Characteristics of boar semen preserved at liquid state

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ABSTRACT
Thirty semen ejaculates collected from five Large White Yorkshire boars were preserved in Beltsville thawing solution (BTS) and SAFEcell plus extenders at 16–18°C over a period of seven days. Preserved semen samples were evaluated for sperm motility, plasma membrane integrity and concentration of lipid peroxidation compound malondialdehyde (MDA) every day during storage. Semen samples preserved in the SAFEcell plus extender had significantly more per cent of sperm cells with total motility, progressive forward motility and plasma membrane integrity, significantly low level of MDA concentration than BTS extender from day 1 to day 7 of storage. It can be concluded that the SAFEcell plus extender is better than BTS to preserve boar semen in the liquid state at 16–18°C.

1. Introduction
Artificial insemination (AI) is a well-adapted biotechnological tool in bovine species. Adoption of AI had helped the dairy industry to achieve faster transmission of genetic merit for milk production all over the world. Bovine sperm cells have better resistance to freezing injuries and are preserved at –196°C. As the metabolic activities of sperm cells are suspended at –196°C, preserved semen can be used for AI over infinite period.

Practice of AI is gaining momentum in the swine industry for genetic upgradation for better pork production. But most of the AI in pigs is carried out using boar semen preserved in the liquid state at 16–18°C. Boar sperm cells are highly sensitive to low temperatures and are usually preserved in the liquid state. Metabolic activities of sperm cells continued during storage at 18°C results in the exhaust of available nutrients and accumulation of metabolic end products including reactive oxygen species (ROS) in the semen. When the ROS levels are elevated, there is oxidative stress to the sperm cells (Bansal & Bilaspuri 2007). Mature spermatozoa have little capacity for repairing oxidative damage because their cytoplasm contains low concentrations of scavenging enzymes (Alvarez & Storey 1989). Seminal plasma is endowed with antioxidant capacity (Jimenez et al. 1990) which has the property of scavenging ROS and protect spermatozoa against oxidative stress. Semen dilution with extenders reduces the potential protective capacity of seminal plasma (Maxwell & Stojanov 1996). The cooling and thawing procedures also cause severe damages and/or death to certain per cent of sperm cells, by generating excess ROS via an aromatic amino acid oxidase-catalysed reaction (Sariözkan et al. 2009) and ultimately results in impaired cell functions and cell death (Bucak et al. 2010). Thus, the quality of preserved semen deteriorates and the conception rate decreases as the storage period advances. Quality of preserved semen can be evaluated by assessing the in vitro sperm characters which has high correlation with conception rate such as sperm motility, plasma membrane integrity and morphology.

Most of the inseminations are carried out using the preserved boar semen within two days of storage. It will be more economical to the swine industry, if the preserved semen can be used for AI purposes up to 4/5 days after collection with optimal conception rate and litter size (Waterhouse et al. 2004). To date, large numbers of boar semen extenders are available, but there exists high variability among different extenders in terms of viability and fertilizing capacity of spermatozoa. Therefore, it is essential to find out the suitable extenders for preservation of boar semen in the liquid condition which may maintain fertilizing potential of sperm cells. The study was conducted to evaluate the efficiency of two commercially available extenders to preserve boar semen at 16–18°C in the liquid state in terms of sperm cell motility, plasma membrane integrity and lipid peroxidation (LPO) status.

2. Materials and methods
2.1. Experimental animals
The experiment was carried out at Research Farm, ICAR – Central Coastal Agricultural Research Institute, Old Goa, Goa, India. The experiment was conducted in accordance with the approval of Institute Research Council. Experimental animals included five sexually mature Large White Yorkshire boars maintained for breeding. The mean age and body weight of boars were 23.33 ± 5.14 months and 195.66 ± 7.08 kg, respectively.

2.2. Management of animals
Breeding boars were housed individually in well-ventilated pens with average temperature range of 25–30°C. Animals...
samples were stored in 250 ml glass beakers at 16°C for 24 h. Technologies France. Each subsamples were diluted to have thawing solution (BTS) and SAFEcell plus sourced from IMV were diluted in two different extenders, namely, Beltsville Ceros II, Hamilton Thorne, Beverly, MA) as per guidelines of computer-assisted sperm analysis (CASA) system (HT CASA – Ceros II, Hamilton Thorne, Beverly, MA) as per guidelines of the supplier. The ejaculates, which met the minimum spermio-
cerance test (the hypoosmotic swelling test)

2.3. Semen collection and dilution
Ejaculates were collected with the ‘gloved hand’ method (Hancock & Hovell 1959) using dummy sow (Instruments de Médecine Vétérinaire (IMV), France). Semen was collected from each boar by the same trained person. Semen was collected not more than twice a week. Semen was filtered to remove the gel fraction. The following parameters were evaluated in fresh boar semen; semen volume, sperm cell motility, concentration, morphology and viability. Volume of the ejaculate was determined by using a graduated measuring cylinder. Sperm cell concentration and motility were analysed using computer-assisted sperm analysis (CASA) system (HT CASA – Ceros II, Hamilton Thorne, Beverly, MA) as per guidelines of the supplier. The ejaculates, which met the minimum spermogram standards for fresh semen, were used for the study.

Six ejaculates from each boar and a total of 30 ejaculates were used in the study. Subsamples from each ejaculates were diluted in two different extenders, namely, Beltsville thawing solution (BTS) and SAFEcell plus sourced from IMV Technologies France. Each subsamples were diluted to have 35 million sperm cells per ml of extended semen. Two hundred and twenty-five millilitres of extended semen samples were stored in 250 ml glass beakers at 16–18°C in a BOD incubator (Nanolab, India) for a period of seven days (day of collection is considered as day 0).

2.4. Evaluation of in vitro sperm characters
Sperm motility and membrane integrity were evaluated initially 2 h after extension of semen (day 0) and then at every 24 h interval over a period of seven days. On each day an aliquot of the sample was taken and kept in a water bath at 37°C for 15 min before evaluation for sperm motility and plasma membrane integrity. Sperm motility was assessed using CASA.

Membrane integrity was assessed using the osmotic resistance test (the hypoosmotic swelling test – HOST) by incubating an aliquot (100 µl) of semen sample with 1 ml of double distilled water at 37°C for 30 min (Nur et al. 2012). After incubation, a pinch of eosin was added; a drop of the well-mixed sample was placed on a glass slide and covered with cover slip. This slide was observed at 400× magnification under the phase contrast microscope. Spermatozoa with swollen tail were counted as HOST positive. A minimum of 200 spermatzoa were observed for tail coiling (Figure 1). The percentage of reactive spermatozoa was then calculated by subtracting the percentage of tail defects recorded in the sperm population before incubation in HOST media was carried out.

The LPO level of spermatozoa was estimated in semen samples by measuring the MDA concentration, using thiobarbituric acid (TBA) as per the method described by Suleiman et al. (1996) with slight modifications in sperm concentration and incubation time. One millilitre of semen samples containing 35 million sperm cells were taken from each stored semen samples. The semen samples were washed twice with phosphate buffered saline (PBS) by centrifugation (500g for 5 min). Then the sperm pellet was re-suspended in 2 ml of PBS (pH 7.2). Lipid peroxide levels were measured in spermatozoa after the addition of 2 ml of TBA and trichlor acetic (TCA) reagent (15% w/v TCA and 0.375% w/v TBA in 0.25 N HCl) to 2 ml of spermatozoa suspension. The mixture was kept in a boiling water bath for 45 min. After cooling, the suspension was centrifuged at 500g for 15 min. The supernatant was separated and the absorbance was measured at 535 nm under a UV spectrophotometer. The MDA concentration was determined by the specific absorbance coefficient (1.56 x 10³/mol cm⁻³).

2.5. Statistical analysis
Prior to conducting least-squares analysis, all the per cent values of parameters were subjected to Arc Sin transformation to overcome the scale effects. The data were analysed following the mixed model least-squares analysis for fitting constants (Harvey 1990). The comparison of different sub-groups mean was made by Duncan’s Multiple Range Test (DMRT) as described by Kramer (1957).

3. Results and discussion
3.1. Sperm motility
Total motility and progressive forward motility of boar semen samples preserved in BTS and SAFEcell plus extenders during different days of storage assessed by CASA are presented in Table 1. Average total motility of the sperm cells was >90% in both the extenders on the day 0 of storage. Total motility as well progressive forward motility of sperm cells got reduced significantly (P < .01) in both extenders as the storage period progressed. Khan et al. (2006) reported similar significant (P < .01) reduction in progressive motility in the semen samples...
presented in different extenders, namely BTS, Modena, Zarnobsa and Guelph.

Though sperm cells preserved in the SAFEcell plus extender had significantly less number of total motile cells than BTS extender on the day 0 of preservation, sperm cells preserved in the SAFEcell plus extender had significantly (P < .01) more numbers of motile cells than the BTS extender from day 1 (91.14 ± 0.07 vs. 86.33 ± 0.06), day 2 (87.55 ± 0.07 vs. 77.07 ± 0.06), day 3 (82.41 ± 0.07 vs. 64.76 ± 0.06), day 4 (78.29 ± 0.07 vs. 44.87 ± 0.06), day 5 (73.14 ± 0.07 vs. 23.67 ± 0.06), day 6 (65.27 ± 0.07 vs. 14.33 ± 0.06) and day 7 (56.14 ± 0.07 vs. 4.05 ± 0.06). The SAFEcell plus extender also had significantly (P < .01) more of sperm cells with progressive forward motility than the BTS extender from day 0 to day 7. In contrast to the present observations, Khan et al. (2006) did not find any significant difference among the different extenders such as BTS, Modena, Zarnobsa and Guelph in the progressive motility of spermatozoa during the storage period of 24, 48 and 72 h. Motility of sperm cell is essential to reach the site of fertilization and to penetrate through the oocyte to form zygote. Estimation of sperm motility by a light microscope is a subjective method. In the present study in addition to sperm motility, the HOST was applied for the assessment of the sperm membrane integrity. This test is based on the semi-permiability of the intact cell membrane that allows the sperm to swell under hypoosmotic conditions (Frydrychova et al. 2009). Perez-Llano et al. (2001) reported a significant correlation of HOST with in vivo fertility and farrowing rate.

### 3.3. Lipid peroxidation

The mechanism of ROS-induced damage to spermatozoa is mainly through an oxidative attack on the sperm membrane lipids that leads to initiation of the LPO cascade (Sharma & Agarwal 1996). The susceptibility of spermatozoa to oxidative stress is more due to the abundance of poly unsaturated fatty acids (PUFA) in the sperm membrane. The presence of double bonds in PUFA molecules make the spermatozoa more vulnerable to free radical attack that initiates the LPO cascade leading to subsequent loss in membrane integrity, impaired cell function, sperm motility and induction of sperm apoptosis (Bucak et al. 2010). Concerning the chemistry of LPO in spermatozoa, it is implied that once this process was initiated, its propagation was impeded, leading to accumulation of lipid peroxides in the sperm plasma membrane (Sharma & Agarwal 1996). Peroxidation of PUFAs in sperm cell membrane was an autocatalytic, self-propagating reaction, which probably gave rise to cell dysfunction associated with the loss of membrane functions and integrity.

### Concentration of lipid peroxide compound – MDA in preserved boar semen samples significantly increased during storage in both

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### Table 1. Mean ± SE of total motility, progressive forward motility, plasma membrane integrity percentage and MDA in boar semen preserved at 18°C in different extenders.

| Extenders       | Day 0   | Day 1   | Day 2   | Day 3   | Day 4   | Day 5   | Day 6   | Day 7   |
|-----------------|---------|---------|---------|---------|---------|---------|---------|---------|
| **Total motility (%)** |         |         |         |         |         |         |         |         |
| SAFEcell plus   | 91.59 ± 0.07a | 91.14 ± 0.07b | 87.55 ± 0.07a | 82.41 ± 0.07a | 78.29 ± 0.07a | 73.14 ± 0.07a | 65.27 ± 0.07a | 56.14 ± 0.07a |
| BTS             | 92.66 ± 0.06ab | 86.33 ± 0.06b | 77.07 ± 0.06ab | 64.76 ± 0.06ab | 44.87 ± 0.06ab | 23.67 ± 0.06ab | 14.33 ± 0.06ab | 4.05 ± 0.06ab |
| **Progressive forward motility (%)** |         |         |         |         |         |         |         |         |
| SAFEcell plus   | 58.98 ± 0.05ab | 52.14 ± 0.05ab | 44.01 ± 0.05ab | 34.82 ± 0.05ab | 27.88 ± 0.05ab | 21.82 ± 0.05ab | 17.03 ± 0.05ab | 11.70 ± 0.05ab |
| BTS             | 48.37 ± 0.04ab | 38.70 ± 0.04ab | 29.34 ± 0.04ab | 21.71 ± 0.04ab | 11.58 ± 0.04ab | 3.96 ± 0.04ab | 0.80 ± 0.04ab | 0.15 ± 0.04ab |
| **Membrane integrity (%)** |         |         |         |         |         |         |         |         |
| SAFEcell plus   | 30.90 ± 0.13ab | 29.54 ± 0.17ab | 29.54 ± 0.16ab | 29.24 ± 0.19ab | 28.01 ± 0.19ab | 20.50 ± 0.21ab | 20.33 ± 0.21ab | 18.34 ± 0.21ab |
| BTS             | 25.86 ± 0.23ab | 21.99 ± 0.15ab | 22.46 ± 0.16ab | 19.80 ± 0.16ab | 18.84 ± 0.19ab | 8.74 ± 0.21ab | 2.24 ± 0.16ab |         |
| **MDA (μ-mol ml−1)** |         |         |         |         |         |         |         |         |
| SAFEcell plus   | 0.613 ± 0.052ab | 0.848 ± 0.071b | 0.976 ± 0.062c | 1.127 ± 0.074d | 1.297 ± 0.074e | 1.613 ± 0.108f | 2.210 ± 0.158h |         |
| BTS             | 0.558 ± 0.068b | 0.760 ± 0.053b | 0.946 ± 0.081c | 1.228 ± 0.100d | 1.509 ± 0.132e | 2.012 ± 0.149f | 2.260 ± 0.122 |         |

Notes: Data shown all mean ± SEM (n = 30). Means with different superscripts a, b, c, d, e, f, g, h in a row differ significantly at P < .01 and P < .05, respectively.
extenders (Table 1). Semen samples preserved in the SAFEcell plus extender had a significantly low level of MDA than the BTS extender on day 3 (1.127 ± 0.074 vs. 1.228 ± 0.10), day 4 (1.297 ± 0.087 vs. 1.509 ± 0.132), day 5 (1.613 ± 0.108 vs. 2.012 ± 0.149), day 6 (1.975 ± 0.141 vs. 2.176 ± 0.142). There was no significant difference between the extenders in the concentration of MDA on day 7 and it may be due death of most sperm cells stored in the BTS extender thus only very few cells were metabolically active to generate ROS.

4. Conclusion

It was observed from the study that the BTS extender could maintain the sperm motility suitable for AI up to four days of storage while the SAFEcell plus extender could maintain up to six days of storage. Comparatively a lower level of lipid peroxide compound MDA concentration in the semen stored in the SAFEcell plus extender indicated that it has better antioxidants. Efforts may be carried out in future to improve the quality of the BTS extender by incorporating antioxidants.

Disclosure statement

No potential conflict of interest was reported by the authors.

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