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Effect of extenders on semen quality of Beetal bucks preserved at 5 °C

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Abstract
A total of 22 pooled ejaculates comprising 94 ejaculates collected using artificial vagina from five adult Beetal bucks maintained at Goat Research Station, AAU, Burnihat were used to study the effect of extenders on the quality of buck semen preserved at 5 ºC. Each pooled ejaculate was split into three equal parts. The parts were extended using three extenders viz., Tris extender containing 20 percent egg yolk, Tris extender containing 1.5 percent soya lecithin and commercial Ovixcell extender by split-sample technique and then preserved in a refrigerator at 5 ºC. The extended semen samples were evaluated about two hours after collection before cooling to 5 °C and then at 24, 48, 72 and 96 hours of preservation at 5 °C for sperm motility, live sperm, intact acrosome and HOST-reacted sperm. The sperm motility and live sperm count in Tris extender containing 20 percent egg yolk were significantly ($P<0.05$) higher than Tris extender containing 1.5 percent soya lecithin and Ovixcell extender. A significant ($P<0.05$) decline was also observed in both sperm motility and live sperm count at each hour of preservation period in all the three extenders. The incidence of intact acrosome and HOST-reacted sperm in Tris extender containing 20 percent egg yolk was significantly ($P<0.05$) higher than the other two extenders and in Tris extender containing 1.5 percent soya lecithin than in Ovixcell extender. Both the acrosome and HOST-reacted sperm were significant ($P<0.05$) decline at each hour of the preservation period in all three extenders. It can be concluded that Tris-egg yolk extender is superior to Tris-soya lecithin and Ovixcell extenders for the preservation of buck semen at 5 °C.

Keywords: Sperm, beetal bucks, HOST-reacted sperm, acrosome, Tris, Ovixcell

1. Introduction

Goat is a multi-purpose animal that plays a significant role in the economy and nutrition of landless and marginal farmers in the country. Artificial insemination in goats is practiced in many countries, and although A.I. with frozen semen could reach acceptable fertility results of 55-65 percent (Salvador *et al*., 2007) [18]. The utilization of chilled liquid semen in a breeding program is an important alternative to A.I. with frozen semen which is more expensive. Moreover, fertility using semen stored at 5 °C is higher than that obtained with frozen semen (Paulenz *et al*., 2005) [15]. Freezing of semen has certain limitations which include decreases motility, interferes morphological integrity, increase embryonic loss and ultimately reduce fertility. Semen can be diluted and chilled as an alternative to freezing when insemination is performed within a short time after collection. However, the cool storage of semen from goat has received little attention during recent years. The media generally used for storage of chilled liquid buck semen (4-5 °C) are skim milk, sodium citrate-egg yolk and Tris-egg yolk diluents. Egg yolk lecithin and low-density lipoprotein has been reported to protect sperm membranes from cold shock and is a common constituent of the extenders used in the preservation of mammalian spermatozoa at low temperature (Leboeuf *et al*., 2000) [11].

The World Organization for Animal Health (OIE), recommended in the 2003 Terrestrial Animal Health Code, that animal origin product used in semen processing should be free of any biological risk (Marco-Jimenez *et al*., 2004) [12]. So, the search for non-animal origin, well-defined and contamination-free medium for extension of semen is highly desirable. Recently, several commercial vegetable origin extenders have been reported to show promising results that can be considered as extenders. Thus, the present study described the effect of the extenders on the quality of buck semen preserved at 5 ºC.

2. Materials and Methods

2.1 Experimental animals and handling

Five sexually mature Beetal bucks aged 2-4 years maintained at Goat Research Station, Assam Agricultural University were included in the present study. The animals were housed in a well-ventilated shed and maintained under uniform managemental practice. Before selection, the animals were thoroughly examined for sexual as well as general health including palpation of the testis and epididymis.

2.2 Collection and pooling of semen

A total of 22 pooled ejaculates comprising 94 ejaculates were used in the study. Semen samples were collected from each buck once or twice a week with the help of a standard artificial vagina using a restrained doe. After collection, semen samples were placed in a water bath at 37 °C and transferred to the laboratory for semen evaluation within 15 minutes. Only ejaculates collected from different bucks having volume 0.5 ml or more, mass activity (0 to 4+ scale) 3+ or more and initial sperm motility 70 percent or more were selected and pooled.

2.3 Preparation of semen extenders

A Tris extender containing 20 percent egg yolk, 1.5 percent soya lecithin and Ovixcell were used in the study. The pH of the extender was adjusted to 6.8. All the constituents of the extender except egg yolk were mixed and kept overnight at 5 ºC. The composition of Tris extenders is mentioned in Table 1.

A soybean lecithin-based commercial extender (Ovixcell) is a commercial extender which contained ultra-pure water, salts, sugar, electrolytes, glycerol, antibiotics and animal-free protein whose concentrations were not revealed as a trade secret.

Table 1: Composition of Tris extenders:

Ingredient	Tris extenders	
	20% Egg yolk	**1.5% Soyabean Lecithin**
Tris (hydroxyl methyl aminomethane)	2.422 g	2.422 g
Citric acid	1.36 g	1.36 g
Fructose	1.0 g	1.0 g
Soya lecithin	-	1.5 g
Distilled water up to	80 ml	100 ml.
Egg yolk	20.0 ml	-
Benzylpenicillin	1, 00, 000 IU/100ml	1, 00, 000 IU/100ml
Streptomycin	100 mg/100 ml	100 mg/100 ml

The pooled semen samples were split into three equal parts and was extended (1:15) separately using the above mentioned three extenders. The extended semen samples were evaluated at room temperature about two hours after collection before cooling to 5 °C for sperm motility, live sperm, intact acrosome and HOST-reacted sperm. Each semen sample was evaluated for sperm motility, live sperm, intact acrosome and HOST-reacted sperm at 24, 48, 72 and 96 hours of preservation at 5ºC.

2.4 Semen Evaluations

2.4.1 Assessment of Sperm motility

A drop of preserved semen was placed on a pre-warmed glass slide (37º C) and examined under coverslip at a magnification of 400**X** using a compound microscope. The sperm motility was recorded from 0-100 based on the percentage of progressively motile sperm.

2.4.2 Assessment of Live sperm

The percentage of live spermatozoa (Fig. 1) in preserved semen was determined using the Eosin-Nigrosin staining technique described by Blom (1977) [2]. The staining solution was prepared by mixing 1part of 5 percent Eosin and 4 parts of 10 percent Nigrosin stain and kept at 5 °C in a refrigerator. One drop of extended semen was mixed with 2 drops of pre-warmed (37 °C) staining solution and allowed to stand for 5 minutes. 200 spermatozoa were examined in different areas of the smear under the oil immersion objective at a magnification of 1000X using a compound microscope for determining the percentage of live spermatozoa. Live and dead spermatozoa were considered as not stained and stained or partially stained respectively.

2.4.3 Assessment of Intact acrosome

A total of two hundred spermatozoa were examined in each smear at a magnification of 1000X under the oil immersion objective of a compound microscope to determine the percentage of the intact acrosome. The incidence of intact acrosome was studied in stained smears of extended semen using the Giemsa staining technique (Watson, 1975) [21].

2.4.4 Assessment of HOST-reacted sperm

The functional membrane integrity was evaluated by the short hypo-osmotic swelling test. A preserved semen (0.1ml) in 1ml of hypo-osmotic solution (100 mOsm/kg), 1.35 g fructose and 0.73 g sodium citrate were dissolved in 100 ml distilled water. This mixture was incubated in a water bath at 37 ºC for 60 minutes. A total of 200 spermatozoa were examined at a magnification of 400X using a phase-contrast microscope for sperm swelling (coiled tail). Swollen spermatozoa having coiled tail was considered as HOST-reacted sperm and percentage was calculated (Fig.2).

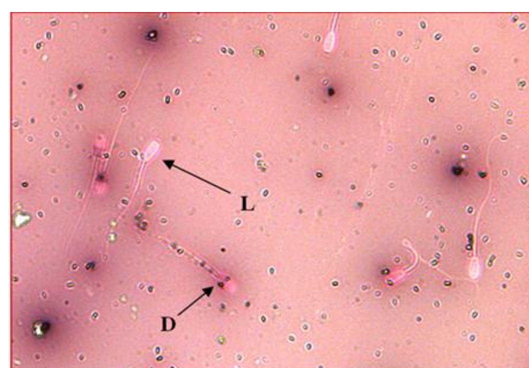

Fig 1: Live (L) and Dead Sperm (D)

Fig 2: Host reacted sperm

2.5 Statistical Analysis
Data obtained were analyzed using SPSS (version 14.0) software. ANOVA test was employed to know the different sperm parameters of Beetal buck semen in different extenders during preservation at 5 °C. Significance level was set at 95% confidence level.

2.6 Results and Discussion
Spermatological characteristics of fresh semen are shown in Table 2. The overall mean ejaculate volume, mass activity and initial sperm motility were 0.94 ± 0.03 ml, 3.26+ and 80.14 ± 0.34 percent respectively. The difference between bucks was significant (*P*<0.05) for ejaculate volume but not for initial motility. The mean ejaculate volume recorded in the present was similar to the values reported by earlier workers (Goswami, 2014) [6]. The variations recorded in ejaculate volume could be due to the difference in the age of the animals used for the study. However, the ejaculate volume differed significantly (P=0.0178) between the bucks. This is in agreement with the findings of Goswami (2014) [6] and Kakati (2017) [9] in Beetal bucks. The mass activity (Scale 0-4) of Beetal goat semen in the present study was lower than that obtained by earlier workers (Goswami, 2014; Kakati, 2017) [6, 9]. The variations recorded in mass activity could be attributed to the difference in season, age, feeding and management and individual variation. The initial sperm motility noted in the present study was close to the values recorded by Dutta Borah (2005) [5] and Raza *et al*. (2006) [16]. However, the mean value of initial sperm motility obtained in the present study was higher than the values recorded by Akela (2006) [1] and Das (2007) [4] and lower than that reported by earlier workers (Sarma *et al*., 2011) [19].

Table 2: Quality of fresh semen (MEAN ± SE) in Beetal buck

Semen characteristics	Beetal					Overall (n=94)
	Buck Ⅰ (n=19)	Buck Ⅱ (n=22)	Buck Ⅲ (n=18)	Buck Ⅳ (n=19)	Buck Ⅴ (n=16)	
Ejaculate volume (ml)	$0.88^{b} \pm 0.04$	$1.11^{a} \pm 0.08$	$0.96^{ab} \pm 0.05$	$0.86^{b} \pm 0.05$	$0.82^{b} \pm 0.05$	0.94 ± 0.03
Mass activity (0 to 4 + Scale)	3.19+	3.21+	3.23+	3.28+	3.39+	3.26+
Initial sperm motility (%)	79.26 ± 0.73	80.77 ± 0.78	79.61 ± 0.71	79.94 ± 0.73	81.12 ± 0.87	80.14 ± 0.34

n= number of ejaculates; a, bMeans bearing different superscripts in a row differ significantly (*P*<0.05).

2.6.1 Effect on sperm motility and live sperm count
The sperm motility in Tris extender containing 20 percent egg yolk was significantly (*P*<0.05) higher than Tris extender containing 1.5 percent soya lecithin and Ovixcell extender (Table 3). Significantly higher sperm motility in Tris extender containing egg yolk than in Tris extender containing soya lecithin was also reported by earlier workers in goat (Phutikanit *et al*., 2011; Yodmingkwan *et al*., 2016) [15, 22] and bull semen (Rehman *et al*., 2014) [17]. Further, there was a significant (*P*<0.05) decline in sperm motility at each hour of preservation period in all the three extenders. The drop in sperm motility at each hour of preservation at 5 °C was also reported in goat (Udeh and Oghenesode, 2011; Parmar *et al.,* 2012) [20, 13] and ram semen (Zohara *et al.,* 2018) [23].

In our study, the live sperm in Tris extender containing 20 percent egg yolk was also significantly (*P*<0.05) higher than that in Tris extender containing 1.5 percent soya lecithin and Ovixcell extender (Table 4). Significantly higher live sperm in Tris extender containing egg yolk than in Tris extender containing soya lecithin was also reported by earlier workers in goat (Phutikanit *et al.*, 2011; Yodmingkwan *et al.,* 2016) [15, 22] and cattle semen (Rehman *et al.,* 2014) [17]. It was also observed in the present study that live sperm count declined significantly (*P*<0.05) as the preservation period increased (Table. 4). The decline in live sperm count at each hour of preservation period was also reported by several earlier workers in goat (Islam, 2006; Parmar *et al*., 2012) [8, 13] and ram semen (Zohara *et al*., 2018) [23].

Table 3: Percent sperm motility (MEAN* ± SE) of Beetal buck semen in different extenders during preservation at 5 °C

Extender	Hours of preservation				
	2†	24	48	72	96
Tris-20% Egg yolk	$80.05^{a} \pm 0.61$	$74.77^{b} \pm 0.65$	$69.66^{c} \pm 0.68$	$62.61^{d} \pm 0.72$	$54.98^{e} \pm 0.76$
Tris-1.5% Soya lecithin	$66.39^{c} \pm 0.74$	$60.75^{d} \pm 0.66$	$56.05^{e} \pm 0.73$	$48.86^{f} \pm 0.79$	$40.07^{g} \pm 1.13$
Ovixcell	$69.91^{c} \pm 0.68$	$56.14^{e} \pm 0.86$	$41.55^{g} \pm 0.96$	$28.07^{h} \pm 0.92$	$15.45^{i} \pm 0.67$

*44 Observations† before cooling to 5 °C; Means bearing different superscripts differ significantly (*P*<0.05).

Table 4: Percent live sperm (Mean* ± SE) of Beetal buck semen in different extenders during preservation at 5 °C

Extender	Hours of preservation				
	2†	24	48	72	96
Tris-20% Egg yolk	$85.59^{a} \pm 0.83$	$79.65^{b} \pm 0.86$	$74.31^{c} \pm 0.71$	$67.05^{de} \pm 0.81$	$59.74^{f} \pm 0.89$
Tris-1.5% Soya lecithin	$71.21^{cd} \pm 1.04$	$65.48^{e} \pm 0.71$	$60.61^{f} \pm 0.85$	$53.95^{g} \pm 0.73$	$45.51^{h} \pm 0.87$
Ovixcell	$74.88^{c} \pm 0.83$	$62.86^{ef} \pm 0.89$	$48.96^{h} \pm 0.97$	$33.86^{i} \pm 1.25$	$20.69^{j} \pm 1.05$

*44 Observations† before cooling to 5 °C; Means bearing different superscripts differ significantly (*P*<0.05).

2.6.2 Effect on intact acrosome and HOST-reacted sperm
The incidence of intact acrosome in Tris extender was significantly (*P*<0.05) higher than the other two extenders, and in Tris extender containing 1.5 percent soya lecithin than in Ovixcell extender (Table 5). The incidence of intact acrosome recorded in the present study in Beetal buck semen preserved in Tris extender containing 20 percent egg yolk, Tris extender containing 1.5 percent soya lecithin and Ovixcell extender at 5 °C could not be compared as the literature in this respect was not available. Further, there was a significant (*P*<0.05) decline in the incidence of intact acrosome at each hour of preservation period in all the three extenders (Table. 5).

HOST-reacted sperm in all the three extenders dropped significantly (*P*<0.05) as the preservation period increased. The HOST-reacted sperm in Tris extender containing 20 percent egg yolk was significantly (*P*<0.05) higher than Tris extender containing 1.5 percent soya lecithin and Ovixcell extender (Table 6). A perusal of available literature revealed no information on the preservation of goat semen at 5 °C in commercial Ovixcell extender, hence the present finding of HOST-reacted sperm in Beetal buck semen extended in Ovixcell extender could not be compared. There was a significant (*P*<0.05) decline in HOST-reacted sperm at each hour of preservation period in all the three extenders (Table. 6). The decline in the functional integrity of spermatozoa with the increase in preservation time during liquid storage at 5 °C was also reported in ram semen (Kasimanickam *et al.,* 2007; Gundogan *et al.,* 2011; Zohara *et al.*, 2018) [10, 7, 23].

Table 5: Percent intact acrosome (Mean* ± SE) of Beetal buck semen in different extenders during preservation at 5 °C

Extender	Hours of preservation				
	2†	24	48	72	96
Tris-20% Egg yolk	$86.48^{a} \pm 0.98$	$81.08^{ab} \pm 1.05$	$76.33^{bc} \pm 1.16$	$68.76^{d} \pm 1.07$	$61.82^{e} \pm 1.27$
Tris-1.5% Soya lecithin	$72.73^{cd} \pm 1.16$	$67.34^{de} \pm 1.16$	$62.42^{e} \pm 1.26$	$54.85^{f} \pm 1.34$	$47.03^{g} \pm 1.18$
Ovixcell	$75.71^{bc} \pm 1.14$	$62.44^{e} \pm 1.37$	$50.90^{fg} \pm 1.72$	$34.84^{h} \pm 2.29$	$20.88^{i} \pm 1.49$

*44 Observations† before cooling to 5 °C; Means bearing different superscripts differ significantly (*P*<0.05).

Table 6: Percent HOST-reacted sperm (Mean* ± SE) of Beetal buck semen in different extenders during preservation at 5 °C

Extender	Hours of preservation				
	2†	24	48	72	96
Tris-20% Egg yolk	$73.48^{a} \pm 0.80$	$68.17^{b} \pm 0.91$	$61.67^{c} \pm 1.08$	$54.31^{d} \pm 1.07$	$46.37^{fg} \pm 0.75$
Tris-1.5% Soya lecithin	$60.82^{c} \pm 1.18$	$54.66^{d} \pm 0.95$	$49.35^{ef} \pm 0.86$	$43.91^{gh} \pm 0.94$	$36.94^{i} \pm 1.02$
Ovixcell	$63.36^{c} \pm 0.83$	$52.62^{de} \pm 0.93$	$41.25^{hi} \pm 0.90$	$28.04^{j} \pm 1.06$	$19.64^{k} \pm 0.94$

*44 Observations† before cooling to 5 °C; Means bearing different superscripts in a row differ significantly (*P*<0.05).

3. Conclusion
Based on the results, it can be concluded that Tris-egg yolk extender is superior to Tris-soya lecithin and Ovixcell extenders for the preservation of buck semen at 5 °C.

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