



Egg yolk powder an alternative to fresh-egg yolk for buffalo semen cryopreservation

MAHAK SINGH¹, NIRMAL CHANDRA BARIK², S K GHOSH³, J K PRASAD⁴, J S RAJORIA⁵, Y K SONI⁶,
ASHOK KUMAR⁷, J K CHAUDHARY⁸ and N SRIVASTAVA⁹

Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243122 India

Received: 22 July 2014; Accepted: 19 September 2014

ABSTRACT

The present work was carried out to evaluate the effect of commercial egg yolk (EY) powder as an alternative to fresh EY on buffalo semen freezability. Ejaculates (12) from 3 bulls (4 from each bull) were collected as per standard procedure. After initial selection of ejaculates on the basis of mass activity (+3 and above) and individual progressive motility (70% and above), ejaculates were divided into 3 parts and diluted with tris dilutor, G1, containing 5% EY powder; G2, containing 10% EY powder, and G3, containing fresh EY (20%). Ejaculates were processed and cryopreserved using automated biological cell freezer followed by examination of frozen samples after 24 h. Semen quality variables (individual progressive motility, viability using propidium iodide, acrosome integrity using FITC-PSA and membrane integrity by HOST) were examined at fresh and post-thaw stage. At fresh stage semen quality variables did not vary among 3 groups.

Key words: Buffalo semen, Cryopreservation, Egg yolk powder, Semen, Sperm

Low density lipoproteins (LDL) present in egg yolk, a commonly used in extender at a concentration of 20% (v/v) for semen cryopreservation in buffalo (Bathgate *et al.* 2006), provides protection against cold shock, preserves sperm motility, reduces the loss of acrosomal enzymes and maintains mitochondrial membranes of sperm during semen freezing (Salamon and Maxwell 1995). In practical terms, harvesting hen egg yolk for use in semen extender is a cumbersome process, involving proper disease screening, disinfection, skillful breaking of the outer shell and inner membrane to separate the yolk from albumin and chalazae (Andrabi *et al.* 2008). Use of egg yolk represents a potential microbiological risk for use in semen cryopreservation due to its animal origin, hence, OIE (2003) in the Terrestrial Animal Health Code recommended that products of animal origin, used to treat sperm, should originate from sources free from any health risks, or should be treated prior to use to make the products safe. This potential contamination can be avoided by using powdered egg yolk in semen extender, instead of fresh egg yolk, as it is pasteurized. The present study was designed to compare powdered vis-a-vis fresh EY in semen extender for the

cryopreservation of buffalo bull semen to meet the requirement of OIE.

MATERIALS AND METHODS

Experimental design: Murrah buffalo bulls (3), 4–6 year-old, maintained at the Germ Plasm Centre of Indian Veterinary Research Institute, Izatnagar, were used for the collection of semen. These bulls were reared under the similar feeding and management conditions during the entire duration of the study. Semen was collected during the morning hours using an artificial vagina as per the standard practice.

Semen collection and evaluation: Ejaculates (12) from 3 bulls (4 from each bull) were selected on the basis of mass activity (+3 and above) and individual progressive motility (70% and above). The mass activity of the semen sample was determined by assessing the motility of the spermatozoa following semen collection. Motility was graded on the scale of 0 to +5 (Salisbury *et al.* 1985). The concentration and individual progressive motility of spermatozoa (%) in semen samples was determined using sperm quality analyzer (Johnston *et al.* 1995). Propidium iodide (PI) and fluorescein-labeled *Pisum sativum* agglutinin (FITC-PSA) were used to analyze sperm viability and acrosomal integrity using adaptations of the original protocols proposed by Way *et al.* (1995). Hypo-osmotic swelling test (HOST) was performed (Jeyendran *et al.* 1984) to evaluate the functional integrity of the sperm membrane.

Present address: ^{1,7}Scientist (mahaksinghivri@gmail.com), Indian Council of Agricultural Research (Regional Centre) for NEH Region, Barapani. ²M.V.Sc. Scholar, ³Principal Scientist, ⁴Senior Scientist, ^{5,8}Ph.D. Scholar, Germ Plasm Centre, Division of Animal Reproduction. ^{6,9}Scientist, Central Institute for Research on Cattle, Meerut.

The swelling pattern and swelling of sperm tail was observed by counting 200 cells for each sample at 400× magnification under a phase contrast microscope.

Semen processing: After initial examination of each ejaculate, semen sample was divided into 3 equal parts, part 1 was mixed with tris extender containing egg yolk powder, 5% (w/v), part 2 with tris extender with 10% (w/v) egg yolk powder, and part 3 with 20% (v/v) fresh egg yolk. A uniform extension rate (40 million progressively motile spermatozoa/ml) in egg yolk tris glycerol (EYTG) extender was followed to ensure that each 0.5 ml French straw contained 20 million progressively motile spermatozoa in each group. Filled straws were then subjected to a combined cooling with an equilibration period of 4 h at 5°C. The rack along with the straws was transferred to biological cell freezer for automated freezing. The freezing in biological cell freezer was carried @ 5°C/min for 4 to -10°C; 40°C/min for -10 to -100°C and 20°C/min for -100 to -140°C. Straws were then plunged into liquid nitrogen (-196°C) for storage until assayed. Following cryopreservation of ejaculates for 24 h, three straws were randomly collected, thawed at 37°C for 30 s and tested for individual progressive motility (post-thaw), viability, acrosome integrity and plasma membrane integrity.

Statistical analysis: To minimize variation in the subjective scoring system used in the present investigation, each sample was evaluated by two and the average was analyzed using SPSS software (SPSS version 17.0) computer program. Independent sample t-test was applied to determine mean, standard error and level of significance.

RESULTS AND DISCUSSION

At fresh stage, individual progressive motility, per cent live spermatozoa, per cent acrosome integrity and membrane integrity spermatozoa were similar among three groups. At post-thaw stage, individual progressive motility, live spermatozoa, acrosome integrity and plasma membrane integrity were significantly ($P<0.05$) higher in GI as compared to other 2 groups (Table 1).

Fresh egg yolk has been used for years in the diluents to freeze semen from different animal species (Salamon and Maxwell 1995). However, because of the heterogeneity in the composition of egg yolk between lots and because of the potential microbiological contamination risk (Bousseau *et al.* 1998), finding an egg yolk substitute is highly

desirable. Powdered egg yolk could be an alternative for fresh egg yolk, as this product undergoes a pasteurization process to destroy bacteria, in agreement with the laws established for the human consumption (Thibier and Guerin 2000), and the lots have a more homogeneous composition. During initial phase of this study another group with 20% egg yolk powder was taken, but due to low solubility of egg yolk powder and lesser microscopic visibility it was discarded for further study. Tris extender in groups 1 and 2 with egg yolk powder were given a heat treatment at 45°C for 15 min for increasing the solubility. With respect to the kinetic parameters, a decrease in the velocity parameters was observed when powdered egg yolk was used in the diluents (Marco-Jiménez *et al.* 2004). The decrease in sperm motility parameters could be because of increase in viscosity of the medium when powdered egg yolk is used. Miranda *et al.* (2000) reported that high temperatures reached during pasteurization process denatured egg yolk proteins, inducing them to have a more gel-like consistency in the medium after reconstitution leading to high diluent viscosity. Sperm motility and plasma membrane integrity are essential parameters used to assess quality of bovine semen. The evaluation of sperm plasmalemma integrity is of particular importance due to its involvement in metabolic exchanges with the surrounding medium (Jeyendran *et al.* 1984). Results in this experiment are in contrast with the earlier reports of use of egg yolk powder for semen cryopreservation of ovine and bull semen (Marco-Jiménez *et al.* 2004 and Ansari *et al.* 2010), in terms of egg yolk concentration i.e. in present experiment egg yolk powder provide better cryoprotection at 5% concentration in contrast to 20% in earlier experiments. The percentages of sperm with intact acrosomes were similar for both egg yolk sources at 20% concentration, but a significant increase in the percentage of total motile sperm (+9%) was observed when the sperm had been frozen in the diluent containing powdered egg yolk compared to fresh egg yolk (Macro-Jimenez *et al.* 2004). Several substances in egg yolk inhibited cell respiration and decreasing in number of motile cells (Pace and Graham 1974). It is likely that the pasteurization inhibits the activity of these compounds.

In conclusion, powdered egg yolk at 5% can be used, in place of fresh egg yolk, in the diluents for freezing buffalo sperm. However, different *in vitro* assays should be performed to study if sperm functionality is altered. At the same time, *in vivo* fertility trials need to be conducted to

Table 1. Effect of powdered vs fresh egg yolk as an additive on semen quality parameters for buffalo semen cryopreservation

Seminal quality parameters	Fresh	Post-thaw		
		Group 1 (with 5% egg yolk powder)	Group 2 (with 10% egg yolk powder)	Group 3 (with 20% fresh egg yolk)
Individual progressive motility	89.34±2.07	54.16±2.74 ^a	47.00±1.43 ^b	46.25±3.37 ^b
Viability	94.02±1.44	65.58±1.92 ^a	54.66±1.32 ^b	55.58±2.80 ^b
Acrosomal integrity	95.16±2.82	62.38±1.92 ^a	54.66±1.39 ^b	55.41±3.00 ^b
Plasma membrane integrity	95.20±2.54	62.25±2.09 ^a	51.08±1.63 ^b	50.00±2.67 ^b

The values with different letters in a row differ significantly ($P<0.05$).

determine if powdered egg yolk can maintain the fertility of cryopreserved buffalo sperm.

REFERENCES

- Andrabi S M H, Ansari M S, Ullah N, Anwar M, Mehmood A and Akhter S. 2008. Duck egg yolk in extender improves the freezability of buffalo bull spermatozoa. *Animal Reproduction Science* **104**: 427–33.
- Ansari M S, Rakha B A, Andrabi S M H and Akhter S. 2010. Usefulness of powdered and fresh egg yolk for cryopreservation of Zebu bull spermatozoa. *Reproductive Biology* **10** (3): 235–40.
- Bahtgate R, Maxwell W M and Evans G. 2006. Studies on the effect of supplementing boar semen cryopreservation media with different avian egg yolk types on *in vitro* post-thaw sperm quality. *Reproduction in Domestic Animals* **41**: 68–73.
- Bousseau S, Brillard J P, Marquant-Le Guienne B, Gue'rin B, Camus A and Lechat M. 1998. Comparison of bacteriological qualities of various egg yolk sources and the *in vitro* and *in vivo* fertilizing potential of bovine semen frozen in egg yolk or lecithin based diluents. *Theriogenology* **50**: 699–706.
- Jeyendran R S, Van der Ven H H, Perez-Pelaez M, Carbo B G and Zaneveld L J. 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *Journal of Reproduction and Fertility* **70**: 219–28.
- Johnston R C, Clarke G N, Liu D Y and Gordon Baker H W. 1995. Assessment of sperm quality analyser. *Fertility Sterility* **63** (5): 1071–76.
- Marco-Jiménez F, Puchades S, Mocé E, Viudes-de-Cartro M P, Vicente J S and Rodriguez M. 2004. Use of powdered egg yolk vs. fresh egg yolk for the cryopreservation of ovine semen. *Reproduction in Domestic Animals* **39**: 438–41.
- Miranda J, Guerrero A F and Partal P. 2000. Reología de derivados de la yema de huevo deshidratada. *Grasas y Aceites* **51**: 244–50.
- OIE, 2003. Terrestrial Animal Health Code. Appendix 3.2.1, Article 3.2.2.4, URL: http://www.oie.int/eng/normes/Mcode/A_summry.htm.
- Pace M M and Graham E F. 1974: Components in egg yolk which protect bovine spermatozoa during freezing. *Journal of Animal Science* **39**: 1144–49.
- Salamon S and Maxwell W M C. 1995. Frozen storage of ram semen. I. Processing, freezing, thawing and fertility after cervical insemination (review). *Animal Reproduction Science* **37**: 185– 249.
- Thibier M and Guerin B. 2000. Hygienic aspects of storage and use of semen for artificial insemination. *Animal Reproduction Science* **62**: 233–51.
- Way L A, Henault M A and Killian G J. 1995. Comparison of four staining methods for evaluating acrosome status and viability of ejaculated and cauda epididymal bull spermatozoa. *Theriogenology* **43**: 1301–16.