Global Journal of Biotechnology & Biochemistry 5 (4): 211-215, 2010
ISSN 2078-466X

Effect of Egg Yolk from Different Avian Species on Cryopreservability of Buffalo Semen

Reda I. El-Sheshtawy, Gamal A. El-Sisy, Alaa A. Mohamed and Walid S. El-Nattat

Department of Animal Reproduction and A.I., National Research Center, Egypt

Abstract: Semen samples were collected from four healthy buffalo bulls. Tris-extender combined with whole and clarified duck, quail or hen egg yolks were used. Semenal samples were frozen and evaluated for visual motility, plasma membrane integrity, sperm morphology, acrosomal integrity, live sperm cells percent, DNA fragmentation. Results revealed that the use of whole and clarified quail egg yolk significantly improved motility (78.0±1.22 and 79.0±3.67), acrosome integrity (75.2±3.27 and 84.8±1.88) and live sperm percent (78.8±3.96 and 82.8±2.85, respectively) compared to duck and hen egg yolks in cooled semen. On the other hand, all parameters were significantly improved in quail egg yolk after freezing-thawing compared to duck and hen egg yolks. We concluded that the quail egg yolk (whole and clarified) are better than chicken or duck egg yolks as an anti-cold shock when added to extenders in cryopreservation of buffalo semen.

Key words: Egg Yolk · Buffalo · Semen · Quail Egg Yolk · Duck Egg Yolks · Chicken Egg Yolks

INTRODUCTION

Cryopreservation has been applied as a routine technique for processing bovine sperm in artificial insemination (AI) and numerous studies have been carried out to evaluate fundamental biological properties [1, 2]. Techniques of cryopreservation resulted in the loss of 40-50% of viable sperm during the freezing-thawing process with little improvement over the last several decades [3-5]. Cold shock, osmotic stress, ice crystal formation or oxidative damage are the main sources of sperm cryoinjury and finally cause loss of sperm viability and fertility [6-9]. High survival and fertility rates of bull sperm in extender with 20% egg yolk have been achieved [10, 11]. Specifically, domestic chicken eggs yolk can help in resisting against cold shock in association with other components [8,12,13]. These protective effects are mainly attributed to the low-density lipoprotein (LDL) in the egg yolk [14-20]. Media containing yolk from eggs of avian species other than domestic chickens have been studied [21-23].

Evaluation of DNA integrity may have a priority in the design of new freezing thawing procedures to increase fertilization rates and ensure good embryo development, health of the offspring and maintenance of the genotypes to be preserved.

The objective of this study was to evaluate the effect of egg yolk of different species on cryopreservation of buffalo semen.

MATERIALS AND METHODS

Semen Collection and Initial Evaluation: Four healthy buffalo bulls maintained under common management conditions at Abassia Buffalo Semen Freezing Center (Animal Reproduction Division, General Organization for Veterinary Services, Ministry of Agriculture, Egypt) were used in the this study. Semen was collected using artificial vagina (AV) at 42°C on weekly intervals for a period of 5 weeks (replicates n = 5) for each experiment.Immediately after collection, semen samples were evaluated and those ejaculate having more than 70% progressive motility were used in processing.

Visual motility of each ejaculate was assessed at 37°C by using microscope attached with a closed circuit television. Sperm concentration was assessed by hemocytometer in a 1:200 dilution.

Ejaculates were pooled in order to have sufficient semen volume for a replicate and to eliminate the bull effect. The semen was kept at 37°C in water bath before dilution.

Corresponding Author: Walid S. El-Nattat, Department of Animal Reproduction and A.I., National Research Center, Egypt.
E-mail: prof_dr_wasamomo@yahoo.com.

Semen Processing

Preparation of Extender: The basic Tris extender consisted of 3.028 g Tris, 1.675 g citric acid monohydrate, 1.25 g fructose, 8 ml glycerol and 92 ml of glass redistilled water combined with 25 ml of egg yolk plus antibiotics (penicillin G sodium-1000 IU/ml; streptomycin sulphate 1000μg/ml).

Duck egg yolk (DEY) and quail (QEY) were used in freezing extender for comparison with hen egg yolk (HEY). Also effect of egg yolk clarification on freezability was studied

Egg yolk was harvested from fresh eggs of different avian species, mixed with distilled water (1:3) and centrifuged at 10,000×*g* for 50 min at 4°C. The supernatant (clarified egg yolk) was used for preparation of extenders, the ingredient of the basic extender were dissolved in 80 ml clarified egg yolk (20% egg yolk) and completed to 100 ml using distilled water as described by Wani *et al.* [24].

Three aliquots of semen were diluted at 37°C in a single step with one of the three experimental extenders (HEY, DEY and QEY) in order to obtain approximately 120×10^6 motile spermatozoa ml^{-1}.

After dilution, the semen was cooled to 4°C 2 h and equilibrated in an equilibration chamber for 4 h at 5°C (Minitub, Germany) before filling in 0.25 ml French straws. After filling and sealing, the straws were placed in a rack at 4cm above liquid nitrogen in the vaporous phase for 10 min and plunged into liquid nitrogen container (-196°C). Straws were stored in liquid nitrogen for 10 days. The frozen semen straws were thawed at 37°C for 30 s before evaluation.

Sperm Functional Assays: Theses assays were conducted on fresh, cooled and frozen-thawed buffalo spermatozoa

Visual Motility: A drop of thawed semen sample was placed on a pre-warmed glass slide and cover slipped. Sperm motility was evaluated at 400× magnification based on the visual estimation of the percentage of sperm possessing progressive motility and the percentage was rounded to nearest 5%.

Plasma Membrane Integrity: Plasma membrane integrity (PMI) of buffalo bull spermatozoa was assessed by hyposmotic swelling (HOS) assay as described earlier [25]. The solution of HOS contained sodium citrate 0.73 g and fructose 1.35 g, dissolved in 100 ml distilled water (osmotic pressure ~190 mOsmol kg^{-1}). The assay was performed by mixing 50 ul of frozen-thawed semen sample to 500 μl of HOS solution and incubated at 37°C for 40 min. After incubation, a drop of semen sample was examined under phase contrast microscope (X 400; Olympus BX40, Japan). Two hundred spermatozoa were counted for their swelling characterized by coiled tail indicating intact plasma membrane

Sperm Morphology: Total sperm morphological abnormalities (head, mid-piece and tail) of buffalo bull were determined.

Acrosomal Integrity: Acrosomal integrity percentage was evaluated at 1000X magnification by giemsa staining method as the procedure given by Watson [15].

Percent Live Sperm Cells Were Evaluated Using Eosin-nigrosin Staining Method [26]

DNA Fragmentation Using Acridine Orange Staining: Acridine orange staining was performed according to the method of Katayose *et al.* [27]. A stock solution of 0.1% acridine orange (3, 6-bis [dimethylamino] acridine, hemi[zinc chloride]salt, Sigma Chemical Co., St. Louis, MO) was made and stored in the dark at 4°C until use. At the time of staining, a working solution was prepared by mixing 4 parts acridine orange stock solution with 16 parts 0.1 M citrate and 1 part 0.3 M Na_2HPO_4 $7H_2O$. The acridine orange final concentration of the resultant phosphate-citrate buffered solution was adjusted to 0.019% (pH 2.5).

Firstly, semen samples were smeared on the glass slide. After being air dried, the samples were treated with acid alcohol (methyl alcohol-glacial acetic acid _ 3:1, vol/vol) for _2 hours. Immediate preparation is necessary to prevent natural oxidation of thiols in sperm nucleoprotein and the acid alcohol was made on the day of the experiment. Immediately after air drying, approximately 1 mL of working solution was mounted on each slide glass for 5 minutes at room temperature and the samples were then washed with distilled water. The samples were observed under an epifluorescent microscope (Olympus U-PMTVC5D 00637, Olympus, Tokyo, Japan) immediately after a cover glass was put in position. A total of 100 to 200 spermatozoa were observed and classified by type as green, red, or yellow, which is the intermediate type, based on differences in their fluorescent color.

Statistical analysis was performed on data of sperm motility, membrane integrity, acrosome integrity, live sperm, sperm abnormalities and fragmented DNA using SPSS statistics program ver. 14.0 (2005). Analysis of variance significance was accepted at least $P<0.05$.

RESULTS

From the present trials, we could conclude that the best results were obtained with diluent containing quail egg yolk and its clarified egg yolk. Improvement in sperm motility was observed when using clarified hen or duck egg yolk with obvious difference between normal and clarified egg yolk obtained with duck egg yolk.In the case of quail egg yolk, no detectable variation between the normal egg yolk and its clarified treatment could be found. The quail egg yolk and its clarified egg yolk gave a significant ($p<0.05$) higher motility than hen and duck egg yolk (Table 1).

The membrane integrity examined by HOS gave best result with raw quail and duck egg yolk than hen egg yolk, but the variation could not reach to the level of significant. Clarified quail egg yolk gave the highest value followed by clarified duck egg yolk and lastly hen egg yolk but without significant variation (Table 1).

Acrosome intactness estimated with geimsa stain revealed the best results with quail egg yolk than hen or duck egg yolk with remarked significant variation. For clarified egg yolk, the highest value obtained with quail egg yolk than hen followed by duck with significant ($p<0.05$) variation between quail and duck clarified egg yolk (Table 1).

The percentage of live sperm of semen samples diluted with extender containing quail egg yolk gave best results than the semen fraction extended with media containing either hen or duck egg yolk and the variation was significant ($p<0.05$). While, semen samples diluted in extender containing clarified egg yolk showed the highest value with quail egg yolk than others but without significant difference between quail egg yolk and duck egg yolk and absence of variation between hen and duck egg yolk (Table 1).

Both total sperm abnormalities and percentage of DNA fragmentation showed best results with quail egg yolk either raw or clarified than either hen or duck egg yolk without significant variation (Table 1).

Post-thaw sperm motility showed significant ($p<0.05$). variation among semen samples extended in different types of egg yolk with the best value obtained with quail egg yolk which was significantly ($p<0.05$). higher than

Table 1: Effect of adding yolks from different avian species in freezing extender on characteristics of buffalo semen after cooling and equilibrium

Treatments	Motility	Membraneintegrity	Acrosomeintegrity	Live sperm%	Sperm abnormalities	Fragmented DNA
HEY	69.0±4.00^{a}	74.8± 2.76^{a}	69.6±3.89^{a}	73.2±2.45^{a}	7.0±0.89^{a}	5.6±0.73^{a}
Clarified HEY	72.0±4.74^{A}	78.4±3.74^{A}	76.8±3.27AB	76.8±3.96^{A}	5.6±1.28^{A}	3.6±0.51^{A}
DEY	69.0±3.31^{a}	79.4±2.42^{a}	69.6±4.01^{a}	74.8±2.70^{a}	7.4±0.51^{a}	5.2±1.07^{a}
Clarified DEY	76.0±1.87^{A}	82.4±5.68^{A}	73.2±2.67^{A}	80.0±2.77^{AB}	5.2±1.16^{A}	3.6±0.51^{A}
QEY	78.0±1.22^{b}	79.2±3.29^{a}	75.2±3.27^{b}	78.8±3.96^{b}	4.4±1.03^{a}	4.0±0.71^{a}
Clarified QEY	79.0±3.67^{B}	85.0±2.91^{A}	84.8±1.88^{B}	82.8±2.85^{B}	3.6±0.68^{A}	3.4±1.67^{A}

HEY = hen egg yolk , DEY= duck egg yolk and QEY= quail egg yolk

Mean with different capital litter mean significant different in means of clarified yolk

Mean with different small litter mean significant different in means of yolk

P<0.05

Table 2: Effect of adding yolks from different avian species in freezing extender on post-thawing semen characteristics in buffaloes

Treatment	Motility	Membrane integrity	Acrosome integrity	Live sperm%	Sperm abnormalities	Fragmented DNA
HEY	36.0±2.91^{a}	48.0±4.20^{a}	43.0±1.50^{a}	59.0±2.5^{a}	18.6±1.36^{a}	12.4±1.66^{a}
Clarified HEY	38.0±2.00^{A}	52.6±3.07^{A}	51.8±5.86^{A}	66.6±0.93^{A}	16.2±1.50^{A}	12.0±1.14^{A}
DEY	37.0±1.22^{a}	58.6±1.75^{b}	51.8±2.98^{a}	57.0±3.04^{a}	16.8±2.42^{a}	12.2±1.46^{a}
Clarified DEY	39.0±1.87^{A}	59.4±1.36^{AB}	57.4±3.56^{AB}	64.6±4.25^{A}	14.0±2.07^{A}	11.2±1.28^{A}
QEY	44.0±2.23^{b}	67.6±2.99^{c}	64.2±3.78^{b}	69.0±1.43^{b}	12.2±1.74^{b}	10.4±0.81^{b}
Clarified QEY	43.0±4.47^{B}	75.4±1.80^{B}	66.0±3.56^{B}	75.8±2.02^{B}	10.8± 0.97^{B}	8.2±1.02^{B}

HEY = hen egg yolk , DEY= duck egg yolk and QEY= quail egg yolk

Mean with different capital litter mean significant different in means of clarified yolk

Mean with different small litter mean significant different in means of yolk

P<0.05

both hen and duck egg yolk. Results for clarified egg yolk showed the same values as raw egg yolk but without significance (Table 2).

Post-thaw membrane integrity showed significant ($p<0.05$). variations among different types of egg yolk with best values obtained for quail egg yolk followed by duck egg yolk and lastly the hen egg yolk. Clarified egg yolk showed no differences between quail and duck egg yolk and no differences between hen and duck egg yolk but there is a difference between hen and quail egg yolk (Table 2).

Post-thaw acrosome integrity showed significant ($p<0.05$). variation among different types of egg yolk with best value obtained for quail egg yolk and the lowest value obtained with hen egg yolk and the same results obtained with clarified egg yolk from the three species with best results with quail egg yolk and the lowest value with hen egg yolk (Table 2).

Post-thaw live sperm percentage, total sperm abnormalities and DNA fragmentation percentage revealed no significant variations among diluents containing hen or duck egg yolk either raw or clarified. In the same time diluents containing quail egg yolk either raw or clarified showed significant difference and best results than other egg yolk (Table 2).

DISCUSSION

The traditional use of chicken egg yolk in cryopreservation of spermatozoa of several mammalian species had given its wide availability. No available reports were found comparing the effects of egg yolk from domestic chicken, domestic duck, domestic goose, Japanese quail or domestic pigeon in extenders on bull sperm cryopreservation. The present study was designed to compare the cryoprotective effects of 20% egg yolk from different avian species (chicken, duck and quail) in extender on buffalo bull sperm cryopreservation. In addition, the effect of clarified egg yolk of different avian species on cryopreservation of buffalo bull semen was studied. The use of quail egg yolk had afforded good results in comparison with the chicken and duck egg yolks. Media containing yolk from eggs of avian species other than domestic chickens resulted in significantly higher motilities and longevities of frozen-thawed boar, Jackass or stallion sperm [21-23], this was attributed to the different components of fatty acid, phospholipids and cholesterol, of egg yolk from the avian of quail, duck or chicken, which resulted in different cryopreservative effects on sperm [28].

The substitution of quail egg yolk for chicken egg yolk for the cryopreservation of poitou jackass sperm has been shown to increase both post-thaw motility and the percentage of intact acrosomes (PIA) [29], this was due to significantly more phosphatidylcholine, less phosphatidylethanolamine and a smaller ratio of polyunsaturated to saturated fatty acids than chicken egg yolk [21].

It was concluded that the quail egg yolk (whole and clarified) are better than chicken or duck egg yolks as an anti-cold shock when added to extenders in cryopreservation of buffalo semen.

REFERENCES

1. Watson, P.F., 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod. Fertil. Dev., 7: 871-891.
2. Martinez, C.O., L. Juarez-Mosqueda Mde, J. Hernandez and J. Valencia, 2006. Cryopreservation of bull spermatozoa alters the perinuclear theca. Theriogenol., 66: 1969-1975.
3. Holt, W.V., 2000. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenol., 53: 47-58.
4. Watson, P.F., 2000. The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci., pp: 60-61, 481-492.
5. Prathalingam, N.S., W.V. Holt, S.G. Revell, S. Mirczuk, R.A. Fleck and P.F. Watson, 2006. Impact of antifreeze proteins and antifreeze glycoproteins on bovine sperm during freeze-thaw. Theriogenol., 66: 1894-1900.
6. Mazur, P., 1984. Freezing of living cells: mechanisms and implications. Am. J. Physiol., 247: C125-C142.
7. Alvarez, J.G. and B.T. Storey, 1992. Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. J. Androl., 13: 232-241.
8. Amirat, L., D. Tainturier, L. Jeanneau, C. Thorin, O. Gerard, J.L. Courtens and M. Anton, 2004. Bull semen *in vitro* fertility after cryopreservation using egg yolk LDL: a comparison with optidyl, a commercial egg yolk extender. Theriogenol., 61: 895-907.

9. Li, Y.H., K.J. Cai, L. Su, M. Guan, X.C. He, H. Wang, A. Kovacs and W.Z. Ji, 2005. Cryopreservation of cynomolgus monkey (*Macaca fascicularis*) spermatozoa in a chemically defined extender. Asian J. Androl., 7: 139-144.
10. Steinbach, J., R.H. Berndtson, M.B. Unal and B.W. Pickett, 1964. Effects of catalase and anaerobic conditions upon the post-thawing survival of bovine spermatozoa frozen in citrate-and Tris-buffered yolk extenders. J. Dairy. Sci., 47: 812-815.
11. Wall, R.J. and R.H. Foote, 1999. Fertility of bull sperm frozen and stored in clarified egg yolk-Tris-glycerol extender. J. Dairy. Sci., 82: 817-821.
12. Bogart, R. And D.T. Mayer, 1950. The effects of egg yolk on the various physical and chemical factors detrimental to spermatozoan viability. J. Anim. Sci., 9: 143-152.
13. Kulaksız, R., C. Cebi, E. Akcay and A. Daskın, 2010. The protective effect of egg yolk from different avian species uring the cryopreservation of Karayaka ram semen. Small Ruminant Res., 88: 12-15.
14. Pace, M.M. and E.F. Graham, 1974. Components in egg yolk which protect bovine spermatozoa during freezing. J. Anim. Sci., 39: 1144-1149.
15. Watson, P.F., 1975. The interaction of egg yolk and ram spermatozoa studied with a fluorescent probe. J. Reprod. Fertil, 42: 105-111.
16. Quinn, P.J., P.Y. Chow and I.G. White, 1980. Evidence that phospholipid protects ram spermatozoa from cold shock at a plasma membrane site. J. Reprod. Fertil, 60: 403-407.
17. Graham, J.K. and R.H. Foote, 1987. Effect of several lipids, fatty acyl chain length and degree of unsaturation on the motility of bull spermatozoa after cold shock and freezing. Cryobiol., 24: 42-52.
18. Babiak, I., J. Glogowski, M.J. Luczynski, M. Luczynski and W. Demianowicz, 1999. The effect of egg yolk, low density lipoproteins, methylxanthines and fertilization diluent on cryopreservation efficiency of northern pike (*Esox lucius*) spermatozoa. Theriogenol., 52: 473-479.
19. Moussa, M., V. Marinet, A. Trimeche, D. Tainturier and M. Anton, 2002. Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen-thawed bull semen. Theriogenol., 57: 1695-1706.
20. Bergeron, A. And P. Manjunath, 2006. New insights towards understanding the mechanisms of sperm protection by egg yolk and milk. Mol. Reprod. Dev., 73: 1338-1344.
21. Trimeche, A., M. Anton, P. Renard, G. Gandemer and D. Tainturier, 1997. Quail egg yolk: a novel cryoprotectant for the freeze preservation of Poitou jackass sperm. Cryobiol., 34: 385-93.
22. Clulow, J., W.M.C. Maxwell, G. Evans and L.H.A. Morris, 2004. A comparison between duck and chicken egg yolk for the cryopreservation of stallion spermatozoa. In: Proc. 15th Int. Cong. Anim. Reprod, Porto Seguro, Brazil, pp: 506(Abstract).
23. Bathgate, R., W.M.C. Maxwell and G. Evans, 2006. Studies on the effect of supplementing boar semen cryopreservation media with different avian egg yolk types on *in vitro* post-thaw sperm quality. Reprod. Dom. Anim., 41: 68-73.
24. Wani, N.A., M. Billah and J.A. Skidmore, 2008. Studies on liquefaction and storage of ejaculated dromedary camel (Camelus dromedarius) semen. Anim. Reproduction Sci., 109: 309-318.
25. Jeyendran, R.S., H.H. van der Ven, M. Perez Pelaez, B.G. Crabo and L.J.D. Zaneveld, 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J. Reprod. Fertil, 70: 219-228.
26. Barth, A.D. and R.J. Oko, 1989. Abnormal Morphology of Bovine Spermatozoa. Ames: Iowa State University Press, pp: 285.
27. Katayose, H., K. Yanagida, S. Hashimoto, H. Yamada and A. Sato, 2003. Use of diamide-acridine orange fluorescence staining to detect aberrant protamination of human-ejaculated sperm nuclei. Fertil Steril, 79(Suppl 1): 670-676.
28. Bair, C.W. and W.W. Marion, 1978. Yolk cholesterol in eggs from various avian species. Poult. Sci., 57: 1260-1265.
29. Trimeche, A., P. Renard and D. Tainturier, 1998. A procedure for poitou jackass sperm cryopreservation. Theriogenol., 50: 793-806.