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Research Article

EFFECT OF SOYBEAN LECITHIN EXTENDER ON POST-THAW SEMEN QUALITY OF BENGAL BUCK

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Semen extenders containing lecithin of animal origin are frequently criticized because of the risk of disease dissemination. To look for some alternative extender, the present study was carried out with the aim to evaluate if egg yolk lecithin could be replaced by soybean lecithin by adding glycerol to the extender either at the temperature of 30°C or 5°C during cryopreservation of buck semen. For the purpose, ejaculates collected from four Bengal bucks by artificial vagina method were diluted and frozen either in a Tris-egg yolk or Tris-soybean lecithin with glycerol added either at 30°C or 4°C. Results revealed that sperm motility and plasma membrane and acrosome integrity were not affected (p>0.005) between the semen fraction frozen in an egg yolk and soybean lecithin based extenders. Moreover, adding glycerol to the extender at 30°C or 4°C did not change significantly (p>0.05) the quality of semen diluted and frozen in both extenders. Therefore, soybean lecithin can properly replace egg yolk lecithin during semen freezing of Bengal bucks and glycerol as a cryoprotector may be added to the extender either at 30°C or 4°C.

Key words: Buck, Freezing-thawing, Glycerol, Lecithin, Semen, Soybean

Freezing of semen is one of the numerous methods of assisted reproductive technologies that contribute significantly to promote artificial insemination and genes exchange in the world. One of the critical points to make more efficient semen doses is to freeze semen in an extender that ensures not only good fertility but also

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maintains animal health without being any source of disease apparition for the female at the time of insemination. Elsewhere, the mostly extenders used to freeze male gametes are prepared based on lecithin from milk and egg yolk that are being questioned now-a-days because of the fact that they constitute a big risk for diseases dissemination between animals. Therefore, recently researches are more focused on testing alternative extenders free of animal proteins like soybean lecithin to preserve semen in cattle bull (Tarig *et al.*, 2017; Lima- Verde *et al.*, 2018), buffalo bull (Chaudhari *et al.*, 2015; Singh *et al.*, 2018), ram (Khalifa and Abdel-Hafez, 2014), dog (Dalmazzo *et al.*, 2018) and human (Mutalik *et al.*, 2014). Because of presence of the egg-yolk coagulating enzyme (EYCE) in buck seminal plasma (Ustuner *et al.*, 2009) and a 5560 kDa glycoprotein lipase secreted from the buck bulbourethral gland (Pellicer-Rubio *et al.*, 1997) that are toxic for sperm when diluted in conventional extenders based on egg yolk and skimmed milk (Pellicer-Rubio and Combarnous, 1998), recently initiatives are being taken to freeze buck semen using soya lecithin-based extender. Recent comparative studies using soya lecithin-vis-à-vis skim milk-based extenders for goat semen cryopreservation showed that soya lecithin addition at different concentrations in the extender preserved the semen quality parameters in a similar manner to the conventional skim milk-based extender (Vidal *et al.*, 2013). Further, no significant difference was observed for sperm progressive motility, viability or plasma membrane integrity when buck sperm was preserved in soybean lecithin- and egg yolk- based medium (Salmani *et al.*, 2013, 2014; Konyak *et al.*, 2018). Addition of different concentrations of glycerol in the extenders at two different temperatures (5°C and 30^0C) showed that particular concentration of glycerol exhibited better results in terms of post-thaw sperm quality at either 5°C or 30^0C (Farshad *et al.*, 2009). It is, therefore, possible that the temperature at which glycerol is added to the soya lecithin-based extender will determine the post-thaw semen quality while buck semen is cryopreserved using soya lecithin based extender. Hence, the aim of the present study was to evaluate whether extenders (soybean lecithin and egg yolk based) and temperature of glycerol addition in diluents (30°C and 4°C) could affect frozen semen quality in buck.

MATERIALS AND METHODS

Animals and semen processing: Four mature and healthy Bengal bucks were selected from the farm of ICAR-National Dairy Research Institute, Eastern Regional Station, Kalyani, Nadia, West Bengal for the present experiment. The study was conducted during February to March, 2018. The Animals were housed in the farm of the Eastern regional station of ICAR-

National Dairy Research Institute, Kalyani, West Bengal (India) and semen samples were collected two times a week by an artificial vagina.

Immediately after each collection, ejaculates were mixed together and split in four equal fractions: EY1, EY2, SL1 and SL2. Then, two fractions EY1 and SL1 were diluted at 30°C in Tris-citric acid-egg yolk -glycerol 6% and Tris-citric acid-soybean lecithin-glycerol 6% in order to reach a final concentration of 400 millions of spermatozoa/mL. After that, semen was cooled to 4°C for 2 h, packed in 0.25 mL French straws and kept at 4°C for another 2 hours more for equilibration before freezing. While other two remaining fractions of semen i.e. EY2 and SL2 were diluted in Tris- citric acid-egg yolk and Tris-citric acid- soybean lecithin extenders, respectively. During the first dilution at 30°C, semen was diluted in correspondent extenders without glycerol to reach a concentration of 800 million of spermatozoa/mL and then, after 2 hours of cooling to 4°C, semen was diluted a second time in the same extenders but containing 12% glycerol in order to reach a final sperm concentration of 400 millions of spermatozoa/mL. Then, semen was packed in French straws and kept for 2 hour more at that temperature for equilibration before freezing

All semen doses were frozen in nitrogen vapor at 4 cm above of nitrogen liquid level for 10 minutes before stocking straws in nitrogen tank for preservation.

The extender composition was 1.74 g citric acid, 3.03 g tris–(hydroxymethyl)-aminomethane, 0.80 g fructose, 1000U/mL penicillin, 1000µg/mL streptomycin, 20% egg yolk and 80 mL distilled water for Egg yolk extender (EY) and for Soybean lecithin extender (SL), it was the same composition as EY extender but 20% egg yolk was replaced by 1% W/V soybean lecithin.
Eight replications of sperm freezing were considered for each extender and temperature of glycerol addition in all the experiments.

Semen assessment: Semen was checked for motility, membrane and acrosome integrity at the following steps: just after dilution at 30°C, 2 hours of cooling to 4°C, 2 hours of equilibration at 4°C and after freezing-thawing straws.

Sperm motility was estimated by visual examination under low power magnification (×40) using a phase-contrast microscope while sperm plasma membrane integrity and acrosome integrity were assessed by Hypo-osmotic swelling test (HOST) as described by Jeyendran *et al.* (1984) and Johnson *et al*. (1976), respectively.

Statistical analysis: Data were analyzed by

the SPSS statistical software (version 20.0). A multivariate analysis of variance was performed to check the significance of extender, temperature of glycerol addition and freezing step effects on the sperm parameters evaluated. Means of the numerical variables were compared each order by Duncan test or a simple Means tests of the same software and P-value less than 0.05 was considered to be statistically significant.

RESULTS

The effects of different studied parameters on individual sperm motility, and acrosome and membrane integrity have been depicted in Table 1. Our results showed that there were no effects of extender and temperature of glycerol addition on semen quality of ejaculates both diluted and frozen. However, the quality of semen varied significantly ($p<0.001$) between the freezing steps even though there was no significant interaction ($P>0.05$) between the groups of effects analyzed on semen quality. Thus, individual motility, and acrosome and membrane integrity of sperm diluted in soybean lecithin or egg yolk extenders with glycerol added at 30°C and 4°C did not differ significantly (Table 2). whereas, the semen parameters were better ($p<0.05$) before cooling and lower after freezing and thawing semen diluted either with soya lecithin or egg yolk extender and independently of glycerol addition temperature in diluents (Table 3). Semen quality did not vary significantly ($p>0.05$) between cooling sperm for 2 hour and its equilibration at 4°C for another 2 hours. Thus, values obtained for individual motility, acrosome integrity and membrane integrity were 78.75±6.46% and 74.16±7.46%, 94.83±4.94% and 92.62±4.04%, and 78.58±6.04% and 75.95±7.21% for 2 hour of cooling and equilibration, respectively.

Table 1. Effects of different studied parameters on individual sperm motility, acrosome and membrane integrity

Effects	Individual motility	Acrosome integrity	Membrane integrity
Extender	0.651	0.569	0.605
Glycerol temperature	0.260	0.662	0.679
Freezing step	0.000 ***	0.000 ***	0.000 ***
Extender*Freezing step	0.664	0.867	0.925
Extender*Glycerol temperature	0.260	0.762	0.776
Glycerol temperature*Freezing step	0.595	0.793	0.688
Extender*Glycerol temperature*Freezing step	0.832	0.926	0.950

*** indicates the level of significance ($P<0.001$)

Table 2. Effects of extender and temperature of glycerol addition in diluents on the quality of semen collected and frozen by Mean test

Extender	Temperature	Individual motility (%)	Acrosome integrity (%)	Membrane integrity (%)
SL	30°C	69.16±16.72	87.20±19.29	69.54±21.14
	4°C	73.33±15.36	86.83±19.76	68.41±19.10
EY	30°C	72.08±15.87	86.45±17.97	69.91±21.52
	4°C	72.08±17.83	84.37±22.64	69.70±20.00
	P-value	0.260	0.762	0.776

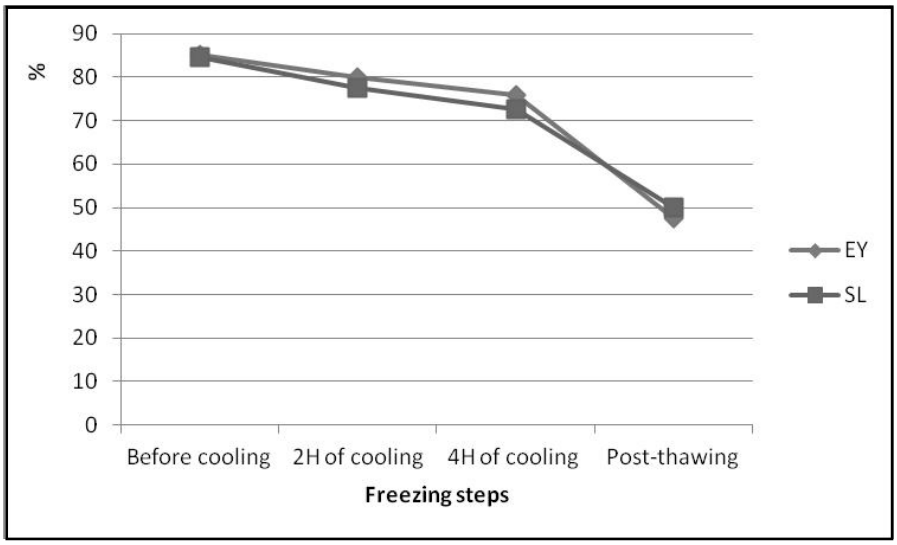
P >0.05, Differences are not statistically significant

Table 3. Variation of semen quality during the process of freezing by Duncan test

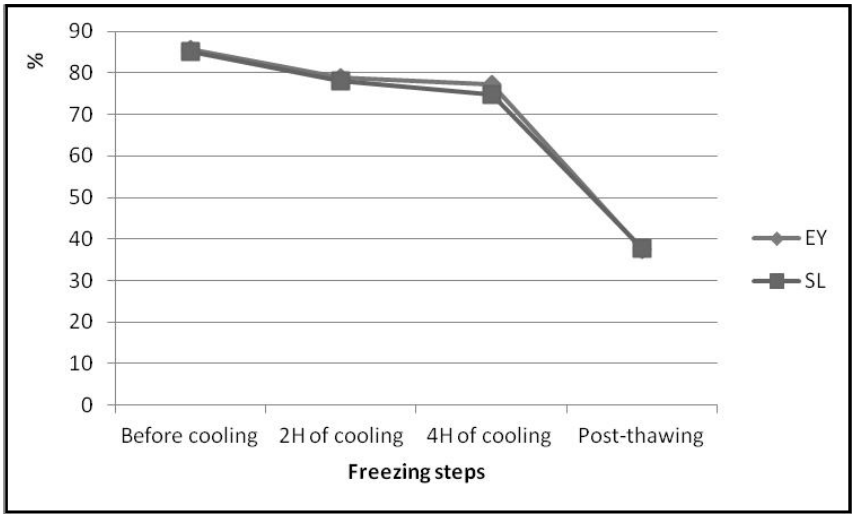
Freezing step	Individual motility (%)	Acrosome integrity (%)	Membrane integrity (%)
Before cooling	85±3.29 [a]	96.83±2.16 [a]	85.41±3.04 [a]
2 h of cooling	78.75±6.46 [b]	94.83±4.94 [a]	78.58±6.04 [b]
4 h of cooling	74.16 ±7.46 [b]	92.62±4.04 [a]	75.95±7.21 [b]
After freezing-thawing	48.75±14.08 [c]	60.58±25.19 [b]	37.62±11.18 [c]

Letters different in the same column means differences are statistically significant (P<0.05)

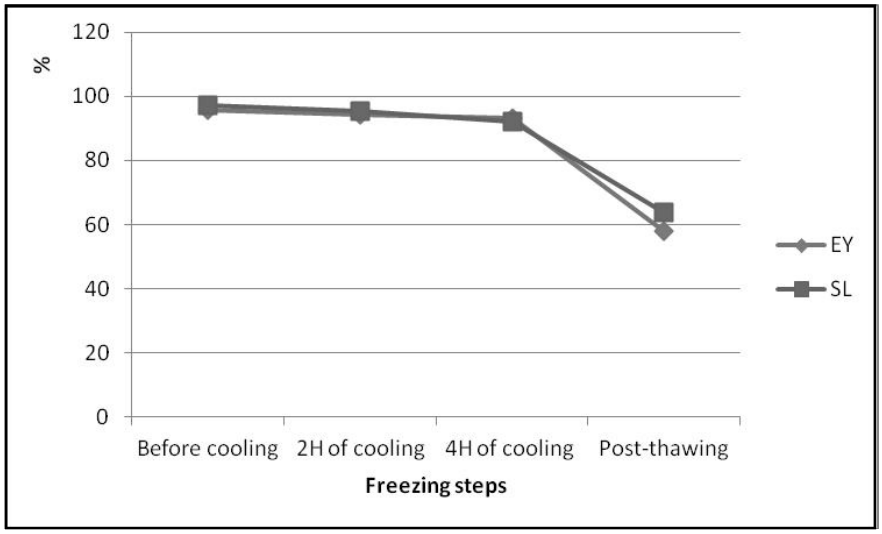
Moreover, Graphs 1, 2, 3, 4, 5 and 6 showed a significant decrease of sperm motility, and acrosome and membrane integrity during the process of semen freezing with the highest and lowest values before cooling sperm and post freezing-thawing semen straws, respectively and these events were independent of extender type used and the temperature used to add glycerol in the diluents. Elsewhere, it was observed an important agglutination of spermatozoa around large precipitates in 66.66% of egg yolk extender samples checked before freezing while in sperm diluted with soybean lecithin, sperm movements were more rapid and faster.



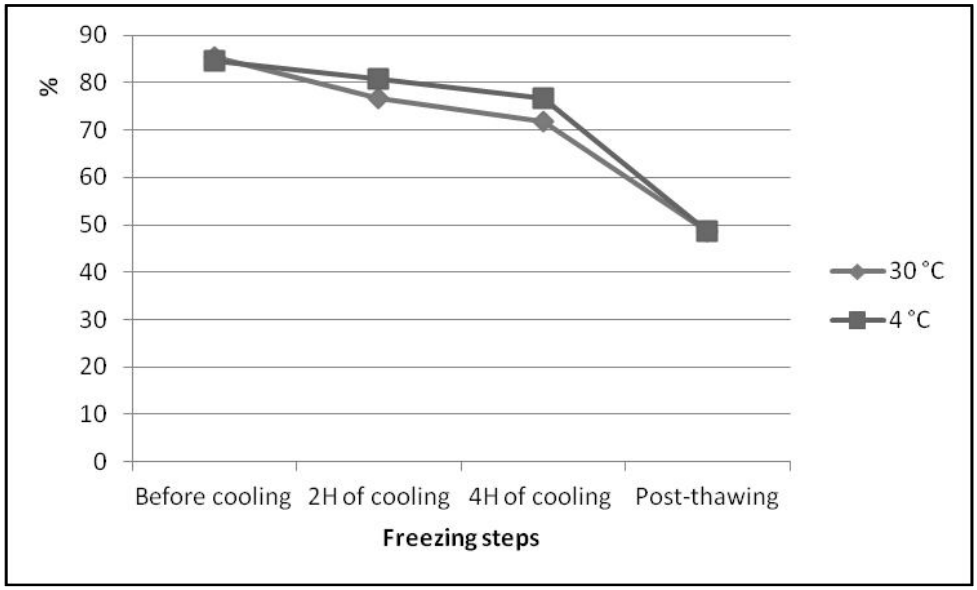
Graph 1. Evolution of individual motility of sperm diluted in both extenders during freezing process



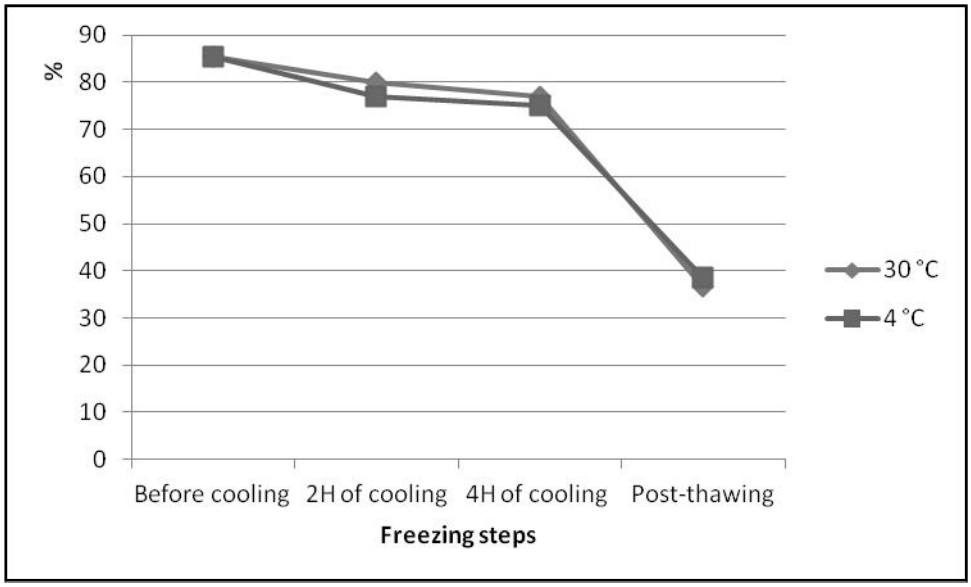
Graph 2. Evolution of sperm membrane integrity in both extenders during freezing process



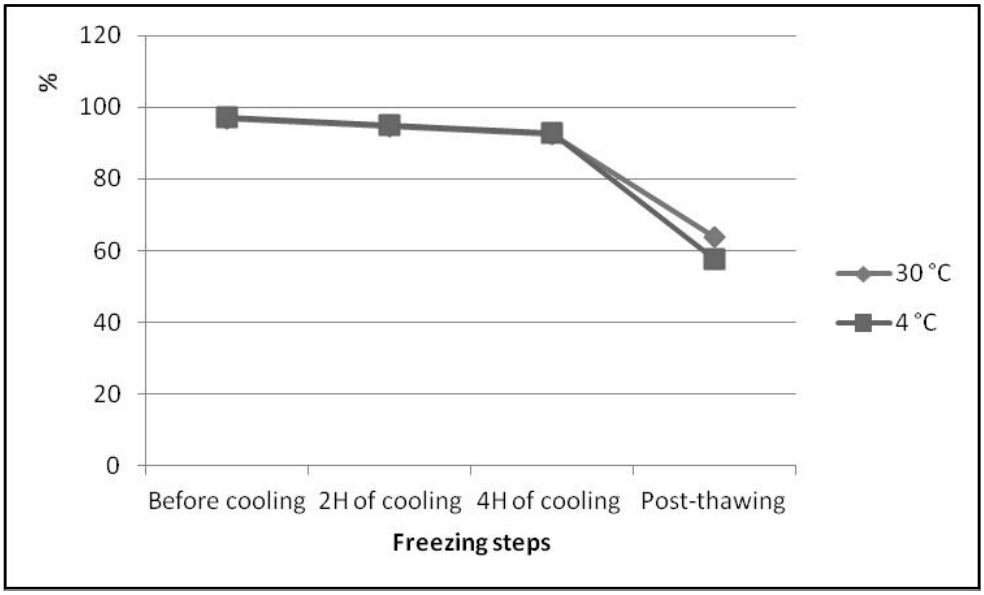
Graph 3. Evolution of sperm acrosome integrity in both extenders during freezing process



Graph 4. Evolution of individual motility of sperm during freezing process the acording of glycerol temperature addition in extender



Graph 5. Evolution of sperm membrane integrity during freezing process the acording of glycerol temperature addition in extender



Graph 6. Evolution of sperm acrosome integrity in both extenders during freezing process the according of glycerol temperature addition in extender

DISCUSSION

In the present study, our results clearly revealed that there were no effects of extender prepared with either egg yolk and soybean lecithin on sperm quality neither in fresh ejaculates diluted nor frozen-thawed semen. This confirms previous studies done in the same species (Vidal *et al*., 2013). Vidal *et al*. (2013) evaluated the effect of different concentrations of soybean lecithin (0.04%, 0.08% and 0.16%) in extenders for goat sperm cryopreservation and they found that all concentrations of soybean lecithin used, preserved the sperm quality parameters in a manner similar to the conventional skim milk-based extender. Salmani *et al.* (2013) and Konyak *et al.* (2018) reported that the percent of total and progressive motility, viability, plasma membrane integrity and normal morphology of goat semen do not differ (P>0.05) between egg yolk and soybean lecithin groups. Furthermore, similar conclusions were drawn by Fukui *et al.* (2008) and they found no significant difference in term of pregnancy and lambing rates and the prolificacy between the semen extenders containing egg yolk and AndroMed extender (a soybean lecithin based extender) when ewes were inseminated intrauterine using ram semen. On contrary, bull semen diluted in the egg yolk based extender exhibited higher total and progressive motility, percentage of rapid sperms and intact membrane cells than soybean lecithin based extender (Aires *et al.*, 2003; Crespilho *et al.*, 2012). Moreover, Ovixcell®, a soybean lecithin based extender was found to be superior to milk-egg yolk extender in preserving chromatin stability and motility during liquid storage of ovine semen (Khalifa *et al*., 2013). Khalifa and Abdel-Hafez (2014) showed that addition of 3.5% soy-lecithin increased progressive motility, viability and pregnancy rate and reduced abnormal acrosomes of ram spermatozoa compared to 15% egg yolk extender.

In the present study, we did not find any difference (p>0.05) on semen quality when glycerol was added to the diluents either at 30°C or 4°C and this was independent of semen freezing steps (Graph 4, 5 and 6). The results as found in the present experiments are in agreement with the findings of Jiménez-Rabadán *et al.* (2013). The workers did not find any differences of semen quality after thawing between both temperatures of glycerol addition i.e. 30°C and 5°C in Blanca-Celtibérica buck semen.

Furthermore, agglutination of spermatozoa in the form of large precipitates in 66.66% cases in the egg yolk-based extender as observed before freezing in the present study may be due to egg yolk disintegration by the egg-yolk coagulating enzyme (EYCE) contained in buck seminal plasma (Ustuner *et al*., 2009).

The present study demonstrated for the first time that use of soybean lecithin instead of conventional egg yolk in the extenders by

adding glycerol as a cryoprotectant at either 30°C or 4°C for Bengal buck semen cryopreservation did not differ semen quality neither in fresh nor in the frozen-thawed semen. Furthermore, use of soybean lecithin ameliorated the problem of sperm agglutination in case of buck semen preservation. Therefore, soybean lecithin can properly replace egg yolk lecithin during sperm freezing of Bengal bucks and glycerol as a cryoprotector may be added to the extender either at 30°C or 4°C.

Conflict of interest: Authors declare that there is no conflict of interest regarding the present research work.

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