Small Ruminant Research 120 (2014) 112–115

Contents lists available at ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres

Trans-scrotal ultrasonography and testicular fine-needle aspiration cytology in the evaluation of ram sperm production

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ARTICLE INFO

Article history:
Received 23 October 2013
Received in revised form 15 April 2014
Accepted 12 May 2014
Available online 22 May 2014

Keywords:
Ram
Semen
Ultrasonography
Testicular cytology
BSE

ABSTRACT

The aim of this study was to assess the predictive value of trans-scrotal ultrasonography and testicular fine-needle aspiration cytology for sperm production in rams. Ultrasonographic examination and testicular fine-needle aspiration cytology were performed on 18 adult rams of proven fertility. Semen was collected twice using electroejaculation with an interval of 1 week, then rams were slaughtered and epididymal spermatozoa were collected. Kinetic analysis of post-thawed semen was performed using a Computer-Assisted Sperm Analysis. Rams were divided in four groups on the base of testicular appearance at ultrasonography. Data were analyzed using one-way ANOVA, and Pearson correlation indexes were calculated to identify correlation among the considered parameters. The best echogenicity score group had a higher Spermatic Index (SI) and a higher semen production than the worst group ($P = 0.047$ for electroejaculation; $P = 0.033$ for epididymal spermatozoa). Several cytological parameters were correlated with sperm production: secondary spermatocytes with electroejaculation collection (0.703; $P = 0.007$), spermatogonia (−0.746; $P < 0.001$) and SI (0.667; $P = 0.003$) with epididymal collection. In conclusion trans-scrotal ultrasonography and testicular fine-needle aspiration can add useful information about breeding soundness examination in ram.

1. Introduction

Breeding soundness examination (BSE) of rams should be carried out before the beginning of the breeding season to eliminate sub-fertile rams (Gouletsou and Fthenakis, 2010; Gouletsou et al., 2003). Routinely BSE of rams includes: libido assessment, physical examination, examination of the reproductive organs (external exam, palpation and measure of testicular dimensions) and semen evaluation (Kimberling and Parsons, 2007; Ley et al., 1990; Ruttle and Southward, 1988). We are aware of few studies (Gouletsou and Fthenakis, 2010; Gouletsou et al., 2003; Ahmad et al., 1991) that reported the importance of ultrasound imaging in ram BSE. In particular Gouletsou et al. (2003) proposed scanning techniques for ultrasonography of ram genitalia and described the normal ultrasonographic appearance of these organs. Testicular fine needle aspiration cytology (TFNAC) has been proposed for the BSE of bulls (Chapwanya et al., 2008), while no report are available on its use in ram, except for a work reporting normal testicular cytology composition in healthy rams (Vencato et al., 2013) with no mention to its relationship with fertility or semen quality. This study was designed to define the correlation of ultrasonography and TFNAC examination with semen quality collected either by electroejaculation or from epididymis and to assess if these techniques can be useful in the BSE of rams.

2. Materials and methods

Eighteen healthy rams of four different breeds ($N = 5$ Alpagota; $N = 5$ Brogna; $N = 4$ Foza; $N = 4$ Lamon) of 5–7 years of age and with proven fertility were used in this study. Animals were housed in a group pen, fed with hay ad libitum and supplemented with a commercial concentrate (Compli Sheep®, TECNOZOO). The study was conducted during the 2012-breeding season in Northern Italy. A general health examination and clinical

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http://dx.doi.org/10.1016/j.smallrumres.2014.05.005
0921-4488/

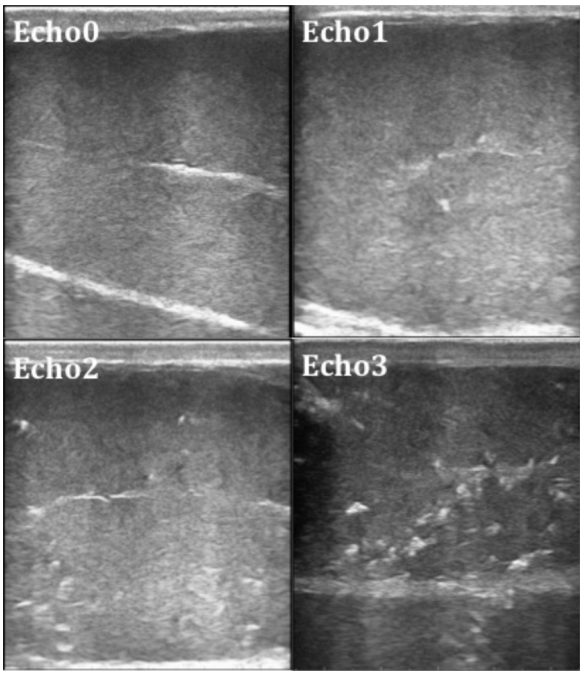

Fig. 1. Testis ultrasonography appearance classes in rams.

examination of genitalia were performed as previously described (Fthenakis et al., 2001) restraining rams in a standing position. Testicular ultrasonography was carried out using an ultrasound scanner (MyLabVet™ One, ESAOTE S.P.A., Genoa, Italy) fitted with a multiple frequency (6.0, 8.0 and 10.0 MHz) linear transducer. Different frequencies were tested in order to define the one providing higher quality images, and 10 MHz frequency was used for the entire study. Animals were restrained using a neck lock system, with an operator lifting the tail; no sedation was required. The testicles were pulled downwards within the scrotum and the operator held each testicle at the site opposite to where the transducer was applied. Sagittal and transverse sections of the testicles were taken as previously described (Gouletsou et al., 2003). Testicular parenchyma appearance was classified using a 0–3 score scale (Fig. 1). Score 0: testes with a homogeneous parenchyma with a coarse medium echo-pattern except for the mediastinum testis; score 1: testes with a less homogeneous parenchyma; score 2: testes with highly echogenic structures in relation to the surrounding tissues occupying less than 10% of the parenchyma; score 3: diffuse highly echogenic structures in the parenchyma.

Testicular fine needle aspiration cytology (TFNAC) was performed in both testicles of all rams as described in previous works in ram and other species (Vencato et al., 2013; Stelletta et al., 2011; Romagnoli et al., 2009). Slides were stained using a modified May-Grünwald-Giemsa staining (Sigma–Aldrich) as described by Vencato et al. (2013). TFNAC slides were evaluated under an optical microscope, Nikon ECLIPSE E600, with a 1000× magnification, counting at least 200 cells per slide. Firstly a descriptive analysis of cell morphology was made, dividing Spermatogonia, Primary Spermatocyte or Spermatocyte I, Secondary spermatocyte or Spermatocyte II, AB spermatids or "early" spermatids, CD spermatids or "late" spermatids, Spermatozoa and Sertoli cells. Relative percentages of each cell types were then determined. In addition, the following indexes were calculated: Spermatic Index (SI) or the percentage of spermatozoa on the total of spermatogenic cells and Sertoli Index (SEI) or the percentage of Sertoli cells on the total of spermatogenic cells, considering the total of spermatogenic cells as the sum of spermatogonia, spermatocytes and spermatids.

Semen was collected using an electroejaculator (Ruakura Ram Probe Plastic Products, Hamilton, New Zealand). To collect semen, rams were restrained as previously described, feces were removed manually from the rectum and a 5 ml bolus of lidocaine 2% was introduced in the rectum. The probe was inserted into the rectum until electrodes were localized above the upper portion of the ampullary region. The electrical stimulation was applied for 3 s and then stopped for 1 s. This cycle was repeated until ejaculate was obtained (usually 6–7 electrostimulations). Semen was collected inside a graduated tube and then placed in a warm water bath (35 °C) and evaluated immediately for volume, concentration, wave motion and total sperm count. Ejaculates were diluted using Tryladil (Minitube International, Tiefenbach, Germany) supplemented with 20% egg yolk and heated at 35 °C, to obtain a final concentration of 40×10^7 spermatozoa/ml. The samples were equilibrated for 2 h, during which the diluted semen was packaged in 0.25 ml straws. These were kept 5 cm above liquid nitrogen for 10 min and then plunged into the nitrogen (−196.8 °C).

Semen was collected twice within a 7-day interval. Seven days after the second collection the rams were slaughtered. Testicles were removed, immediately placed in 5 °C saline solution, as reported by Lone et al. (2011) and transported to the lab within 1 h. The cauda epididymis was excised from the testicle and several longitudinal incisions were made to expose the spermatozoa to the outer environment. Each cauda epididymis was washed with 3 ml of Tryladil supplemented with 20% egg yolk. Concentration and progressive motility were evaluated. Samples showing ≥60% motility were extended to reach final concentration of 40×10^7 spermatozoa/ml. Semen was cryopreserved as described previously. Following thawing semen was analyzed using a Computer-Assisted Sperm Analysis (CASA) with a Hamilton Thorne Bioscience device. The parameters considered were: motility, progressive motility, average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN), elongation (ELONG) and area. The number of 0.25 ml straws containing 10×10^7 spermatozoa was also calculated for each semen collection. Mean values and standard deviations of all parameters taken into account were calculated. One-way ANOVA was performed using the GLM procedure of the software SIGMASTAT 2.03 in order to identify the predicting values of echogenicity score on TFNAC, total sperm, straws production, motility and progressive motility. Pearson correlation indexes were calculated using the software SIGMASTAT 2.03 to establish correlations between ultrasonography imaging, testicular cytology and semen quality.

3. Results

No general health problems were recorded during the clinical examination of the rams. Median body condition score recorded was 3.5 (range 2.75–3.75) considering a score from 0 to 5 (Rankins and Pugh, 2012). Scrotal circumference in all subjects was >33 cm, satisfying the requirement normally considered in the BSE (Van Metre et al., 2012). No abnormalities were detected in their genitalia. Semen collection was achieved both by electroejaculation and by recovery from the epididymis.

Ultrasonographic imaging was obtained using 10 MHz frequency probe with a 60 mm scanning depth. To standardize the exam the images were acquired when the mediastinum testis was evident. The classification of parenchyma was as follows: 27.8% (5/18) of animals had an echogenicity score of 0 (Echo0), 33.3% (6/18) had a score of 1 (Echo1), 27.8% (5/18) had a score of 2 (Echo2) and 11.1% (2/18) had a score of 3 (Echo3).

Table 1 shows the results for TFNAC, total sperm, semen straw production, sperm motility and progressive motility for each echogenicity score class. The mean values for the percentages of spermatogonia, spermatocyte I, spermatocyte II, AB spermatids and CD spermatids were not significantly different between the four echogenicity score classes. The SI declined from the Echo0 (49.8 ± 7.45) to the Echo3 (38.6 ± 13.9) class with values of 45.9 ± 3.97 for Echo1 and 45.3 ± 5.24 for Echo2. The difference between Echo0 and Echo3 was significant ($P = 0.031$). The SEI showed some differences between the four classes (no significant).