

Effect of Different Levels of Egg Yolk on Cryopreservation of Black Bengal Buck Semen in Tris Egg yolk Citrate Fructose Glycerol Extender

Research Article

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ABSTRACT

This study was conducted to assess the physical and morphological characteristics of fresh, pre-freeze and post-thaw black Bengal buck (*Capra hircus*) semen processed in Tris-egg yolk-citrate-fructose-glycerol (TEYCFG) extender containing 4 different concentrations (2.5, 5, 7.5 and 10%) of egg-yolk. Ejaculates were collected once a week for eight weeks from six mature bucks (48 ejaculates) using an artificial vagina. It was found that a general decrease in values of pre-freeze and post-thaw semen parameters such as progressive sperm motility (%), live sperm (%), acrosomal integrity (%) and positive sperm (%) in the hypo-osmotic swelling (HOS) test, but an over-all increase in abnormal spermatozoa compared to fresh semen for extenders containing different concentrations of egg-yolk. Best values for all the semen parameters were obtained using extender with 2.5% egg-yolk. Of all egg-yolk concentrations, the 10% egg-yolk supplement caused the highest percentage of abnormal sperm, which was significant ($P < 0.05$), compared to 2.5% and 5% egg-yolk containing extenders, but non-significantly ($P > 0.05$) different compared with 7.5% egg-yolk containing extender. Only progressive motility was significantly different ($P < 0.05$) within extenders containing 2.5%, 5%, 7.5% and 10% egg-yolk. We conclude that Black Bengal buck semen can be cryopreserved effectively with tris-egg yolk-citrate-fructose-glycerol extender containing 2.5% egg yolk (V/V).

KEY WORDS buck, cryopreservation, glycerol, semen extender.

INTRODUCTION

Buck are used either in natural service or as contributors of ejaculate for use in artificial insemination (AI). In natural service, each ejaculate are used to breed a single doe but in case of AI a large number of doses of semen could be prepared from single ejaculate and utilized for insemination of several females. This is one of the major advantages of artificial insemination (AI) over natural service. However, the fertility or reproductive ability of the individual buck is an important factor to determine the reproductive performance

of does. Buck with good breeding soundness and proven fertility are essential for breeding the doe to obtain the large number of offspring. The commonly used components of semen extenders for cryopreservation include egg-yolk, buffer, sugars, glycerol and antibiotics, each providing a complex and important role in protecting the spermatozoa (Purdy, 2006). Egg yolk used in semen extenders plays a major role during the freezing steps of buck sperm cryopreservation (Aboagla and Terada, 2004) and provide sperm cells with nutrients like protein (Ritar *et al.* 1990). On the other hand, egg yolk might have a negative effect on frozen

buck semen due to the presence of phospholipase, an enzyme present in the seminal plasma which catalyzes the hydrolysis of lecithin in egg yolk to fatty acids and lysolecithins, which proved toxic to spermatozoa and cause coagulation of the storage medium (Iritani and Nishikawa, 1972). The cryopreserved sperm cells can be stored for a long period of time before being used. Cryopreservation of buck semen also extends the reproductive life of a buck after his own life (Rahman *et al.* 2008). Here buck semen has been cryopreserved, using tris-based extender, which contains a wide margin of egg yolk. Hence, the present work was carried out with the objective to assess the spermatoc parameters of the Black Bengal buck semen cryopreserved in extender containing different levels of egg-yolk.

MATERIALS AND METHODS

Animals

The experiment was conducted at the Department of Veterinary Gynaecology and Obstetrics, West Bengal University of Animal and Fishery Sciences, Belgachia, Kolkata-37. The experiment was carried out from February to July, 2012.

Six sexually matured black Bengal bucks aged 2¹/₂ to 4 years were used for semen collection. All experimental animals used in this experiment were maintained by the Department of Livestock Production and Management, WBUAFS, under appropriate and uniform conditions of feeding and management. Before selection, all the bucks were thoroughly examined for general and reproductive health conditions. Proper deworming and vaccination were carried out. Experimental animals were grazing on natural pasture from 7 a.m. to 3 p.m. every day. Water was provided *ad libitum* throughout the study period.

Semen collection and evaluation

Semen was collected once a week for 8 weeks by artificial vagina (AV) method. A total number of 48 ejaculates were used in this study. Semen samples collected from each buck were processed individually and evaluated after i) collection, ii) before freezing and iii) after thawing for progressive motility, live and dead sperm percentage, sperm abnormalities, acrosome intactness and functional membrane integrity. The ejaculates were assessed and accepted for experiments if the following criterion was met: volume between 0.5-2 mL; minimum sperm concentration $\geq 2.5 \times 10^9$ sperm/mL; motility $\geq 80\%$.

Progressive motility as an indicator of semen quality was assessed using a phase contrast microscope (Olympus, India), fitted with a warm stage at 37 °C. The viability was evaluated by eosin-nigrosin staining according the method described by Rao (1957). The percentage of sperm with abnormal morphology was determined according to

Hancock (1951). Acrosomal integrity was assessed according to the method of Hancock (1952), modified by Sarma (1995). The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails. The test was done according to Jeyendran *et al.* (1984).

Semen processing and evaluation

Tris-citrate-egg-yolk based cryodiluent was used as an experimental extender in this study and it was composed of 2.422 g Tris-hydroxymethyl-aminomethane, 1.36 g citrate, 1.00 g fructose, 90.5- 83 mL triple distilled water, 2.5%, 5%, 7.5% and 10% (V/V) egg yolk, 7% (V/V) glycerol. Antibiotics viz., penicillin (100000 IU), streptomycin sulphate 100 mg/100 mL were added to the extender.

Extension of semen

Immediately after evaluation, the ejaculate was divided into 4 equal aliquots and diluted in one step at room temperature, to a concentration of 100×10^6 sperm/mL, with this extender. The pH of the diluents was adjusted to 6.8 with an appropriate amount of citrate.

Equilibration

The extended semen samples from each buck were kept in a cold handling cabinet at +5 °C for 3 h of equilibration period, and subsequently, the different parameters of semen were evaluated.

Freezing, thawing and evaluation

Straws (0.25 mL, French) were filled with extended semen using the comb and hand pump (IMV, India) immediately after equilibration. Then the straws were sealed by polyvinyl alcohol (PVA). The straws were then racked before and exposed to LN₂ vapour by placing those 5 cm above the LN₂ in a thermo box for 10 min and then plunged the straws into LN₂. Thawing of the straws was carried out individually after 24 h of freezing at 37 °C for 30 s in water bath for post-thaw sperm evaluation.

Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA), followed by the Duncan's multiple range test to determine significant difference in all parameters between groups using the SPSS software system (SPSS, 2011). Differences with values of $P < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

In the fresh semen, the average values of progressive motility (%), live (%), abnormal spermatozoa (%), intact acrosome (%) and HOS positive sperm (%) was depicted in

Table 1. There was a general decrease trend in values of pre-freeze and post thawed semen parameters such as progressive motility, live sperm, intact acrosome and HOS positive sperm, but mainly, increase in percentage of abnormal sperm in the semen diluted in extenders containing different concentrations of egg-yolk in comparison to fresh semen. The percentage of progressive motility, live sperm, intact acrosome and HOS positive sperm were significantly ($P<0.05$) higher, when 2.5% egg-yolk was added to the extender. Conversely, the lower percentage of abnormal sperm ($P<0.05$) was obtained in the same group. The 10% egg-yolk containing extender showed all parameters presented lower results than other treatments, except sperm abnormality (Table 1 and 2).

Cryopreservation as a technique for storage of buck semen has advantages but the freezing and thawing processes induce detrimental effects on the sperm ultra-structure, and the biochemical and functional integrity (Watson, 2000).

Egg yolk, is a crude lipid and is a common component for sperm refrigeration, the role of which is protecting sperm plasma membrane against cold shock, and interacts with sperm plasma membrane (Watson, 1975; Watson, 1976; Foulkes, 1977).

The present study showed that 2.5% egg-yolk in TEYCFG extender provided better pre-freeze sperm protection than all other egg-yolk concentrations tested. The findings of pre-freeze seminal parameters are in close agreement with values of a study undertaken by Priyadharsini *et al.* (2011). On the contrary, a study conducted by Yimer *et al.* (2014), showed that progressive motility (%) at 10% omega-3 egg-yolk in citrate extender was better than 2.5% omega-3 egg-yolk in citrate extender during cryopreservation of buck semen.

Current study revealed that progressive sperm motility (%) after thawing remains above 50% only when using the 2.5% egg-yolk concentration. Similar trend of changes in respect to sperm motility have been reported by Shamsuddin *et al.* (2000). The decline in sperm motility after thawing may be due to formation of peroxides from free radicals. This oxidative stress causes damage to biomolecules and cellular components (Halliwell, 1991). The sperm membrane contains a high number of unsaturated fatty acids, which are predisposed to damage due to peroxidation, which destroys the structural integrity of plasma membrane leading to loss in motility (Aitken *et al.* 1989; Salamon and Maxwell, 2000). The toxicity of cryoprotective agent glycerol in semen diluents causes a reduction in sperm motility and to alter the acrosome integrity by interfering with the permeability of the sperm membrane (Maxwell and Salamon, 1993; Maxwell and Watson, 1996).

The percentage of post-thaw motility of spermatozoa was decreased significantly ($P<0.05$) between 2.5% to 10% egg-

yolk extender in this study. Similar trend of changes in sperm motility have been reported by Ritar *et al.* (1990). These findings may signify that when egg yolk level increased in the extended semen, hydrolysis of lecithin led to further deterioration of sperm motility. The current results came in line with those obtained by El-Maghraby (2007) and Ashmawy *et al.* (2010) who found an optimal level of 2.5% egg yolk with tris-based extender for dilution of buck semen.

Bispo *et al.* (2011) has also observed that low level inclusion of egg yolk significantly improved post-thaw physical-morphological seminal parameters compared to inclusion of 20% egg yolk in the extender. This result coincide with those Cabrera *et al.* (2005) has included 1.5, 6 and 12% egg yolk for freezing canary buck semen. The finding with respect to the quality of post-thawed spermatozoa in the present study was in disagreement with the findings of Cabrera *et al.* (2005).

Ranjan *et al.* (2009) investigated the effect of 2.5, 5, 7.5 and 10% egg yolk level and concluded that 10% showed higher post-thaw motility and liveability in sirohi buck semen held at refrigeration temperature for 72 h. On the other hand, our study exhibited that progressive motility and viability at 2.5% egg-yolk level in TEYCFG extender was highest compared to other extender, which was in discrepancy with the result of Ranjan *et al.* (2009). The comparative study of extender containing egg-yolk at elevated concentration hampered the seminal parameters suggesting that egg yolk could contain some deleterious components which are potent to reduce sperm motility at higher concentrations.

The ingredients of egg-yolk defend the spermatozoa from deleterious effect of cryopreservation but not all of its components are favourable. Addition of more amount of egg-yolk increases the concentration of these deleterious components and that might be a cause for the excess damage caused by 10% egg-yolk level in this trial. Moreover, egg yolk increases the risk of microbial contamination and thereby releases excess endotoxin which may reduce the fertilizing capacity of spermatozoa (Aires *et al.* 2003; Bousseau *et al.* 1998).

On evaluation of effect of freezing, it was observed that, the motility of spermatozoa were significantly influenced by freezing and thawing, which reiterates earlier findings in Boer cross and Barbari bucks (Sundaraman and Edwin, 2005).

All the sperm parameters were significantly reduced in the post-thawing semen. Changes in the osmotic pressure during semen processing for cryopreservation critically affect the spermatozoa. This may be the most important deterrent to sperm survival during cryopreservation (Watson, 1995).

Table 1 Mean \pm SEM of fresh and pre-freeze semen quality parameters (%) extended with different concentrations of egg-yolk in the extender

Parameters (%)	Fresh semen	Egg-yolk level (%)			
		2.5	5	7.5	10
Progressive motility	84.14 \pm 0.30 ^a	82.39 \pm 0.42 ^a	79.27 \pm 0.38 ^b	76.33 \pm 0.40 ^c	67.87 \pm 0.41 ^d
Live sperm	86.85 \pm 0.24 ^a	84.66 \pm 0.37 ^a	78.70 \pm 0.36 ^b	73.60 \pm 0.31 ^c	70.27 \pm 0.26 ^d
Morphological abnormalities	6.44 \pm 0.25 ^c	6.98 \pm 0.22 ^c	8.10 \pm 0.22 ^b	8.8 \pm 0.34 ^{ab}	9.58 \pm 0.49 ^a
Acrosomal integrity	93.83 \pm 0.25 ^a	93.35 \pm 0.26 ^a	92.22 \pm 0.31 ^b	89.64 \pm 0.51 ^c	87.16 \pm 0.46 ^d
HOS positive	85.37 \pm 0.85 ^a	83.00 \pm 0.34 ^a	82.23 \pm 0.36 ^a	72.58 \pm 0.33 ^b	68.58 \pm 0.25 ^c

HOST: hypo-osmotic swelling test.

SEM: standard error of the means.

The means within the same row with at least one common letter, do not have significant difference ($P > 0.05$).**Table 2** Mean \pm SEM of fresh and post-thaw buck semen quality parameters (%) extended with different concentrations of egg-yolk in the extender

Parameters (%)	Fresh semen	Egg-yolk level (%)			
		2.5	5	7.5	10
Progressive motility	84.14 \pm 0.30 ^a	52.35 \pm 0.59 ^b	47.37 \pm 0.56 ^c	44.77 \pm 0.65 ^d	40.89 \pm 0.65 ^e
Live sperm	86.85 \pm 0.24 ^a	59.68 \pm 0.57 ^b	54.62 \pm 0.62 ^c	52.29 \pm 0.73 ^d	50.48 \pm 0.50 ^e
Morphological abnormalities	6.44 \pm 0.25 ^d	13.37 \pm 0.36 ^c	14.52 \pm 0.41 ^b	15.85 \pm 0.39 ^a	16.81 \pm 0.43 ^a
Acrosomal integrity	93.83 \pm 0.25 ^a	71.62 \pm 0.63 ^b	66.06 \pm 0.78 ^c	63.37 \pm 0.85 ^d	62.00 \pm 0.81 ^d
HOS positive	85.37 \pm 0.85 ^a	58.20 \pm 0.58 ^b	52.66 \pm 0.71 ^c	48.20 \pm 0.59 ^d	47.02 \pm 0.58 ^d

HOST: hypo-osmotic swelling test.

SEM: standard error of the means.

The means within the same row with at least one common letter, do not have significant difference ($P > 0.05$).

Furthermore, membrane destabilization can occur when the sperm plasma membrane undergoes a phase transition from the liquid crystalline phase to the gel phase due to a decrease in temperature (Barrea-Compean *et al.* 2005). The irreversible changes in the sperm membrane induced by lipid phase transitions during cooling warming may possibly affect the movement characteristics of spermatozoa during semen processing for cryopreservation (Deleeuw *et al.* 1990). In addition, frozen-thawed sperm are more vulnerable to oxidative stress due to peroxidation than sperm in freshly diluted semen (Neild *et al.* 2005). As semen is diluted many fold in the extender it reduces the total antioxidant concentration in the medium and cells (Kumar and Das, 2005). Many sperm are killed during cryopreservation. Thus, it is likely that cryopreserved sperm cells are posed to more reactive oxygen species concentration and therefore many of the surviving cells post-thaw exhibit as if they are capacitated or acrosome reacted (Bailey *et al.* 2000). The overall effects of these events may adversely affect quality of post-thaw semen.

It is most likely that the cryo damages are due to the irreversible destruction of individual components of the structural organization of sperm cells. Sperm are subjected to major changes in osmotic pressure during freezing and thawing. The stress on sperm membranes is dependent upon the basic extender used and the concentration of cryoprotectant as they interact with the freezing and thawing rates (Curry and Watson, 1994; Curry *et al.* 2000).

The post-thaw viability was highest in 2.5% egg-yolk followed by 5%, 7.5% and 10% egg-yolk and lowest in 10% egg-yolk diluents. There was highly significant difference of sperm liveability between 2.5%, 5%, 7.5% and 10%

egg-yolk. The live sperm percentages of post-thaw spermatozoa of the present study were in disagreement with the findings of Priyadharsini *et al.* (2011), who reported that the post-thaw liveability of Jakhrana buck semen in TEYCFG extender diluted with 10% and 20% egg-yolk levels were 53.4 ± 0.80 and 53.2 ± 1.12 , respectively.

The proportion of abnormality of post-thaw spermatozoa was lowest at the 2.5% egg-yolk, increased with 5%, 7.5% and 10% egg-yolk extender. There was a highly significant difference ($P < 0.05$) in morphological abnormality of spermatozoa of post-thaw semen between 2.5% and 5% egg-yolk and non-significant difference between 7.5% and 10% egg-yolk. The present findings of sperm abnormality of post-thaw semen were in agreement with the observation by Apu *et al.* (2012) and disparate with the findings of Batista *et al.* (2009).

The most probable reason for this morphological abnormality seems to be the physical and chemical environments to which a spermatozoon is exposed during the preservation. On the other hand, Medeiros *et al.* (2002) and Ozkavucu *et al.* (2008) noted that the sperm cell water exchange during the early stages of the preservation causes swellings and shrinkages which may be intolerable for the majority of organelles and might predispose to spermatozoon morphological abnormality.

It was noteworthy that number of abnormal spermatozoa were significantly increased ($P < 0.01$) as the number of motile spermatozoa decreased following preservation.

The values of post-thaw acrosomal integrity were highest at 2.5% egg-yolk followed by 5%, 7.5% and 10% egg-yolk diluents in the present study. There was highly significant difference ($P < 0.05$) of acrosomal integrity between 2.5%

and 5% egg-yolk diluents but there was non-significant ($P>0.05$) difference of acrosomal integrity between 7.5% and 10% egg-yolk extender. The present observations of acrosomal integrity were in close agreement with the findings of Batista *et al.* (2009) and Nally *et al.* (2010).

The values of HOS positive sperm of post-thaw were highest in 2.5% egg-yolk and followed by 5% and 7.5% egg-yolk and lowest in 10% egg-yolk. There was highly significant difference of HOS positive sperm between 2.5% and 5% egg-yolk.

There was no significant difference of HOS positive sperm between 7.5 and 10% egg-yolk. Pramanik and Raina (2001) studied hypo-osmotic swelling response of buffalo spermatozoa in various extenders. The value of HOS positive at post-freezing stage in skim milk extender (SEM) and Tris were $60.25 \pm 1.44\%$ and $57.75 \pm 2.66\%$ respectively and showed significantly ($P<0.01$) better performance over egg-yolk-citrate ($46.75 \pm 2.87\%$). Our findings of HOS positive sperm was in close agreement with the findings of Priyadharsini *et al.* (2011), who reported that post-thaw HOS positive of Jakhrana buck semen in TEYCFG extender diluted with 10% and 20% egg-yolk levels were 60.56 ± 1.31 and 52.7 ± 1.87 respectively.

CONCLUSION

On the basis of the present result, it is concluded that 2.5% egg-yolk level used in tris-egg yolk-citrate-fructose-glycerol (TEYCFG) extender was best for cryopreservation of black Bengal buck semen.

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