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Effect of different extenders on recovery and storage of epididymal ram spermatozoa

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Keywords

milk; tris; sodium citrate; caproic acid

Abstract

This experiment was conducted to determine effect of different extender on recovery and storage of epididymal ram spermatozoa. Testes were obtained from eight mature rams which were slaughtered at four different occasions, a pair in each session. Caudal epididymis was isolated and divided to four equal parts. Spermatozoa recovery from each part of the caudal epididymis was performed by milk (M), Tris (T), Tris plus caproic acid (T-C) and sodium citrate (S) extenders. Spermatozoa in each extender were divided into four parts and then the amount of 0 (E0), 5 (E5), 10 (E10) and 15% (E15) egg yolk were added. Samples were chilled to 4 °C and stored for 7 days. The evaluation of sperm motility, viability, membrane integrity and acrosome integrity were performed daily. The results showed that there was interaction between egg yolk and storage time and also egg yolk and extender on sperm motility ($p < 0.05$). Sperm motility was highest at 15 % egg yolk (25.6 %) on 7 d ($p < 0.05$). Sperm motility was higher in ME15 (57.8 %) and T-CE15 (62.3 %) than TE15 (53.4 %) and SE15 (41.9 %; $p < 0.05$). There was interaction among egg yolk, extender and storage time on acrosome integrity, membrane integrity and sperm viability ($p < 0.05$). On 7 d, there were no differences among ME15, T-CE15 and TE15 on sperm viability and membrane integrity. Acrosome integrity was higher in T-CE15 (75.25 %) than ME15 (62.5 %) on 7 d ($p < 0.05$). Therefore, Tris caproic extender was superior to other extender for recovery and storage of ram epididymal spermatozoa.

Abbreviations

M: Milk extender
T: Tris extender
T-C: Tris plus caproic acid extender
S: Sodium citrate extender
E0: 0% Egg yolk
E5: 5 % Egg yolk
E10: 10% Egg yolk
E15: 15 % Egg yolk

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Introduction

In sheep, acceptable fertility was obtained by using uterine insemination with frozen-thawed ram spermatozoa via laparoscopy (Kumar & Naqvi, 2014). Sheep breeders because of low cost prefer vaginal insemination as compared to intra-uterine insemination. Nowadays, vaginal insemination is performed normally by fresh or stored ram semen in liquid form. Therefore, optimizing extenders is an essential issue for the proper storage of sperm at 4 °C.

The in vitro production of mammalian embryos is used commonly for research, genetic improvement or commercial purposes. It was well known that epididymal spermatozoa can fertilize oocytes in vitro (Kaabi et al., 2003) and also in vivo (Morris et al., 2002). However, the technique of obtaining spermatozoa from caudal epididymis is available for best sires which strike neuroskeletal disorders during breeding season to fertilize female gamete (Kaabi et al., 2003). In addition, epididymal spermatozoa are used in the propagation and conservation of animal specimens with high genetic values from dead animals (Dong et al., 2008) and endangered species (Santiago-Moreno et al., 2006).

It was mentioned that epididymal ram spermatozoa are resistant to chilling and other environmental stresses, when compared to ram ejaculated spermatozoa (Varisli et al., 2009). However, there are few comparative studies about the effect of extenders routinely used in ram ejaculated spermatozoa cryopreservation on epididymal sperm viability, and there are no data about effects of different extenders on the recovery of ram epididymal spermatozoa. Therefore, the aim of the present study was to evaluate effect of different extenders on the recovery and storage of ram epididymal sperm at 4°C.

Materials and Methods

This experiment was conducted at The University of Guilan, Faculty of Agricultural Sciences, Education Research and Practice Farm, South of Rasht (it is located at 37° 12′ North latitude and 49° 39′ longitudes) during autumn.

Four extenders were used in this study included: Tris extender (36.3 mg/mL tris[hydroxymethyl] aminomethane, 19.9 mg/mL citric acid monohydrate, 5 mg/mL d-(+)-Glucose and 25 µg/mL gentamycin at pH 7.0), Tris plus caproic acid (0.03125% caproic acid added to Tris extender), sodium citrate (2.37% aqueous solution of trisodium citrate, 0.5% glucose and 25 µg/mL gentamycin) and standard milk extender (11% w/v non-fatty milk powder, 0.5% glucose and distilled water were mixed, heated to 95°C for 10 min, and then mixture was cooled to room temperature and 25 µg/mL gentamycin was added).

Experimental design

Eight mature Taleshi rams, aged between 3 and 4 years, were used. They were healthy and fertile. A ram was slaughtered in each session and after that the testes were separated and placed in sterile plastic containers, containing normal saline (0.9% NaCl), and the lids tightly sealed. They were transported to the laboratory at 4-6 °C (Lone et al., 2011).

The procedure of sperm recovery and preparation and also sperm treatment were performed at room temperature. To perform recovery of epididymal spermatozoa, caudal epididymis of each testis was carefully isolated by scalpel and divided into four equal parts. Each part was suspended in Petri dish containing 5 mL milk (M), Tris (T), Tris plus caproic acid (T-C) and sodium citrate (S) extenders. The samples were agitated and incubated at room temperature for 10 min; liquid phase of different extenders (M, T, T-C and S) containing epididymal spermatozoa was collected.

Samples were evaluated to possess acceptable motility (>70%) and spun for 10 min at 700×g at room temperature; the supernatant was removed. The pellets were diluted up to 1×10^9 cell/mL with the same extender which was used in procedure of epididymal sperm recovery. The obtained spermatozoa from each right or left epididymis were pooled in each of four extenders. Each sperm sample colleted in each individual extender was also split into four tubes and the amounts of 0 (E0), 5 (E5), 10 (E10) or 15% (E15) egg yolk were also added. The final concentration of spermatozoa was 500×10^6 sperm/mL (Fig 1). The samples were chilled to 4 °C (0.25 °C/min) and stored for 7 days in Test Chamber (KATO, Japan). Sperm viability, motility, membrane integrity and acrosome integrity were evaluated daily.

Sperm assessment

The concentration of spermatozoa was determined by means of a Neubauer haemocytometer.

The percentage of sperm motility was assessed by phase-contrast microscopy (400× magnification) on a warm stage at 37 °C. Samples were diluted with 1:8, and then, a wet mount was made using a 5 µL drop of semen placed directly on a microscope slide and covered by a cover slip. Sperm motility was estimated in 3-5 different microscopic fields for each sample. The subjective estimations were approximated to the nearest 5 % by a technician. The mean of the successive estimations was recorded as the final motility score (Evans and Maxwell 1987).

The hypo-osmotic swelling test (HOST) was used to evaluate the integrity of the sperm membrane. HOST was performed by incubating 5 µL of semen with 500 µL of a 100 mOsm hypo-osmotic solution (7.35 g sodium citrate dehydrate and 13.51 g fructose in 1 L distilled water) at 37 °C for 30 min. One drop of the mixture was placed on a pre-warmed slide, covered with a cover slip and examined under a phase-contrast microscope (Olympus IX70, 400 × magnifications). The sperm with the coiled swollen tails

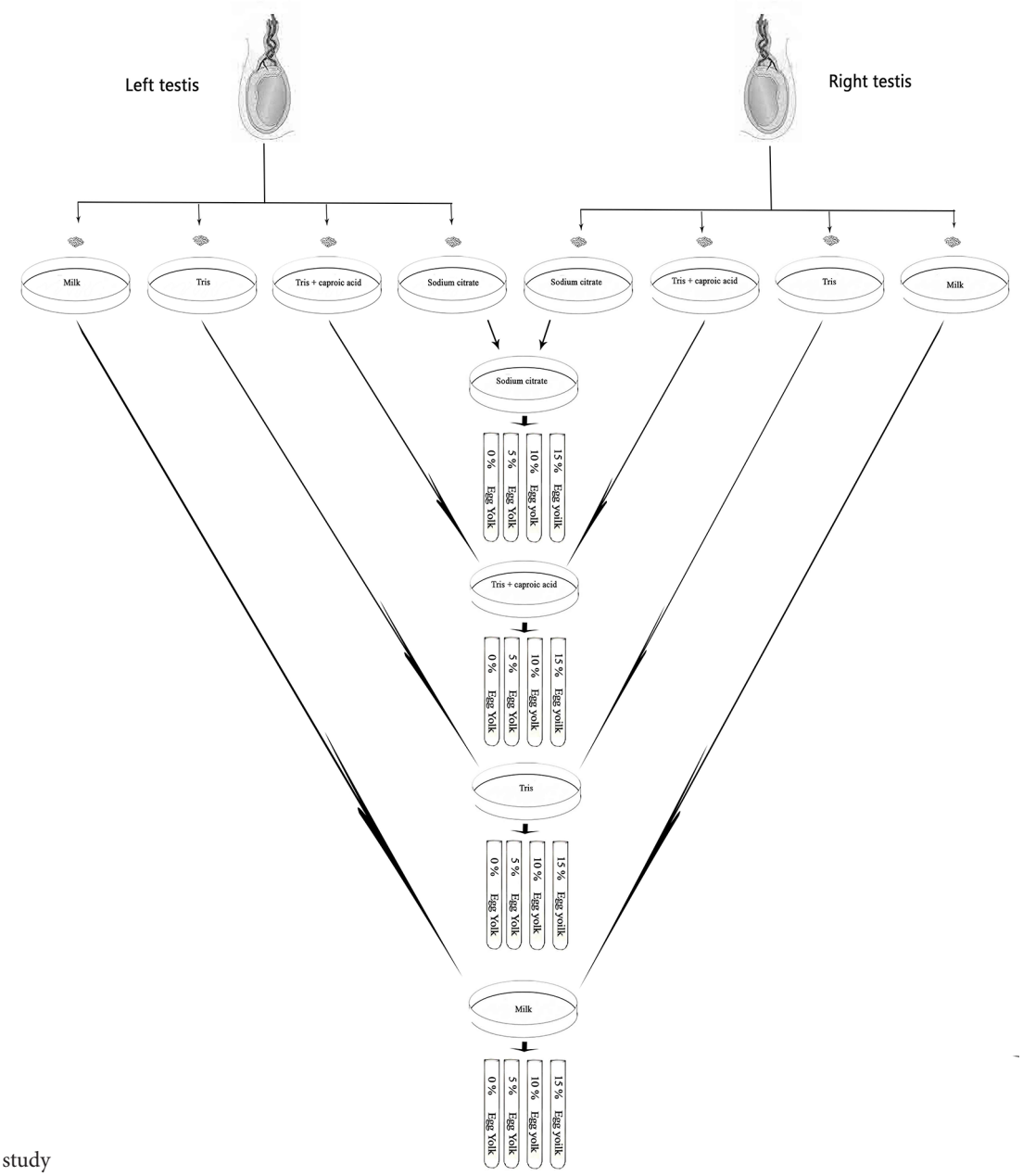

Figure 1
Design of this study

were considered intact (Jeyendran et al., 1992). To assess the percentages of intact sperm, a total of 100 sperm were evaluated from at least five different microscopic fields.

Spermatozoa viability was assessed by the fix vital stain method (De Leeuw et al., 1991). Briefly, sample was mixed with an equal volume of glutaraldehyde fixative solution (2% glutaraldehyde in phosphate-buffered saline) then it was mixed with an equal volume of 20 µg/mL bisbenzimide H33258. A smear was prepared after 10 minutes of incubation at room temperature. Two hundred spermatozoa per smear were evaluated in 3-7 different microscopic fields for each sample using an Olympus IX70, phase-contrast microscope (high- pressure mercury illuminator, UG1 excitation filter, U dichroic mirror, L420 barrier filter; Olympus, Tokyo, Japan). The procedure was performed by epifluorescence microscopy combined with bright-field illumination. Light intensity of the microscope is set at an optimum for visualization of both spermatozoa and fluorescence of H33258-labeled nuclei (Roostaei-Ali Mehr et al. 2015).

Acrosome integrity was determined by phase-contrast microscope (600× magnification) in a flat drop of sample after glutaraldehyde-fixation of spermatozoa. The spermatozoon head was scored as intact or missing (reacted), 200 spermatozoa were counted for each slide and percentage of spermatozoa with normal acrosome was noted (Varisli et al. 2009).

Statistical analysis

All data on motility, viability, functional membrane integrity and acrosome integrity of sperm were recorded from 0 to 7 d after incubation at 4 °C and analyzed by the

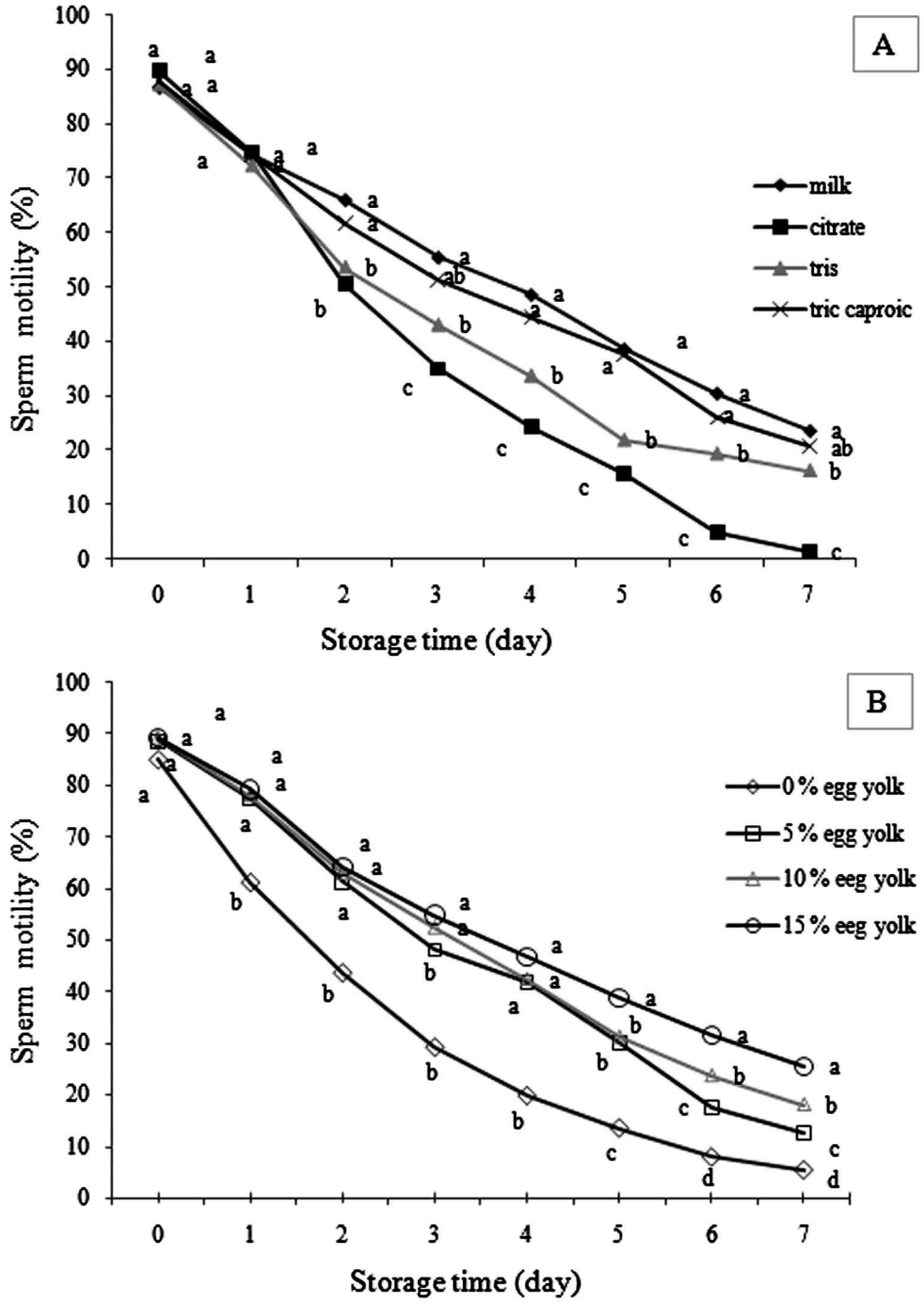

Figure 2
Interaction effect between extender and storage time (A) and between egg yolk and storage time (B) on motility of epididymal spermatozoa. a-d Different superscripts indicate significant differences within incubation time ($p < 0.05$).

MIXED procedure of SAS (SAS Institute Inc 2002). The statistical design was a 4×4 factorial arrangement of the sixteen treatment combinations as fixed effects and the eight incubation times as a repeated measure. Obtained epididymal spermatozoa were considered as subjects in this experiment. The egg yolk levels, extenders, storage time and their interactions 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 effects of extender, egg yolk and storage time on sperm motility, viability, membrane integrity and acrosome integrity are presented in Table 1. Sperm motility was higher in milk and Tris plus caproic acid extenders than Tris and sodium citrate extenders ($p < 0.05$). Sperm viability, response to HOST and acrosome integrity were lowest and highest in sodium citrate and milk extenders, respectively ($p < 0.05$). Sperm motility, viability, membrane in-

tegrity and acrosome integrity were lowest at 0 % egg yolk ($p < 0.05$). Sperm motility was highest at 15 % egg yolk ($p < 0.05$). In the presence of egg yolk, there was no difference among egg yolk levels on sperm viability, membrane integrity and acrosome integrity ($p > 0.05$). Sperm motility, viability, membrane integrity and acrosome integrity were highest and lowest on day 0 and day 7 of storage time ($p < 0.05$).

There was interaction between extender and storage time (Fig. 2 A) and also between egg yolk and storage time (Fig. 2 B) on sperm motility ($p < 0.05$). After 4, 5 and 6 days storage at 4 °C, sperm motility was higher in M (48.8±1.98, 38.8±1.98 and 30.6±1.98 %, respectively) and T-C (44.4±1.98, 37.5±1.98 and 25.9±1.98 %, respectively) than other extenders ($p < 0.05$). On day 5, 6 and 7, sperm motility was highest at 15 % egg yolk (38.8±1.98, 31.6±1.98 and 25.6±1.98 %, respectively; $p < 0.05$).

There was interaction between extender and egg yolk (Fig. 3) on sperm motility ($p < 0.05$). The highest sperm motility was observed in M (49.4 ± 2.23) at 0 % egg yolk ($p < 0.05$). At 5, 10 and 15% concentrations of egg yolk, sperm motility was higher in M (51.6 ± 2.23, 53.8 ± 2.23 and 57.8 ± 2.23 %, respectively) and T-C (53.1 ± 2.23, 57.5 ± 2.23 and 62.3 ± 2.23 %, respectively) than other extenders ($p < 0.05$).

There was interaction among extender, egg yolk and storage time on sperm viability, membrane integrity and acrosome integrity (Fig. 4, $p < 0.05$). At 0 % egg yolk, sperm viability was highest in M (72.8 ± 4.22 %) on 7 d ($p < 0.05$). At 5 % egg yolk, sperm viability was higher in M (75.7 ± 4.22 %) and T-C (69.5 ± 4.22 %) than other extenders on 7 d ($p < 0.05$). After 7 d storage at 4 °C and in the presence of 10 or 15 % egg yolk, the lowest sperm viability (13.8 ± 4.22 % and 15.8 ± 4.22 %, respectively) and the lowest membrane integrity (10.0 ± 4.80 % and 13.8 ± 4.80 %, respectively) were in S ($p < 0.05$) and there was no difference among other treatments ($p > 0.05$). At 5 % egg yolk, the highest membrane integrity was achieved by M (63.3 ± 4.80 %) on 7 d ($p < 0.05$).

On day 6 and 7, the highest acrosome integrity was observed in M (76.5 ± 4.39 % and 70.0 ± 4.39 %, respectively) at 0 % egg yolk (Fig. 4, $p < 0.05$). In presence of 5 % egg yolk, acrosome integrity was higher in T-C (69.0 ± 4.39 %) and M (73.0 ± 4.39 %) than other treatments after 7 days storage ($p < 0.05$). At 10 % egg yolk, acrosome integrity was lowest in S (17.5 ± 4.39 % and 16.25±4.39 %, respectively) on 6 and 7 d ($p < 0.05$) and there was no difference among other treatments ($p > 0.05$). On 6 and 7 d, acrosome integrity was higher in T-C (78.5 ± 4.39 % and 75.25±4.39 %, respectively) than M (64.5 ± 4.39 % and 62.5 ± 4.39 %, respectively) in the presence of 15 % egg yolk ($p < 0.05$).

Discussion

Oxidative, osmotic or acidity stress is an important consideration when preparing spermatozoa for long-term cold storage. Detrimental effects of these stresses can be reduced considerably using an appropriate medium.

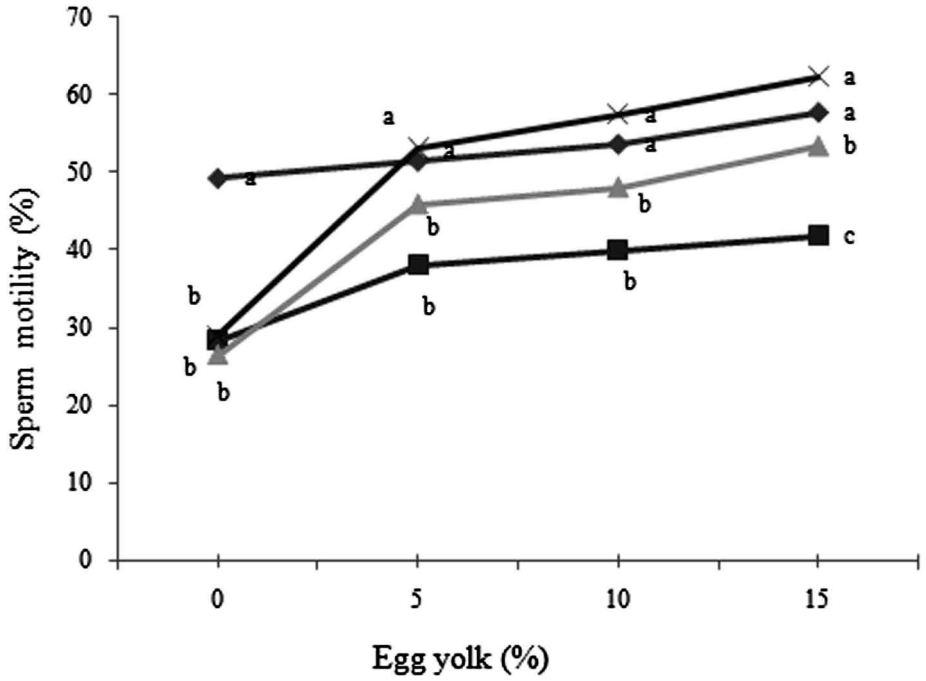

Figure 3
Percentage of motility of epididymal spermatozoa recovered and stored with either skim milk (♦), Tris (▲), Tris plus caproic acid (X) or sodium citrate (■) and either 0, 5, 10 % or 15 % egg yolk. a-c Different superscripts indicate significant differences within egg yolk level ($p < 0.05$).

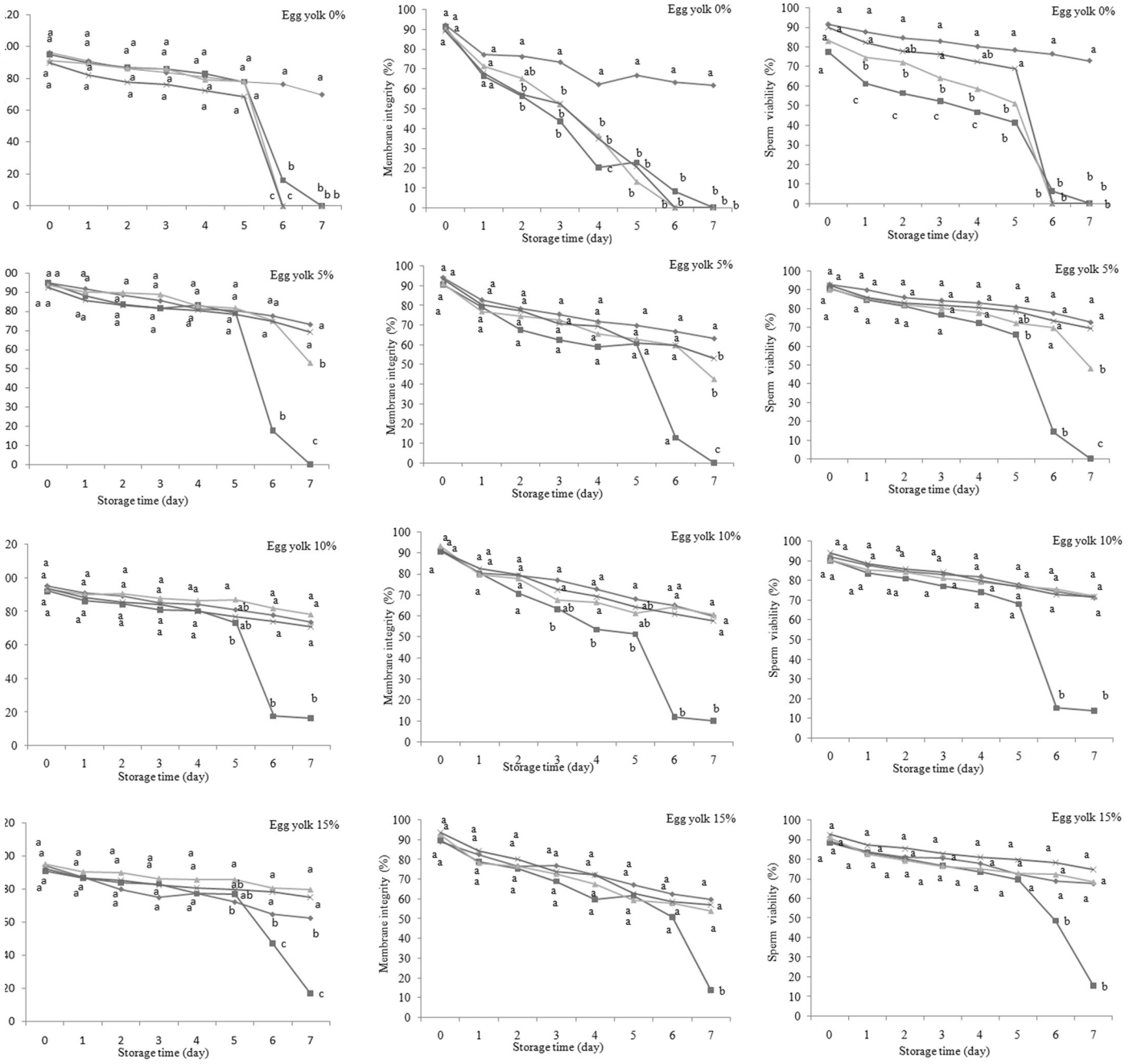

Figure 4

Percentage of viability, membrane integrity and acrosome integrity of epididymal spermatozoa recovered and stored with either skim milk (♦), Tris (▲), Tris plus caproic acid (X) or sodium citrate (■) and either 0, 5, 10 % or 15 % egg yolk for 7 d. a-d Different superscripts indicate significant differences within incubation time ($p < 0.05$).

The results showed sperm motility was higher in M and T-C extender than T and S extender after 4 d storage at 4 °C (Fig. 2 A). After two-day storage, sperm motility was more than 60 % in skim milk and Tris plus caproic acid extender only. Therefore, our data demonstrate that the application of skim milk and Tris plus caproic acid extender for recovery and short-term storage of epididymal ram spermatozoa is superior to Tris and sodium citrate extender. Sperm motility is an important criterion in sperm evaluation in vitro, although, there is some controversy over correlation between sperm movement characteristics and sperm fertility (Wierzbowski et al., 1993). In vitro and in vivo studies showed that skim milk and commercial Tris-based extenders did not differ significantly in performance of fresh ram semen (O'Hara et al., 2010; Roostaei-Ali Mehr et al., 2013). But, it was reported that sperm motility and fertility were significantly higher in milk and Tris plus caproic acid extenders compared to Tris extender after storing ram semen more than 24 h at 5 °C (López-Sáez et al., 2000; Roostaei-Ali Mehr et al., 2013). Moreover, Paulenz et al. (2002) showed significant decrease in sperm motility after using Tirs extender over time under

Table 1
Main effects of extender, egg yolk and storage time on motility, viability, membrane integrity (HOST) and acrosome integrity of ram epididymal spermatozoa during six days of storage at 4°C (LSMean ± SE)

Variables		Motility (%)	Viability (%)	Acrosome Integrity (%)	Host (%)
Extender	Skim milk	53.1 [a] ± 1.12	81.0 [a] ± 1.19	82.1[a] ± 1.04	73.6 [a] ± 1.19
	Tris-Caproic	50.5 [a] ± 1.12	75.8 [b] ± 1.19	75.9 [c] ± 1.04	64.0 [b] ± 1.19
	Tris	43.5 [b] ± 1.12	71.0 [c] ± 1.19	79.9 [b] ± 1.04	62.0 [b] ± 1.19
	Sodium citrate	37.1 [c] ± 1.12	58.3 [d] ± 1.19	67.5 [d] ± 1.04	52.2 [c] ± 1.19
Egg Yolk (%)	0	33.4 c ± 1.12	58.2 [b] ± 1.19	68.0 [b] ± 1.04	47.9 [b] ± 1.19
	5	47.2 [b] ± 1.12	75.1 [a] ± 1.19	78.2 [a] ± 1.04	67.1 [a] ± 1.19
	10	49.8 [b] ± 1.12	76.7 [a] ± 1.19	80.0 [a] ± 1.04	67.9 [a] ± 1.19
	15	53.9 [a] ± 1.12	76.1 [a] ± 1.19	79.3 [a] ± 1.04	63.6 [a] ± 1.19
Time (day)	0	88.1 [a] ± 0.99	89.7 [a] ± 1.05	93. 6 [a] ± 1.10	91.5 [a] ± 1.20
	1	74.1 [b] ± 0.99	83.2 [b] ± 1.05	88.5 [b] ± 1.10	78.0 [b] ± 1.20
	2	58.1 [c] ± 0.99	80.3 [c] ± 1.05	85.8 [c] ± 1.10	73.0 [c] ± 1.20
	3	46.3 [d] ± 0.99	77.5 [d] ± 1.05	83.5 [d] ± 1.10	67.2 [d] ± 1.20
	4	37.8 [e] ± 0.99	74.6 [e] ± 1.05	81.0 [e] ± 1.10	59.4 [e] ± 1.20
	5	28.4 [f] ± 0.99	70.7 [f] ± 1.05	78.5 [f] ± 1.10	54.6 [f] ± 1.20
	6	20.2 [g] ± 0.99	51.5 [g] ± 1.05	53.8 [g] ± 1.10	43.9 [g] ± 1.20
	7	15.5 [h] ± 0.99	44.9 [h] ± 1.05	46.2 [h] ± 1.10	37.1 [h] ± 1.20

a-h: Different superscripts within each main effect denote significant differences ($p < 0.05$).

storage at 5 °C. It is well known that increased duration of the liquid storage of ram semen is associated with decrease in sperm motility and fertility (O'Hara et al., 2010). It has been shown that casein micelles isolated from milk can protect stallion (Batellier et al., 1997), goat (Leboeuf et al., 2003), ram (Choong & Wales, 1962; O'Shea & Wales, 1966) and bull (O'Shea & Wales, 1966) ejaculated spermatozoa during cold storage. It was mentioned that milk proteins and peptides can act as antioxidants via primary and secondary mechanisms (Cervato et al., 1999; Kitts, 2005). It was reported that caproic acid inhibits production of lactic acid (Vijayaraghavan et al., 1989) and increase protective potency of Tris extender during sperm storage at 5 °C (Roostaei-Ali Mehr et al., 2013). To the best of our knowledge, this is the first report using Tris plus caproic acid and skim milk extender for recovery and storage of ram epididymal spermatozoa; until now, skimmed milk extender has been used only for recovery of equine epididymal spermatozoa (Guasti et al., 2013).

As shown in Fig. 4 sperm viability, membrane integrity and acrosome integrity were lowest in T, T-C and S in the lack of egg yolk after 7 d storage. But, sperm motility was improved in the presence of egg yolk (Fig. 2B). The highest sperm motility was observed in 15% egg yolk after 5-7 days storage at 4 °C, although, the main effect of egg yolk showed that there was no difference among levels of 5, 10 and 15% egg yolk on sperm viability, membrane integrity and acrosome integrity. Therefore, the application of 15% egg yolk may be necessary to store ram epididymal spermatozoa. Two major roles of protective effect of egg yolk were reported in sperm preparation. This included antioxidant effect and protective action on sperm membranes against cold shock during storage at 5 °C (Watson & Martin, 1975). Therefore, the supplementation of extender with egg yolk, improved longevity of ram epididymal spermatozoa as showed in rat epididymal spermato-

zoa (Varisli et al., 2009).

The data showed there was interaction between egg yolk and extender on sperm motility (Fig. 3). Sperm motility was higher in ME15 and T-CE15 than TE15 and SE15. There was no difference among ME15, ME10 and ME5. But, sperm motility was higher in T-CE15 than ME10 and ME5. Moreover, acrosome integrity was higher in T-CE15 than ME15 on day 6 and 7 of storage at 4 °C. Therefore, longevity of recovered ram epididymal spermatozoa can be improved by supplemented Tris extender with caproic acid and 15% egg yolk under long-term cold storage. It was reported that acrosome integrity is higher in preserved sperm with Tris extender than milk extender under liquid storage (Rakha et al., 2013). It was mentioned that lactate, as a substrates of lactate dehydrogenase X, stimulate calcium uptake via increase in the intracellular levels of NADH in spermatozoa; whereas, caproic acid, as a nonphysiological but effective substrate of lactate dehydrogenase X, reverses the effect of lactate and inhibits calcium uptake (Coronel et al., 1983; Vijayaraghavan et al., 1989). Increase in calcium uptake was paralleled by increased oxygen consumption, thus expediting the metabolism rate of spermatozoa (Simpson et al., 1987). It was reported that increase in intracellular calcium concentration can induce apoptosis and cell aging (Gordo et al., 2002; Martin et al., 2005). Moreover, calcium is probably the key messenger in the acrosome reaction and intracellular calcium plays a central role in the regulation of this exocytotic process (Witte & Schäfer-Somi, 2007). Therefore, the supplementation of Tris with caproic acid may delay premature acrosome reaction and improve quality of spermatozoa during storage at 4 °C.

In conclusion, it can be said that Tris caproic extender is superior to other extenders for recovery and storage of ram epididymal spermatozoa.

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