IJBPAS, July, 2015, 4(7): 5034-5044

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

EVALUATION OF DIFFERENT EXTENDERS FOR STORAGE OF GHEZEL RAM SPERMATOZOA

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ABSTRACT

The present study was performed to evaluate the effects of different extenders on Ghezel ram spermatozoa in pre and post freezing conditions. Semen samples were collected by electro-ejaculator from Six healthy and mature Ghezel rams. Suitable semen of individual animals were pooled and divided into three aliquots for dilution with the experimental extenders (Tris–citric acid, Sodium citrate and Skim milk) at 37^0C. Extended semen was cooled from 37^0C to 5^0C in 2h, and characteristics of spermatozoa were assay. Then the cooled semen samples were packaged into0. 5ml straws. Straws were frozen in the vapor of liquid nitrogen and thawed ten days and the characteristics of spermatozoa were examined. Results of this study showed that the effects of extenders on some parameters of spermatozoa such as motility, progressive motility, viability and plasma membrane integrity, were significant (P<0.05), and the higher percentages of this treats were observed in Tris–citric acid extender compared to Sodium citrate and Skim milk based extender. The effects of extenders on normal spermatozoa were not significant, but the highest abnormal of spermatozoa were obtained in Skim milk extender.In conclusion, Tris–citric acid extender appears to be abetter option compared with Milk and Sodium citrate extenders for liquid and frozen storage ofGhezel ram semen.

Keywords: Tris–Citric Acid, Sodium Citrate, Skim milk Extender, Ghezel ram

INTRODUCTION

The success of Artificial insemination (AI) in sheep depends on maintenance of viability, motility and fertility of spermatozoaduring storage. Despite the improvements, post-thaw viability and fertility of the cryopreserved sperm are stillreduced, because of accumulated cellular injuries that increase throughout the cryopreservation process

(Medeiros *etal*, 2002). Therefore the success of semen cryopreservation depends on severalfactorssuch as, the initial quality of the semen samples, cryopreservation process and chilling and freezingextenders (Mocé and Vicente, 2009).Although several researchers have developed different extenders and protocols for freezing–thawing ram semen, in general, results of fertility are not comparable to those observed with fresh semen and natural mating (Fiser and Fairfull, 1984).

Thediluents used for survival of ram semen, as for other species, generally should have adequate pH andbuffering capacity, suitable osmolality, and should protect spermatozoa fromcryogenic damages. It is suggestedthat extender type used to dilution semen is the important and effective factors on successful storage ofspermatozoa, and as well as sugar added extenders play important role in the viability of frozen and non-frozenspermatozoa(Paulenz*et al*, 2002).Many extenders have been applied forfreezing ram semen. Semen has been usually dilutedwith Tris plus egg yolk, glucose phosphate solution,egg yolk-citrate solution, homogenized whole milk,fresh and dried skim milk, lactose solution, andcommercial diluents (Salamon and Maxwell, 2000). Lopez etal (1999) were observed that there were no differences between sodium citrate, Tris- or milk-based extenders when ovine liquid semen was stored at 5 degrees C during a short period (2 days). However, viability of spermatozoa was better when semen was stored for longer time, in the sodium citrate-based extender, butPaulenz*et al*(2012) and Rakha*et al*(2013) reported thatTris-based extenders, preserved sperm viability better than both the sodium citrate- and the milk-based extender for liquid storage.Emamverdi*et al*(2013) showed that TSL extender (Tris-based extender containing 1.5% (w/v) soybean lecithin) may provide stabile milieu and conditions for ram sperm cryopreservation compared with Bioxcell and TEY (Tris extender egg yolk) extenders.

Most of the research work has been conducted on semen extenders, but there is a need tofurther investigate the short-term and long-termpreservability of Ghezel ram spermatozoa for theextensive utilization of this valuable germplasm by artificial insemination. Therefore the presentstudy was designed to evaluate the effects of variousextenders on semenquality of Ghezel ram.

METHOD and MATERIAL

Animals and semen collection

In this experiment, six 3-4 years oldsexually mature and healthy Ghezel rams was selected for semen collection. Semenwas collected 2

times in aweek during the breeding season by electro ejaculator. The ejaculates wereplaced in a thermos (37ºC) immediately after collection, and semen quality was assessed andpooled. Thepooled semen meansmacroscopically suitablequality, were selected for this experiment (Table1). Thus, the pooled ejaculate divided into 3 equal parts and diluted with Tris,Sodium and Milkextenders. The extended semen was then cooledto 5ºC during 2h by placing in a refrigerator. The cooled semenwas evaluated. The remaining part of the samples was packaged intoplastic straws (0.5ml) andprepared to cryopreservation, which done by N2 vapor for 8 minute and stored in liquid nitrogen. Semen wasthawed in a thermostatic bath at 37ºC for 45 second and evaluated after ten days.(Salamon and Maxwell, 2000).

Extender T (Tris-citric acid)

Tris-citric acid egg yolk extender was prepared byusing 3.63 g of Tris-(hydroxymethyl)-aminomethane,1.99g of citric acid, 0.5g of glucose, and 20% egg yolkin 80mL of distilled water (Evans and Maxwell,1987).

Extender SC (Sodium citrate)

Sodium citrate extender was prepared from a 2.9%aqueous solution of tri-sodium citrate and 20% eggyolk in 80mL of distilled water(Evans and Maxwell,1987).

Extender M (Milk)

Milk extender was prepared by using 10g ofskimmed milk powder and 0.9g of glucose in 100mL of distilled water, heated to 95°C for 10min, andthen cooled to room temperature before the additionof 10% egg yolk (Evans and Maxwell,1987).

Semen evaluation

Volume

The volume of semen was measured by reading the graduated tube (Biswas*et al*., 2002).

Sperm Concentration

The sperm concentration was assay by means of a haemocytometer (Smith and Mayer, 1955).

Mass motility

To investigate the mass motility, a drop (25µl) of semen was placed on a pre-warmed slide without any cover slip and examined under microscope magnification (100X). The mass activity was scored into 5 scales: 1= no movement, 2= free spermatozoa moving without forming any waves, 3=small, slow moving waves, 4= vigorous movement with moderately rapid waves and eddies and 5= dense, very rapidly moving waves and eddies (Biswas*et al*, 2002).

Motility

Sperm motility was assay by using light microscope (×400 magnification), with a

warm stage maintained at 37°C. 2µL of semen placed directly on a microscopeslide and covered by a cover slip. For each sample, at least 5 microscopic fields were examined (Ax *et al*, 2000).

Progressive motility

A drop of diluted semen in was placed on pre warmed slide (37ºC) and cover slip. The motility was determined by eye-estimation of the proportion of spermatozoa moving progressively straight forward at higher magnification (400X).

Sperm viability

Sperm viability was studied by a dualstaining procedure (Kovacs*et al*, 1992). The supravital stain trypan-blue was used to distinguish live and dead spermatozoa. equal drops of trypan-blue and semen wereplaced on a slide and mixed quickly. Smears were air-dried andslides werefixed with formaldehyde–neutral red for 5min. Trypan-blue penetratednon-viable, dead spermatozoa with disrupted membrane,which were appeared, stained in blue, whereas livespermatozoa appeared unstained. One hundred spermatozoa wereevaluated separately for sperm viability under microscopemagnification(400X).

Sperm morphology

For evaluated of sperm morphology, a smalldrop of semen was added to Eppendorftubes containing 0.5mL of Hancock's solution (Schafer and Holzmann,2000). Asingle drop of this mixture was put on a microscopeslide and covered with a cover slip. The percentageof abnormal sperm (detached heads, acrosomalaberrations, abnormal mid-pieces, or tail defects) wasrecorded by counting a total of 200 spermatozoa underphase contrast microscopy (1000X magnification; oilimmersion).

Sperm plasma membrane integrity (HOST)

Sperm plasma membrane integrity was determined using a hypo-osmotic swelling. HOS solution consisted of 0.73g sodiumcitrate and 1.35g fructose dissolved in 100mldistilled water (osmotic pressure:-190mOsmol/Kg) for investigate the sperm tail plasma membraneintegrity, semen (50µl) was mixed with HOS solution(500µl) and incubated for 30 minutes at37˚C before examination with a phase contrast microscope(×400).Two hundred spermatozoa were assessed for theirswelling ability in HOS. The swollen spermatozoadetermined by coiling of the tail were consideredto have an intact plasma membrane (Jeyendran*et al*, 1984).

Sperm Motility Recovery Rate

The sperm motility recovery rate was study by comparing the motility of pre freeze (Mpr) and post thaw (Mps) spermatozoa using the

formula: Recovery rate = Mps/Mpr× 100% (Li *et al*, 2005).

Sperm progressive Motility Recovery Rate

The sperm progressive motility recovery rate was assay by comparing the progressive motility of pre freeze (PMpr) and post thaw (PMps) spermatozoa. Recovery rate = PMps/PMpr × 100%.

Sperm viability Recovery Rate

The sperm viability recovery rate was evaluated by comparing the viability of pre freeze (Vpr) and post thaw (Vps) spermatozoa. Recovery rate = Vpr/Vps × 100%.

Statistical analysis

The results were expressed as mean ± standard error of mean (S.E.M.). Means were analyzed by one-way analysis of variance, followed by the Duncan post hoc test to determine significant differences in all the parameters among all groups using the SPSS/PC computer program (Version15.0; SPSS,). Differences with values of $P<0.05$ were considered to be statistically significant.

RESULTS

The characteristics of fresh spermatozoa are shown in Table 1.The influence of extenders on the cooled semen parameters of *Ghezel rams* were shown in Table 2. Spermatozoa motility was significantly higher ($P<0.05$) in Tris extender (75±1.63) compared with Skim milk (60±1.32), andsodium citrate (68±1.43). The effects of extenders in the post freezing on sperm characteristics were shown in Table 3. The percentages of motility, progressive motility, viability and plasma membrane integrity, were improved in Tris extender pre and post freezing. But the effects of extenders were not significant on morphological defects ($p\geq 0.05$). Recovery rate of motility and viability of spermatozoa are shown in Table4. The highest motility (36 ± 1.32), progressive motility (38.28 ± 1.17) and viability recovery rate (38.31 ± 1.64) were observed in Tris extender ($p<0.05$).

Table 1: Characteristics of Ghezel ram spermatozoa, under fresh condition

Volume (ml)	Mass motility	Motility (%)	Progressive Motility(%)	Viability (%)	Minor Defects(%)	Normal Sperm(%)	Concentration (ml)
1.43	4.5	80	74	84	4.6	95.4	3.4×10^9

Table 2: Characteristics of Ghezel ram spermatozoa, stored at 5°C in different extenders (mean ±S.E.M)

Extenders	Motility (%)	Progressive Motility (%)	Viability (%)	Minor Defects (%)	Normal sperm (%)	HOST (%)
Tris	75±1.63[a]	70±1.37[a]	78.3±1.45[a]	8.23±0.97	91.77±1.02	75±1.53[a]
Sodium citrate	68±1.43[b]	64±1.52[b]	70±1.35[b]	9.47±1.31	90.53±1.23	66±1.72[b]
Skim milk)	60±1.32[c]	58±1.37[c]	63±1.02[c]	10.23±1.48	89.77± 1.1	60±1.39[c]

Different superscript letters (a toc) within the same column showed significant differences among the groups (P<0.05).

Table 3: Characteristics of Ghezel ram spermatozoa after frozen-thawed in different extenders (mean ±S.E.M)

Extenders	Motility (%)	Progressive Motility (%)	Viability (%)	Minor Defects (%)	Normal sperm (%)	HOST (%)
Tris	27±1.32[a]	24±1.46[a]	30±1.13[a]	17±1.63	82.2±1.54	20±1.43[a]
Sodium citrate	20±1.22[b]	17±1.93[b]	24.3±1.63[b]	20.2±1.74	79.8±1.70	15±1.24[b]
Skim milk)	15±1.11[c]	12.7±1.37[c]	19±1.07[c]	21.43±1.12	78.57± 1.68	9±1.33[c]

Different superscript letters (a to c) within the same column showed significant differences among the groups (P<0.05).

Table 4: Recovery rate of Characteristics of Ghezel ram spermatozoa, in different extenders (mean ±S.E.M)

Extenders	Recovery Of Motility (%)	Recovery Of Progressive motility (%)	Recovery Of Viability (%)
Tris	36 ± 1.32[a]	38.28 ± 1.17[a]	38.31 ± 1.64[a]
Sodium citrate	29.41 ± 1.47[b]	26.56 ± 1.33[b]	34.71± 1.32[b]
Skim milk)	25 ± 1.03[c]	21.89 ± 1.12[c]	30.15 ± 1.24[c]

Different superscript letters (a toc) within the same column showed significant differences among the groups (P<0.05).

DISCUSION

Ram semen has proven to be more difficult to cryopreserve than that of other farm animals (Abdelhakeam*et al*, 1991). Different extenders composition, cryopretective agents and cooling and freeze-thaw protocol have been explained for cryopreservation of ram semen (Nr *et al*., 2010).The extenders compositions helpin stabilizing the cell during the freezing and thawing process (Liu *et al*, 1998). From the present results it was observed that the typeof extender used in diluting Ghezelram semenis an important factor in the successful preservation(at 4°C in

the refrigerator) and cryopreservation (at−196°C in liquid nitrogen) of ram spermatozoa.In this study,Trisextender protect sperm motility, better than other extenders. Assessment of motility is the most widely-used measure ofsemen quality both before artificial insemination and in thelaboratory to investigate the effects of experimental procedures. The presence of motile spermatozoa after cooling/freezing and storage indicates that less injury hastaken place during preservation (Cormier*et al*, 1997).

These findings were in agreement withthe results of Paulenz*et al* (2003) and Gündoğan (2009)that reported better spermatozoa motility and membraneintegrity rates in a Tris-based extender than in thesodium citrate and skimmed milk extenders. Contrary to our results were obtained by Lopez-Saez*et al*(2000) and Kulaksız*et al*(2011) that observed Skimmed milkextenders preserve sperm motility better than other extenders on ram semen. Lopez et al. (1999) observed no differences between sodiumcitrate-, Tris-, and milk-based extenders whensubjected to liquid storage at 4°C. But Fischer (1990) found that,Trisextender containing 2% egg yolk was the best in protection of acrosomal integrity and motility after freezing-thawing.In the present study, the percentage of live spermatozoa was higher in Tris–citric acid extendercompared to skim milk and sodium citrate extender in pre and post freezing conditions. This results agreement with finding ofPaulenz*et al*(2002) in liquid storage of ram spermatozoa.

Spermare subjected to major changes in osmotic pressureduring cooling and freezing. The stress on spermmembranes is related to the basic extenderused and the concentration of cryoprotectant asthey interact with the freezing and thawing rates(Curry and Watson, 1994).The present study showed that for preservationof plasma integrity, therewas a significant difference between extenders, maybe due to the great variation indensity in semen diluted in Tris extenders**.** Injury of sperm membranes is mainly due to the loweringof temperature and/or by aging of spermatozoa during the liquidstorage (Andrabi*et al*, 2008).

A disruption ofmembrane integrity after the freezing/thawing process was related to lower osmotic resistance ofspermatozoa diluted with Sucrose and Lactose, indicating that these extenders was less effective at preservation ofspermatozoa during the freezing/thawing process.The Tris extender contains some types of buffer substancessuch as Trisand the egg yolk. The elevated concentration of buffer solutions in the extender decreases

thedeleterious effects of the great amount of hydrogenic ions wasproduced from the metabolic activity ofspermatozoa. In addition, egg yolk is known that it contains many specific components like lecithin,phospholipids and lipoprotein fractions that bonding with deleterious proteins in seminal plasma (Demianowiczand Strzezek, 1996). Moreover decreased pH on the extender solution increaseddamages of spermatozoa membrane, consequently interfering with fertilizing capacity ofinspermatozoa.Rakha*et al*(2013) found that, the percentage ofspermatozoa with intact sperm plasma membrane was higherin Tris–citric acid than in sodium citrate extender at days 2 and 3of storage in liquid form.Similarly,Kasimanickam*et al*(2007) reported that, (Tris–citric acid compared tomilk based extenders, was the best diluter formaintaining the intact of ram sperm plasma membranefor up to 8 days at 4 8C .

Morphological examination of sperm conducted at determining both qualitative and quantitative changes is one of the most fundamental and unbiased methods of semen quality evaluation(Tabatabaei and Aghaei, 2010). In our study the effects ofextenders on morphological defects of spermatozoa were not significant but the higher percentages of normal spermatozoa were observed in Tris extender.Also the effect of extenders on recovery rate of motility, viability and progressive motility were significant and the higher this treats were obtained in Tris extender.

CONCLUSION

In conclusion, Tris–citric acidextender appears to be a better option compared with skim milkand sodium citrate extenders for short and long term storage of Ghezel ramsemen.

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