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Effect of different extenders on cryopreservation of mithun semen

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Semen extenders function to protect the fertility of sperm and to increase the total volume of a sperm dose to a usable and practical size. Many semen extenders contain milk or egg yolk (EY) as primary ingredients because components in each protect sperm from cold shock (Garcia and Graham 1987). Conception rate by using cryopreserved semen is decreased as it induces physical, physiological shock and oxidative stress which results into damages to spermatozoa structure which inturn decreased post-thawed quality of spermatozoa (Ozkavukcu *et al*. 2008) finally caused loss of sperm viability and fertility. Therefore it is needed to investigate the methodology or inclusion/deletion of the components in the extender to minimize the cryodamage to increase the fertility rate in bovine species. EY of hen is a component commonly included in the semen extenders due to the presence of the low density lipoprotein (LDL) (Moussa *et al*. 2002). Despite the significant benefits of EY on cryopreservation, it has many adverse effects as represents potential microbiological risks, high level of calcium ions which induces the premature acrosome reaction and capacitation (Watson and Martin 1976). LDL extracted from EY of hen protects the sperm membrane by sequestration of BSP proteins in seminal plasma, thus preventing binding of BSP proteins on the sperm surface at ejaculation. Thus cholesterol and phospholipids efflux from the sperm membrane is minimized, minimizing membrane damage and cryoinjury (Nauc and Manjunath 2000). LDL alone attenuates the toxicity of glycerol in cryopreservation of semen. Instead of whole EY, LDL has improved the freezability and fertility in many species at different concentration (Hu *et al*. 2011, Akhter *et al*. 2011, Perumal *et al.* 2016). Whole milk is used in extenders are also to protect sperm during storage which could bind BSP proteins and protect sperm. Casein micelles from milk could interact with BSP proteins, the detrimental factors to sperm membranes (Bergeron *et al.* 2007). Binding of the casein micelles with BSP proteins leads to prevent the extraction of phospholipid and cholesterol from sperm plasma membrane as BSP proteins induce the release these components form sperm membrane inturn loss of fertility (Bergeron *et al.* 2007). Perusal of literatures revealed no information on effect of milk extender (MYG) on improvement of SQPs and CASA parameters in cryopreservation of mithun semen in comparison with TGFL and TGFY. Therefore, the present study was designed to evaluate the effect of different extenders in cryopreservation of mithun semen.

Three apparently healthy mithun bulls of 4–6 years of age with good body condition score (5–6) and body weight ranged 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 16 ejaculates collected through rectal massage method from three healthy mithun bulls (six ejaculates in each bull). These ejaculates were allowed to study the preliminary evaluation. 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 splited into three equal aliquots: Gr 1, Tris-glycerol-fructose-egg yolk-extender (TGFY; 20% egg yolk); Gr 2, Tris-glycerol-fructose-LDL-extender (TGFL; 8% LDL) and Gr 3, Milk-egg yolk-glycerol-extender (MYG; 73% double heated milk). Each sample was diluted (to get final concentration of 60 million spermatozoa per ml) with the respective extender. Diluted semen samples of experimental groups were cooled simultaneously from 37 to 5°C at a rate of 0.2 - 0.3°C/ min in a cold cabinet and maintained at 5°C for 2h. 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 quality parameters, viz. sperm motility (Nikon, Eclipse 80i; magnification 400×),

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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 three 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.

The semen samples were selected and used in the present study had almost uniform SQPs and CASA parameters at the fresh stage or neat semen. SQPs such as total motility, livability, functional membrane integrity and vanguard distance travelled by sperm in estrus bovine cervical mucus were significantly ($P<0.05$) higher and sperm, nuclear and acrosomal abnormalities were significantly ($P<0.05$) lower in Gr 2 (TGFL) followed by Gr 3 (MYG) and 1 (TGFY) (Table 1). Correspondingly CASA parameters, viz. total motility, forward progressive motility, straightness, amplitude of lateral head displacement and beat cross frequency were significantly ($P<0.05$) higher and static sperm percentage was lower in Gr 2 followed by Gr 3 and 1 (Table 2).

Semen extenders function to protect the fertility of sperm and to increase the total volume of a sperm dose to a usable and practical size. Optimal protection of sperm cell membrane integrity, mitochondrial function and chromatin quality should be primary considerations when selecting an extender for cryopreservation of sperm. Many semen extenders contain milk or egg yolk as primary ingredients because components in each protect sperm from cold shock (Garcia and Graham 1987). Semen preservation in ultra low temperature (–196°C) is a routine procedure in frozen semen bank and germ plasma centre. In overall, the fertility rate with cryopreserved bull semen is generally acceptable in spite of the cryopreservation techniques resulted in the loss of 40–50% of viable sperm during the freezing-thawing procedure. Critical factors in cryopreservation of semen, viz. cold shock, osmotic stress and oxidative are major sources of cryoinjury to sperm which inturn leads to loss of fertility. High fertility rates of sperm in extender with EY in different concentration have been reported by various workers in different species (Wall and Foote 1999). There are many extenders developed for preservation of bovine semen in liquid as well as cryopreserved form. But still

Table 1. Effect of different extenders on semen quality parameters of cryopreserved mithun semen (Mean ± SE)

Seminal quality parameter	TGFY	8% LDL	Milk extender
Total motility (%)	42.12 ±1.96^{a}	47.52 ±1.68^{b}	45.76 ±1.72^{b}
Livability (%)	54.92 ±2.21^{a}	59.98 ±2.35^{b}	56.38 ±1.93^{a}
Acrosomal integrity (%)	58.92 ±2.34^{a}	66.20 ±2.15^{c}	61.99 ±2.21^{b}
Total sperm abnormality (%)	20.86 ±1.26^{b}	15.91 ±1.21^{a}	16.30 ±1.39^{a}
Plasma membrane integrity (%)	54.64 ±2.12^{a}	63.64 ±1.83^{c}	58.92 ±2.16^{b}
Nuclear integrity (%)	66.98 ±1.55^{a}	74.91 ±1.60^{c}	71.14 ±2.23^{b}
Cervical mucus penetration test (mm/hr)	20.40 ±1.26^{a}	25.12 ±1.38^{c}	23.72 ±1.30^{b}

Means bearing different superscripts within rows differ significantly ($P<0.05$), n = 16. TGFY, Tris-glycerol-fructose-egg yolk-extender; LDL, Low density lipoprotein; Cervical mucus penetration test (mm/hr): vanguard distance travelled by sperm in bovine estrus cervical mucus.

Table 2. Effect of different extenders on mobility and velocity parameters of cryopreserved mithun semen (Mean ± SE)

Mobility and velocity parameter	TGFY	8% LDL	Milk extender
Progressive forward motility (%)	28.25 ± 2.21^{a}	31.17 ± 2.35^{b}	29.08 ±1.88^{ab}
Total motility (%)	41.16 ± 1.78^{a}	45.94 ± 1.92^{b}	44.17 ± 1.73^{b}
Static sperms (%)	58.84 ± 1.81^{b}	54.06 ± 1.88^{a}	55.83 ± 1.76^{a}
Curvilinear velocity (μm/sec)	125.68 ± 3.72^{ab}	130.73 ± 3.63^{b}	120.37 ± 3.40^{a}
Straight line velocity (μm/sec)	92.94 ± 3.78	90.94 ± 3.40	92.57 ± 3.23
Average path velocity (μm /sec)	118.50 ± 3.70^{b}	112.06 ± 3.55^{ab}	109.38 ± 3.31^{a}
Linearity (%)	73.65 ± 1.89^{b}	69.50 ± 2.16^{a}	76.88 ± 1.60^{c}
Straightness (%)	78.17 ± 1.91^{a}	84.72 ± 2.06^{c}	81.18 ± 2.24^{b}
Wobble (%)	94.24 ± 1.60^{c}	85.67 ± 1.72^{a}	90.89 ± 1.95^{b}
Amplitude of lateral head displacement (μm)	3.34 ± 0.99^{a}	4.72 ± 0.87^{b}	3.80 ± 0.96^{a}
Beat/Cross frequency (Hz)	22.48 ± 1.37^{a}	25.24 ± 1.22^{b}	22.23 ± 1.13^{a}

Means bearing different superscripts within rows differ significantly ($P<0.05$), n = 16. TGFY, Tris-glycerol-fructose-egg yolk-extender; LDL, Low density lipoprotein.

research is undergoing to find out suitable composition of the extender, additives, methodology to process, instrument for processing, preservation and analysis of the quality to get better post thaw motility and fertility in reproductive biotechnology. Therefore it is needed to investigate the methodology or inclusion/deletion of the components in the extender to modify or to form a new extender to minimize the cryodamage to increase the fertility rate in bovine species.

Hen's egg yolk is an important component which is widely used in semen extenders for cryopreservation of semen for various species over a period of decades to prevent cold shock damages to enhance the fertilizing potential of the sperm as the cold shock absorbent (Bogart and Mayer 1950) and its protective action is mainly due to the presence of the low density lipoprotein (Moussa *et al*. 2002, Perumal *et al.* 2016). Although the EY has significant beneficial effects on semen cryopreservation, it has many deleterious effects on morphological structures as because it is an animal origin may represent potential microbiological risks, alter the sperm chromatin structure, subsequently leads to poor viability and fertility (Akhter *et al.* 2011) and also it is heterogenic in nature. Moreover, the quality & composition of EY vary between the batches and various other factors present in the EY. Further, various factors present in the whole EY inhibit the sperm respiration and its metabolic function leads to reduction of motile sperm percent (Pace and Graham 1974). Low density lipoprotein extracted from egg yolk of hen protects the sperm membrane by sequestration of BSP proteins in seminal plasma, thus preventing binding of BSP proteins on the sperm surface at ejaculation. Thus cholesterol and phospholipids efflux from the sperm membrane is minimized, minimizing membrane damage and cryoinjury (Nauc and Manjunath 2000). On the other side, LDL forms a coat overlaying sperm membrane and protects from cold shock and cryoinjury of spermatozoa. LDL alone attenuates the toxicity of glycerol in cryopreservation of semen. Instead of whole EY, LDL extracted from EY has improved the freezability and fertility in many species at different concentration (Hu *et al*. 2011, Akhter *et al*. 2011, Perumal *et al.* 2016). Therefore in the present study, the SQPs and CASA parameters were significantly higher in LDL treated group than 20% egg yolk or milk extender.

Homogenized whole milk or skimmed milk has been proposed for the preservation of semen, egg yolk is still commonly used for cryopreservation of bovine spermatozoa worldwide. Casein micelles present in milk are responsible for sperm protection during storage of bull semen. The protective action of milk on sperm is analogous to the protective action of egg yolk, which is widely used as an extender in the storage of bull semen (Bergeron *et al.* 2007). The reports on viability evaluations indicate that milk was a more hospitable incubation medium than egg yolk-citrate for maintaining mitochondrial function post thaw and cell membrane integrity was best preserved during incubation by egg yolk-citrate (Karabinus *et al.* 1991). Because optimal maintenance of cell membrane integrity and mitochondrial function of the sperm are presumably desirable, these results suggest that an extender combining the attributes of milk and egg yolk-citrate would best serve both purposes (Karabinus *et al.* 1991).

As in the case of egg yolk, milk prevents the binding of BSP proteins to sperm and reduces sperm lipid loss, while maintaining sperm motility and viability during storage. Interestingly, while sperm protection by egg yolk involves the sequestration of BSP proteins by the LDLs present in egg yolk, the protection afforded by milk does not involve the participation of lipids or lipoproteins (Bergeron *et al.* 2007). It is the casein micelles present in milk that sequesters the BSP proteins. Thus, sperm protection by milk may involve a BSP protein-casein micelle (protein-protein) interaction. The prevention of BSP binding to sperm was probably not due to the binding of milk proteins to sperm during storage and it's due to an interaction between BSP proteins and casein micelles (Bergeron *et al.* 2007). Reports of fertility trials have shown little difference between milk and egg yolk citrate extenders when used separately or in combination for unfrozen semen or for frozen-thawed semen (Schenk *et al.* 1987). Extenders containing lactose-egg yolk glycerol showed better performance during laboratory evaluation, but milk-egg yolk glycerol extender gave higher conception rate when frozen semen was inseminated. It is not clear why sperm cryopreserved in milk versus egg yolk citrate differed for chromatin structure, cell membrane integrity and maintenance of mitochondrial function (Karabinus *et al.* 1991). Differences in composition between the extender components may have been a possible cause. Milk and egg yolk differ in levels of minerals, vitamins, lipid, and amino acids (Posati and Orr 1976). Milk which have all required nutrients for maintaining the liveability of spermatozoa when used as extender for diluting semen (4–7°C) was found superior over Tris and EYC in linearity, lateral head displacement (ALH), average path velocity (VAP), and motility (Pramanik and Raina 1998).

It was concluded that 8% LDL in TGF extender holds a clear advantage and higher benefits over egg yolk 20% in TGF extender and MYG extender in cryopreservation of mithun semen on SMAs and CASA parameters. The results of this preliminary study indicate that further, larger scale investigations of extender effects on semen quality traits should be conducted in conjunction with fertility trials. Better understanding of extender effects on semen quality may result in enhanced post thaw semen quality through modified semen processing and cryopreservation techniques.

SUMMARY

A study was carried out to assess the effect of different extenders on semen quality parameters (SQPs) and mobility and velocity attributes measured by computer assisted sperm analyser (CASA) in cryopreserved mithun semen. Ejaculates (16) were collected through rectal massage

method from 3 healthy mithun bulls (6 ejaculates in each bull). The ejaculates having concentration: >500 million/ml, mass activity: 3+ or above, individual motility: >70% and total sperm abnormality: <10% were selected for the present study. Ejaculates were splited into 3 equal aliquots: Gr 1, Tris-glycerol-fructose-egg yolk-extender (TGFY); Gr II, Tris-glycerol-fructose-LDL-extender (TGFL) and Gr III: Milk-egg yolk-glycerol-extender (MYG). SQPs and mobility and velocity parameters were measured following cryopreservation and thawing of semen. Statistical analysis revealed significant improvement was observed in Gr 2 followed by Gr 3 and Gr 1. It was concluded that 8% LDL in TGF extender holds a clear advantage and higher benefits over 20% egg yolk in TGF extender and MYG extender in cryopreservation of mithun semen.

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