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Effect of certain extenders on semen quality of boars during preservation at 17°C

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ABSTRACT

The present study was designed to determine the effect of three selected extenders on liquid preservation of boar semen at 17°C. Ejaculates (30) were collected from three boars (one ejaculate/boar/week) housed at University pig farm, Ludhiana using dummy sow. Only ejaculates having ≥70% initial progressive motility were extended in Beltsville Thawing Solution (BTS), Safe Cell (SFC) and Tris-Egg Yolk (TEY) extenders in the ratio 1:4 and preserved in a BOD incubator at 17°C for 120 h. The semen was evaluated for different sperm attributes and lipid peroxidation (MDA) at 0, 24, 48, 72, 96 and 120 h of preservation. The mean percentage of sperm motility, viability, plasma membrane integrity and acrosome integrity were significantly higher in BTS as compared to SFC and TEY at different hours of storage period. At 120 h of preservation, highest percentage of *in vitro* capacitation/acrosome reaction was seen in semen stored in BTS followed by SFC and lowest in TEY after 6 h of incubation. Eventually, significantly lower levels of MDA were noticed in semen extended in BTS than in their contemporary extenders (SFC and TEY) at 96 and 120 h of storage period. The mean percentage of most sperm parameters decreased gradually from day of collection (0 h) up till 120 h in all the extenders. In conclusion, Beltsville Thawing Solution was better than Safe Cell and Tris-Egg Yolk extenders in liquid preservation of boar semen at 17°C owing to improved sperm characteristics and reduced oxidative stress.

Key words: Boar, Extender, Liquid preservation, Semen, Sperm characteristics

Artificial insemination in most gilts/sows (>99%) is performed using extended liquid semen whether used on same day and/or within 24 h of fresh collection (Bielas *et al*. 2017). Until recent past, there has been no major development in the use of frozen boar semen owing to peculiar plasma membrane composition due to its high sensitivity to sudden cooling and freezing (-196°C) immediately after collection followed by subsequent thawing resulting in poor fertility rates (Govindasamy *et al*. 2016). This sensitivity to ultra-low temperature requires storage at moderately low temperatures (16-20°C). The key advantage of using liquid semen over frozen semen is that fertility is maintained even with low sperm count. However, use of different extenders for preservation of liquid semen evaluated on short-term basis exhibited variable success in terms of fertilizing capacity of spermatozoa (Mapeka *et al*. 2012). Validation is the most common observation for use of liquid extenders. Development of more effective and validated liquid extenders has still not been fully explored (Roca *et al*. 2011).

Another major drawback of liquid semen *vis-à-vis* type of extender used is their compromised quality resulting in reduced fertility especially during prolonged storage period (Frydrychová *et al*. 2010). The viability of preserved boar semen depends upon the composition of extender used (Bresciani *et al*. 2013). In addition, the shelf-life of diluted semen also plays an important role since the quality of extended semen frequently decreases as the preservation period increases and ultimately affects the success of AI (Waberski *et al*. 2008). Thus, many weaknesses still persist in liquid preservation of boar semen which needs to be improved prior to its routine use in commercial pig farms on a large scale (Mercadoa *et al*. 2009). In India, development of effective liquid extenders to extend and stabilize the functional life of spermatozoa is still obscure in boars. Therefore, the present study was conducted to determine the effect of certain selected extenders on semen quality of boars at 17°C.

MATERIALS AND METHODS

Semen collection, extension, evaluation, processing and preservation: The study was approved by the Institutional Animal Ethics Committee (IAEC) of Guru Angad Dev Veterinary and Animal Sciences University (GADVASU/2020/IAEC/54/19). The present study was conducted on 30 ejaculates obtained from three trained, apparently healthy adult Large White Yorkshire breeding boars (aged 2-3 years) housed at pig farm, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. The study was conducted from February through May during which the mean daily temperature and relative humidity

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was 23.65±2.7°C and 56.0±6.5% inside shed. Semen from each boar (one ejaculate/boar/week) was collected in a pre-warmed (37°C) sterilized thermos flask using gloved hand technique in morning hours (8:00 AM - 9:00 AM). Only ejaculates (fresh semen) having initial motility ≥70% and concentration ≥100 million/ml were selected for further evaluation and preservation. Each filtered ejaculate was gently homogenized, split into three equal parts and extended with pre-warmed (37°C) extenders viz. Beltsville thawing solution (BTS; Karunakaran *et al*. 2017), Safe Cell (SFC, IMV Technologies, L'Aigle, France; Bielas *et al*. 2017) and Tris-Egg Yolk (TEY; Igboeli 1970) in the ratio 1:4 in three sterile glass beakers. The extended semen was preserved at 17ºC for 120 h in a BOD incubator. The SFC, a commercial product, is ready to use extender and kept at 5°C. Both BTS and Tris buffer were prepared 12 h prior to use and kept in a refrigerator at 5°C. On the day of use, egg yolk (20%) was added to Tris buffer. Immediately before use, all the extenders were placed in hot water bath (37°C) for further extension, evaluation and preservation of semen.

Analysis of sperm parameters: In the extended semen at 0 h (immediately after extension), 24, 48, 72, 96 and 120 h of preservation, the selected functional sperm characteristics were determined.

Sperm motility: The sperm motility was subjectively assessed under 400× magnification using a light microscope in three different fields by the same observer. Motility was expressed as the percentage of progressively motile spermatozoa.

Sperm viability: Exactly 10 µl semen was mixed with 10 µl Eosin-Nigrosin stain. A thin smear was prepared from the semen-stain mixture on a clean glass slide and examined at 100× using light microscopy. The sperm which did not stain were considered live whereas partially stained and/or completely stained sperm were considered dead. Two hundred spermatozoa in different fields were counted and sperm viability was calculated in percentage.

Plasma membrane integrity: To 1.0 ml hypo-osmotic solution (100 mosm/l), 100 µl semen sample was mixed and incubated at 37°C for 1 h. After incubation, 10 µl semen was kept on a glass slide and examined under the high power magnification (400×) using bright-field microscopy. In different fields, about 200 sperm with curled and non-curled tails were counted and the plasma membrane integrity was expressed in percentage.

Acrosome integrity: From the semen (10 µl), a smear was prepared, stained in solution containing 2 ml 0.1 M PBS, 3 ml Giemsa stain stock solution and 35 ml distilled water for 120 min. After staining, the slide was air dried and examined under oil immersion (100×). Two hundred sperm with intact acrosome and damaged acrosome (partially or totally) were counted in various fields and estimated in percentage.

In vitro capacitation/acrosome reaction: Briefly, 250 µl semen was centrifuged at 1000 rpm for 5 min with 2 ml energy rich TALP media and again suspended in 0.5 ml energy rich TALP media. The sperm suspension was incubated at 37°C for 6 h. A thin smear of semen with working solution of Giemsa stain was prepared at 0 h and 6 h and about 200 spermatozoa were counted for *in vitro* capacitation/acrosome reaction under 40× magnification using bright-field microscope. The acrosome displaying shedding, vesiculation and swelling were considered *in vitro* capacitated/acrosome-reacted and expressed in percentage.

Measurement of lipid peroxidation (LPO): The estimation was done through analysis of malondialdehyde (MDA) in the preserved semen at 0, 24, 48, 72, 96 and 120 h. Briefly, extended semen (2 ml) was centrifuged at 3000 rpm for 2 min. Following centrifugation, the pellet was re-suspended in PBS (100 µl, pH 7.4) to which 150 mM Tris HCL (100 µl, pH 7.1) was added. The sperm suspension was incubated at 37°C for 20 min. After incubation, 10% trichloroacetic acid (0.5 ml) and 0.375% thiobarbituric acid (1.0 ml) were added to sperm suspension and kept in boiling water bath at 100°C for 20 min. After cooling, the mixture was centrifuged for 15 min at 5000 rpm. Finally, absorbance in supernatant was read at 532 nm and MDA was calculated as:

$$\text{MDA } (\mu\text{mole}/10^9 \text{ sperm}) = \frac{\text{OD} \times \text{Volume of assay mixture}}{\text{Extinction coefficient} \times \text{Volume of sample}}$$

Statistical analysis: Statistical evaluations were carried out using SPSS (Statistical Package for the Social Sciences, version 26) program. Response variables like motility, live sperm count, plasma membrane integrity, acrosome integrity and *in vitro* capacitation/acrosome reaction were expressed as percentages. The proportionality data were corrected using angular transformation. All variables were analyzed by one way analysis of variance (ANOVA) and a confidence level of $P<0.05$ was considered to be significant. ANOVA was also applied to determine possible effects of extenders on LPO. When the ANOVA test showed statistical differences, the mean values of response variables and LPO were compared using Tukey test. The results were expressed in mean±SE.

RESULTS AND DISCUSSION

The effect of different extenders on sperm characteristics and oxidative stress in boar semen preserved for different hours at 17°C are given in Table 1.

Effect of different extenders on sperm motility: Analysis of variance revealed that the mean sperm motility was significantly higher ($P<0.05$) in BTS than in SFC and TEY at 0, 24, 48, 72, 96 and 120 h of preservation (Table 1). A similar trend ($P<0.05$) of sperm motility was also seen in SFC compared to TEY. Amongst BTS, SFC and TEY, the sperm motility differed significantly ($P<0.05$) at all hours of preservation. These findings are in agreement with the observations of earlier studies (Lange-Consiglio *et al*. 2013, Chutia *et al*. 2014) who demonstrated significantly ($P<0.05$) higher sperm motility in BTS as compared to its

Table 1. Sperm characteristics (Mean±SE) in liquid semen extended in selected extenders

Extender	Preservation period (h)					
	0	24	48	72	96	120
Individual progressive motility (%)						
BTS	82.3±1.4$^{a!}$	71.5±0.9$^{a@}$	58.5±1.6$^{a\#}$	47.2±2.1$^{a\wedge}$	41.2±0.7^{a*}	24.0±0.8^{a+}
SFC	77.1±0.9$^{b!}$	65.8±1.4$^{b@}$	51.0±1.2$^{b\#}$	40.7±0.8$^{b\wedge}$	33.6±1.1^{b*}	18.3±0.6^{b+}
TEY	57.0±0.5$^{c!}$	42.9±1.2$^{c@}$	29.7±0.9$^{c\#}$	17.2±1.7$^{c\wedge}$	10.8±0.8^{c*}	6.3±1.5^{c+}
Viability (%)						
BTS	91.7±0.9$^{a!}$	86.1±1.2$^{a@}$	80.1±1.4$^{a\#}$	71.5±1.9$^{a\wedge}$	63.1±1.3^{a*}	56.5±0.9^{a+}
SFC	83.5±0.8$^{b!}$	74.9±0.8$^{b@}$	69.1±0.8$^{b\#}$	63.1±1.1$^{b\wedge}$	44.5±1.8^{b*}	38.9±0.7^{b+}
TEY	66.7±1.1$^{c!}$	58.8±1.4$^{c@}$	49.9±0.7$^{c\#}$	43.4±1.5$^{c\wedge}$	36.1±2.1^{c*}	30.2±1.2^{c+}
Plasma membrane integrity (%)						
BTS	67.5±1.5$^{a!}$	62.4±0.8$^{a@}$	54.8±1.2$^{a\#}$	53.5±1.4$^{a\wedge}$	37.2±1.2^{a*}	31.1±1.4^{a+}
SFC	60.8±1.3$^{b!}$	46.5±1.6$^{b@}$	42.3±0.7$^{b\#}$	36.8±1.2$^{b\wedge}$	31.5±1.5^{b*}	27.7±0.7^{b+}
TEY	44.7±0.9$^{c!}$	37.4±1.2$^{c@}$	29.5±1.5$^{c\#}$	24.4±0.9$^{c\wedge}$	15.8±1.9^{c*}	10.7±1.6^{c+}
Acrosome integrity (%)						
BTS	87.4±0.6$^{a!}$	77.7±1.4$^{a@}$	71.5±1.7$^{a\#}$	62.8±1.5$^{a\wedge}$	52.9±1.3^{a*}	46.8±0.8^{a+}
SFC	80.7±0.7$^{b!}$	69.6±0.9$^{b@}$	64.5±0.6$^{b\#}$	57.3±1.6$^{b\wedge}$	44.5±1.9^{b*}	41.2±1.8^{a+}
TEY	74.1±1.5$^{c!}$	61.2±1.6$^{c@}$	56.1±1.3$^{c\#}$	41.2±1.8$^{c\wedge}$	36.5±0.7^{c*}	29.8±1.4^{c+}

Values having different superscripts between column (alphabets) and within row (symbols) differ significantly (P<0.05).

contemporary extenders. Eventually, presence of citrate and ethylenediamine tetra acetic acid (EDTA) in BTS over SFC and TEY played a pivotal role in providing energy to sperm cells necessary for sustaining higher percentage of motile spermatozoa until 120 h of preservation (Gadea 2003, Chutia *et al*. 2014). Furthermore, during preservation excessive generation of reactive oxygen species (ROS) by spermatozoa also has been linked to reduced sperm motility owing to plasma membrane damage (Kumaresan *et al*. 2009).

Effect of different extenders on sperm viability: Observations on 30 ejaculates revealed that BTS presented significantly (P<0.05) higher proportion of viable sperm than SFC and TEY (Table 1). Likewise, SFC exhibited significantly (P<0.05) higher percentage of viable sperm as compared to TEY extender. From the day of collection (0 h) until 120 h, sperm viability decreased gradually in all the extenders. Similar findings of Saravia *et al*. (2007) have shown the superiority of BTS over SFC and TEY owing to the presence of potassium chloride in the chemical composition of BTS that plays a key role in maintaining higher proportion of viable spermatozoa until 120 h of preservation. A decline in sperm viability over the storage period could be attributed to LPO reaction which has been incriminated as a main cause of sperm membrane damage (Flores *et al*. 2008).

Effect of different extenders on plasma membrane integrity: The plasma membrane integrity was significantly (P<0.05) higher in semen extended in BTS than in SFC and TEY; as well as in SFC when compared to TEY at all hours of preservation (Table 1). Throughout the storage period (0-120 h), the proportion spermatozoa with intact plasma membrane decreased progressively in all the extenders. Similarly, Lalrintluanga *et al*. (2016) also recorded high sperm plasma membrane integrity in semen extended in BTS than in egg yolk based extender at all hours of preservation. Comparative studies on semen extended in BTS and SFC for plasma membrane integrity are not available in literature. Individual studies using BTS (Sa *et al*. 2013) and SFC (Sangma *et al*. 2020) extenders separately determined that plasma membrane integrity in BTS and SFC could be maintained upto day 4 and day 6 of preservation, respectively. In the current study, all extenders exhibited reduced plasma membrane integrity as the preservation period progressed. Decreased sperm biochemical activity, ROS production and lower cytoplasmic component containing antioxidants have been linked to plasma membrane damage during preservation (Kumaresan *et al*. 2009).

Effect of different extenders on acrosome integrity: In the present study, the acrosome integrity was significantly (P<0.05) higher in BTS than in SFC and TEY throughout the storage period (Table 1). Similarly, the percentage of intact acrosome was significantly (P<0.05) higher in SFC as compared to that in TEY at all hours of preservation. The results of present study are in consonance with the findings of Sa *et al*. (2013) that BTS compared to commercial extenders provided better protective action against acrosomal impairment. Moreover, presence of tri-sodium citrate in BTS helped in contributing higher proportion of intact acrosomes over SFC and LEY (Gadea 2003). During storage, the concentration of cholesterol and phospholipids in seminal plasma and peroxidation effect seemed to be potent factors for higher acrosomal damage (Dimitrov *et al*. 2009, Frydrychová *et al*. 2010). Correspondingly, the sperm acrosome integrity decreased as the storage period increased in all extenders in the present study.

Effect of different extenders on in vitro capacitation/ acrosome reaction: Changes in mean percentages of *in vitro* capacitation patterns exhibited no significant (P>0.05)

Table 2. Percentage of *in vitro* capacitation/acrosome reaction (Mean±SE) in liquid semen extended in different extenders

Extender	Acrosome reaction	Preservation period (h)					
		0	24	48	72	96	120
BTS	0 h	1.7±0.4$^{!}$	2.6±0.5$^{!}$	3.8±0.3$^{@}$	5.1±0.7$^{\#}$	5.7±0.6$^{\#}$	6.5±0.8$^{\#}$
	At 6 h	5.1±1.0$^{!}$	6.3±1.1$^{!}$	9.4±1.4$^{@}$	15.2±1.8$^{\#}$	21.1±2.2$^{\wedge}$	31.9±1.6^{a+}
	Final acrosome reaction (%)	3.4±0.6$^{!}$	3.7±0.6$^{!}$	5.6±1.1$^{@}$	10.1±1.1$^{\#}$	15.4±1.6$^{\wedge}$	25.4±0.8^{a+}
SFC	0 h	1.4±0.6$^{!}$	2.3±0.4$^{!}$	3.9±0.7$^{@}$	5.3±0.5$^{\#}$	5.9±0.8$^{\#}$	6.2±0.4$^{\#}$
	At 6 h	5.6±0.8$^{!}$	6.9±0.9$^{!}$	10.1±1.1$^{@}$	12.7±2.1$^{@}$	18.8±1.9$^{\wedge}$	27.7±1.7^{b+}
	Final acrosome reaction (%)	4.2±0.2$^{!}$	4.6±0.5$^{!}$	6.2±0.4$^{@}$	7.4±1.6$^{\#}$	12.9±1.1$^{\wedge}$	21.5±1.3^{b+}
TEY	0 h	2.1±0.3$^{!}$	3.1±0.6$^{!}$	3.6±0.5$^{!}$	4.8±0.3$^{\#}$	6.3±0.7$^{\#}$	6.7±1.0$^{\#}$
	At 6 h	5.4±1.0$^{!}$	6.7±1.2$^{!}$	9.6±0.9$^{@}$	12.9±1.5$^{\#}$	18.5±2.1$^{\wedge}$	22.8±2.2$^{c\wedge}$
	Final acrosome reaction (%)	3.3±0.7$^{!}$	3.5±0.6$^{!}$	6.0±0.4$^{@}$	8.1±1.2$^{\#}$	12.2±1.4$^{\wedge}$	16.1±0.8^{c+}

Values having different superscripts within row (symbols) differ significantly (P<0.05). a vs b vs c (P<0.05); from corresponding values in a column.

Table 3. Lipid peroxidation (MDA, µmole/10^9 sperm) levels (Mean±SE) in liquid semen extended in selected extenders

Extender	Preservation period (h)					
	0	24	48	72	96	120
BTS	0.31±0.04$^{!}$	0.44±0.07$^{@}$	0.58±0.05$^{\#}$	0.73±0.09$^{\wedge}$	0.85±0.06^{a*}	1.04±0.10^{b+}
SFC	0.39±0.08$^{!}$	0.53±0.05$^{@}$	0.66±0.07$^{\#}$	0.81±0.04$^{\wedge}$	1.08±0.02^{b*}	1.24±0.05^{a+}
TEY	0.36±0.11*	0.51±0.02$^{@}$	0.69±0.06$^{\#}$	0.80±0.03$^{\wedge}$	1.14±0.09^{b*}	1.37±0.04^{a+}

Values having different superscripts between column (alphabets) and within row (symbols) differ significantly (P<0.05).

difference among the three extenders during respective preservation time at 0 h of observation throughout the study period (Table 2). After 6 h of incubation, the *in vitro* capacitation/acrosome reaction was similar (P>0.05) in semen extended in all the extenders at 0-48 h of preservation. At 72 and 96 h (P>0.05) of storage, the acrosome reaction was higher (P>0.05) in semen extended in BTS than in SFC and TEY after 6 h of incubation. At 120 h of preservation, the percentage of acrosome reacted spermatozoa were significantly (P<0.05) highest in BTS followed by SFC (P<0.05) and lowest in TEY after 6 h of incubation. In all the extenders, the *in vitro* capacitation/acrosome reaction increased at all storage times both at 0 h of observation and following 6 h of incubation. BTS contains bicarbonate in its composition. Previous studies (Conejo-Nava *et al*. 2003) have shown that the presence of bicarbonate promotes quick and reversible organizational modifications in lipid bilayer of sperm membrane, enhances calcium ion influx and stimulates acrosome reaction. The authors further demonstrated that bicarbonate has a destabilizing effect on sperm membranes at temperatures as low as 16°C. Accordingly, sodium hydrogen carbonate (Sodium bicarbonate) in BTS, might have contributed toward changes in structural membrane of spermatozoa leading to progressively enhanced capacitation status following preservation for upto 120 h in the current study.

Effect of different extenders on LPO: Amongst different extenders, the MDA concentrations were lower (P>0.05) in semen preserved in BTS as compared to that in SFC and TEY at 0-72 h of storage period (Table 3). At 96 and 120 h of storage period, significantly (P<0.05) lower levels of MDA were perceived in semen extended in BTS than in SFC and TEY. Nevertheless, the MDA production increased exponentially (P<0.05) in all the extenders at 0, 24, 48, 72, 96 and 120 h of storage. Limited studies (Karunakaran *et al*. 2017) demonstrated no significant differences in MDA concentrations in BTS and SFC extenders at day 5 of preservation. In contrast, our results showed significantly (P<0.05) lower MDA levels in BTS than in SFC at similar time of storage. The increased (P<0.05) MDA production in all the extenders at all hours of preservation was in accordance with the findings of Kumaresan *et al*. (2009) who reported lower MDA levels in semen extended in BTS at the start of the preservation which gradually increased up to the 96 h of storage. The ROS-induced sperm destruction triggered the LPO cascade resulting in sperm death (Bucak *et al*. 2010). The lower increment of MDA in BTS during the period of storage could be due to the scavenging action of citrate and EDTA on free radicals, thus, protecting the plasma membrane from impairment (Chutia *et al*. 2014).

This suggested that liquid preservation of boar semen in Beltsville Thawing solution (BTS) exhibited better sperm attributes and reduced lipid peroxidation.

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