Effect of season on scrotal circumference, semen characteristics, seminal plasma composition and spermatozoa motility during liquid storage in INRA180 rams

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A B S T R A C T

The present study was undertaken to assess the effect of seasons on scrotal circumference, semen characteristics, seminal plasma composition, and sperm motility during liquid storage of INRA180 rams. The semen was collected from five mature INRA180 rams (2–3 years of age) during one year (from April 2014 to March 2015). Scrotal circumferences, semen characteristics, some biochemical parameters of seminal plasma were evaluated. Immediately after collection and evaluation, the semen was pooled and extended in skim milk (SM) at 15°C to reach 0.8 × 109 spermatozoa/ml. Thereafter, samples were evaluated at different storage times (0, 8, and 24 h). The results showed that scrotal circumference, semen quality and the concentration of total protein in seminal plasma were relatively constant during the year (P>0.05). However, total lipid and cholesterol concentrations increased significantly (P<0.001) in winter and summer. The result showed also that progressive motility was higher in winter and summer after 24 h of storage (P<0.01). In contrast, no difference was recorded regarding total motility (P>0.05). To conclude, the INRA180 rams have the ability to produce semen with high quality all over the year. The only parameters showing seasonal variations are cholesterol, total lipid, and progressive motility.

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1. Introduction

Increasing the number of lambs marketed per ewe and per year, is a major approach to improve the efficiency of meat production in sheep. To crossbreed the local breeds with highly prolific ones is a well-known practice in several countries. In Morocco the D’Man breed is the only prolific sheep that show a long breeding season and sexual precocity. After the creation of the INRA180, a synthetic breed resulting from D’Man and Timahdit crosses, the list of Moroccan prolific sheep has been extended (El Fadili, 2012). The breed was shown to inherit characteristics such as the prolificacy, sexual precocity and the non-seasonality of ewes (Chafri and Mahouechi, 2009; El Fadili, 2011, 2012). It might be suitable for farmers planning to intensify lambs’ production. Nevertheless, the INRA180 breed is considered as one of the most endangered sheep in Morocco (FAO,
2013), as the overall number of existing heads does not exceed 400, mainly conserved through an in situ program at INRA-El Koudia station and at different farms.

Stored liquid semen represents one way to preserve this breed through connections of various farms as well as semen transfer from the experimental stations or artificial insemination centers to different farms. To ensure this dissemination on a large scale covering distant locations and a large number of animals in a short period of time, the liquid semen must have a good quality for at least 24 h of storage (O’Hara et al., 2010). During this process the season is one factor among those (Cárdenas-Gallegos et al., 2012) that could have an influence on testicular volume, hormonal profiles, sexual behavior, semen quality changes, and semen preservation in rams (Colas, 1980; D’Alessandro and Martemucci, 2003). Furthermore, the biochemical composition of ram seminal plasma (Cardozo et al., 2006; Gündogan, 2006) and fertilizing capacity (Mann and Lutwak-Mann, 1981; Luna et al., 2015; López-Pérez and Pérez-Clariget, 2012) can also be affected by the season.

Up to the present and to the best of our knowledge the INRA180 male has never been studied before as all research efforts have been focused on the female reproduction. Recently, FAO (2013) highlighted that summarizing information on breeds, especially sexual parameters and their fluctuation under seasons, is an important step for animal genetic resources preservation. Thus, the present study aimed to determine the seasonal variations in scrotal circumference, semen characteristics, seminal plasma composition and motility of stored semen in skim milk (SM) based extender at 15 °C of INRA180 rams.

2. Material and methods

2.1. Animals and management

The study was carried out from April 2014 to March 2015. Four seasons were defined as follow: Autumn (21 September to 20 December), Winter (21 December to 20 March), Spring (21 March to 20 June), Summer (21 June to 20 September). Animals were housed under semi-arid conditions at the National Institute of Agricultural Research center of Settat; Morocco (32° latitude).

At the onset of the experiment, five mature INRA180 rams (2–3 years of age), were maintained under conventional and similar feeding, housing and lighting conditions (lighting varied with the day length). Animals were clinically examined regarding the health of their external genitalia. All animals were kept indoors in a covered shelter and allowed to walk freely. Rams were fed a mixture of straw–hay, barley and sunflower as a concentrate. Daily amounts provided per head were 1000 g of hay and 1200 g of concentrate given in two equal quantities.

2.2. Scrotal circumference

Scrotal circumference (SC) was measured monthly for each ram by grasping the neck of the scrotum with the hand using the fingers to push the testicles ventrally. The measuring metal taper was passed around the scrotum and tightened at the greatest width of the two testicles and measured in centimeters.

2.3. Semen collection and evaluation

Semen was collected monthly from each ram, using an artificial vagina (AV) with temperature ranging from 42 to 43 °C. Immediately after collection, all ejaculates were placed in a water-bath at 37 °C. Semen samples from each ram, were evaluated for volume (ml), sperm concentration (×10⁹ spermatozoa/ml), mass motility (arbitrator scale from 0 (immotile) to 5 (vigorous motility)) and individual motility (from 0 to 100%). The proportion of live and dead spermatozoa was determined using the eosin-nigrosin staining technique by counting at least 200 spermatozoa cells with bright-field microscopy (400x). Spermatozoa showing partial or complete purple stained head were considered non-viable and only spermatozoa showing strict exclusion of the stain were considered to be alive (Kulaksiz et al., 2010). The percentage of abnormality was assessed using a Diff-Quik staining (Automatic Diagnostic Systems, S.L., Barcelona, Spain). Briefly, a smear of 3 μl of diluted semen (20 × 10⁹ spermatozoa/ml) was performed on a slide and air-dried for 4 min. Then the slide was dipped for 1 min in Diff-Quik fixative solution (0.002 g/l of Fast Green in Methanol) prior to be stained with Diff-Quik solution 1 (1.22 g/l of Eosin Y in phosphate buffer at pH 6.6 and 0.1% (w/v) sodium azide as preservative) for 50 s and with Diff-Quik solution 2 (1.1 g/l of Thiazine day in phosphate buffer at 6.6) for 50 s. Between the fixing step and each of the Diff-Quik solutions, the excess was dried from the slides by placing them vertically on absorbent paper. The morphology of at least 200 spermatozoa was evaluated using a UB203 microscope (400x magnification).

2.4. Seminal plasma assessment

After each initial evaluation, seminal plasma from the five ram ejaculates was separated by centrifugation at 13000rpm for 10 min at 4 °C and the supernatants were pooled and stored at −20 °C until analysis. Total protein concentrations were measured colorimetrically using the Lowry method (Lowry et al., 1951) based on bovine serum albumin (BSA) as standard. The total lipids were estimated according to the method of Woodman and Price (1972). Cholesterol concentrations were measured by a colorimetric method (Wybenga et al., 1970).

2.5. Semen conservation and quality assessments

Immediately after collection and initial evaluation, 250 μl from each ejaculate of the five rams were pooled, then extended in skim milk to reach 0.8 × 10⁹ spermatozoa/ml and evaluated (T0). The extended semen was allowed to equilibrate at 15 °C and stored for 24 h. Thereafter, total and progressive motility were evaluated at different storage times (0, 8, and 24 h) using a computer-assisted sperm motility analysis (CASA; ISAS, version 1.0.17, Proiser, Valencia, Spain) as described by Yániz et al. (2008). For each sample, the semen was diluted in phos-
phosphate buffered saline supplemented with bovine serum albumin (1 mg/ml) to achieve $20 \times 10^6$ spermatozoa/ml.

2.6. Statistical analysis

The results were tabulated as mean ± standard error (S.E.). All analyses were carried out using a statistical software program JMP SAS 11.0.0 (SAS Institute Inc., Cary, NC, USA).

Variance analysis (one-way ANOVA) was performed by using Tukey’s post hoc test. It was used subsequently for mean value comparison for scrotal circumference, semen quality parameters and seminal plasma composition at different seasons. Differences between seasons were considered significant when $P<0.05$. For each month, the 5 rams were assessed individually for semen quality and SC. While for seminal plasma a pool gathering the 5 rams samples was used.

The data of stored sperm motility were analyzed by a factorial design ANOVA. The statistical model included the fixed effect of seasons and storage periods (0, 8, and 24 h). When statistically significant differences were detected, the Tukey’s post hoc, was used to compare the means and standard errors, considering the significance level of $P<0.05$.

For semen liquid storage a pool gathering the 5 rams samples in each month was used.

3. Results

3.1. Scrotal circumference and semen characteristics

No significant difference was observed between seasons regarding scrotal circumference (37.08 ± 0.33 cm), semen volume (1.52 ± 0.05 ml), concentration (3.39 ± 0.06 $10^6$ spermatozoa/ml), mass motility (4.66 ± 0.05), individual motility (92.31 ± 0.25%), viability (93.89 ± 0.27%) and abnormality (5.11 ± 0.19%) of INRA180 rams.

3.2. Seminal plasma assessment

The seasonal variations in seminal plasma total protein, total lipid and cholesterol are presented in Table 1. The concentration of total protein in seminal plasma was relatively constant during the year ($P>0.05$). However, total lipid ($P<0.001$) and cholesterol ($P<0.001$) concentrations increased significantly in winter and summer.

3.3. Semen liquid storage

The results of sperm total and progressive motility during liquid storage are presented in Fig. 1 and 2.

For Total motility, no significant difference was recorded between the seasons, whatever the storage time was ($P>0.05$). Similarly, within the seasons, no variation has been observed, except for winter where the total motility, decreased from 0 h to 8 h then stabilized at 24 h ($P<0.05$).

Regarding progressive motility, no significant difference was observed between different seasons at 0 h of storage ($P>0.05$). Whereas in winter and summer the highest progressive motility values were obtained at 8 h and 24 h of conservation, compared to spring and autumn ($P<0.01$). Within each season, a clear decrease in progressive motility was detected in autumn and spring after 8 h of storage ($P<0.05$), then stabilized until 24 h ($P>0.05$). However, no significant decrease was recorded concerning storage time, in winter and summer.

4. Discussion

This work is the first to cover the seasonal changes in some reproductive parameters of INRA180 rams reared in Morocco. Nowadays, it is known that the sensitivity to photoperiodic signals or the way these signals are conveyed to generate seasonal reproductive changes in sheep appears to be different between breeds. Such difference is associated to the latitude origin (Gómez-Brunet et al., 2008; Coelho et al., 2006). However, in mid-latitudes (from $>30^\circ$ N to $<45^\circ$ N) including the one of our experimental stations (32 $^\circ$ N), controversial ram sexual performances were obtained (Azawi and Ismaeel, 2012; Hötz et al. 2003; Martin et al., 2002; Tibary et al., 1988).

In the present study, the rams were in the same range of age, fed in the same way during the whole experiment and the semen collected at the same time with the same frequency. Thus, it should be supposed that only season can affect the studied semen properties. The effect of season on scrotal circumference and semen quality was demonstrated in different ovine breeds (Colas, 1980; Boland et al., 1985; Kafi et al., 2004). Surprisingly, our findings showed that there is no effect of season on SC and on fresh semen quality, when the study was conducted in a region with latitude of 32 $^\circ$ N. Similar results have been recorded in D’Man ram breed (the origin of INRA180 rams) (Tibary et al., 1988) when reared in latitudes of 32.6 $^\circ$ N, in semi-arid climate or in a region with latitudes of 36.18 $^\circ$ N and Mediterranean climate (Chafri and Mahouachi, 2009). These findings suggest that INRA180 ram breed may have inherited the no seasonality character from the D’Man breed.

Comparing the results observed for the INRA180 ram with other sheep breeds, it is found that, the overall mean recorded for SC was higher to that observed in D’man breed (Tibary et al., 1988) and for other breeds during their sexual season (Gündogan, 2006; Mickelsen et al., 1981). This may be considered as an excellent indicator of sperm production in the INRA180 ram (Tee et al., 2000).

The means of INRA180 semen volume and concentration recorded during the year in our experiment are in agreement with those showing an average volume of ram semen ranging from 0.5 to 2 ml and a mean of concentration with 3 billion/ml (Salamon, 1976; Tibary et al., 1988; Menchaca et al., 2005; Olah et al., 2013). Regarding INRA180 sperm viability and individual motility, our findings exhibit values exceeding those described by Salamon, (1976) and Gergátz, (2007). The mean of INRA180 sperm mass motility was in the same range recorded during the breeding season of Karakul rams (Kafi et al., 2004). This value was higher to that recorded for D’man breed (Chafri and Mahouachi, 2009). Our results showed no seasonal effects on sperm cell abnormalities of INRA180 rams and
Table 1
Effect of season on biochemical parameters of seminal plasma (total protein, cholesterol, total lipid) for INRA180 rams semen (X ± s.e).

<table>
<thead>
<tr>
<th>Season</th>
<th>Autumn</th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
<th>Annual Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Proteins (g/l)</td>
<td>24.93 ± 0.08</td>
<td>25.25 ± 0.22</td>
<td>25.20 ± 0.42</td>
<td>25.55 ± 0.11</td>
<td>25.24 ± 0.12</td>
</tr>
<tr>
<td>Cholesterol (g/l)</td>
<td>1.59 ± 0.01b</td>
<td>1.75 ± 0.01a</td>
<td>1.60 ± 0.02b</td>
<td>1.76 ± 0.03a</td>
<td>1.68 ± 0.03</td>
</tr>
<tr>
<td>Total Lipids (g/l)</td>
<td>3.53 ± 0.07b</td>
<td>3.96 ± 0.06a</td>
<td>3.48 ± 0.05b</td>
<td>3.83 ± 0.03a</td>
<td>3.70 ± 0.07</td>
</tr>
</tbody>
</table>

Different superscripts within rows indicate a significant effect of season within each parameter (p < 0.05).

Fig. 1. Overall mean values of INRA180 rams’ sperm total motility maintained at 15°C after different storage time (0, 8 and 24 h) during the four seasons of the year (X ± s.e).
a,b,c. Different superscripts within bars indicate an effect of storage duration within each season (P < 0.05).
A, B, C. Different superscripts within bars indicate an effect of season for each storage duration (P<0.05).

the mean value was lower to those found by Cárdenas-Gallegos et al. (2012) and Sarlós et al. (2013) respectively, for Hair and Racka rams. However, it was higher to that found for Akkaraman and Awasi rams in their sexual season (Gündogan, 2006).

Scrotal circumference and semen characteristics are not sufficient for semen assessment in the current practice of commercial artificial insemination (Gündogan, 2006). For this reason, biochemical analysis of the seminal plasma are also used for semen evaluation (Ashworth et al., 1994). As it was indicated for SC and semen characteristics, seminal
plasma constitutes could be also affected by seasonal variations (Gündogan, 2006; Dogan et al., 2009). It is known as a mixture of secretions from the epididymis and the accessory sex glands and plays a critical role in the fertilizing ability of spermatozoa (Soleilhavou et al., 2014). Among these constitutes, the proteins are known to protect spermatozoa during ejaculation and their low concentration is associated with poor semen quality (Ashworth et al., 1994; White et al., 1987). The INRA180 ram seminal plasma protein content was not affected by season. The average value of protein concentration in this study is in agreement with those found by Gündogan (2006) for the Akkaraman and Awassi rams during the sexual season.

It is documented that seminal plasma lipids and cholesterol have special relevance in the structure and function of the plasma membrane of spermatozoa (Cross, 1998). They also might play a significant role in the sperm structure, metabolism, capacitation, and fertilization of female gametes (Hafer, 1987). Taha et al. (2000) and Kelso et al. (1997) reported that a decrease in sperm concentration and motility was associated with a decline in seminal plasma lipids. On one hand, our result showed that the annual mean of total lipid and cholesterol concentrations, recorded for INRA180 seminal plasma are in concordance with previous studies in different ram breeds (Juynena and Stelletta, 2012). On the other hand, both parameters (lipids and cholesterol) significantly increased in winter and in summer compared to the spring and autumn. The recorded variation did not follow the tendency of the SC and sperm quality parameters. The latest observation is difficult to explain as. It is probably due to many uncontrollable factors, such as the assimilation rate of nutrients.

In this study, the results of INRA180 ram semen liquid storage in SM showed that the total motile spermatozoa was not affected by the season after 24 h of storage. On the other hand, that the sperm progressive motility, decreased in spring and autumn, then increased in winter and summer. This finding is positively associated to the seminal plasma cholesterol and total lipids. In fact, seminal plasma has been shown to stabilize the membrane of ram spermatozoa during in vitro processing through its protective effects against damages induced by high dilution, freezing and oxidative stress (Leahy et al., 2010). This suggests that seminal plasma may influence the seasonal resistance of the spermatozoa during liquid storage. However, this finding cannot be generalized, because of the limited number of seminal plasma samples. The decrease recorded for progressive motility in spring and autumn after 24 h of conservation did not affect the possibility to use conserved semen during the whole year. This implies the possibility to predict a successful fertility for INRA180 dissemination program (Li et al., 2016; Herrara et al., 2005).

5. Conclusion

The results of the present study suggest that rams do not show a reproductive seasonality (SC, sperm quality and total proteins of seminal plasma) which implies that this breed may have inherited the no seasonality character from its origin (D’Man breed). However, this work will gain large advantages if the breed shows its non seasonality when tested in different Moroccan regions. Total lipid and cholesterol concentrations increased in winter and summer. The stored semen in SM at 15 °C from 0 h to 24 h throughout the twelve months showed a preference for winter and summer regarding the motility parameters. The relation between total lipid and cholesterol concentrations of seminal plasma and progressive motility of liquid stored ram semen merits further research.

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