Semen quality and fertility of liquid stored and frozen-thawed semen in crossbred pigs of North-Eastern India

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ABSTRACT
Study was conducted to compare the semen quality and fertility of liquid stored semen for three days and frozen-thawed semen in the north-eastern region of India. For liquid semen, the semen ejaculates were extended in Beltsville Thawing Solution (BTS) extender and preserved at 17°C for three days. For cryopreservation, semen was diluted Lactose-egg yolk-glycerol extender and frozen in straw using programmable freezer with freezing rate of 40°C/min from -6 to -140°C. The preserved evaluated for sperm motility, viability, plasma membrane integrity and fertility. The results revealed that the liquid stored semen has maintained the sperm motility and viability up to day 3 without significant reduction. Similarly the plasma membrane integrity did not differ significantly up to day 2, but it was significantly (P<0.05) reduced on days 3 in liquid stored semen. After freezing and thawing, the mean sperm motility, viability and plasma membrane integrity were 58.25 ± 2.96%, 64.75 ± 2.47% and 47.06 ± 2.02%, respectively. These parameters were significantly (P<0.01) lower as compared to the liquid stored semen from day 0 to day 3. After insemination with liquid semen, the farrowing rate was 77.7%, 80.76%, 73.07% and 69.8%, respectively from day 0, day1, day 2 and day 3. The pregnancy rate, farrowing rate and litter size did not differ significantly among different days of liquid storage. These parameters were significantly (P<0.01) lower in frozen semen as compare to that of liquid stored semen. The study concluded that the liquid semen stored up to three days is more efficient than frozen-thawed semen in terms of preserving sperm quality and fertility.

Key words: Artificial insemination, Fertility, Frozen-thawed semen, Liquid semen, Pig.

INTRODUCTION
Artificial Insemination (AI) is extensively used for dissemination of superior germplasm of pig throughout the world. Over the past decade, there has been a tremendous improvement in the field of AI services in the majority of countries concerned with pig production. AI is considered to be an effective tool in crossbreeding programs of non-descript indigenous pigs with superior germplasm to enhance productivity in the developing countries including India (Visalvethaya et al., 2010; Kadirvel et al., 2013). More than 99 per cent of the estimated 19 million inseminations in pig are either with freshly extended liquid semen on the same day or with that stored at 15°C-20°C for 1 to 5 days (Khalifa et al., 2014). Liquid semen has the advantage of maintaining fertility equal to natural service even with 2-3 billion sperm per insemination and superior storage competence (Garcia et al., 2007). Insemination with liquid semen has improved the reproductive efficiency as well as economic profit in the swine industry (Waterhouse et al., 2004). However, the major drawback of liquid semen is short term storage for 3-7 days. Several studies have shown the decline in fertility during storage periods in relation to the type of extenders used (De Ambrogi et al., 2006; Frydrychova et al., 2010).

On the other hand, frozen-thawed boar semen holds the potential to have an impact on the future of the swine industry because of its long-term storage. The frozen semen can be transported to longer distance for different distant countries. Frozen semen technology has improved the ability of a swine producer to access top-tier genetics from around the world to improve the efficiency, profitability and the quality of product to meet the consumer demands. Historically, these benefits have been masked by reduction in fertility measures particularly reduction in litter size with frozen thawed semen as compared to liquid semen. The pig producers/breeders will get higher benefits by using this technology, if the fertility rate following AI with frozen-thawed semen is comparable with that of liquid semen. Therefore, the objective of the present study was to compare the semen quality and fertility of three days stored liquid semen and frozen-thawed boar semen in crossbred pigs.

MATERIALS AND METHODS
Animals, semen collection and evaluation: The experimental animals were maintained and the study was designed as per the guidelines of Institutional Animal Ethics Committee. The present study was carried out in six adult (2-4 years) crossbred boars (indigenous- Sniang Megha X
Hampshire) routinely used for breeding programs in the pig breeding farm of the institute. The breeding boars were maintained under uniform management conditions, fed with the balanced concentrate rations and fed twice daily. The boars were trained for semen collection by gloved hand technique with a dummy sow (IMV Technologies, France). A total of 126 semen ejaculates, 21 ejaculates from each boar, were collected twice weekly. Ejaculates were collected in a sterilized plastic bottle and the gel fraction was strained using a Buchner funnel with gauze. Immediately after collection, the semen was evaluated for the semen quality parameters and those ejaculates having more than 70% sperm motility were selected. Out of 126 ejaculates, 112 samples were selected for further processing and preservation.

**Semen Processing and preservation**

**Liquid semen:** The semen ejaculate was extended in Beltsville Thawing Solution (BTS, Johnson et al., 1988) at the rate of 1:3 to 1:4 depending upon the sperm concentration. Approximately 95 ml of the extended semen containing ~3 billion sperm was packed in a sachet using a filling and sealing machine (IMV Technologies, France) and preserved at 17°C in a biochemical oxygen demand incubator (BOD, Narang Scientific Works-NSW-152, New Delhi, India). The extended and preserved semen was evaluated for sperm motility, viability and plasma membrane integrity and fertility from day 0 to 3 of storage.

**Frozen semen:** Semen ejaculates were processed and frozen following the straw freezing procedure as described earlier (Westendorf et al., 1975) with the minor modifications. In brief, the ejaculates were diluted with BTS extender at 1:1 ratio, held at 24°C for 2 hours and then at 18°C for 1 hour in the BOD incubator. After a period of three hours of holding, sperm pellet was re-suspended in fraction I of lactose-egg yolk extender (11% lactose and 20% egg yolk) to a concentration of 2 x 10^9 sperm/ml. The extended semen was cooled to 5°C for 90 min in the BOD incubator and then was transferred to cold handling cabinet (IMV Technologies, France) maintained at 5°C and mixed with equal volume fraction II of lactose-egg yolk-glycerol extender (11% lactose, 20% egg yolk and 6% glycerol) to make the final concentration of 1.0 x 10^9 sperm/ml. Then the extended semen was equilibrated for 60 min at 5°C in cold handling cabinet. The cooling rate of 3°C/min from 5°C to -6°C with 1 minute hold at -6°C followed by freezing of rate of 40°C/min from -6 to -140°C were used for freezing using programmable freezing machine (Cryo-Med Freezer, Thermo Scientific Model 7452, Ohio, US). The frozen semen was thawed in warm water at 70°C for six seconds for further evaluation and insemination.

**In-vitro assessment of sperm motility, viability and plasma membrane integrity:** Sperm motility was assessed under high power magnification (40X) using a phase contrast microscope (Olympus-BX51, Tokyo, Japan) as per the standard procedure and sperm with normal, vigorous and forward linear motion was subjectively assessed to the nearest 5%. Sperm viability was assessed by the Eosin-Nigrosin staining technique (Campbell et al., 1953). Two hundred spermatozoa were examined under an oil immersion (100X) using the phase contrast microscope. The functional integrity of the sperm plasma membrane was assessed by Hypo-Osmotic Swelling Test (HOST) as per the method described by Revell and Mrode (1994) using a Hypo-Osmotic Solution (HOS) containing 150mOsm/L of sodium citrate dehydrate and fructose.

**In-vivo fertility assessment:** The crossbred sows maintained under standard management conditions in the pig breeding farm of the institute were used for fertility assessment. Estrus detection was carried out twice daily and the sows exhibiting standing estrus were inseminated with golden pig catheter (IMV Technologies, France). A total of 104 sows/gilts, 27, 26, 26 and 25 were inseminated with liquid semen stored on day 0, 1, 2 and 3 respectively. For the frozen thawed semen, total of 10 straws were thawed and mixed with 60 ml pre-warmed BTS extender and total volume of 65 ml thawed semen was inseminated per sow/gilt within 5 min of thawing. In the present study, 32 sow/gilts were inseminated with frozen thawed semen. Double cervical insemination was carried out at 30 and 42 hours following onset of oestrus. After insemination, non-cycling sow/gilts were diagnosed for pregnancy after 6 weeks by the Doppler method using a trans-abdominal probe (EXAGO, Asha Medical & CO, New Delhi). The farrowing rate and litter size at birth were calculated after farrowing.

**Statistical Analysis:** The data were analysed statistically with the standard methods as per the Snedecor and Cochran (1994). The data on semen quality and fertility parameters were analyzed through ANOVA with a generalized linear model (SPSS version. 13) using Student–Newman-Kuels (SNK) multiple range test. Differences with values of P<0.05 were considered to be significant.

**RESULTS AND DISCUSSION**

Recently AI has been widely implemented in pig production with liquid semen and limited extend with frozen thawed semen. Frozen boar semen is valued for maintaining swine genetics in the cryopreserved state and facilitating opportunities for global gene distribution (Bailey et al., 2008). Although the frozen semen has many advantages, due to reduced fertility rate in comparison to liquid extended semen, frozen-thawed semen is used in less than 1% of all inseminations (Johnson et al., 2000). The semen quality is essential for the optimum conception rate, farrowing rate and litter size in pig husbandry. In the present study, comparison was made on the semen quality and fertility rate between liquid semen stored for three days and frozen-thawed semen. In the liquid semen storage, the study showed that there was no significant reduction in percentage of sperm motility and
viability from day 0 to day 3 although there was linear decline in percentage of sperm motility, viability and plasma membrane integrity with the advancement of storage days (Table 1). Similar report was observed in the earlier studies (Maepka et al., 2012, Kadirvel et al., 2016). The sperm motility reduces in the liquid semen storage over the period due to the fact that there was a decrease in membrane integrity and thereby initiating the acrosome reaction during storage. The lower semen storage temperature during liquid storage affects the plasma and acrosomal membrane integrity, ultrastructure and biochemical components of sperm (Gączarzewicz et al., 2015). The sperm plasma membrane integrity was reduced significantly (P<0.05) on day 3 as compare to day 0 in the present study. Similar to the present study, there was a lower percentage of sperm with intact plasma membranes with the advancement of storage days as reported in the earlier studies (Frydrychova et al., 2010). However, liquid semen can satisfactorily maintain the motility of boar sperm at 18°C for 8 days in long-term extender (Kaeoket et al., 2010). The BTS extender used in the study for liquid storage is the short-term extender. The advantage of the BTS extender is low-cost, easy to prepare and less variation among the different batches.

In frozen semen, the study obtained the mean post-thaw sperm motility and viability of boar semen was 58.25 ± 2.96% and 64.75 ± 2.47%, respectively (Table 1). There was reduction in sperm motility and viability by 20-25% after freezing. In agreement with our results, similar range of post-thaw sperm motility and viability were recorded recently (Baishya et al., 2015). Liquid semen maintained significantly (p<0.01) higher sperm motility, viability and plasma membrane integrity from day 0 to 3 as compared to frozen-thawed semen (Table 1). This reduction of sperm motility in frozen-thawed semen may be due to the damage to plasma and acrosomal membrane during the cryopreservation which in turn altered the function of plasma membrane, flagellum leads to leakage of intracellular sperm components (Corcuera et al., 2007).

In vivo fertility parameters such as the pregnancy rate, farrowing rate and litter size at birth following AI with liquid semen on different days of storage revealed there was non-significant difference among days from day 0 to 3 (Table 2). However, the litter size at birth was significantly (p<0.05) reduced after day 3. This observation in the present study is in accordance with the earlier reports (De AmbrogI et al., 2006). Recently, Kadirvel et al. (2016) also reported that the farrowing rate was significantly superior for semen that used from initial days of preservation as compared to aged semen. The pregnancy rate and farrowing rate recorded in the present study for frozen-semen is in close agreement with Baishya et al. (2015) recorded recently, but higher than that reported by Apic et al. (2015) and lower than that reported by Didion et al. (2011). The study recorded significantly (p<0.01) lower pregnancy rate, farrowing rate and litter size in frozen thawed semen as compared to liquid semen (Table 2). Inseminations with liquid semen within two days after collection did not reduce fertility rate but it was decreased with semen stored for more than 48 to 87 h (Garcia et al., 2007).

The study concluded that liquid semen stored for day 3 was more efficient than frozen-thawed semen in terms of preservation of sperm characteristics and fertility for AI in pigs. Further study would be required to improve the pregnancy rates and litter sizes with frozen-thawed semen.

**ACKNOWLEDGEMENT**

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**Table 1**: Sperm motility, viability and plasma membrane integrity of liquid stored and frozen-thawed boar semen.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Liquid semen</th>
<th>Day (0)</th>
<th>Day (1)</th>
<th>Day (2)</th>
<th>Day (3)</th>
<th>Frozen thawed semen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm motility</td>
<td>83.25±0.34^aa</td>
<td>80.75±1.59^aa</td>
<td>79.35±1.76^aa</td>
<td>77.68±1.81^aa</td>
<td>58.25±2.96^a*</td>
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<tr>
<td>Viability</td>
<td>85.75±0.93^aa</td>
<td>82.67±1.75^aa</td>
<td>80.65±0.84^aa</td>
<td>79.60±1.96^aa</td>
<td>64.75±2.47^b*</td>
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<tr>
<td>Plasma membrane integrity</td>
<td>86.62±0.17^aa</td>
<td>83.76±1.21^aa</td>
<td>76.55±1.71^ab</td>
<td>74.45±1.89^bc</td>
<td>47.06±2.02^c*</td>
<td></td>
</tr>
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</table>

Data shown as Mean ± SE, values with different superscript in the column differ significantly (P<0.05), *values differ significantly (P<0.01) in the respective column.

**Table 2**: Pregnancy rate, farrowing rate and litter size of liquid stored and frozen-thawed boar semen.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Liquid semen</th>
<th>Day (0)</th>
<th>Day (1)</th>
<th>Day (2)</th>
<th>Day (3)</th>
<th>Frozen thawed semen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animal inseminated</td>
<td>27</td>
<td>26</td>
<td>26</td>
<td>25</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Pregnancy rate (%)</td>
<td>81.48^aa</td>
<td>80.76^aa</td>
<td>76.92^aa</td>
<td>76.00^aa</td>
<td>53.12^a*</td>
<td></td>
</tr>
<tr>
<td>Farrowing rate (%)</td>
<td>77.77^aa</td>
<td>80.76^aa</td>
<td>73.07^aa</td>
<td>66.66^aa</td>
<td>50.00^a*</td>
<td></td>
</tr>
<tr>
<td>Litter size at birth</td>
<td>9.35 ± 0.3^aa</td>
<td>9.59 ± 0.6^aa</td>
<td>9.14 ± 0.5^ab</td>
<td>8.98 ± 0.8^bc</td>
<td>7.36 ± 0.18^c*</td>
<td></td>
</tr>
</tbody>
</table>

Data shown as Mean ± SE, values with different superscript in the column differ significantly (P<0.05), *values differ significantly (P<0.01) in the respective column.
REFERENCES


