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Effect of egg yolk powder on cryopreservation of mithun semen

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ABSTRACT

The present study was conducted to assess the effect of the egg yolk powder in place of fresh egg yolk (EY) in the semen diluent on semen morphological attributes (SMAs) and mobility & velocity profiles measured by computer assisted sperm analyser (CASA) in cryopreserved mithun semen. A total of 25 ejaculates were collected through rectal massage method from five healthy mithun bulls (five ejaculates from each bull) and diluted with the standard tris citrate glycerol (TCG) extender and were splited into four equal aliquots, viz. Gr I, control (20% EY); Gr II, III and IV contained 5, 8 and 10% EY powder, respectively. SMAs, CASA parameters were evaluated following freezing-thawing of semen. Result revealed significant ($P<0.05$) improvement was observed in these SMAs and CASA parameters in Gr II and III as compared to Gr I and IV. It was concluded that inclusion of 5% or 8% EY powder in semen diluent holds a clear advantage and higher benefits over 20% fresh EY in cryopreservation of mithun semen.

Key words: Cryopreservation, Egg yolk powder, Mithun, Mobility and velocity parameters, Semen, Sperm morphological attributes

The conception rate in frozen-thawed semen is low in bovine species. Cryopreservation exerts damages to spermatozoa and decreased its post-thawed quality (Perumal *et al.* 2011). EY of hen is a component commonly included in the semen extenders as a cold shock absorbent in cryopreservation of semen. Protective effect of EY is mainly due to the presence of the low density lipoprotein (LDL) (Moussa *et al*. 2002). In mithun, it was proved that 8% LDL was suitable for semen preservation (Perumal *et al.* 2016a, Perumal *et al*. 2016b). Despite the significant benefits of EY on cryopreservation, it has many adverse effects as it is an animal source and hence may represent a potential microbiological risks, change the sperm chromatin structure leading to poor viability and fertilizing ability (Akhter *et al.* 2008). It contains high level of calcium ions which induces the premature acrosome reaction and capacitation leading to poor fertility of semen (Watson and Martin 1976). Components in the EY interfere the laboratory biochemical assays and metabolic investigations (Wall and Foote 1999). These disadvantageous effects of EY can be overcome by inclusion of the sterilized, pasteurised, homogenous EY powder in the semen extender in place of fresh EY. Earlier workers reported that EY powder has improved the freezability at 5% in bubaline semen (Singh *et al*. 2015). Perusal of literatures revealed no information on EY powder as a replacement for fresh EY on improvement of SMAs and CASA parameters in cryopreservation of mithun semen. Therefore, the present study was designed to evaluate the EY powder as a substitute for fresh EY in TCG extender in cryopreservation of mithun semen.

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MATERIALS AND METHODS

Five apparently healthy mithun bulls of 4–6 years of age with good body condition score (5–6) and body weight ranging from 495–510 kg were selected from the mithun farm, ICAR-NRC on Mithun, Medziphema, Nagaland, India. The experimental animals were fed, watered and maintained as per the farm routine schedule. A total of 25 ejaculates were collected through rectal massage method from five healthy mithun bulls (five ejaculates from each bull). These ejaculates were allowed to study the preliminary evaluation, viz. volume, colour, mass activity, concentration, individual motility and total sperm abnormality. The individual ejaculates having concentration: >500 million/ml, mass activity: 3+ or above, individual motility: >70% and total sperm abnormality: <10% were processed further. These ejaculates were diluted with the standard tris citrate glycerol (TCG) extender and were splited into four equal aliquots, viz. Gr I, Control (20% EY); Gr II, III and IV contained 5, 8 and 10% EY powder, respectively. Each sample was diluted (to get final concentration of 60 million spermatozoa per ml) with the TCG extender. Diluted semen samples of control and

treatment groups were cooled simultaneously from 37 to 5°C at a rate of 0.2 - 0.3°C per minute in a cold cabinet and maintained at 5°C for 2 h. Polyvinyl chloride straws (0.5 ml) were filled and maintained in a cold cabinet at 5°C for 2.5 h. Subsequently, these straws were wipe-cleaned, dried and spread over the freezing rack. The rack containing straws was kept in biological programmable freezer for freezing (final temperature maintained at –124°C, 12 min) followed by plunging of straws into the liquid nitrogen (–196°C) and was stored therein. At the time of evaluation, the stored semen straws were taken out of the cryo-cans and thawed in water at 37°C for 30 sec. Semen morphological attributes, viz. sperm motility (Nikon, Eclipse 80i; magnification 400×), velocity and mobility parameters by computer based analyser (Hamilton Thorne Sperm Analyser, Version 11), viability and total sperm abnormality by eosin–nigrosin staining, acrosomal integrity by Giemsa staining, plasma membrane integrity by hypo-osmotic swelling test, nuclear integrity by Feulgen staining technique and vanguard distance travelled by the sperm in the estrus bovine cervical mucus were determined with standard procedure. The results were analysed statistically with one way analysis of variance followed by the Tukey's post hoc test to determine significant differences between the four experimental groups using the SPSS/PC computer program (version 17.0; SPSS, Chicago, IL) and differences with values of $P<0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

The samples which had similar SMAs and CASA parameters for the four experimental groups at fresh semen were selected. The SMAs such as total motility, viability, plasma membrane, nuclear & acrosomal integrity and vanguard distance travelled by sperm in estrus bovine cervical mucus were significantly ($P<0.05$) higher and total sperm abnormality was significantly ($P<0.05$) lower in Gr II (5%) and III (8%) than Gr I (control; 20% EY) and Gr IV (10%) (Table 1). Similar results were observed in CASA attributes that the total motility, forward progressive motility, straightness, linearity, amplitude of lateral head displacement and beat cross frequency were significantly ($P<0.05$) higher in 5 and 8% EY powder treated samples than samples treated with 20% whole EY or 10% EY powder (Table 2).

Cryopreservation is a routine methodology for

Table 1. Effect of egg yolk powder on semen morphological attributes of cryopreserved mithun semen (Mean±SE)

Semen morphological attributes (%)	Fresh semen	Group I (20% Egg yolk)	Egg yolk powder		
			Group II (5%)	Group III (8%)	Group IV (10%)
Total motility (%)	71.54 ± 2.56	43.47 ±1.72^{a}	46.33 ±1.80^{b}	47.75 ± 1.87^{b}	44.25 ± 1.76^{a}
Livability (%)	74.98 ± 2.34	50.03 ±1.98^{a}	57.63 ± 2.24^{b}	58.32 ± 2.53^{b}	55.20 ±2.36^{ab}
Acrosomal integrity (%)	82.23 ± 1.68	57.70 ±2.09^{a}	63.69 ± 2.12^{b}	64.66 ± 2.45^{b}	61.17 ±2.25^{ab}
Total sperm abnormality (%)	9.78 ± 1.41	21.88 ±1.11^{b}	17.02 ±1.78^{a}	16.54 ± 1.53^{a}	20.14 ± 1.13^{b}
Plasma membrane integrity (%)	79.33 ± 2.34	56.75 ± 1.87^{a}	62.87 ±2.36^{b}	61.49 ± 2.66^{b}	57.41 ±1.87 ^{a}
Nuclear integrity (%)	83.47 ± 2.45	67.29 ±1.18^{a}	70.60 ±2.62^{b}	72.65 ± 2.58^{b}	68.16 ± 1.42^{a}
CMPT (mm/hr)	23.55 ± 1.42	20.31 ±1.36^{a}	23.47 ±1.35^{b}	24.74 ± 1.40^{c}	21.25 ± 1.26^{a}

Means bearing different superscripts within rows for different percentages of egg yolk powder and egg yolk differ significantly ($P<0.05$), n = 25. CMPT, vanguard distance travelled by sperm in bovine estrus cervical mucus (cervical mucus penetration test).

Table 2. Effect of egg yolk powder on mobility and velocity parameters of cryopreserved mithun semen (Mean±SE)

Mobility and velocity parameters	Fresh semen	Group I (20% Egg yolk)	Egg yolk powder		
			Group II (5%)	Group III (8%)	Group IV (10%)
PFM (%)	52.15 ±2.37	27.32 ±1.92^{a}	30.38 ±2.26^{b}	32.42 ±2.39^{b}	28.35 ±2.42^{ab}
TM (%)	76.47 ±2.40	39.57 ±1.65^{a}	43.36 ±2.32^{bc}	45.25 ±2.16^{c}	42.35 ±1.82^{b}
SM (%)	33.63 ±2.66	68.43 ±1.53^{c}	56.64 ±2.16^{ab}	54.75 ±2.22^{a}	57.65 ±1.65^{b}
VCL (µm/sec)	132.79 ±3.85	136.51 ±2.69^{c}	131.96 ±3.31^{b}	132.37 ±3.73^{b}	121.69 ±3.47^{a}
VSL (µm/sec)	98.12 ±3.51	86.54 ±2.96^{a}	93.52 ±3.16^{b}	98.84 ±3.53^{c}	92.52 ±3.65^{b}
VAP (µm /sec)	123.75 ±3.46	101.54 ±3.09^{a}	124.07 ±3.64^{c}	113.45 ±3.82^{b}	110.16 ±3.31^{b}
LIN (%)	73.77 ±1.71	63.27 ±1.87^{a}	74.68 ±1.78^{c}	76.84 ±1.54^{d}	69.79 ±2.06^{b}
STR (%)	79.14 ±1.80	75.18 ±1.27^{a}	81.52 ±2.20^{b}	84.96 ±2.04^{c}	79.53 ±1.69 ^{b}
WOB (%)	93.38 ±1.73	74.26 ±1.90^{a}	90.59 ±1.95^{c}	93.91 ±1.56 ^{d}	85.66 ±1.71^{b}
ALH (µm)	6.89 ±1.18	2.67 ±0.78^{a}	3.71 ±0.95^{b}	4.62 ±0.90^{c}	3.73 ±0.93^{b}
BCF (Hz)	22.70 ±1.12	19.86 ±1.03^{a}	23.26 ±1.26^{c}	24.89 ±1.35^{d}	21.81 ±1.47^{b}

Means bearing different superscripts within rows for different percentage of egg yolk powder and egg yolk differ significantly ($P<0.05$), n = 25. PFM, Progressive forward motility; TM, total motility; SM, static sperms; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; bcf, beat/cross frequency.

processing of bovine sperm in artificial breeding/ insemination centre. Fertility with cryopreserved bull semen was generally acceptable in spite of the fact that cryopreservation techniques resulted in the loss of 40–50% of viable sperm during the freezing-thawing process. However, some improvement has been reported over the last several decades (Prathalingam *et al*. 2006). Various factors such as cold shock, osmotic stress, ice crystal formation and oxidative damage were main sources of sperm cryoinjury, finally caused loss of sperm viability and fertility (Li *et al.* 2005). High survival and fertility rates of bull sperm in extender with 20% EY had been reported by various workers (Wall and Foote 1999). However, it is needed to investigate the process/methodology or technique or inclusion/deletion of the components in the EY or extender to minimize the cryoinjury to increase the fertility rate in bovine species.

EY derived from hen is a common component which is widely used in the preparation of semen extenders for cryopreservation of semen for various species over a period of decades to minimize or prevent cold shock damages and to enhance the fertilizing potential of the sperm (Bogart and Mayer 1950) and its protective action is due to presence of the LDL (Moussa *et al*. 2002, Perumal *et al.* 2016a, Perumal *et al.* 2016b). Although the EY has significant beneficial effects on semen cryopreservation, it has many adverse effects on sperm morphological structures because it is an animal origin and hence may represent potential microbiological risks, alter the sperm chromatin structure, subsequently leading to poor viability and fertility (Akhter *et al.* 2008) and also it is heterogenic in nature. Moreover, the quality & composition of EY vary between the batches. Further, various factors present in the whole EY inhibit the sperm respiration and its metabolic function leading to reduction of motile sperm percent (Pace and Graham 1974). Whereas the EY powder is uniform in composition and was undergoing sterilization, pasteurization followed by quality control checks which leads to reduction or elimination of microbiological risks and inhibits the activities of the deleterious factors (Thibier and Guerin 2000). Therefore, the EY powder is desirable in place of whole fresh EY in preparation of semen extender for cryopreservation in mithun species.

In the present study, the SMAs and CASA profiles were increased in the extender that contained EY powder from 5% to 8% and then decreased to 10% as because excessive amount of EY than optimum may in turn to higher fluidity of plasma membrane of sperm, creating the sperm more prone to plasma membrane and acrosomal damages and also inclusion of high dosage leads to deleterious effects on the spermatozoa as due to alteration in osmotic, physiological and physical condition of diluent. Singh *et al.* (2015) reported that 5% EY powder is optimum for buffalo semen cryopreservation whereas in mithun it was 5–8% in the present study. But the EY powder concentration in the diluent if higher than required amount leads to deleterious and toxic effect to spermatozoa (Singh *et al.* 2015). However, reduced concentration also altered the sperm structures and its functions. Therefore, based on the present study, SMAs and CASA parameters were increased maximum upto EY powder 8% then reduced to 10%. Moreover, during the sterilization and pasteurization process, the high quality EY protein denatures due to high temperature leading to gel like consistency in the semen extender which increases the viscosity of the extender and alters the osmolarity of the extender subsequently affecting the SMAs and CASA parameters (Miranda *et al.* 2000). Further, EY powder especially at 5–8% in semen extender showed significantly ($P<0.05$) higher beneficial effects in semen cryopreservation than EY at 20% in the present study.

It was concluded that EY powder at 5–8% is suitable in place of fresh whole EY at 20% in semen extender for cryopreservation of mithun semen as 5–8% has significantly ($p<0.05$) higher beneficial effects on SMAs and CASA parameters in mithun semen cryopreservation. However, *in vivo* fertility trials and estimation of biochemical & antioxidant profiles are warranted to confirm the present findings in this mountain ox.

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