Iranian Society of Animal Science
Journal of Livestock Science and Technologies
Shahid Bahonar University of Kerman
ISSN: 2322-3553 (Print) ISSN: 2322-374X (Online)

Feasibility of long-term ovine epididymal spermatozoa preservation in a simple extender containing egg yolk

Ebrahim Ahmadi[1*], Hassan Nazari[1], Najmeh Davoodian[1] and Ali Kadivar[1, 2]

[1]Research Institute of Animal Embryo Technology, Shahrekord University, Shahrekord, Iran.
[2]Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran.

***Corresponding author,**
E-mail address:
eahmadi@sku.ac.ir

Received: **06 Aug. 2019,**
Accepted: **15 Jun. 2020,**
Published online: **01 Jul. 2020.**

Abstract Spermatozoa contained in the cauda epididymis could be recovered and used at post-mortem in the situations that the ejaculated sperm are not available such as the sudden death of genetically invaluable livestock males and in endangered wild species. To benefit from the potentials of epididymal spermatozoa, it is important to have suitable protocols for handling and storage of this type of spermatozoa, because the quality of epididymal spermatozoa is affected by the storage conditions. Therefore, the present study was aimed to investigate whether ram epididymal spermatozoa could be preserved in a simple extender containing egg yolk for 120 h at 5˚C. Epididymal spermatozoa were collected from the tails of the epididymides of slaughtered rams and diluted in Tris-citric acid-fructose extenders containing 0, 10, or 20% EY at $1×10^8$ sperm mL^{-1}. Afterwards, the diluted samples were stored at 5˚C for 120 h. The motility, functional membrane integrity, and morphology of spermatozoa were assessed at 0, 2, 24, 48, 72, 96, and 120 h of storage period. The results showed that storing ovine epididymal spermatozoa without EY was not possible. In the extender containing 20% EY, membrane integrity was similar to the fresh samples up to 72 h, normal morphology up to 96 h, and progressive motility up to 72 h.

Keywords: cauda epididymis, liquid storage, membrane integrity, motility, morphology

Introduction

Spermatozoa contained in the cauda epididymis could be recovered and used at post-mortem in the situations that the ejaculated sperm are not available such as the sudden death of genetically invaluable livestock males and in endangered wild species (Lopes et al., 2015). The epididymal and ejaculated spermatozoa differ in several respects (Garcia-Macias et al., 2006); however, artificial insemination of either fresh or frozen–thawed epididymal spermatozoa showed that their fertility was similar to the ejaculated ones (Ehling et al., 2006). To benefit from the potentials of epididymal spermatozoa, it is important to have suitable protocols for handling and storage of this type of spermatozoa, because, immediate insemination or cryopreservation of epididymal semen is not always possible and it has to be transported or stored for a while before being used. Transportation temperature (Lone et al., 2011) and handling conditions (Kaabi et al., 2003) and epididymal sperm storage method and duration

(Tamayo-Canul et al., 2011; Tamayo-Canul et al., 2011; Abella et al., 2015) have substantial effects on the quality of spermatozoa.

The easiest way to store epididymal spermatozoa is to refrigerate the whole epididymis (Barati et al., 2009; Tamayo-Canul et al., 2011; Hoseinzadeh-Sani et al., 2013). Preserving ram epididymides at 5˚C resulted in a fertility rate comparable to the fresh ejaculated and epididymal spermatozoa until 24 h post-mortem and a good viability rate until 48 h post-mortem (Kaabi et al., 2003). Epididymal degeneration starts as early as 12 h postmortem (Songsasen et al., 1998) which could change the microenvironment of the epididymis, thus harming the spermatozoa. Therefore, extracting the epididymal sperm from the epididymis as soon as possible and storing them either at diluted or undiluted state may be one strategy for extending the fertile lifespan of the epididymal spermatozoa (Tamayo-Canul et al., 2011; Tamayo-Canul et al., 2011; Abella et al., 2015). In this regard, Fernández Abella et al (2015) extracted epididymal sperm and stored them for 24 to 96 hours at undiluted state. They cryopreserved the stored spermatozoa and observed that their fertility ranged from 55% to 24% after the intrauterine insemination, depending on the duration of storage before freezing. Storage of diluted epididymal spermatozoa in suitable extenders is another option for preserving the epididymal sperm. The availability of suitable storage protocols to protect the sperm cells against the harmful effects of epididymal degeneration may extend the fertile lifespan of the epididymal sperm (Tamayo-Canul et al., 2011). This approach may be valuable for the situations in which the immediate freezing of epididymal spermatozoa is not possible (Fernández-Santos et al., 2009).

The hen egg yolk (EY) is the most effective agent in protecting the spermatozoa against the cold shock, which improves sperm functions and preserve fertility after storage in liquid or frozen state (Bergeron et al., 2004; Gholami et al., 2012; Rajabi-Toustani et al., 2014). Considering the inconsistent outcomes of storing diluted ovine epididymal spermatozoa in extenders with different formulations (Tamayo-Canul et al., 2011; Tamayo-Canul et al., 2011; Abella et al., 2015), and regarding the fact that the effects of EY have not thoroughly been tested for the storage of ovine epididymal spermatozoa, in the present study we aimed to evaluate ram epididymal spermatozoa stored for 120 h in the presence or absence of EY.

Materials and methods

All chemicals were obtained from Sigma Chemicals Co. (St. Louis, MO, USA) unless otherwise stated.

In the present study, ram cauda epididymal sperm were diluted in 3 extenders containing 0 (EY0), 10 (EY10), or 20 (EY20) percent (v/v) centrifuged egg yolk. Extended samples were stored at 5˚C for 120 h (5 days). The motility, functional membrane integrity, and morphology of spermatozoa were analyzed at 0, 2, 24, 48, 72, 96, and 120 h of storage period.

The extender was composed of 300 mM L^{-1} Tris, 95 mM L^{-1} citric acid, and 27.8 mM L^{-1} fructose. Osmolarity and pH were set at 320-330 mOsm/kg H_2O and 6.8, respectively. Egg yolk was centrifuged for 90 minutes at 20000 ×g and the clear supernatant was added to the extenders at the desired concentrations. The three extenders were divided into 1 mL aliquots and stored at -20˚C state until use.

The testes of sexually mature rams were immediately removed at slaughter and transported to the laboratory at the 20-25˚C. After removing the tunica albuginea over the cauda epididymis, an incision was made on the tail by using a scalpel blade and the contents were collected. Epididymal contents from 3 testes of different rams were pooled and diluted at 1×10^8 sperm mL^{-1} in the 1 mL of thawed aliquots of the 3 extenders. Diluted samples were kept at the room temperature for 30 minutes. The sperm motility was then analyzed by computer-assisted sperm analysis system (CASA, Hooshmand Fanavar, Iran). After CASA analysis, two smears were prepared for Diff-quick staining and 30 µL of each sample were subjected to hypo-osmotic swelling test. Finally, microtubes of samples were put in a beaker containing 500 mL of 25˚C-water and the beaker was placed in a 5˚C-refrigerator. The temperature of water reached to 5˚C in 2 h. For evaluating spermatozoa at each time point, samples were thoroughly re-suspended by gentle pipetting and then a 100 µL aliquot of each sample was removed, warmed by 10 minutes incubation in a 37 ˚C-incubator, and used for the analyses.

Assessment of motility parameters was carried out by using a computer-assisted sperm analysis system. Five µL of re-warmed sperm suspension were loaded on a pre-warmed Spermmeter semen analysis chamber (Sperm Processor Pvt. Ltd., Aurangabad, India; 10 µm depth) and then evaluated. For each sample, six optical fields were analyzed with 30 frames per second. At ×4 Magnification for the objective lens. The motility

descriptors were: total motility (TM; %), progressive motility (PM; %), curvilinear velocity (VCL; μm s^{-1}), linear velocity (VSL; μm s^{-1}), mean velocity (VAP; μm s^{-1}), frequency of head displacement (BCF; Hz), and linearity coefficient (LIN; %).

The functional membrane integrity was assessed using hypo-osmotic swelling test (HOST) according to the WHO laboratory manual for the examination and processing of human semen (2010). In brief, a 30 μL aliquot of the diluted sample was added to the 300 μL of swelling solution (25 mM L^{-1} sodium citrate dehydrate and 75 mM L^{-1} D-fructose) and incubated at 37 ˚C for 30 minutes. Then, a 10 μL aliquot was placed on a grease-free microscope slide, covered by a coverslip, and examined by a phase-contrast microscope using a 40× objective. Two slides per sample were prepared and 200 sperm were counted on each slide. The percentage of spermatozoa with intact membrane was calculated by dividing the number of spermatozoa with swollen tail (intact membrane) to the total counted sperm (Who, 2010)

Sperm morphology assessment was performed after Diff-Quick staining (Heidari Nasirabadi et al., 2019). From each sample, duplicate smears were prepared on the grease-free glass slides and air dried. The smears stained with staining solutions according to the instruction of Diff-Quick staining kit (Avicenna Institute, Tehran, Iran). In Brief, slides were consecutively dipped in fixative solution, first stain solution, second stain solution, and distilled water, each for 5 seconds and the total procedure were lasted 20 seconds. After air drying, the morphology of at least 200 spermatozoa per slide was evaluated using a bright field microscope at 100× magnification. Presence of protoplasmic droplets was not considered a morphological abnormality. The percentages of normal spermatozoa, as well as the percentage of the spermatozoa with the head, midpiece, or tail abnormalities were calculated by dividing the number of normal spermatozoa and the number of spermatozoa with the head, midpiece, or tail abnormalities to the total evaluated spermatozoa.

Statistical analysis

Statistical analysis was performed using the IBM-SPSS-22 Software Package in a model containing the effects of extenders (EY0, EY10, and EY20) and storage time (0, 2, 24, 48, 72, 96, and 120 h). Before analysis, TM, PM, LIN, membrane integrity percentages, and morphological indices were subjected to an arc-sine transformation. Data normality was tested using the Kolmogorov–Smirnov test. Differences between extenders or storage times were assessed using a one-way ANOVA, followed by the Tukey's post-hoc test. The data were expressed as the mean ± SEM. Differences were considered significant at the level of $P \leq 0.05$.

Results

Because of the substantial decrease in the motility and viability, the EY0 extender was only evaluated up to 48 h. The data on motility assessments are presented in Table 1. After 2 h, the motility and kinematic parameters in EY0 extender were significantly lower than those of other groups and lower than their pre-cooling values ($P \leq 0.05$). Moreover, the motility and kinematic parameters of spermatozoa in EY0 extender severely decreased after 24 h and reached almost 0 after 48 h. In EY containing extenders, however, motility was preserved for a longer time than in the EY0 extender. In comparison to the values before storage (0 h), a significant reduction in motility and other kinematic parameters was observed at 72 or 96 h in EY20 extender and at 24 or 48 h in EY10 extender ($P \leq 0.05$). Although EY and its concentrations had no significant effect on the motility of spermatozoa at 0 h, its presence or concentrations led to significant differences in the motility parameters up to 72 h storage. In this regard, EY20 and EY0 recorded the highest and lowest values, respectively.

The results of functional membrane integrity assessments (HOST) are presented in Table 2. A significant decrease in membrane integrity was observed in EY0 group after 24 and 48 h storage of spermatozoa at 5˚C ($P \leq 0.05$). Similar to motility, membrane integrity was preserved for a longer time in EY containing extenders than in EY0 extender. Compared to the initial 0 time values, a significant decrease in membrane integrity was first observed at 96 h and 24 h in EY20 and EY10 extenders, respectively ($P \leq 0.05$). Membrane integrity was similar in all 3 extenders before storage, but was the lowest in EY0 at 24 and 48 h. Moreover, from 24 h onwards, this parameter was significantly higher in EY20 extender than in EY10 one ($P \leq 0.05$).

The results of morphology assessments (normal spermatozoa) are presented in Table 2. At each time-point, morphologically normal spermatozoa were significantly lower in EY0 extender compared to other extenders and compared to its pre-cooling value ($P \leq 0.05$). The normal morphology of spermatozoa was better preserved in EY20 extender than in other extenders. As shown in Table 3, morphological defects

Table 1. Effect of egg yolk concentration on motility parameters of the ovine epididymal spermatozoa stored at 5˚C for up to 120 h (mean±SEM)

Parameter	Extender	0 h	2 h	24 h	48 h	72 h	96 h	120 h
TM	EY0	82.4±3.43^{A}	69.5±2.71^{Ba}	30.7±1.25^{Ca}	14.8±1.5^{Da}	NA	NA	NA
(%)	EY10	86.9±1.82^{A}	84.4±3.28^{Ab}	69.8±3.90^{Bb}	65.6±4.18^{BCb}	53.5±3.55^{CDa}	43.9±4.17^{D}	39.6±5.19^{D}
	EY20	87.5±2.97^{A}	85.1±2.88^{Ab}	83.5±3.24^{ABc}	78.5±3.32^{ABc}	69.2±3.92^{BCb}	56.8±5.72^{CD}	47.7±6.31^{D}
PM	EY0	60.3±3.60^{A}	37.5±4.72^{Ba}	18.7±1.27^{Ca}	6.0±0.69^{Da}	NA	NA	NA
(%)	EY10	66.8±2.78^{A}	63.9±3.15^{Ab}	52.6±3.74^{ABb}	48.2±4.27^{Bb}	37.8±3.16^{BCa}	30.2±3.95^{C}	26.4±4.88^{C}
	EY20	66.0±3.76^{A}	65.6±3.63^{AB}	67.5±4.05^{Ac}	62.0±3.85^{Ac}	52.9±3.68^{ABb}	41.1±5.61^{B}	32.9±5.80^{B}
VCL	EY0	91.4±6.18^{A}	70.2±4.67^{Ba}	29.5±1.24^{Ca}	23.5±1.49^{Da}	NA	NA	NA
(µm/s)	EY10	96.3±4.07^{A}	91.5±4.12^{Ab}	74.3±6.02^{Bb}	64.6±5.24^{Bb}	52.7±4.58^{BCa}	44.1±3.33^{C}	38.2±3.93^{C}
	EY20	97.9±4.82^{A}	93.0±5.29^{Ab}	94.7±6.31^{Ac}	83.0±5.79^{ABc}	70.1±6.08^{BCb}	56.3±5.84^{C}	46.4±5.32^{C}
VSL	EY0	34.7±3.15^{A}	15.6±2.91^{Ba}	6.5±0.55^{Ca}	3.6±0.34^{Ca}	NA	NA	NA
(µm/s)	EY10	37.4±2.48^{A}	36.2±2.09^{Ab}	31.0±3.55^{ABb}	25.9±3.19^{BCb}	18.5±2.31^{CDa}	14.2±2.08^{D}	11.2±2.12^{D}
	EY20	37.5±2.85^{AB}	37.8±3.83^{Ab}	41.3±3.54^{Ab}	35.5±3.15^{ABc}	27.8±3.27^{BCb}	20.1±3.32^{C}	15.1±3.13^{C}
VAP	EY0	51.1±3.06^{A}	39.7±2.44^{Ba}	11.9±0.72^{Ca}	8.1±0.52^{Da}	NA	NA	NA
(µm/s)	EY10	54.9±2.89^{A}	52.8±3.59^{Ab}	42.6±4.24^{ABb}	36.8±3.98^{BCb}	27.9±3.07^{CDa}	22.1±2.70^{D}	18.3±2.92^{D}
	EY20	55.6±3.46^{A}	54.0±3.17^{Ab}	55.7±4.42^{Ac}	49.0±4.15^{Ac}	40.1±4.37^{ACb}	30.2±4.35^{C}	23.6±4.16^{C}
BCF	EY0	4.2±0.45^{A}	2.5±0.35^{Ba}	0.7±0.11^{Ca}	0.3±0.08^{Ca}	NA	NA	NA
(Hz)	EY10	4.6±0.26^{A}	4.6±057^{Ab}	3.5±0.51^{ABb}	2.8±0.46^{BCb}	1.9±0.30^{CDa}	1.5±0.23^{CD}	1.1±0.23^{D}
	EY20	4.8±0.30^{A}	4.7±0.32^{Ab}	5.1±0.50^{Ac}	4.4±0.46^{Ac}	3.3±0.50^{ABb}	2.4±0.44^{B}	1.7±0.36^{B}
LIN	EY0	35.8±2.47^{A}	23.6±1.62^{Ba}	20.4±1.89^{Ba}	17.2±2.61^{Ba}	NA	NA	NA
(%)	EY10	37.6±1.96^{A}	37.4±2.05^{Ab}	34.2±1.76^{ABb}	32.5±1.80^{ABCb}	28.1±1.35^{BCDa}	24.5±2.04^{D}	24.9±1.68^{CD}
	EY20	38.1±1.85^{A}	37.9±1.90^{Ab}	39.2±1.72^{Ab}	37.3±1.22^{Ab}	33.4±1.30^{ABb}	28.4±2.10^{B}	26.1±2.10^{B}

$^{A-D}$within rows, indicate significant differences between the evaluation time-points for each extender (P≤0.05).
$^{a-c}$within columns, indicate significant differences between extenders at each evaluation time-point (P≤0.05).
EY0, EY10, and EY20: extenders with 0, 10, and 20% egg yolk, respectively; TM: total motility; PM: progressive motility; VCL: curvilinear velocity; VSL: linear velocity; VAP: mean velocity; BCF: frequency of head displacement; LIN: linearity coefficient.

Table 2. Effect of egg yolk concentration on the functional membrane integrity and morphology of ovine epididymal spermatozoa stored at 5˚C for up to120 h (mean±SEM)

Parameter	Extender	0 h	2 h	24 h	48 h	72 h	96 h	120 h
Intact	EY0	86.9±2.01^{A}	79.7±4.80^{A}	53.1±2.79^{Ba}	32.5±1.85^{Ca}	NA	NA	NA
Membrane	EY10	86.3±1.82^{A}	86.5±2.01^{A}	74.0±3.90^{Bb}	63.2±4.04^{BCb}	49.6±2.93^{CDa}	41.1±3.40^{Da}	33.1±5.14^{Da}
	EY20	85.8±2.27^{A}	86.2±2.64^{A}	86.5±2.98^{Ac}	76.8±4.35^{ABc}	71.9±3.73^{ABCb}	59.7±5.53^{BCb}	51.8±5.84^{Cb}
Normal	EY0	92.2±0.58^{A}	86.5±1.01^{Ba}	68.1±1.71^{Ca}	65.3±1.54^{Ca}	NA	NA	NA
morphology	EY10	92.4±0.80^{A}	92.7±0.98^{Aa}	89.8±2.02^{ABb}	86.2±1.14^{ABCb}	84.1±1.92^{BCDa}	79.5±2.10^{CDa}	75.9±2.95^{D}
	EY20	92.9±1.01^{A}	92.8±1.26^{Aa}	92.9±1.27^{Ab}	91.3±1.82^{Ab}	89.8±1.58^{ABb}	87.0±2.33^{ABb}	83.1±2.17^{B}

$^{A-D}$within rows, indicate significant differences between the evaluation time-points for each extender (P≤0.05).
$^{a-c}$within columns, indicate significant differences between extenders at each evaluation time-point (P≤0.05).
EY0, EY10, and EY20: extenders with 0, 10, and 20% egg yolk, respectively.

due to the storage mostly occurred in the sperm midpiece, then in the tail.

Discussion

In the present study, we aimed to investigate the impacts of egg yolk on the longevity of ram epididymal spermatozoa during a long-term liquid storage (120 h). Our results showed that the presence of EY, as well as its concentration affected the motility, membrane integrity, and morphology of stored spermatozoa.

What named aging starts as the differentiation of the spermatozoon completes in the testis. From that point, the spermatozoon starts to deteriorate due to oxidative, osmotic, and temperature damage, as well as ATP depletion (Reinhardt, 2007). Amongst them, oxidative stress has been suggested as the main cause of sperm aging (Aitken and Baker, 2004; Aitken et al., 2014). In a population of normal spermatozoa, the reactive oxygen species (ROS) are mainly produced in the oxidative phosphorylation process which takes place in the sperm mitochondria (Amaral et al., 2013). In the male reproductive tract, the oxidative damage to sperm and aging is slowed down by various protective mechanisms. In the epididymis, spermatozoa are stored at a relatively quiescent state which reduces the

Table 3. Effect of egg yolk concentration in the extender on the morphological abnormalities (%) of the ovine epididymal spermatozoa stored at 5˚C for up to 120 h (mean±SEM)

Parameter	Extender	0 h	2 h	24 h	48 h	72 h	96 h	120 h
Abnormal heads	EY0	1.7±0.21^{A}	1.8±0.34^{A}	6.3±0.25^{Ba}	6.1±0.38^{Ba}	NA	NA	NA
	EY10	1.8±0.25^{A}	1.8±0.40^{A}	2.1±0.46^{ABb}	2.3±0.19^{ABb}	2.8±0.38^{ABC}	3.5±0.21^{BC}	4.0±0.47^{C}
	EY20	1.7±0.29	1.8±0.31	1.6±0.30^{b}	1.7±0.36^{b}	1.9±0.25	2.1±0.41	2.7±0.37
Abnormal midpieces	EY0	3.6±0.50^{A}	7.9±0.67^{Ba}	16.5±1.25^{Ca}	17.7±0.91^{Ca}	NA	NA	NA
	EY10	3.3±0.27^{A}	3.4±0.36^{Ab}	5.5±1.23^{ABb}	7.9±0.77^{BCb}	8.8±0.98^{BCDa}	11.5±1.33^{CDa}	13.3±1.49^{D}
	EY20	3.1±0.34^{A}	3.1±0.39^{Ab}	3.6±0.63^{Ab}	4.8±0.95^{ABb}	5.3±0.82^{ABb}	7.2±1.34^{BCb}	9.5±1.09^{C}
Abnormal tails	EY0	2.5±0.46^{A}	3.8±0.60^{A}	9.1±0.32^{Ba}	10.9±0.34^{Ba}	NA	NA	NA
	EY10	2.2±0.62^{A}	2.1±0.55^{A}	2.6±0.40^{Ab}	3.5±0.30^{ABb}	4.1±0.64^{ABC}	5.5±0.75^{BC}	6.8±1.13^{C}
	EY20	2.3±0.29^{AB}	2.3±0.47^{AB}	1.9±0.40^{Ab}	2.2±0.54^{ABb}	2.9±0.51^{AB}	3.7±0.68^{BC}	4.8±0.79^{C}

$^{A-D}$within rows, indicate significant differences between the evaluation time-points for each extender (P≤0.05).
$^{a-c}$within columns, indicate significant differences between extenders at each evaluation time-point (P≤0.05).
EY0, EY10, and EY20: extenders with 0, 10, and 20% egg yolk, respectively.

rate of metabolism, and hence ROS production (Jones and Murdoch, 1996). Moreover, the temperature of external testes is maintained lower than the ambient temperature, which in turn reduces the metabolic rate (Djakiew and Cardullo, 1986).

Upon exiting from the epididymis and mixing with seminal plasma or further dilution in extenders, spermatozoa gradually become activated and their metabolism increases. This increase is a necessary step for the activation of motility and other fertilization related phenomena in sperm (Reinhardt, 2007), but restricts the longevity of spermatozoa outside the epididymis. Under *in vitro* conditions, the temperature at which spermatozoa are stored has a substantial impact on their lifespan and functionality, especially for longer storage periods. In one study, ram semen extended with different extenders and stored even at 15˚C for 72 h had significantly lower motility and viability than those stored at 5˚C (O'hara et al., 2010). Therefore, spermatozoa are usually stored at refrigerator temperature (2-10˚C) for long-term storage (Salamon and Maxwell, 2000). By reducing the storage temperature another problem emerges, the risk of cold shock (Salmon et al., 2016). Cold shock is probably related to the phase transition of membrane lipids and may result in phase separation and loss of selective permeability (Drobnis et al., 1993). The most obvious consequence of cold shock is loss of motility which is not restored when the sample is rewarmed (reviewed by White, 1993). The susceptibility of spermatozoa to the cold shock is related to the ratio of unsaturated: saturated fatty acids in phospholipids and to the cholesterol content of their plasma membrane (Mocé et al., 2010). Cooling sperm samples slowly and supplementing the diluents with components such as the egg yolk are the main measures for overcoming the cold shock problem (White, 1993; Paulenz et al., 2002).

In comparison with the ejaculated spermatozoa, ram epididymal sperm cells were considered more resistant to cold shock (Varisli et al., 2009). As a result, we hypothesized that long-term storage of epididymal spermatozoa might be possible without supplementation of the diluent with EY. However, in our study condition, we observed that epididymal spermatozoa could not be stored in the absence of EY. Considering the method used in the present study to cool the sperm samples, the decrease in the sperm quality was not possibly related to the cold shock. All damages due to the storage at low temperatures, which can be called as the chilling injury, to distinguish it from the cold shock, resulted in a severe reduction in the quality of stored spermatozoa. Deleterious effects of cold storage began from the start of the storage period. In EY0 extender, the obvious effects of chilling were motility reduction, changes in the pattern of motion, and increased abnormalities in midpiece (bent midpiece). Bent midpiece was also the main morphological abnormality observed at the subsequent evaluation time points. In EY-containing extenders, this abnormality appeared later (at 72 h in EY10 and at 96 h in EY20) than it did in EY0 extender. In this regard, it has been shown that the cold-induced midpiece bending is a consequence of local membrane lesions at this site. These lesions act on the axonemal structure of the flagellum and cause its bending (Holt et al., 1988). Therefore, this morphological defect may have a relationship with the motility disorders observed in the cooled spermatozoa. Moreover, local membrane lesions may propagate over time and result in the loss of membrane function as that we observed in its most severe state in EY0 extender. The plasma membrane covering the sperm cell maintains the chemical gradient of ions

and other soluble components by its semi-permeable features. Spermatozoa without functionally intact sperm plasma membranes are considered dead and do not possess fertilizing ability (Silva and Gadella, 2006).

As mentioned earlier, long-term extra-epididymal storage of ovine epididymal spermatozoa has not thoroughly been sufficiently studied. In this regard, Tamayo-Canul and coworkers published two papers in 2011in which they evaluated the effects of storage method (intra-epididymal, extra-epididymal diluted, or extra-epididymal undiluted) and extender osmolality (320, 370, add 420 mOsm/kg) on the quality (Tamayo-Canul et al., 2011) and freezability (Tamayo-Canul et al., 2011) of ovine epididymal spermatozoa stored for 72 h. It is noteworthy that the osmolality of their complete extenders could not have been the above values because according to their articles, they added 8% (about 1100 mM) glycerol into their extenders; therefore, the final osmolality of their extenders should have been much more than 420 mOsm/kg. However, they concluded that storing the spermatozoa in the epididymis at 5˚C is a good strategy for maintaining sperm quality in ram, at least for 48 h, and that is a better option than extracting the sperm mass and keeping it undiluted. Moreover, storing the diluted sperm mass in a suitable extender was superior to storing it at undiluted state. In contrast, Abella et al. (2015) observed that the motility of ovine epididymal spermatozoa was better preserved in the undiluted epididymal fluid than when the epididymis itself was preserved in refrigerator or when epididymal spermatozoa were diluted in classic ovine extenders such as skim milk. In that study, unlike the present study and two reports of Tamayo-Canul et al. in 2011, the motility of diluted spermatozoa rapidly declined over time. This difference could have been related to the different composition of extenders used in the studies. Our extender was composed of Tris, citric acid, fructose, and 10 or 20% EY. The extender used by Tamayo-Canul et al. (2011) contained TES, Tris, fructose, 20% EY, and glycerol. Abella et al. (2015) used either skim milk or an extender composed of glucose, potassium bicarbonate, glutamic acid, glycine, proline, myo-inositol, D-mannitol, bovine serum albumin and 20% EY. Altogether, it can be concluded that the extenders based on TES-Tris-fructose or Tris-citric acid-fructose are desirable extenders for liquid storage of ovine epididymal spermatozoa.

In the present study, we used clarified EY to facilitate motility analysis. Moreover, in extenders containing clarified EY, spermatozoa exhibit better motility than in un-processed EY (un-published data) possibly because the coarse particles and droplets of un-processed EY disturb the normal motion of spermatozoa. Our results showed that EY on its own was able to protect spermatozoa against the detrimental effects of cold storage, and hence, it prolonged the lifespan of stored spermatozoa. As the case of red deer epididymal spermatozoa (Fernández-Santos et al., 2006), our results reconfirmed the importance of EY in the storage of the ovine epididymal spermatozoa. Our results showed that 20% EY provided substantially better protection for stored spermatozoa than that obtained by 10% EY. The mechanisms involved in sperm protection by EY against storage, cooling, and freezing damages are not clear. It is obvious that EY exerts its protective effect at the plasma membrane level and stabilizes the structure of this organelle (De Leeuw et al., 1993). Because of the absence of glycerol, which may have detrimental side effects on ovine spermatozoa, our extender was possibly superior to the extenders used by Tamayo-Canul et al.(2011). It has been shown that glycerol accelerates the induction of the acrosome reaction in ovine spermatozoa (Slavik, 1987). Moreover, after 3 days of storage of bovine spermatozoa, glycerol, supplemented into a Tris-EY extender, appeared to be toxic in comparison to the non-supplemented one (Vera-Munoz et al., 2011). Therefore, it could be inferred that storing diluted ovine epididymal spermatozoa in the absence of glycerol may be preferred. If the final goal is freezing spermatozoa, it may be possible that glycerol or other cryoprotectants be added at the time of freezing.

Gradual deterioration of sperm during storage even in the presence of EY may have several reasons. The negative impacts of chilling on the metabolic processes of spermatozoa were likely to be one of the reasons. Although storage at lower temperatures reduces the metabolic activity, not all the changes associated with lower temperatures are beneficial to the spermatozoa. For example, the activity of the Na^+/K^+-ATPase pump decreases with reduced temperatures to a such extent that is unable to cope with the diffusion of ions across the cell membrane and results in the increased concentration of intracellular Na^+, which is detrimental to the survival of spermatozoa (reviewed by Vishwanath and Shannon, 2000). Another contributing factor to sperm quality reduction perhaps could have been the oxidative stress. It was shown that ROS production significantly increased during the cold storage of ram semen

and there was a weak significant negative correlation between the ROS levels and sperm kinematic parameters during storage (Falchi et al., 2018). It is noteworthy that extenders based on Tris and EY have low antioxidant capacity (Bilodeau et al., 2002).

In conclusion, our results showed that ovine epididymal spermatozoa could be stored in Tris-citric acid-fructose extender containing 20% centrifuged EY without significant quality reduction up to 72 h. Freezability and fertility of stored spermatozoa need further studies.

Acknowledgements

The authors would like to thank Shahrekord University for financial support and the Research Institute of Animal Embryo technology, Shahrekord University for technical cooperation.

Author Contributions

Conceived and designed the experiments: EA. Performed the experiments: EA, HS, ND, and AK. Analyzed the data: EA. Wrote the paper: EA.

Conflict of Interest

The authors declare that they have no competing interests.

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