Short Paper

Effect of different levels of egg yolk on ram sperm coating and preserving at 5°C

Rajabi-Toustani, R.[1]; Roostaei-Ali Mehr, M.[2*] and Motamedi-Mojdehi, R.[1]

[1]Graduated from Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran; [2]Department of Animal Science, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran

[*]***Correspondence:*** *M. Roostaei-Ali Mehr, Department of Animal Science, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran. E-mail: roostaei@guilan.ac.ir*

(Received 17 Aug 2013; revised version 3 Dec 2013; accepted 9 Dec 2013)

Summary

Experiment was conducted to evaluate the effect of different levels of egg yolk for coating and storing spermatozoa. Ejaculates were collected from four rams. In each session, second ejaculates (n=4) were collected in a tube containing 1 mL coating buffers which were prepared by 10, 15 or 20% egg yolk plus Tris-fructose. Samples were pooled, centrifuged and the supernatant removed in the laboratory. The pellets were diluted with Tris-fructose containing egg yolk which was equal to its concentration in coating. To perform four replicates for each sample, diluted sample was split into four parts then each part was split into two fractions. One of them (cold-shock) was suddenly put on ice water and the other was chilled at 0.25°C/min until 5°C. Aliquots were kept at 5°C for 36 h and sperm motility, viability and functional membrane integrity were determined at 0, 12, 24 and 36 h. The results showed that functional membrane integrity was highest in 20% yolk egg under gradual cooling at 0, 24 and 36 h (85.7, 75.9 and 71.7%, respectively; $P<0.05$). In the presence of 20% egg yolk, sperm viability was highest under cold-shock (74.8%) and gradual cooling (78.5%) and sperm motility was lower in cold-shock than gradual cooling at all storage times (76.9, 60.7, 36.2 and 27.7% vs. 85.4, 73.1, 54.6 and 40.8%, respectively; $P<0.05$). Therefore, 20% egg yolk can improve coated sperm longevity in normal gradual cooling; but it does not prevent the destructive effect of cold-shock from taking place.

Key words: Coated spermatozoa, Cold-shock, Egg yolk, Ram

Introduction

Several methods are demonstrated to improve the storage of cooled ram spermatozoa such as using different buffer, anti oxidative agent, applying different cooling methods, modifying the lipid content of the plasma membrane, etc. (Salamon and Maxwell, 2000; Motamedi-Mojdehi *et al*., 2013). It was reported that egg yolk plasma can replace egg yolk in stallion freezing extenders (Pillet *et al*., 2011). *In vitro*, coating spermatozoa with egg yolk, as a simple method, was shown to be useful for storing bull semen (De Pauw *et al*., 2003). In this, the method of coating spermatozoa, semen was collected in tubes containing the commercial diluents supplemented with egg yolk and then seminal plasma was removed. Recently it has been reported that longevity of frozen-thawed ram spermatozoa was improved by coating spermatozoa with egg yolk (Roostaei-Ali Mehr and Sharafi, 2013). Therefore, the aim of the present study was to determine the optimal level of egg yolk for coating and storing ram spermatozoa at 5°C.

Materials and Methods

Animals and semen collection

The study was performed on four healthy Taleshi rams aged between 3 and 5 years. Semen collection was performed by artificial vagina twelve times with two-day intervals between sessions in the breeding season. In each session, two ejaculates per ram were collected. In this experiment, second ejaculates (n=48) were used. To obtain coated spermatozoa, second ejaculates were collected in a tube containing 1 mL coating buffers. Coating buffers were prepared by 10, 15 or 20% egg yolk, 269 mM tris[hydroxymethyl]aminomethane, 89 mM citric acid monohydrate, 52 mM fructose, 2000 IU/mL penicillin G and 0.4 mg/mL streptomycin, (pH = 7.0). Sampling was repeated four times for each concentration of egg yolk. After collection, samples were transferred to laboratory in an insulated Styrofoam box (33°C) as quickly as possible.

Coated sperm preparation

The initial dilution was considered and all diluted ejaculates were tested to possess acceptable volume (>0.5 mL), motility (>70%) and concentration (>2.5 × 10^9 sperm/mL; Motamedi-Mojdehi *et al*., 2013). Samples were pooled, centrifuged for 10 min at 720 × g at room temperature and the supernatant removed (Roostaei-Ali Mehr and Sharafi, 2013). The pellets were diluted with Tris-fructose containing egg yolk up to 600 × 10^6 sperm/mL. The egg yolk concentration of Tris extender was equal to its concentration in the coating buffer that

had been used. To perform four replicates for each sample, diluted sample was split into four parts then each part split into two fractions (Fig. 1). One of them (cold-shock) was suddenly put on ice water for 15 min and other (no cold-shock) was gradually (0.25°C/min) chilled by Test Chamber (EG53AH, KATO, Japan) until 5°C. Aliquots were kept at 5°C for 36 h and sperm motility, viability and functional membrane integrity were determined at 0, 12, 24 and 36 h storage.

Sperm assessment

The concentration of spermatozoa was determined by means of a Neubauer haemocytometer. The percentage of sperm motility was assessed subjectively by phase-contrast microscopy (×400) on a warm stage at 37°C (Evans and Maxwell, 1987). The viability was assessed by means of a one-step eosin-nigrosin staining (Björndahl *et al*., 2003). The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane. The procedure was described by Jeyendran *et al*. (1992) and adapted for ram semen by García-Artiga (1994).

Statistical analysis

All data were analysed by the MIXED procedure of SAS (2002). The experiment was conducted by completely randomized design in a 3 × 2 factorial arrangement of the six treatment combinations as fixed effects and the four times of storage as a repeated measure. Pooled semen was considered as subjects in these experiments. The egg yolk levels, cooling method, time and their interaction effects were defined as class variables. Results are reported as least squares means (LSM) ± SE. Differences were considered to be statistically significant at $P<0.05$.

Results

The main effect of cooling rate showed that motility, viability and functional membrane integrity of spermatozoa in cold-shock were lower than gradual cooling (Table 1). There was an interaction between egg yolk concentration, cooling method and storage time on sperm motility (Fig. 2, $P<0.05$) and functional membrane integrity (Fig. 3, $P<0.05$). After 36 h under cold-shock, sperm motility in 20% egg yolk was higher (27.7 ± 3.9%) than 10% egg yolk (11.9 ± 3.5%, $P<0.05$). Under cold-shock and gradual cooling, functional membrane integrity was highest in 20% egg yolk at 0, 24 and 36 h ($P<0.05$). There was an interaction between egg yolk concentration and cooling method on sperm viability (Fig. 4, $P<0.05$). Sperm viability was highest in 20% egg yolk and there was no difference between cold-shock (74.8 ± 1.4%) and gradual cooling (78.5 ± 1.4%, $P>0.05$). There was an interaction between egg yolk concentration and storage time on sperm viability (Fig. 5, $P<0.05$). In each storage time, sperm viability was highest in 20% egg yolk ($P<0.05$).

Discussion

In this study, there was interaction between egg yolk concentration and storage time on sperm viability. Consequently, on sperm viability, ranking egg yolk

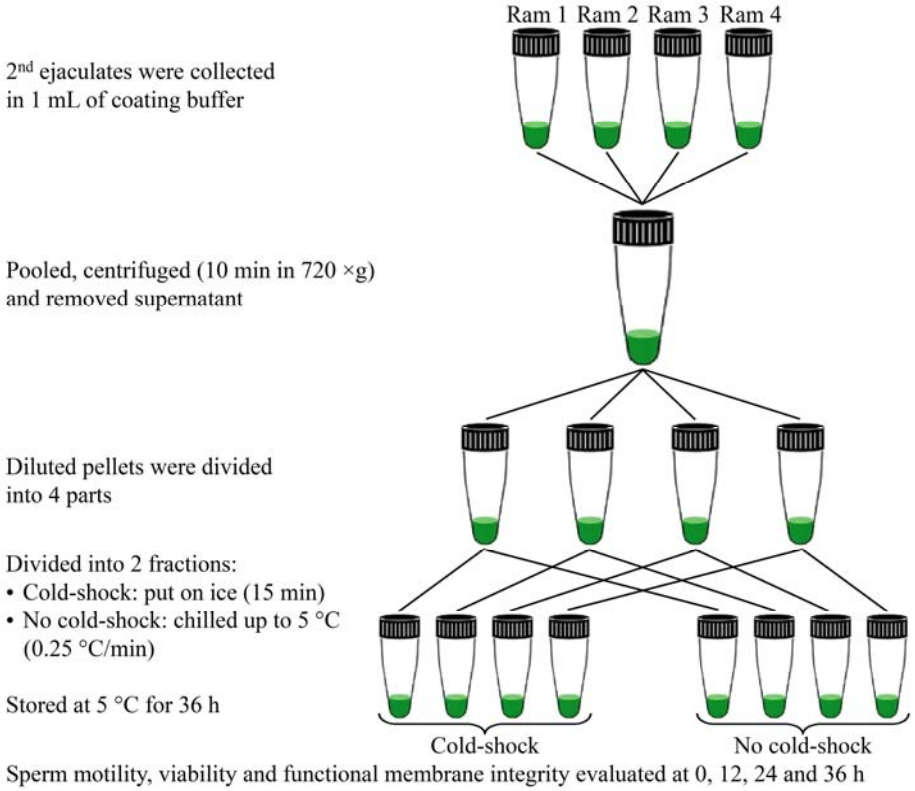

Fig. 1: Design of experiment

Table 1: Main effect of cooling rate and concentration of egg yolk on motility, viability and functional membrane integrity (HOST) of ram coated spermatozoa

Source of variation		Motility (%)	Viability (%)	HOST (%)
Cooling rate	No cold-shock	63.5 ± 1.4^{a}	72.4 ± 0.7^{a}	73.1 ± 0.8^{a}
	Cold-shock	45.5 ± 1.4^{b}	63.0 ± 0.7^{b}	60.7 ± 0.8^{b}
Egg yolk (%)	10	49.8 ± 1.7^{b}	63.6 ± 0.9^{b}	61.0 ± 0.9^{c}
	15	56.8 ± 1.7^{a}	62.7 ± 0.9^{b}	65.9 ± 0.9^{b}
	20	56.9 ± 1.9^{a}	76.6 ± 1.0^{a}	73.9 ± 1.0^{a}

Different superscripts within main effect denote significant differences (P<0.05). Presented data are LSM ± SE

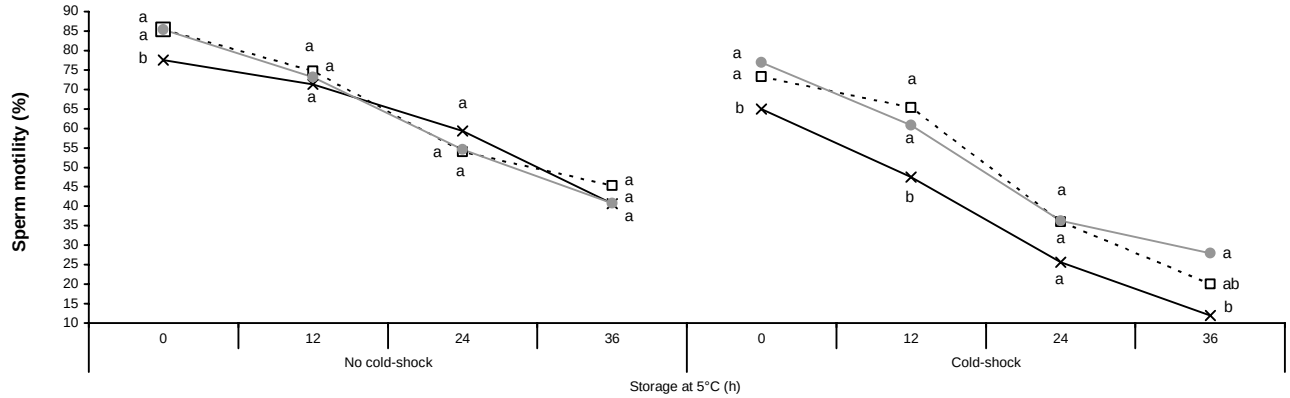

Fig. 2: Percentage of motility of ram spermatozoa were coated and stored with 10 (×), 15 (-□-) or 20% (●) egg yolk. Different superscripts indicate significant differences among treatments at each storage time (P<0.05)

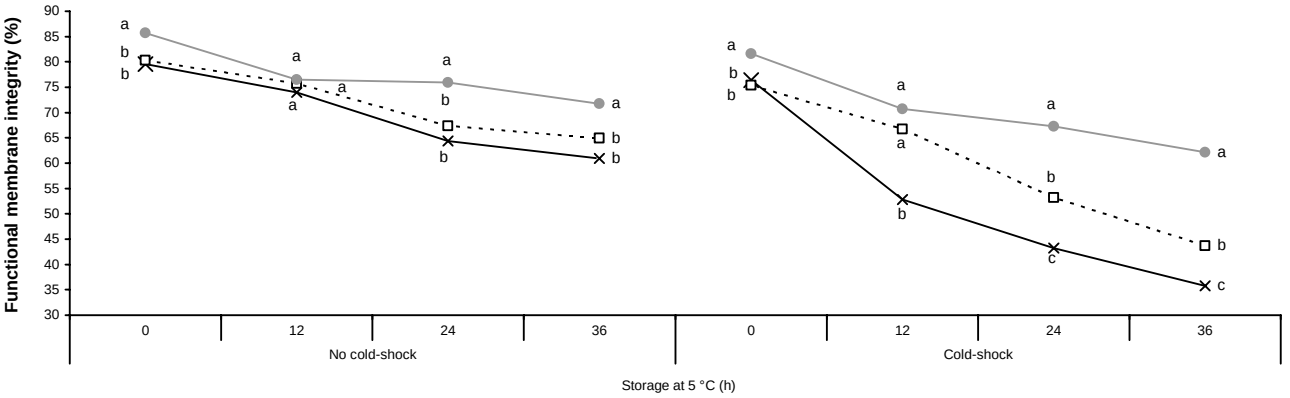

Fig. 3: Percentage of functional membrane integrity of ram spermatozoa were coated and stored with 10 (×), 15 (-□-) or 20% (●) egg yolk. Different superscripts indicate significant differences among treatments at each storage time (P<0.05)

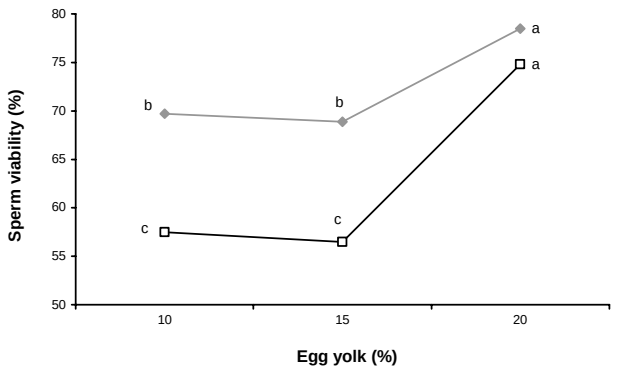

Fig. 4: The percentage of viability of ram spermatozoa were coated and stored with different levels of egg yolk under cold-shock (□) and no cold-shock condition (♦). Different superscripts indicate significant differences among treatments (P<0.05)

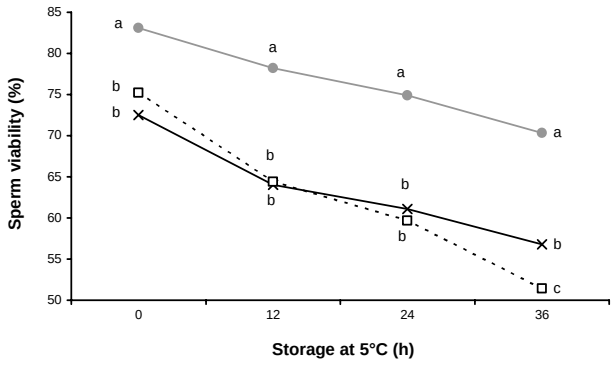

Fig. 5: The percentage of viability of ram spermatozoa coated and stored with 10 (×), 15 (-□-) or 20% (●) egg yolk during storage at 5°C. [a-c] Different superscripts indicate significant differences among treatments within storage time (P<0.05)

concentrations in different storage times were not the same (Zar, 1999). However, 20% egg yolk improved sperm viability and functional membrane integrity, although there was no difference between 15 and 20% egg yolk on sperm motility. We conclude that using 20% egg yolk can be helpful to prepare and store coated ram spermatozoa. It was reported that sperm coating by 20% egg yolk can preserve sperm viability and penetrating capacity of fresh bovine spermatozoa up to 6 days (De Pauw *et al*., 2003). Moreover, in the process of freezing-thawing, longevity of ram spermatozoa is improved by using 15% egg yolk in sperm coating (Roostaei-Ali Mehr and Sharafi, 2013). It was illustrated that ram seminal plasma proteins form stable complexes with low-density fraction of egg yolk (Bergeron *et al*., 2005). Moreover, the availability of seminal plasma proteins is altered by egg yolk (Leahy *et al*., 2010). It has been shown that the components of low-density fraction of egg yolk bind rapidly to seminal plasma proteins (Manjunath *et al*., 2002; Manjunath and Thérien, 2002) and compete with detrimental seminal plasma cationic peptides 5 kDa in binding to the sperm membrane (Vishwanath *et al*., 1992; Manjunath *et al*., 2002; Bergeron *et al*., 2004). According to one report, long-term exposure of bull sperm to bovine seminal plasma (BSP) proteins or exposure to too large concentrations of them can be deleterious to the sperm membrane (Manjunath *et al*., 2002). Furthermore, it is suggested that egg yolk acts as a counteracted factor with detrimental components of ram seminal plasma (Roostaei-Ali Mehr and Sharafi, 2013). Therefore, it seems that 20% egg yolk may be superior to lower concentration of egg yolk to prepare liquid ram semen via sperm coating.

The results showed that the functions of coated sperm were decreased after cold-shock at all of the egg yolk concentrations, although sperm viability was not decreased significantly in the presence of 20% egg yolk. Consequently, it seems that the high level of egg yolk (20%) cannot prevent the detrimental effect of cold-shock on ram spermatozoa from taking place. During cooling, the architecture of the plasma membrane undergoes modifications and there is a redistribution of membrane proteins that are excluded from the phospholipids regions when phospholipids change from fluid to gel phase (Pena *et al*., 2003b). These membrane changes have been shown to impair the function of calcium ion channels and the Ca^{2+} pump, keeping Ca^{2+} concentration under the critical threshold and triggering acrosome reaction and subsequently cell death (Pena *et al*., 2003a).

In conclusion, it can be said that the high concentration of egg yolk (20%) is helpful to prepare ram spermatozoa via coating method and normal gradual cooling. But, sudden cooling induced distractive effect on coated spermatozoa in presence of 20% egg yolk.

References

Bergeron, A; Crête, MH; Brindle, Y and Manjunath, P (2004). Low-density lipoprotein fraction from hen's egg yolk decreases the binding of the major proteins of bovine seminal plasma to sperm and prevents lipid efflux from the sperm membrane. Biol. Reprod., 70: 708-717.

Bergeron, A; Villemure, M; Lazure, C and Manjunath, P (2005). Isolation and characterization of the major proteins of ram seminal plasma. Mol. Reprod. Dev., 71: 461-470.

Björndahl, L; Söderlund, I and Kvist, U (2003). Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. Hum. Reprod., 18: 813-816.

De Pauw, IMC; Van Soom, A; Maes, D; Verberckmoes, S and de Kruif, A (2003). Effect of sperm coating on survival and penetrating ability of *in vitro* stored bovine spermatozoa. Theriogenology. 59: 1109-1122.

Evans, G and Maxwell, WMC (1987). Handling and examination of semen. In: Evans, G and Maxwell, WMC (Eds.), *Salamon's artificial insemination of sheep and goats*. (1st Edn.), Butterworths, Sydney, Australia, Butterworth. PP: 97-98.

García-Artiga, C (1994). Test de endósmosis en ovino. In: *7th International Meeting on Animal Reproduction*. Murcia, Spain. PP: 77-81.

Jeyendran, RS; Van-der-Ven, HH and Zaneveld, LJ (1992). The hypoosmotic swelling test: an update. Arch. Androl., 29: 105-116.

Leahy, T; Marti, JI; Evans, G and Maxwell, WM (2010). Seasonal variation in the protective effect of seminal plasma on frozen-thawed ram spermatozoa. Anim. Reprod. Sci., 119: 147-153.

Manjunath, P; Nauc, V; Bergeron, A and Ménard, M (2002). Major proteins of bovine seminal plasma bind to the low-density lipoprotein fraction of hen's egg yolk. Biol. Reprod., 67: 1250-1258.

Manjunath, P and Thérien, I (2002). Role of seminal plasma phospholipid binding proteins in sperm membrane lipid modification that occurs during capacitation. J. Reprod. Immunol., 53: 109-119.

Motamedi-Mojdehi, R; Roostaei-Ali Mehr, M and Rajabi-Toustani, R (2013). Effect of different levels of glycerol and cholesterol-loaded cyclodextrin on cryosurvival of ram spermatozoa. Reprod. Dom. Anim., 49: 65-70.

Pena, A; Lugilde, LL; Barrio, M; Becerra, JJ; Quintela, LA and Herradon, PG (2003a). Studies on the intracellular Ca^{2+} concentration of frozen-thawed dog spermatozoa: influence of Equex of different sources, two thawing diluents and post-thaw incubation in capacitating conditions. Reprod. Dom. Anim., 38: 27-35.

Pena, A; Lugilde, LL; Barrio, M; Herradon, PG and Quintela, LA (2003b). Effects of Equex from different sources on post thaw survival, longevity and intracellular Ca^{2+} concentration of dog spermatozoa. Theriogenology. 59: 1725-1739.

Pillet, E; Duchamp, G; Batellier, F; Beaumal, V; Anton, M; Desherces, S; Schmitt, E and Magistrini, M (2011). Egg yolk plasma can replace egg yolk in stallion freezing extenders. Theriogenology. 75: 105-114

Roostaei-Ali Mehr, M and Sharafi, F (2013). Influence of sperm coating and seminal plasma on frozen-thawed spermatozoa. Iranian J. Vet. Res., 14: 305-312.

Salamon, S and Maxwell, WMC (2000). Storage of ram semen. Anim. Reprod. Sci., 62: 77-111.

SAS (2002). *SAS® User's Guide: Statistics*. Version 9, SAS Institute Inc., Cary, NC, USA.

Vishwanath, R; Shannon, P and Curson, B (1992). Cationic extracts of egg yolk and their effects on motility, survival and fertilizing ability of bull sperm. Anim. Reprod. Sci., 29: 185-194.

Zar, JH (1999). *Biostatistical analysis*. 4th Edn., Upper Saddle River, New Jersey, USA, Prentice Hall. P: 282.