( $P < 0.05$ ) higher plasma membrane integrity was observed at initial stage in buffalo semen extended in Optixcell than Andromed, and the TFYG was the intermediate of the two commercial extenders. Andromed showed comparatively poor results as compared to Optixcell and TFYG extenders. Hinsch *et al.* (1997) and Meena *et al.* (2010) did not find significant differences between the extenders in terms of acrosome status of spermatozoa, which is in agreement with our observations. Crespilho *et al.* (2012) and Chaudhari *et al.* (2015) also found significantly higher post-thaw plasma membrane integrity of sperms frozen in TFYG than Andromed and Bioxcell extenders, respectively. The results of the study, however, are contrary to those reported by Singh *et al.* (2013) and Sharma and Atreja

**Table 3:** Mean ( $\pm$  SE) percentage of segment wise sperm abnormalities in Gir cattle and Surti buffalo bulls' semen at different stages of cryopreservation in three extenders.

Freezing stage	Extender	Gir cattle				Surti buffalo			
		Head	Mid-piece	Tail	Total	Head	Mid-piece	Tail	Total
Initial	TFYG	1.29 $\pm$ 0.27	0.79 $\pm$ 0.23	3.17 $\pm$ 0.23	5.25 $\pm$ 0.36	1.25 $\pm$ 0.27	0.88 $\pm$ 0.30	2.54 $\pm$ 0.18	4.67 $\pm$ 0.37
	Optixcell	1.17 $\pm$ 0.29	0.71 $\pm$ 0.26	3.13 $\pm$ 0.26	5.01 $\pm$ 0.41	1.33 $\pm$ 0.24	0.92 $\pm$ 0.26	2.71 $\pm$ 0.24	4.96 $\pm$ 0.39
	Andromed	1.29 $\pm$ 0.27	0.67 $\pm$ 0.29	3.38 $\pm$ 0.35	5.34 $\pm$ 0.39	1.38 $\pm$ 0.29	0.58 $\pm$ 0.25	2.71 $\pm$ 0.21	4.67 $\pm$ 0.38
Pre-freeze	Average	<b>1.25<math>\pm</math>0.16</b>	<b>0.72<math>\pm</math>0.15</b>	<b>3.23<math>\pm</math>0.16</b>	<b>5.20<math>\pm</math>0.22*</b>	<b>1.32<math>\pm</math>0.15</b>	<b>0.79<math>\pm</math>0.15</b>	<b>2.65<math>\pm</math>0.12</b>	<b>4.76<math>\pm</math>0.22*</b>
	TFYG	1.76 $\pm$ 0.30	1.25 $\pm$ 0.28	4.25 $\pm$ 0.31	7.26 $\pm$ 0.42	1.47 $\pm$ 0.26	1.03 $\pm$ 0.26	3.50 $\pm$ 0.22	6.00 $\pm$ 0.39
	Optixcell	1.59 $\pm$ 0.27	1.04 $\pm$ 0.31	3.88 $\pm$ 0.25	6.51 $\pm$ 0.33	1.34 $\pm$ 0.23	1.07 $\pm$ 0.26	3.75 $\pm$ 0.23	6.16 $\pm$ 0.32
Post-thaw	Andromed	1.68 $\pm$ 0.32	1.04 $\pm$ 0.32	4.00 $\pm$ 0.26	6.72 $\pm$ 0.39	1.47 $\pm$ 0.26	0.73 $\pm$ 0.26	3.38 $\pm$ 0.23	5.58 $\pm$ 0.34
	Average	<b>1.68<math>\pm</math>0.17</b>	<b>1.11<math>\pm</math>0.17</b>	<b>4.04<math>\pm</math>0.16</b>	<b>6.83<math>\pm</math>0.20*</b>	<b>1.43<math>\pm</math>0.14</b>	<b>0.94<math>\pm</math>0.15</b>	<b>3.54<math>\pm</math>0.13</b>	<b>5.91<math>\pm</math>0.20*</b>
	TFYG	2.77 $\pm$ 0.22	2.03 $\pm$ 0.35	6.26 $\pm$ 0.22	11.06 $\pm$ 0.34	2.60 $\pm$ 0.20	1.73 $\pm$ 0.24	5.80 $\pm$ 0.21	10.13 $\pm$ 0.33
Post-thaw	Optixcell	2.94 $\pm$ 0.25	1.82 $\pm$ 0.33	6.31 $\pm$ 0.22	11.07 $\pm$ 0.34	2.56 $\pm$ 0.23	1.77 $\pm$ 0.27	6.05 $\pm$ 0.26	10.38 $\pm$ 0.29
	Andromed	2.94 $\pm$ 0.25	1.82 $\pm$ 0.32	6.47 $\pm$ 0.27	11.23 $\pm$ 0.37	2.72 $\pm$ 0.27	1.43 $\pm$ 0.19	5.68 $\pm$ 0.21	9.83 $\pm$ 0.32
	Average	<b>2.88<math>\pm</math>0.14</b>	<b>1.89<math>\pm</math>0.19</b>	<b>6.35<math>\pm</math>0.14</b>	<b>11.12<math>\pm</math>0.21*</b>	<b>2.63<math>\pm</math>0.13</b>	<b>1.64<math>\pm</math>0.13</b>	<b>5.84<math>\pm</math>0.13</b>	<b>10.11<math>\pm</math>0.18*</b>

Means bearing different superscripts between freezing stages (x,y,z) differ significantly ( $P < 0.05$ ), but not between dilutors.

(2014), wherein they reported higher plasma membrane integrity and acrosomal integrity of sperms at post-thaw stage in egg yolk free soya bean based extender than in standard TFYG extender.

The benefits of lecithin based bovine semen extenders over milk and/or egg yolk regarding hygienic issues are undeniable. According to Bousseau *et al.* (1998), the use of lecithin may stop the contamination with bacteria and mycoplasma. The efficacy of lecithin based extenders is still a matter of debate. Many previous studies have reported either comparable or higher sperm motility and plasma membrane and acrosomal integrity (Thun *et al.*, 2002; Amirat *et al.*, 2005; Rastegarnia *et al.*, 2013; Singh *et al.*, 2013; Chaudhari *et al.*, 2015; Kumar *et al.*, 2015; Ansari *et al.*, 2016; Layek *et al.*, 2016) with similar or even better fertility rates (Bousseau *et al.*, 1998; Gil *et al.*, 2000; Akhter *et al.*, 2010; Akhter *et al.* 2012; Beura *et al.*, 2014) for bovine semen cryopreserved using lecithin or plant derived soya bean based commercial extenders, while some studies showed better efficiency of egg yolk based extenders over soya lecithin based extenders (Celeghini *et al.*, 2008; Veerabramhaiah *et al.*, 2011; Crespilho *et al.*, 2012).

While relating TFYG and soya lecithin based Andromed extenders, Aires *et al.* (2003) favoured soya lecithin extender in terms of good quality parameters and higher conception rate. Similarly Meena *et al.* (2010) supported soybean based Biociphos extender than TFYG because of improved visualization and low bacterial load. However Thun *et al.* (2002) documented contradictory findings and showed better protective capacity of egg yolk based TFYG extender than soybean based Biociphos-plus with higher *in vivo* fertility results as well. Veerabramhaiah *et al.* (2011) and Crespilho *et al.* (2012) demonstrated better cryoprotective ability of TFYG extender than Biociphos-plus and Botu-Bov-Soy lecithin extenders. The results of present study and of many others thus indicate that until soybean based or other animal protein free extender is universally proved better and economically viable, we need to continue using TFYG extender for cryopreservation of cattle and buffalo semen.

**Post-Thaw longevity of spermatozoa:** The pooled mean percentages of motile spermatozoa observed on post-thaw incubation of semen at 37°C, irrespective of extenders, at 0, 30, 60, 120 and 180 min were 40.31 $\pm$ 0.85, 34.51 $\pm$ 0.82, 28.40 $\pm$ 0.91, 17.78 $\pm$ 0.86 and 9.44 $\pm$ 0.72 for Gir bull semen, and 40.45 $\pm$ 1.04, 34.87 $\pm$ 1.02, 28.01 $\pm$ 0.99, 18.40 $\pm$ 1.01 and 10.51 $\pm$ 0.93 for Surti buffalo semen, respectively. The incubation survival of sperms was significantly ( $P < 0.05$ ) better in Optixcell, followed by TFYG and Andromed extender at all the stages of evaluation particularly at 60, 120 and 180 min of post-thaw incubation in both the species (Table 4). The results display that all three extenders could sustained better and tolerable levels of sperm survival at least

**Table 4:** Mean ( $\pm$  SE) post-thaw longevity (%) of Gir cattle and Surti buffalo bulls' spermatozoa at different post-thaw incubation interval at 37°C in three extenders.

Incubation time	Extender	Post-thaw sperm longevity (%)	
		Gir bulls	Surti bulls
0 min	TFYG	40.37 $\pm$ 1.36	40.19 $\pm$ 1.77
	Optixcell	42.41 $\pm$ 1.47	42.69 $\pm$ 1.76
	Andromed	38.15 $\pm$ 1.54	38.46 $\pm$ 1.86
	Average	<b>40.31<math>\pm</math>0.85<sup>p</sup></b>	<b>40.45<math>\pm</math>1.04<sup>p</sup></b>
30 min	TFYG	34.82 $\pm$ 1.37 <sup>ab</sup>	34.42 $\pm$ 1.70
	Optixcell	36.67 $\pm$ 1.41 <sup>b</sup>	37.50 $\pm$ 1.74
	Andromed	32.04 $\pm$ 1.39 <sup>a</sup>	32.69 $\pm$ 1.80
	Average	<b>34.51<math>\pm</math>0.82<sup>q</sup></b>	<b>34.87<math>\pm</math>1.02<sup>q</sup></b>
60 min	TFYG	29.07 $\pm$ 1.56 <sup>ab</sup>	27.50 $\pm$ 1.60 <sup>ab</sup>
	Optixcell	31.11 $\pm$ 1.52 <sup>b</sup>	31.15 $\pm$ 1.67 <sup>b</sup>
	Andromed	25.00 $\pm$ 1.46 <sup>a</sup>	25.39 $\pm$ 1.73 <sup>a</sup>
	Average	<b>28.40<math>\pm</math>0.91<sup>r</sup></b>	<b>28.01<math>\pm</math>0.99<sup>r</sup></b>
120 min	TFYG	18.15 $\pm$ 1.26 <sup>b</sup>	16.92 $\pm$ 1.50 <sup>a</sup>
	Optixcell	21.67 $\pm$ 1.13 <sup>b</sup>	22.89 $\pm$ 1.69 <sup>b</sup>
	Andromed	13.52 $\pm$ 1.64 <sup>a</sup>	15.39 $\pm$ 1.73 <sup>a</sup>
	Average	<b>17.78<math>\pm</math>0.86<sup>s</sup></b>	<b>18.40<math>\pm</math>1.01<sup>s</sup></b>
180 min	TFYG	8.70 $\pm$ 0.91 <sup>a</sup>	9.04 $\pm$ 1.18 <sup>a</sup>
	Optixcell	13.52 $\pm$ 1.09 <sup>b</sup>	15.19 $\pm$ 1.89 <sup>b</sup>
	Andromed	6.11 $\pm$ 1.32 <sup>a</sup>	7.31 $\pm$ 1.31 <sup>a</sup>
	Average	<b>9.44<math>\pm</math>0.72<sup>t</sup></b>	<b>10.51<math>\pm</math>0.93<sup>t</sup></b>

Means bearing different superscripts between extenders (a,b,c) at each stage and between stages/time (p,q,r,s,t) differ significantly (P<0.05).

for 1 hr after thawing, and only Optixcell up to 2 hrs, therefore semen frozen in such extenders should be used effectively in AI within specified time after thawing with better expected conception rates.

These results on post-thaw longevity of cryopreserved bovine spermatozoa and the effect of extenders to some extent accorded well with many previous reports in cattle (Rana *et al.*, 2003; Beran *et al.*, 2012) and buffalo (Dhami *et al.*, 1994; Chaudhari *et al.*, 2015) semen. Muralinath *et al.* (1990) and Taraphder *et al.* (2001) observed good post-thaw incubation (37°C) sperm survival in buffalo semen in Tris diluent till 3-4 hrs, while Muino *et al.* (2007) observed sperm survival up to 9 hrs and concluded that use of Biladyl results in higher sperm survival and longevity than the use of Andromed or Biociphos-Plus, which is in agreement with current findings in terms of trends between dilutors. Beran *et al.* (2012) in HF bull observed post-thaw motility up to 2-hr and found better result in ionized egg yolk based than soybean based Andromed and Bioxcell. Chaudhari *et al.* (2015) in Surti bulls found acceptable post-thaw longevity up to 1-hr of incubation (37°C) and revealed that soybean based Optixcell was superior over TFYG and soybean based Bioxcell. However, the current findings are on the contrary to those reported by Asr *et al.* (2011) and Rastegarnia *et al.* (2013) in buffalo bulls stating higher post-

thaw motility and viability of semen till 6 and 4 hrs of incubation in Bioxcell and Andromed than in standard TFYG extender, respectively. These variations in post-thaw longevity of bull spermatozoa in different extenders and different studies may be attributed to breeds, age and nutritional status of bulls, initial and post-thaw quality of semen, season, climate, extender-additives used, freezing-thawing protocols and even post-thaw incubation conditions employed in their studies.

In general, except motility and post-thaw longevity, no significant differences were observed between different extenders in preserving sperm viability, sperm morphology, and acrosomal and plasma membrane integrity of sperm. We therefore inferred that egg yolk free commercial Optixcell extender (with better transparency) and egg yolk based TFYG extender (cost-wise cheaper) were at par in terms of most sperm quality parameters evaluated, hence any one of them can be preferred over Andromed by commercial semen production stations for successful routine cryopreservation of cattle and buffalo semen.

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#### REFERENCES

- Aires, V.A., Hinsch, K.D., Schloesser, F.M., Bogner, K., Schloesser, S.M. and Hinsch, E. (2003). *In vitro* and *in vivo* comparison of egg yolk-based and soybean lecithin based extenders for cryopreservation of bovine semen. *Theriogenology*, **60**: 269-279.

- Akhter, S., Ansari, M.S., Andrabi, S.M.H., Rakha, B.A., Ullah, N. and Khalid, M. (2012). Soya-lecithin in extender improves the freezability and fertility of buffalo (*bubalus bubalis*) bull spermatozoa. *Reprod. Dom. Anim.*, **47**(5), 815-819
- Akhter, S., Ansari, M.S., Rakha, B.A., Andrabi, S.M.H., Iqbal, S. and Ullah, N. (2010). Cryopreservation of buffalo (*Bubalus bubalis*) semen in Bioxcell® extender. *Theriogenology*, **74**: 951-955.
- Amirat, L., Anton, M., Tainturier, D., Chatagnon, G., Battut, I. and Courtens, J. (2005). Modifications of bull spermatozoa induced by three extenders: Biociphos, low density lipoprotein and Triladyl, before, during and after freezing and thawing. *Reproduction*, **129**: 535-543.
- Ansari, M.S., Rakha, B.A., Akhter, S. and Ashiq, M. (2016). Optixcell improves the post-thaw quality and fertility of buffalo bull sperm. *Theriogenology*, **85**(3): 528-532.
- Asr, S.J., Beheshti, R. and Kohram, H. (2011). The evaluations of Tris-citrate-egg yolk or Bioxcell extenders on the post-thawed buffalo sperm parameters. *Annals Biol. Res.*, **2**(4): 360-365.
- Beran, J., Stadnik, L., Bezdicek, J., Louda, F., Citek, J. and Duchacek, J. (2012). Effect of sire and extender on sperm motility and share of live or dead sperm in bulls' fresh ejaculate and in AI doses after thawing. *Archiv Tierzucht*, **55**(3): 207-218.
- Beura, D., Mishra, P.C., Das, P., Mohanty, D.N., Barik, A.K. and Biswal, S.S. (2014). Effect of Bioxcell and Tris citric egg yolk extender on post thaw semen quality and *in vivo* fertility in cross bred Jersey bull. *Indian J. Anim. Reprod.*, **35**(1): 29-32.
- Bousseau, S., Billard, J.P., Marquan-Le, Guienne, B., Gurien, B., Camus, A. and Lechat, M. (1998). Comparison of bacteriological qualities of various egg yolk sources and the *in vitro* and *in vivo* fertilizing potential of bovine semen frozen in egg yolk free or lecithin based diluents. *Theriogenology*, **50**: 699-706.
- Celeghini, E.C.C., Arruda, R.P., Andrade, A.F.C., Nascimento, J., Raphael, C.F. and Rodrigues, P.H.M. (2008). Effects that bovine sperm cryopreservation using two different extenders has on sperm membranes and chromatin. *Anim. Reprod. Sci.*, **104**: 119-131.
- Chaudhari, D.V., Dhami, A.J., Hadiya, K.K. and Patel, J.A. (2015). Relative efficacy of egg yolk and soya milk-based extenders for cryopreservation of buffalo semen. *Vet. World*, **8**(2): 239-244.
- Chaudhari, D.V., Dhami, A.J., Patel, J.A. and Hadiya, K.K. (2017). Evaluation of comparative efficacy of egg yolk and soya based extenders for refrigeration preservation (5°C) of buffalo semen. *Indian J. Anim. Res.*, **51**(1): 21-24. DOI:10.18805/ijar.v0i0f.3808.
- Crespilho, A.M., Filho, M.F., Aqua, J.A., Nichi, M., Monteiro, G.C., Avanzi, B.R., Martins, A. and Papa, F.O. 2012. Comparison of *in vitro* and *in vivo* fertilizing potential of bovine semen frozen in egg yolk or new lecithin based extenders. *Livestock Sci.*, **149**: 1-6.
- Dhami, A.J., Jani, V.R., Mohan, G. and Sahni, K.L. (1994). Effect of extenders and additives on freezability, post-thaw thermo resistance and fertility of frozen Murrah buffalo semen under tropical climate. *Buffalo J.*, **10**(1): 34-45.
- Gil, J., Januskauskas, A., Haard, M., Johanisson, A., Soderquist, L. and Rodriguez-Martinez, H. (2000). Functional sperm parameters and fertility of bull semen extended in Biociphos-Plus and Triladyl. *Reprod. Dom. Anim.*, **35**: 69-77.
- Hinsch, E., Hinsch, K., Boehm, J., Schill, W. and Mueller-Schloesser, F. (1997). Functional parameters and fertilization success of bovine semen cryopreserved in egg yolk containing extenders. *Reprod. Dom. Anim.*, **32**: 143-149.
- Jayendran, R.S., Van der Ven, H.H., Perez-Pelaez, M., Crabo, B.G. and Zaneveld, L.J.D. (1984). Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J. Reprod. Fertil.*, **70**: 219-228.
- Jimenez, F., Puchades, S., Moce, E., Cartro, M.P., Vicente, J.S. and Rodriguez, M. (2004). Use of powdered egg yolk vs. fresh egg yolk for the cryopreservation of ovine semen. *Reprod. Dom. Anim.*, **39**: 438-441.
- Kumar, P., Saini, M., Kumar, D., Balhara, A.K., Yadav, S.P., Singh, P. and Yadav, P.S. (2015). Liposome-based semen extender is suitable alternative to egg yolk-based extender for cryopreservation of buffalo (*Bubalus bubalis*) semen. *Anim. Reprod. Sci.*, **159**: 38-45.
- Layek, S.S., Mohanty, T.K., Kumaresan, A. and Parks, J.E. (2016). Cryopreservation of bull semen: evolution from egg yolk based to soybean based extenders. *Anim. Reprod. Sci.*, <http://dx.doi.org/10.1016/j.anireprosci.2016.04.013>
- Leeuw, A.M., Haring, R.M., Lansbergen, L.M. and Daas, J.H. (2000). Fertility results using bovine semen cryopreserved with extenders based on egg-yolk and soybean extract. *Theriogenology*, **54**: 57-67.
- Leite, T.G., Filhoa, V.R., Arrudab, R.P., Andradeb, A.F., Emericka, L.L., Zaffalonb, F.G., Martinsa, J.A. and Andradec, V.J. (2010). Effects of extender and equilibration time on post-thaw motility and membrane integrity of cryopreserved Gyr bull semen evaluated by CASA and flow cytometry. *Anim. Reprod. Sci.*, **120**: 31-38.
- Meena, G.S., Raina, V.S., Gupta, A.K., Mohanty, T.K. and Bishist, R. (2010). Comparative performance of Biociphos and Tris-egg yolk based extenders for buffalo semen preservation. *Indian J. Anim. Sci.*, **80**: 414-417.
- Muino, R., Fernandez, M. and Pena, A.I. (2007). Post-thaw survival and longevity of bull spermatozoa frozen with an egg yolk-based or two egg yolk-free extenders after an equilibration period of 18 hours. *Reprod. Dom. Anim.*, **42**: 305-311.
- Muralinath, E., Murthy, A.S.N., Rao, A.V.N., Harnath, G.B., Reddy, B.B. and Rao, V.H. (1990). Effect of supplementation of prostaglandins on post-thaw motility and *in vitro* motility in cervical mucus of frozen buffalo spermatozoa. *Indian J. Anim. Reprod.*, **11**(1): 10-13.
- Rana, C.M., Dhami, A.J., Zalu, G.K., Vibhakar K.V. and Landani, N.G. (2003). Preservation of Gir (*Bos indicus*) and Jafarabadi (*Bubalus bubalis*) semen at 5°C and ultralow temperature using Sephadex filtration technique. *Indian J. Anim. Sci.*, **73**: 858-863.
- Rastegarnia, A., Shahverdi, A., Rezaei, T. and Shafiepour, V. (2013). *In vitro* comparison of soybean lecithin-based extenders for cryopreservation of buffalo (*Bubalus bubalis*) semen. *Comp. Clin. Path.*, **22**: 1-10.

- Sharma, S. and Atreja, S.K. (2014). A comparative evaluation of spermatozoa quality after cryopreservation of bovine semen in conventional Egg Yolk Tris Citrate (EYTC) versus "in house" Soya Milk Tris (SMT) extender. *Compendium, National Seminar on Biotechnological Approaches to Challenges in Animal Health and Production*, DUVASU, Mathura, India, 6-7 March, pp. 88-89.
- Singh, V.K., Singh, A.K., Kumar, R., and Atreja, S.K. (2013). Development of soya milk extender for semen cryopreservation of Karan Fries (crossbreed cattle). *CryoLetters*, **34**(1): 52-61.
- Snedecor, G.W. and Cochran, W.G. (1994). *Statistical Methods*. 8<sup>th</sup> edn. Iowa State University Press, Ames, Iowa, USA.
- Taraphder, S., Gupta, A.K., Raina, V.S. and Tomar, S.S. (2001). Effect of incubation (37°C) on post-thaw motility of Murrah buffalo bull spermatozoa. *Indian J. Dairy Sci.*, **54**(2): 80-83.
- Thun, R., Hurtado, M. and Janett, F. (2002). Comparison of Biociphos-Plus and Tris egg yolk extender for cryopreservation of bull semen. *Theriogenology*, **57**: 1087-1094.
- Veerabramhaiah, K., Rao, A.S., Rao, V.H., Naidu, K.V. and Rao, S.T.V. (2011). Efficacy of the Tris and Biociphos-Plus extenders on the freezability of Punganur bull semen. *Indian J. Anim. Reprod.*, **32**(2): 1-4.
- Watson, P.F. (1975). Use of Giemsa stain to detect changes in the acrosome of frozen ram spermatozoa. *Vet. Rec.*, **97**: 12-15.