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Study on Keeping Quality and Cryopreservability of Buffalo Semen in Egg Yolk and Soybean Based Extenders

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Abstract

The study was aimed to compare efficacy of egg yolk based diluent (TFYG) with soybean based commercial diluents (Bioxcell® and Optixcell®, IMV, France) for refrigeration and cryopreservation (-196°C) of six Surti buffalo semen. Each qualified ejaculate (n=8/bull, >70% initial motility) was split-diluted @ 100 $\times 10^6$ sperm ml^{-1} at 34°C with 3 diluents. Part of each aliquot was filled in French mini straws and rest transferred to refrigerator for gradual cooling to 4-5°C. After 4 h of equilibration at 4-5°C in cold handling cabinet, the straws were frozen using programmable bio-freezer. The initial mean motility, acrosome integrity and hypo-osmotic reactive sperm per cent of fresh semen were 78.54±0.30, 94.40±0.20 and 79.35±0.42%, respectively. Sperm progressive motility at 24 hrs of refrigeration storage was 68.33±0.45, 66.25±0.46 and 70.31±0.46 %, and at 72 hrs of refrigeration storage 52.50±0.68, 48.54±0.84 and 53.85±0.75 % in TFYG, Bioxcell and Optixcell diluents, respectively. Acrosomal integrity and hypo-osmotic reactivity at 24 hrs of refrigeration storage were 88.15±0.18, 87.25±0.21, 89.27±0.20% and 66.04±0.50, 63.81±0.45, 68.10±0.46%, whereas at 72 hrs of refrigeration, the values were 81.67±0.23, 80.60±0.30, 82.83±0.27% and 51.35±0.60, 47.35±0.68, 53.40±0.68%, respectively, in above 3 diluents. The pre-freeze sperm progressive motility recorded in TFYG, Bioxcell and Optixcell diluents was 69.48±0.37, 68.02±0.49 and 70.94±0.38%, and post-thaw motility was 47.71±0.79, 44.38±0.85 and 49.90±0.90%, respectively. Per cent sperm acrosomal integrity and hypo-osmotic reactivity in TFYG, Bioxcell and Optixcell diluents were 89.54±0.18, 88.58±0.22, 90.52±0.21% and 67.96±0.32, 65.65±0.42, 70.23±0.37% at pre-freeze stage, whereas 76.83±0.23, 75.90 ±0.27, 78.50±0.25% and 45.02±0.84, 42.31±0.82, 47.81±0.90% at post-thaw stage, respectively. The post-thaw longevity after 60 minutes of incubation (37°C) was 35.52±0.79, 31.15±0.85, 37.19±0.81%, respectively, in above 3 diluents. Among the three semen diluent percent sperm progressive motility, acrosomal integrity and hypo-osmotic reactivity at particular stage were higher in Optixcell showing at par results in TFYG, whereas Bioxcell showed significantly ($P<0.05$) lower findings than other two diluents. Optixcell is a commercial product much costlier than TFYG, hence there is a need to develop local soybean based semen extender which can give comparable results with TFYG.

Key Words: Buffalo semen, Cryopreservation, Refrigeration, Quality traits, Soybean extender.

Introduction

Semen as a high valued male product had likely inspired the investigators to search methods for its preservation for a long-term survival to be used on demand (Foote, 2002). Nowadays, Sperm cryopreservation and storage are of a great challenge for conserving the superior genetic origins of the males, the expansion of assisted reproductive technologies such as AI and *in vitro* fertilization and clinical medicine. The consequences of sperm cryoinjury caused by the cryopreservation procedure are impaired transport and poor survival in the female reproductive tract (Salamon and Maxwell, 1995). Hence, a variety of extenders and additives have been used to protect spermatozoa from deleterious effects of preservation and for improving freezability/keeping quality and fertility of bull semen. Egg yolk has been used as a basic component of extenders for bovine ejaculate since 1939 (Amirat *et al.,* 2004) and has still remained popular. Nevertheless, the use of egg yolk as a cryoprotectant has recently been restricted in some countries for reasons of immunologic and hygienic risks (Thun *et al.,* 2002). Moreover, Amirat *et al.* (2005) have demonstrated that extenders based on egg yolk can have negative effects on sperm respiration and motility due to other specific substances they contain. An alternative to replace the components of animal origin in semen extender is the soy lecithin. Therefore, this investigation was envisaged to evaluate the comparative efficacy of standard egg yolk based and commercially available soybean based extenders for refrigeration and cryopreservation of buffalo semen.

Materials and Method

The study was conducted during favorable breeding season on semen of six sexually mature 4-6 years old Surti buffalo bulls. The bulls were maintained under uniform standard nutritional and managerial practices at the Central Sperm Station of the College in Anand. The bulls were under regular twice a week semen collection schedule using artificial vagina. The ejaculates (8/bull, total 48) soon after collection were transferred in to a water bath at 34°C and evaluated for gross quality, motility, and sperm concentration. Only the ejaculates with >70% initial motility were used for further processing and freezing.

Preparation of extenders

The standard Tris-citrate-fructose-egg yolk-glycerol (TFYG) extender containing 73 ml of Tris buffer (Tris 3.048 g, citric acid 1.67 g, fructose 1.25 g in 100 ml Milli-Q water) + 20 ml fresh egg yolk + 7 ml glycerol was prepared fresh daily and antibiotics benzyl penicillin 1000 IU/ml and streptomycin sulfate 1000 μg/ml were added as recommended. The commercial Bioxcell® and Optixcell® were prepared fresh for use by diluting 2 and 4 times, respectively, with Milli-Q water according to manufacturer's instructions (IMV, France). The commercial extenders contained carbohydrates, mineral salts, buffer, antioxidant, phospholipids, ultra-pure water, glycerol and antibiotics (gentamicin, tylosin, lincomycin, and spectinomycin) conforming to European Regulations CEE 88/407. They do not contain protein of animal origin but have plant proteins.

Semen processing

Qualifying ejaculates from each bull were split into three equal aliquots and diluted at 34°C with each of three extenders @ 100×10^6 sperm/ml and were evaluated for progressive sperm motility. From each aliquot, part of the diluted semen was preserved at refrigeration temperature (4-5°C). Rest of the semen were filled in French mini straws using automatic filling, sealing and printing machine (IS4 System, IMV Technologies) and cooled to 4-5°C within 60-90 min and further equilibrated at the same temperature for 4 h in cold handling cabinet. Freezing of straws was carried out in liquid nitrogen (LN2) vapor using a programmable bio-freezer (Digitcool 5300 CE ZH 350, IMV, France). The straws were then plunged in LN2 ("196°C) for overnight storage. Semen straws were thawed next day in a water bath at 37°C for 30 second.

Assessment of sperm quality

Semen samples were evaluated at initial, 24 and 72 hrs of refrigeration as well as pre-freeze and

post-thaw stages. The sperm progressive motility was determined at 37°C temperature under high power magnification (×40) of phase contrast microscope fitted with a biotherm stage and a closed circuit television. The percentages of spermatozoa with intact acrosome were assessed using Giemsa stain. The plasma membrane integrity of spermatozoa was assessed using a HOS test employing 150 mOs/L solutions of sodium citrate and fructose with 30 min of incubation at 37°C (Rasul *et al.,* 2000). The wet preparations of semen were then evaluated using a phase contrast microscope (×40). Nearly 200 spermatozoa were assessed from different fields for coiling of tail, i.e. plasma membrane integrity. The post-thaw longevity was assessed after 30 and 60 minutes of incubation at 37°C. The data were analyzed statistically using IBM SPSS Statistics version 20.00.

Results and Discussion

The comparative performance of standard Tris-citrate-fructose-egg yolk-glycerol (TFYG) extender and commercially available soybean based extenders (Bioxcell and Optixcell, IMV, France) at different stages of refrigeration and cryopreservation of buffalo semen in terms of percentage motility, acrosome integrity and HOS reactivity are presented in Tables 1-3. The statistical analysis of data revealed that each extender showed significant differences ($p<0.05$) in sperm quality parameters at each stage for both preservation practice.

Table 1: Sperm parameters (Mean ± SE) at different stages of refrigeration preservation (5°C) in different extenders

Storage stage	Extender	Progressive sperm motility (%)	Acrosomal integrity (%)	HOST reactivity (%)
Initial	--	78.54±0.30	94.40±0.20	79.35±0.42
24 hrs	TFYG	68.33 ± 0.45^{q}	88.15 ± 0.18^{q}	66.04 ± 0.50^{q}
	Bioxcell	66.25 ± 0.46^{r}	87.25 ± 0.21^{r}	63.81 ± 0.45^{r}
	Optixcell	70.31 ± 0.46^{p}	89.27 ± 0.20^{p}	68.10 ± 0.46^{p}
72 hrs	TFYG	52.50 ± 0.68^{x}	81.67 ± 0.23^{y}	51.35 ± 0.60^{y}
	Bioxcell	48.54 ± 0.84^{y}	80.60 ± 0.30^{z}	47.35 ± 0.68^{z}
	Optixcell	53.85 ± 0.75^{x}	82.83 ± 0.27^{x}	53.40 ± 0.68^{x}

Within column different superscripts between extenders at 24 hrs (p,q,r) and 72 hrs (x,y,z) differ significantly ($p<0.05$)

Table 2: Sperm parameters (Mean ± SE) at different stages of cryopreservation (-196°C) in different extenders

Freezing stage	Extender	Progressive sperm motility (%)	Acrosomal integrity (%)	HOST reactivity (%)
Initial	--	78.54±0.30	94.40±0.20	79.35±0.42
Pre-freeze	TFYG	69.48 ± 0.37^{q}	89.54 ± 0.18^{q}	67.96 ± 0.32^{q}
	Bioxcell	68.02 ± 0.49^{r}	88.58 ± 0.22^{r}	65.65 ± 0.42^{r}
	Optixcell	70.94 ± 0.38^{p}	90.52 ± 0.21^{p}	70.23 ± 0.37^{p}
Post-thaw	TFYG	47.71 ± 0.79^{x}	76.83 ± 0.23^{y}	45.02 ± 0.84^{y}
	Bioxcell	44.38 ± 0.85^{y}	75.90 ± 0.27^{z}	42.31 ± 0.82^{z}
	Optixcell	49.90 ± 0.90^{x}	78.50 ± 0.25^{x}	47.81 ± 0.90^{x}

Within column different superscripts between extenders at pre-freeze (p,q,r) and post-thaw (x,y,z) stage differ significantly ($p<0.05$)

The mean sperm progressive motility observed at 24 and 72 hrs of refrigeration in Optixcell extender were significantly higher than in Bioxcell extender, and the values in TFYG extender were

Table 3: Post-thaw longevity of sperm (Mean ± SE) at 30 and 60 minutes of incubation in different extenders

Extender	Progressive sperm motility (%)		
	0 min post-thaw	30 min post-thaw	60 min post-thaw
TFYG	47.71 ± 0.79^{x}	41.98 ± 0.80^{x}	35.52 ± 0.79^{x}
Bioxcell	44.38 ± 0.85^{y}	38.44 ± 0.82^{y}	31.15 ± 0.85^{y}
Optixcell	49.90 ± 0.90^{x}	44.58 ± 0.93^{x}	37.19 ± 0.81^{x}

Within column different superscripts between extender (x,y,z) differ significantly ($p<0.05$).

intermediate and at par with Optixcell. Similar trend was obtained during cryopreservation of semen at both pre-freeze and post-thaw stages. The results were in agreement with Meena *et al.* (2010). However, Akther *et al.* (2011) recorded non-significant difference in motility for semen extended with Tris-egg yolk and Bioxcell extenders. Singh *et al.* (2012) and Rehman *et al.* (2014) observed significantly higher motile sperms for refrigeration storage in conventional egg yolk-Tris extender and extender with 25 % soy milk than extenders with lower or higher percentages of soy milk. In our study, higher visualization and velocity of sperms were observed in both soybean based semen extender (Bioxcell and Optixcell) in comparison to egg yolk-based extender. The components in egg yolk like lecithin, phospholipids and lipoprotein fractions may form globules that interfere in microscopic observation and impair sperm movement (Moussa *et al.,* 2002). This might be the reason for hindrance in motility and microscopic evaluation.

The acrosome integrity and HOS reactive sperm were maintained at significantly higher level in Optixcell and TFYG extender as compared with Bioxcell extenders at different stages of both preservation practices, and cuncurred with Bousseau *et al.* (1998) and Meena *et al.* (2010). Asr *et al.* (2011) found higher acrosomal and plasma membrane integrity of sperm immediately after thawing as well as after 6 hrs of incubation in Bioxcell than in standard TFYG extender. While comparing 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) also supported soybean based Biociphos extender better than TFYG, because of better visualization and low bacterial load, while Thun *et al.* (2002) and Veerabramhaiah *et al.* (2011) found better protective capacity of egg yolk based TFYG extender and higher *in vivo* fertility results as well than Biociphos-plus extender.

Based on reports of various authors it seems that different extenders have different protective capacity. In our findings newly launched ready to use commercial soybean based extender 'Optixcell' proved better than TFYG and Bioxcell in terms of visualization and quality parameters to some extent as well. On other side, though Bioxcell extender is soya protein based extender, it has less protective capacity. According to Singh *et al.* (2012) and Rehman *et al.* (2014) optimal soya lecithin concentration in the extender is prerequisite for protection of spermatozoa during temperature variations. Concentration of soybean below or above the optimal may be harmful and this might be the situation with Bioxcell extender. Optixcell is newly launched product and proved better than Bioxcell in our experiment. Ultimately, the composition of semen extenders strongly influences sperm survival (Chaveiro *et al.,* 2006). Actually the exact composition of any commercial extender is not disclosed by the manufacturer; otherwise it would be easier to draw valid conclusion based on the findings.

In the present study, Optixcell gave relatively better performance than the TFYG. But the Optixcell is a commercial product much costlier than TFYG. So there is a need to develop local soybean based semen extender which can give comparable results with TFYG for long term preservation of bovine semen.

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Conflict of Interest: All authors declare no conflict of interest.

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