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°C. Extended semen was cooled from 37°C to 5°C in 2h, and characteristics of spermatozoa were assayed. Then the cooled semen samples were packaged into 0.5ml 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 treat 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 a better option compared with Milk and Sodium citrate extenders for liquid and frozen storage of Ghezel 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 spermatozoa during storage. Despite the improvements, post-thaw viability and fertility of the cryopreserved sperm are still reduced, because of accumulated cellular injuries that increase throughout the cryopreservation process.
Therefore the success of semen cryopreservation depends on several factors such as, the initial quality of the semen samples, cryopreservation process and chilling and freezing extenders (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).

The diluents used for survival of ram semen, as for other species, generally should have adequate pH and buffering capacity, suitable osmolality, and should protect spermatozoa from cryogenic damages. It is suggested that extender type used to dilute semen is the important and effective factors on successful storage of spermatozoa, and as well as sugar added extenders play important role in the viability of frozen and non-frozen spermatozoa (Paulenz et al., 2002). Many extenders have been applied for freezing ram semen. Semen has been usually diluted with Tris plus egg yolk, glucose phosphate solution, egg yolk-citrate solution, homogenized whole milk, fresh and dried skim milk, lactose solution, and commercial diluents (Salamon and Maxwell, 2000). Lopez et al. (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, but Paulenz et al. (2012) and Rakha et al. (2013) reported that Tris-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 stable 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 to further investigate the short-term and long-term preservability of Ghezel ram spermatozoa for the extensive utilization of this valuable germplasm by artificial insemination. Therefore the present study was designed to evaluate the effects of various extenders on semen quality of Ghezel ram.

**METHOD and MATERIAL**

**Animals and semen collection**

In this experiment, six 3-4 years old sexually mature and healthy Ghezel rams was selected for semen collection. Semen was collected 2
times in a week during the breeding season by electro ejaculator. The ejaculates were placed in a thermos (37°C) immediately after collection, and semen quality was assessed and pooled. The pooled semen means macroscopically suitable quality, were selected for this experiment (Table 1). Thus, the pooled ejaculate divided into 3 equal parts and diluted with Tris, Sodium and Milk extenders. The extended semen was then cooled to 5°C during 2h by placing in a refrigerator. The cooled semen was evaluated. The remaining part of the samples was packaged into plastic straws (0.5 ml) and prepared to cryopreservation, which done by N2 vapor for 8 minute and stored in liquid nitrogen. Semen was thawed in a thermostatic bath at 37°C for 45 second and evaluated after ten days. (Salamon and Maxwell, 2000).

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

**Semen evaluation**

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

**Sperm Concentration**
The sperm concentration was assayed 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 (Biswa et al., 2002).

**Motility**
Sperm motility was assayed 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 prewarmed 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 were placed on a slide and mixed quickly. Smears were air-dried and slides werefixed with formaldehyde–neutral red for 5 min. Trypan-blue penetrated non-viable, dead spermatozoa with disrupted membrane, which were appeared, stained in blue, whereas live spermatozoa appeared unstained. One hundred spermatozoa were evaluated separately for sperm viability under microscopemagnification(400X).

**Sperm morphology**
For evaluated of sperm morphology, a small drop of semen was added to Eppendorftubes containing 0.5mL of Hancock’s solution (Schafer and Holzmann, 2000). A single drop of this mixture was put on a microscopeslide and covered with a cover slip. The percentage of abnormal sperm (detached heads, acrosomal aberrations, abnormal mid-pieces, or tail defects) was recorded 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 membrane integrity, semen (50μl) was mixed with HOS solution(500μl) and incubated for 30 minutes at 37°C before examination with a phase contrast microscope(×400). Two hundred spermatozoa were assessed for their swelling ability in HOS. The swollen spermatozoa were determined by coiling of the tail were considered to 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 = \( \frac{M_{ps}}{M_{pr}} \times 100\% \) (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 (PMPpr) and post thaw (PMps) spermatozoa. Recovery rate = \( \frac{PM_{ps}}{PM_{pr}} \times 100\% \).

**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 = \( \frac{V_{pr}}{V_{ps}} \times 100\% \).

**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), and sodium 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 Table 4. 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**      | **Mass** | **Motility** | **Progressive** | **Viability** | **Minor** | **Normal** | **Concentration** |
| **(ml)**        | **motility** | **Motility** | **Motility** | **(%)** | **Motility** | **Defects** | **Sperm** | **(%)** | **(ml)** |
| 1.43            | 4.5     | 80       | 74       | 84      | 4.6      | 95.4      | 3.4×10^7 |

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

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

Different superscript letters (a to c) 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)

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

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)

<table>
<thead>
<tr>
<th>Extenders</th>
<th>Recovery Of Motility (%)</th>
<th>Recovery Of Progressive motility (%)</th>
<th>Recovery Of Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>36 ± 1.32a</td>
<td>38.28 ± 1.17a</td>
<td>38.31 ± 1.64a</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>29.41 ± 1.47b</td>
<td>26.56 ± 1.33b</td>
<td>34.71± 1.32b</td>
</tr>
<tr>
<td>Skim milk)</td>
<td>25 ± 1.03c</td>
<td>21.89 ± 1.12c</td>
<td>30.15 ± 1.24c</td>
</tr>
</tbody>
</table>

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

DISCUSSION

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 semen is 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 of semen quality both before artificial insemination and in the laboratory to investigate the effects of experimental procedures. The presence of motile spermatozoa after cooling/freezing and storage indicates that less injury has taken place during preservation (Cormier et al., 1997).

These findings were in agreement with the results of Paulenzen etc. (2003) and Gündoğan (2009) that reported better spermatozoa motility and membrane integrity rates in a Tris-based extender than in the sodium 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 milk extenders preserve sperm motility better than other extenders on ram semen. Lopez et al. (1999) observed no differences between sodiumcitrate-, Tris-, and milk-based extenders when subjected 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 extender compared to skim milk and sodium citrate extender in pre and post freezing conditions. This results agreement with finding off Paulenzen et al. (2002) in liquid storage of ram spermatozoa.

Sperm are subjected to major changes in osmotic pressure during cooling and freezing. The stress on sperm membranes is related to the basic extender used and the concentration of cryoprotectant as they interact with the freezing and thawing rates (Curry and Watson, 1994). The present study showed that for preservation of plasma integrity, there was a significant difference between extenders, maybe due to the great variation in density in semen diluted in Tris extenders. Injury of sperm membranes is mainly due to the lowering of temperature and/or by aging of spermatozoa during the liquid storage (Andrabi et al., 2008).

A disruption of membrane integrity after the freezing/thawing process was related to lower osmotic resistance of spermatozoa diluted with Sucrose and Lactose, indicating that these extenders was less effective at preservation of spermatozoa during the freezing/thawing process. The Tris extender contains some types of buffer substances such as Tris and egg yolk. The elevated concentration of buffer solutions in the extender decreases
The deleterious effects of the great amount of hydrogenic ions was produced from the metabolic activity of spermatozoa. 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 (Demianowicz and Strzezek, 1996). Moreover decreased pH on the extender solution increased damages of spermatozoa membrane, consequently interfering with fertilizing capacity of spermatozoa. Rakha et al. (2013) found that, the percentage of spermatozoa with intact sperm plasma membrane was higher in Tris–citric acid than in sodium citrate extender at days 2 and 3 of storage in liquid form. Similarly, Kasimanickam et al. (2007) reported that, (Tris–citric acid compared to milk based extenders, was the best diluter for maintaining the intact of ram sperm plasma membrane for up to 8 days at 4°C. 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 of extenders 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 acid extender appears to be a better option compared with skim milk and sodium citrate extenders for short and long term storage of Ghezel ram semen.

**REFERENCES**


