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Effect of egg yolk of different avian species on semen quality parameters in mithun

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Various steps of cryopreservation such as processing, freezing and thawing exert physiological, physical as well as chemical stress on the sperm membrane (Chatterjee *et al*. 2001) and cryo-damage to the sperm is the main source of deleterious effect on the sperm functions and fertilizing ability (Perumal *et al*. 2011). Therefore various research studies are undergoing to minimize the cryo-injury like alteration of timing and temperature of different stages of freezing (Khan *et al*. 2017), inclusion of cryo-protector (Bora *et al*. 2015), cold shock absorber (Perumal *et al*. 2015), various chemical antioxidants/additives (Perumal *et al*. 2013, Perumal *et al*. 2015) or herbal antioxidants (Perumal and Rajkhowa 2015) in the semen extender or feeding of antioxidants (Jayaganthan *et al*. 2013) or season (Rajoriya *et al*. 2014). In general, 20% EY of chicken is commonly used as a membrane protector or cold shock absorber in semen preservation due to the presence of phospholipids, cholesterol and low density lipoprotein (Perumal *et al*. 2016). Further, it was reported the composition and concentration of different chemical components present in EY varied with different poultry species which in turn have different capability to protect the sperm during the cryopreservation (Rauch 2013). Earlier workers had reported that EY from different poultry species differed significantly in the cryoprotective effects (Rauch 2013). However, no such reports or studies were conducted in mithun semen preservation. Therefore the present study was designed to assess the effect of the EY of three different avian species such as chicken, turkey and quail on freezability and post thaw SQPs in mithun.

Three mithun bulls of good health, 4 to 6 yr of age, 495 to 510 kg with good body condition (score 5–6 of 9) were selected from Mithun Research Farm, ICAR-National Research Centre on Mithun, Medziphema, Nagaland, India for the present study (Westendorf *et al.* 1988). These bulls were maintained under uniform housing, feeding and managemental conditions. Each and every animal in the experiment was fed as per the farm schedule and offered *ad lib.* clean drinking water.

The extender used in the present study contained 3.028 g Tris (hydroxymethyl) amino methane, 1.675 g citric acid, 1.250 g fructose, 7 ml glycerol (7%), 1000 µg/ml streptomycin sulphate, 1000 IU/ml penicillin G sodium and 20% egg yolk of different poultry species (chicken, turkey and quail, for Gr I, II and III, respectively) for 100 ml deionized water. Ejaculates (30) were collected from the mithun (attempted twice a week) and semen ejaculates of similar properties were pooled to eliminate individual differences. Immediately after collection, the samples were preserved at 37°C in a water bath and evaluated for the routine SQPs. Samples were allowed to the preliminary dilution with pre-warmed (37°C) Tris citrate glycerol extender (TCG). The ejaculates were evaluated and accepted for evaluation if the following criteria were met: concentration >500×10^6/ml, mass activity >3+, individual motility >70% and total sperm abnormality <10%.

Each pooled ejaculate was split into 3 equal aliquots and diluted (@ 60×10^6 cells/ml) with the TCG extender (Gr 1, extender with 20% EY of chicken; Gr 2, extender with 20% EY of turkey and Gr 3, extender with 20% EY of quail). Diluted semen samples were processed and filled in Polyvinyl chloride (PVC) straws (0.5 ml) (IMV, France) and stored into the liquid nitrogen (–196°C). The stored semen straws were thawed at 37ºC for 30 sec after 24 h. The percentage of sperm motility (Nikon, Eclipse 80i; magnification 400× with thermo stage maintained at 37°C), viability by eosin nigrosin staining (Tomar 1997), total sperm abnormality (Tomar 1997), acrosomal integrity by Giemsa staining (Watson 1975), the plasma membrane integrity by hypo-osmotic swelling test (HOST) (Jeyendran *et al*. 1984), nuclear integrity by Feulgen's staining technique (Barth and Oko 1989) and vanguard distance travelled by sperm in the bovine cervical mucus (Matouseket *et al*. 1989) were determined as per standard procedure. Sperm motility and velocity parameters were assessed by computer assisted sperm analyser (CASA) by Hamilton Thorne Sperm Analyser (HTM-IVOS, Version 10.8, Hamilton Thorne Research, Beverly, MA, USA). CASA parameters such as progressive forward motility (%), total motility (%), curvilinear velocity (VCL; µm/sec), straight line velocity (VSL; µm/sec), average path velocity

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(VAP; μm/sec), linearity (LIN; %), straightness (STR; %), wobble (WOB; %), amplitude of lateral head displacement (ALH; μm) and beat/cross frequency (BCF; Hz) were estimated. A minimum of 200 spermatozoa were analysed with minimum of 2 different drops from each semen sample for each ejaculates.

The results were analysed statistically and expressed as the mean±SEM Means were analyzed by one way analysis of variance (ANOVA), followed by the Tukey's post hoc test to determine the significant differences between the 3 experimental groups using the SPSS/PC computer program (version 15.0; SPSS, Chicago, IL). Differences with values of P<0.05 were considered to be statistically significant after arcsine transformation of percentage data.

The result of the present study revealed that the SQPs such as total motility, viability, acrosomal integrity, plasma membrane integrity, nuclear integrity and vanguard distance travelled by the sperm in oestrus bovine cervical mucus were significantly (P<0.05) higher in sperm treated with extender containing quail EY followed by turkey and lowest with chicken EY (Table 1). Similarly, CASA parameters such as forward progressive motility, total motility, VSL, STR, ALH and BCF were significantly (P<0.05) higher in semen extender prepared by using the quail EY; VCL and VAP were significantly (P<0.05) higher in semen extender prepared by using the turkey EY and LIN and WOB were significantly (P<0.05) higher in chicken EY treated extender (Table 2).

Cryopreservation is a primary important process in artificial insemination industry and in this process, the sperm is preserved as unnatural. The sperm suffers various adverse effects in cryopreservation process, mainly cryoinjury or cryodamage (Perumal *et al*. 2011). This cryoinjury can be overcome by inclusion of glycerol and egg yolk as the cold shock absorbers or cold shock protectors in the cryopreservation process. There is a lot of research still

Table 1. Mean (±SE) physico-morphological attributes of mithun semen at post thaw stage of preservation treated with egg yolk of various poultry species

Seminal parameter	Type of extender		
	Chicken EY	Quail EY	Turkey EY
Post thaw motility (%)	41.20±1.92^{a}	47.52±1.68^{c}	44.76±1.70^{b}
Livability (%)	50.42±2.15^{a}	59.75±2.48^{c}	54.27±1.84^{b}
Acrosomal integrity (%)	54.77±2.29^{a}	66.20±2.25^{c}	60.65±2.19^{b}
Total morphological abnormality (%)	22.79±1.23^{c}	15.91±1.35^{a}	17.29±1.42^{b}
Plasm membrane integrity (%)	55.08±2.01^{a}	63.58±1.83^{c}	58.97±2.04^{b}
Nuclear integrity (%)	66.12±1.51^{a}	74.91±1.60^{c}	71.42±2.15^{b}
CMPT (mm/hr)	19.44±1.25^{a}	25.22±1.38^{c}	23.64±1.30^{b}

Means bearing different superscripts within rows differ significantly (P<0.05), n = 30. EY, egg yolk; CPMT, cervical mucus penetration test.

Table 2. Mean (±SE) computer assisted sperm analysis of mithun semen at post thaw stage of preservation treated with egg yolk of various poultry species

Velocity parameters	Type of extender		
	Chicken EY	Quail EY	Turkey EY
Progressive forward motility (%)	25.41±2.19^{a}	31.17±2.36^{b}	28.03±1.92^{a}
Total motility (%)	40.23±1.83^{a}	45.94±1.88^{c}	42.54±1.59^{b}
Curvilinear velocity (VCL) (μm/sec)	111.69±3.67^{a}	122.96±3.63^{b}	130.73±3.64^{c}
Straight line velocity (VSL) (μm/sec)	92.94±3.78^{ab}	94.24±3.74^{b}	90.74±2.94^{a}
Average path velocity (VAP) (μm/sec)	115.27±3.69^{a}	118.50±3.70^{b}	122.81±3.34^{c}
Linearity (LIN) (%)	84.19±1.99^{b}	74.11±2.36^{a}	73.65±1.89^{a}
Straightness (STR) (%)	74.12±2.42^{a}	81.54±1.95^{b}	81.18±2.23^{b}
Wobble (WOB) (%)	103.32±2.00^{c}	85.67±1.72^{a}	100.12±2.24^{b}
Amplitude of lateral head displacement (μm)	3.44±0.96^{a}	4.72±0.87^{b}	4.35±0.88^{b}
Beat/Cross frequency (BCF) (Hz)	17.54±1.43^{a}	25.24±1.16^{c}	22.15±1.09^{b}

Means bearing different superscripts within rows differ significantly (P<0.05), n = 30. EY, egg yolk.

continuing to standardize the freezing protocol and semen extender in different species to get higher freezability and post-thaw fertility. A major component is responsible for cold shock protection is egg yolk. Commonly the EY from hen or chicken is used in semen cryopreservation for bovine species in the semen processing centre or frozen semen bank due to presence of low density lipoprotein, cholesterol and phospholipids and it protects the sperm from adverse effects during the cryopreservation process (Perumal *et al*. 2016). In the present study, the semen quality parameters and CASA profiles were significantly (P<0.05) different among the EY of poultry species and were significantly (P<0.05) higher in the extender treated with EY of quail than turkey followed by chicken as also reported by Rauch (2013). These results indicated that the components of EY not only vary between the avian species but also between breeds within the species (Nisianakis *et al*. 2009) and also dependent upon feeds fed to the poultry. This may also be due to difference in composition and proportion of the phospholipids, cholesterol and polyunsaturated fatty acids in the EY of different species of poultry. For instance, duck EY have higher monosaturated fatty acids than in chicken EY and lowest is in quail EY (Bathgate *et al*. 2006) while EY of quail contains significantly higher amount of phosphatidylcholine and significantly lower amount of phosphatidylethanolamine with a smaller ratio of polyunsaturated to saturated fatty acids than in EY of chicken (Trimeche *et al*. 1997, Rauch 2013) and turkey EY is thicker than the EY of chicken, pigeon and quail. The higher semen quality parameters and motility and velocity parameters of CASA in the present study indicated that the EY of quail has protected the integrity of plasma membrane, acrosomal membrane and nuclear membrane and

mitochondrial membrane potential of sperm during the cryopreservation process which in turn increased the motility, viability and velocity parameters than EY of turkey or chicken. Moreover, the protocol used for the preparation of the extender, had been developed for chicken egg yolk (Moussa *et al*. 2002) and it is needed to modify the protocol according to the species of poultry. The high content of cholesterol in turkey EY was believed to increase the progressive motility of stallion semen after freeze-thawing (Burris and Webb 2009). In the cryopreservation of donkey semen, quail EY was superior to chicken EY in protecting sperm, which is due to its higher ratio of phosphatidylcholine and polyunsaturated fatty acids (Trimeche *et al*. 1997). Moreover, no analysis of chemical composition of the EY was performed; hence the results of the present study cannot be directly correlated with the presence and composition of phospholipid, cholesterol and polyunsaturated fatty acid content. The chemical analysis of EY of different avian species will be very helpful to study the mechanism of EY on protection of sperm and come to strong conclusion.

It was concluded that the quail egg yolk gave higher protection in *in-vitro* fertility tests than turkey or chicken EY. Further, it is required that more number of samples need to be tested, more *in-vitro* tests including measurement of post-thaw motility and semen quality parameters, *in-vivo* fertility trials need to be conducted and modification or refinement of the freezing protocol as per the EY of avian species to confirm the results and need to develop species specific EY based extender for semen preservation.

SUMMARY

A study was conducted to assess the effect of egg yolk (EY) of different avian species on semen quality parameters (SQPs) such as motility, viability, integrity of plasma membrane, acrosome and nucleus, and vanguard distance travelled by sperm in bovine cervical mucus. Velocity and motility parameters were measured by computer assisted sperm analyser (CASA) in the semen extender of mithun semen. Ejaculates (30) were collected from the 3 mithun bulls by transrectal massage method. These ejaculates were divided into 3 equal aliquots and grouped into Gr 1 (20% EY of chicken), Gr 2 (20% EY of turkey) and Gr 3 (20% EY of quail in TCG extender). The semen straws were processed, frozen with standard freezing protocol and stored at ultra low temperature (–196°C). The semen quality parameters and CASA attributes were evaluated after thawing (37°C for 30 sec). Statistical analysis revealed that the SQPs and CASA parameters were significantly higher in extender containing EY of quail than turkey followed by chicken.

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