Available online at www.sciencedirect.com
SCIENCE DIRECT
Theriogenology
ELSEVIER
Theriogenology 64 (2005) 1756–1765
www.journals.elsevierhealth.com/periodicals/the

Effect of semen collection method on pre- and post-thaw Guirra ram spermatozoa

F. Marco-Jiménez [a,*], S. Puchades [b], J. Gadea [c], J.S. Vicente [b], M.P. Viudes-de-Castro [a]

[a] *Animal Research and Technology Center, Valencian Institute of Agrarian Research, Ctra. Náquera-Moncada Km 4.5, 46113 Moncada, Valencia, Spain*
[b] *Laboratory of Biotechnological Reproduction, Department of Animal Science, Polytechnic University of Valencia, Valencia 46071, Spain*
[c] *Department of Physiology, School of Veterinary, University of Murcia, Murcia 30071, Spain*

Received 24 February 2005; received in revised form 11 April 2005; accepted 12 April 2005

Abstract

In this study, we evaluated the potential effect of the method of recovery (artificial vagina or electroejaculation) on the production and quality of Guirra ram spermatozoa cryopreserved for the possible constitution of a sperm bank. In order to address this question, we evaluated the effect of semen collection method on fresh semen quality parameters, including: volume, concentration, production, microscopic analysis (abnormal sperm and intact apical ridge) and sperm motility parameters determined by CASA system. For frozen–thawed semen, we evaluated motility parameters by CASA and intact apical ridge, acrosomal status, assessed by dual staining by IP and FITC-PNA and capacitation status, assessed by M540 and Yo-pro1, using flow cytometry. The main findings from this study were: (i) that electroejaculation resulted in a lower recovery efficiency (80% of the cases), as a consequence of contamination with urine or lack of response to the electrical stimulation; (ii) the fresh seminal quality was not significantly different between recovery methods, except for the concentration of spermatozoa, but total number of spermatozoa and the consequent number of possible seminal doses for artificial insemination were similar; and, (iii) a higher number of stable and functional spermatozoa (higher number of live non-capacitated cells, higher live acrosome intact cells and live acrosome reacted cells) were found for frozen–thawed spermatozoa collected by electro ejaculation than by artificial vagina. According to our results, we are able to develop both methodologies in the creation of the Guirra sperm bank. Assuming the advantages and

* Corresponding author. Tel.: +34 96 342 40 07; fax: +34 96 342 40 07.
E-mail address: framarj1@doctor.upv.es (F. Marco-Jiménez).

0093-691X/$ – see front matter
doi:10.1016/j.theriogenology.2005.04.006

limitations of both methodologies, in Guirra breed, would enable the rapid constitution of a sperm bank including samples from a large number of non-trained rams in a short period of time, which will increase the genetic variability, and so guarantee the conservation of this breed.

Keywords: Electroejaculation; Methods collection; Ram-spermatozoa; Flow cytometry

1. Introduction

The Levantina Red (Guirra) population has been decreasing in recent years, and effective procedures must be applied for the preservation of the valuable genetic patrimony. Cryopreservation of spermatozoa, combined with artificial insemination, may become a more powerful technology for the storage and regeneration of valuable germplasm [1]. Genetic resource banks are used in combination with reproductive technologies for the conservation of endangered species [2].

The Levantina Red (Guirra) sheep is an autochthonous breed distributed in the southeast of Spain. The Guirra is an African ancestry breed, characterized by the reddish coat color, the high degree of wool greasiness and a convex frontonasal profile [3]. The Levantine Red breed shows special qualities, such as adaptability to aridity and drought, a good maternal character and its dual main uses (meat and milk). Nowadays, the total population size is 4850 females and 421 males and the population trend is decreasing (DAD-IS, 2005; EAAP Animal Genetic Data Bank, 2005).

The first step in the creation of the cryopreserved semen bank is the use of an effective method of semen recovery. Semen collection from large numbers of untrained rams makes sperm bank constitution difficult. Semen can be collected from live animals by artificial vagina (AV) and electrical stimulation (EE) [4]. Diverse studies have evaluated the benefits and limitation of both methods of recovery [4–11], but nothing is known about this autochthonous breed. The collection of semen with an artificial vagina resembles natural service, but usually requires a preliminary training period [11], whereas for obtaining semen from a large number of rams, electroejaculation could be a useful procedure [12].

The quality as well as the viability of ram spermatozoa deteriorates as a consequence of freezing and thawing [13]. Ram spermatozoa are sensitive to extreme temperature changes during freezing processes [14] and procedures used to cryopreserve sperm cells have been shown to induce damage to the sperm plasma membrane [15–18]. Gillan et al. [19] suggest that dilution, cooling, freezing and thawing (cryopreservation process) induced structural changes leading to capacitation process. When cryopreserved sperm cells are used in artificial insemination, eight times more sperm cells are required to achieve normal fertilization rates [20], explained by the lower viability, reduced motility and increased abnormal apical ridge. On the other hand, it may occur that dead cells lose acrosomal content due to the breakdown of the membranes and so may present a similarity with acrosomal changes [21].

During the last decade, the discovery of a variety of fluorochromes and compounds conjugated to fluorescent probes has enabled a more widespread analysis of sperm attributes and in conjunction with flow cytometry. [22]. Flow cytometry has been very

helpful in evaluating sperm quality by providing a specific, objective, accurate and reproducible method compared to traditional microscopy-based methods [23]. On the other hand, Computer-assisted sperm analysis (CASA) has also provided an objective and accurate means of evaluating overall sperm motility [24].

The main aim of this study was to evaluate the potential effect of the method of recovery (artificial vagina or electroejaculation) on the production and quality of Guirra ram spermatozoa cryopreserved.

2. Materials and methods

All animal care and procedures used to collect semen were performed in accordance with the Spanish Animal Protection Regulation, RD233/1988, which conforms to European Union Regulation 86/609 and adheres to guidelines established for care and use of Laboratory Animals promulgated by the Society for the Study of Reproduction.

All the chemicals, unless otherwise stated, were reagent grade and purchased from Sigma-Aldrich Química S.A. (Alcobendas, Madrid, Spain). The experiment was carried out at the Department of Animal Science (Universidad Politécnica de Valencia. Spain).

3. Semen collection

Semen was routinely collected from eight mature fertile Guirra rams using the AV and electroejaculation (EE) method alternately, once per week, during 10 weeks. For the electroejaculation, Electrojac IV® stimulator (Minitub Iberica SL, Reus, Spain) was used with a rectal probe of 22 cm long, 2.5 cm in diameter and with three electrodes. Animals received an intramuscular injection of xylazine (0.2 mg kg^{-1} body weight; Rompun 2%, Bayer S.A. Barcelona, Spain) 15 min before electroejaculation. The rectal probe was lubricated and gently inserted into rectum, and orientated so that the electrodes were positioned ventrally. The Electrojac IV® was used in automatic setting, applied for 2 s with 2-s rest intervals between stimuli, increasing the voltage stimuli by one volt at a time. The penis was prolapsed beyond the prepuce, and semen was collected into a graduated collection vial attached to an artificial vagina at room temperature.

For collecting semen with an AV, rams were penned with estrual ewes, in the presence of a handler with an artificial vagina. Temperature of the water in the lining of the AV ranged from 40 to 44 °C at the time of seminal collection.

4. Freezing and thawing protocol

Semen samples were diluted 1:1 at 30 °C with a tris-glusose-citric acid extender supplemented with egg yolk (15%, v/v, [24]) and cooled to 4 °C for 45 min. Then, spermatozoa were diluted 1:5 with the same medium with 5% glycerol (v/v) and equilibrated at 4 °C for 2 h. Later they were packaged in 0.25 mL straws (IMV®, L'Aigle,

France), sealed with modeling paste and frozen in liquid nitrogen vapor, 5 cm above the liquid nitrogen level for 10 min, before being plunged into the liquid nitrogen for storage.

Thawing was carried out by immersing in circulating water bath at 42 °C for 12 s.

5. Sperm analysis

After collection (either by AV or EE), volume of each ejaculate was measured in a graduated tube. The proportion of spermatozoa with an intact apical ridge was evaluated after fixation in a buffered 2% glutaraldehyde solution and examined under differential interference contrast microscope (Normaski contrast) in a Leica Leitz DM IRB (Leica, Barcelona, Spain) microscopy (magnification ×750) to analyze acrosomes [25].

The sperm concentration (10^9 spermatozoa mL^{-1}) was determined using a Thoma-Zeiss counting cell chamber (Marienfield, Germany). Total number of spermatozoa per ejaculate (10^9 spermatozoa) was calculated as the product between sperm concentration and volume of the ejaculate.

The percentage of abnormal spermatozoa (considering all normal forms in sperm head, intermediate piece and tail) was estimated at a magnification of 400× with a phase contrast microscope (Eclipse E400 Nikon, Izasa S.A., Barcelona, Spain).

6. Motion parameters

Motion parameters were assessed using a CASA system (Vimas, Imagesp®, Barcelona, Spain). The CASA-derived motility characteristics studied were total motile sperm cells (%), curvilinear velocity (VCL, $\mu m\ s^{-1}$), straight-line velocity (VSL, $\mu m\ s^{-1}$), average-path velocity (VAP, $\mu m\ s^{-1}$), linearity (LIN = [VSL/VCL] × 100, %), amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz) were evaluated.

To assess sperm motion, aliquot semen was diluted (dilution 1:20) in DPBS supplemented with BSA (1 mg mL^{-1}). A 10 μL drop of sperm was placed on warmed (37 °C at 100× using a phase contrast microscope) slide and covered with a cover slip (20 mm × 20 mm). The main software settings were: 25 frames per sequence, minimum of 15 frames per object and 20 $\mu m\ s^{-1}$ as velocity limit for immobile objects. For each sample, four microscopic fields were analyzed and a minimum of 200 sperms were evaluated.

7. Analysis of seminal parameters by flow cytometry

Flow cytometric analyses were performed using a Coulter Epics XL cytometer (Beckman Coulter Inc., Miami, Florida, USA). A 15 mW argon laser tuned to 488 nm. Fluorescence from 10,000 cells was measured and 4 readings per sample were recorded. Flow cytometric data were analyzed with the software Expo32ADC (Beckman Coulter Inc.) using a gate in forward and side scatter to exclude eventual remaining debris and aggregates from the analysis.

8. Assessment of capacitation status

The detected increase in sperm plasma membrane lipid packing disorder was evaluated with the stains merocyanine 540 (M540, 1 mM solution in DMSO) and Yo-Pro 1 (25 μM solution in DMSO; Molecular Probes). Sperm were diluted after thawing to 5–10×10^6 mL^{-1} with a DPBS diluent, and 2.7 μL M540 (final concentration: 2.7 μM) and 1 μL of Yo-Pro (final concentration: 25 nM) were added to 1 mL of diluted sperm. Fluorescence was measured using a FL-2 sensor, a 575 nm band-pass filter to detect M 540, and a FL-1 sensor and a 525 nm band-pass filter to detect Yo-Pro 1. Cells were classified in low merocyanine fluorescence (live cells, non-capacitated), high merocyanine fluorescence (live cells, capacitated) or Yo-Pro-1 positive (dead).

9. Assessment of acrosomal integrity

Sperms were diluted after thawing to 5–10×10^6 mL^{-1} with a DPBS diluent, and 2 μL propidium iodide (PI; 100 μg mL^{-1} solution in distilled water) and 2 μL of fluorescein-labeled lectin from the peanut plant, *Arachis hypogaea* (FITC-PNA, 10 μg mL^{-1} solution in saline solution) was added to 1 mL of diluted sperm. Samples were incubated for 10 min at room temperature. Fluorescence was measured using a FL-1 sensor, a 525 nm band-pass filter to detect FITC-PNA, and a FL-2 sensor and a 575 nm band-pass filter to detect PI. Four sperm sub-populations were detected: live acrosome intact, live acrosome damaged, dead acrosome intact and dead acrosome damaged.

10. Experiment design

10.1. Effect of collection method on quality parameters for fresh sperm

To examine the effect of method of semen collection on quality parameters, 10 ejaculates from 8 rams were evaluated for:

(a) Volume, concentration and total number of spermatozoa per ejaculate.
(b) Percent of motility and motion parameters by CASA.
(c) Percent intact apical ridge and abnormal spermatozoa by microscopic observation.

10.2. Effect of the method of semen collection on frozen–thawed sperm quality

To examine the effect of method of semen collection on thawed quality parameters, spermatozoa from each male were frozen and three straws per male and method collection were thawed for each week of collection. Seminal samples were evaluated for:

(a) Percent of motility and motion parameters by CASA.
(b) Percent intact apical ridge by microscopic observation.

(c) Capacitation status by merocyanine and Yo-Pro1 by flow cytometry.
(d) Acrosomal integrity by propidium iodide and FICT-PNA by flow cytometry.

11. Statistical analysis

The sperm parameters of each experiment were compared by ANOVA using the general linear models (GML) procedure of the Statgraphics®Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA). We considered the fixed effects were the collection method, male and method collection by male interaction. Week of collection had initially been included in the model, but was excluded as no differences were shown. The results are presented as least square means (LSM) ± standard error of the means (S.E.M.). Significance level was set at $P < 0.05$.

12. Results

When the artificial vagina was used, all the recoveries were effective (40 ejaculates) whereas when semen was collected by electroejaculation, 38 ejaculates (from 40 attempts) were obtained and 3 ejaculates (from different males) then suspected of being contaminated with urine were eliminated from the study (finally 35 ejaculates were processed and evaluated). The mean voltage required to produce ejaculation was 6.8 ± 0.1 V, being similar between males.

12.1. Effect of method collection on quality parameters for fresh sperm

In relation to the collection method, the seminal parameters were similar for both methodologies and only sperm concentration was affected by the collection method, with significantly lower numbers of sperm per milliliter in ejaculates obtained by EE than by AV (5.2×10^9 sperm mL^{-1} versus 6.2×10^9 sperm mL^{-1}, $P = 0.020$, Table 1). The volume, concentration and total number of spermatozoa per ejaculate showed great variation between individual rams ($P < 0.001$). However, microscope evaluation of intact apical ridge, abnormal spermatozoa and motion parameters measured by CASA was not affected by male. Male and method interactions were not detected for any semen parameters evaluated.

12.2. Effect of the semen collection method on frozen–thawed sperm quality

The capacitation and acrosome status evaluated by flow cytometry was affected by collection method, so a higher number of stable and functional spermatozoa were found for frozen–thawed spermatozoa from the electroejaculation collection. The percentages of non-capacitated viable and acrosome intact viable cells were higher in EE than in AV ($P < 0.001$, Table 2), while the opposite was found for the percentage of acrosome reacted viable spermatozoa with lower numbers in EE than in AV ($P < 0.001$). A significant ram effect was detected for some of the parameters studied in the frozen–thawed spermatozoa

Table 1
Effect of semen collection method on quality parameters of fresh sperm (LSM ± S.E.M.)

Parameter	Artificial vagina	Electroejaculation	*P*-value
Volume (mL)	1.2 ± 0.1	1.0 ± 0.1	0.174
Concentration ($\times 10^9$ spermatozoa mL^{-1})	6.2 ± 2.0[a]	5.2 ± 2.1[b]	0.020
Production (10^9 sperm per ejaculate)	6.5 ± 2.3	5.8 ± 2.0	0.192
Morphological parameters			
Abnormal sperm (%)	9.1 ± 1.0	8.1 ± 1.1	0.497
Intact apical ridge (%)	93.3 ± 0.9	93.2 ± 0.9	0.950
Sperm motility parameters			
Total motile cells (%)	69.8 ± 2.5	71.9 ± 2.7	0.579
Curvilinear velocity (μm s^{-1})	172.7 ± 7.2	159.4 ± 7.2	0.208
Straight-line velocity (μm s^{-1})	74.8 ± 3.4	77.6 ± 3.7	0.587
Average-path velocity (μm s^{-1})	96.1 ± 3.8	102.5 ± 4.0	0.252
Linearity (%)	39.8 ± 3.8	44.6 ± 4.1	0.396
Amplitude of lateral head displacement (μm s^{-1})	9.7 ± 0.8	9.2 ± 0.8	0.646
Beat cross frequency (Hz)	12.6 ± 0.4	11.9 ± 0.4	0.258
Number of samples	40	35	

LSM: least square means. S.E.M.: standard error means; values in the same row with different superscripts (a and b) are statistically different ($P < 0.05$).

Table 2
Effect of semen collection method on quality parameters of frozen–thawed sperm (LSM ± S.E.M.)

Parameter	Artificial vagina	Electroejaculation	*P*-value
Intact apical ridge (Nomarski contrast, %)	39.8 ± 1.4	42.1 ± 1.4	0.256
Sperm motility parameters			
Total motile cells (%)	40.3 ± 1.7	39.6 ± 1.7	0.576
Curvilinear velocity (μm s^{-1})	128.1 ± 3.3	126.6 ± 3.2	0.607
Straight-line velocity (μm s^{-1})	68.2 ± 1.8	65.8 ± 2	0.375
Average-path velocity (μm s^{-1})	84.3 ± 1.7	80.2 ± 1.7	0.107
Linearity (%)	54.1 ± 2.2	60.3 ± 2.2	0.101
Amplitude of lateral head displacement (μm)	6.9 ± 0.4	6.5 ± 0.4	0.346
Beat cross frequency (Hz)	12.6 ± 0.3	13.1 ± 0.3	0.224
Capacitation status (M540/Yo Pro 1)			
Live cells, capacitated	7.8 ± 1.0	6.2 ± 0.7	0.669
Live cells, non-capacitated	20.7 ± 1.1[b]	24.2 ± 0.8[a]	0.000
Dead cells	71.5 ± 1.3	69.6 ± 1.0	0.321
Acrosome integrity (FITC-PNA/PI)			
Live acrosome reacted	3.4 ± 0.4[a]	1.4 ± 0.4[b]	0.000
Live acrosome intact	21.2 ± 1.2[b]	26.0 ± 1.0[a]	0.000
Dead	72.6 ± 1.2	75.4 ± 1.4	0.149
Number of samples	40	35	

LSM: least square means. S.E.M.: standard error means; M540: merocyanine 540; PI: propidium iodide; FITC-PNA: fluorescein-labeled lectin from the peanut plant, *Arachis hypogaea*. Values in the same row with different superscripts (a and b) are statistically different ($P < 0.05$).

(VSL, VAP, percentage of non-capacitated viable and acrosome intact viable cells, $P < 0.05$).

13. Discussion

In this study we evaluated the effects of semen collection methods on fresh and frozen–thawed spermatozoa in order to adapt a swift methodology for the creation of the Guirra cryopreserved sperm bank. The main findings from this study were: (i) that electroejaculation resulted in a lower recovery efficiency (80% of the cases), as a consequence of contamination with urine or lack of response to the electrical stimulation; (ii) the fresh seminal quality was not significantly different between recovery methods, except for the concentration of spermatozoa, but total number of spermatozoa and the consequent number of possible seminal doses for artificial insemination were similar; and, (iii) a higher number of stable and functional spermatozoa was found for frozen–thawed spermatozoa from the electroejaculation collection than by artificial vagina.

Previous studies reported that electroejaculation resulted in larger volumes [7,26–28] and lower concentration [29]. The physiological response to electrical stimuli resulted in large volume, probably from contribution of the accessory sex glands [7,8,10] and the urinary losses caused in rams by the retrograde flow of spermatozoa [27]. However, the total number of spermatozoa in the ejaculate seems to be similar [7,27]. On the other hand, electroejaculation is known to alter semen characteristics [26,30], diminishes semen quality [8,9,10] and is worse for predicting fertility [29].

Flaws in recovery methodology have been shown when electrical stimulation was used [7]. In this study, different physiological responses for attempted recovery were observed only when electroejaculation was used (80% efficiency versus 100% for artificial vagina). Electroejaculation could affect the ejaculation reflex as a consequence of the aggressiveness of these techniques, stress management or insufficient stimulation in rectal mucose [7].

The survival of frozen–thawed ram sperm is affected by many factors, such as male, extenders, cryoprotectants, packaging, freezing and thawing rates, as well as the quality of the sperm used for freezing [14]. Cryopreservation process is associated with sperm function damage, including swelling and breakage, loss of membrane selective permeability and changes in membrane fluidity, leakage and aggregation of phospholipids and proteins, reduction in motility, enzyme activity and viability [31] and it seems that cryopreservation induced modifications in sperm membranes, making them more prone to capacitation Watson [16]. Quinn et al. [32] suggested that spermatozoa collected with an artificial vagina were more resistant to cold shock than when ejaculated electrically. The post-thaw resulted, for both methods, in a similar seminal quality as measured by both microscopic analysis and CASA. In this study, a significant effect on capacitation status and acrosomal integrity was detected; percentage of non-capacitated viable and acrosome intact viable cells were higher in EE, while the opposite was found for the percentage of acrosome reacted viable spermatozoa with lower number in EE than in AV. Sperm capacitation and acrosome reaction are two key steps in the fertilization process. Thus, an

evaluation of these processes would be of paramount importance in assessing sperm fertilizing ability [19,35]. Nevertheless, to elucidate the biological meaning of these in vitro differences a wide fertility trial must be developed.

In order to explain how the collection method affects the frozen–thawed sperm function, one main hypothesis could be the differences in seminal plasma composition secreted from sex glands [26,32]. The addition and removal of a variety of proteins during epididymal maturation and at ejaculation play important roles in the capacitation of sperm and fertilization of the egg [33], and Barrios et al. [34] suggested that the acquisition of seminal plasma proteins by adsorption to the sperm cell surface modifies the functional characteristics of damaged spermatozoa.

According to our results, we are able to develop both methodologies in the creation of the Guirra sperm bank. Assuming the advantages and limitations of both treatments: quick application of electroejaculation methodology, without the need for a training period, providing better thawed sperm quality, permitting a fast performance for the conservation of Guirra breed without detriment to cryopreserved seminal quality.

Acknowledgements

This study was funded by the INIA (Project RZOO-014-C3-01) and a special grant from the Polytechnic University of Valencia.

References

[1] Holt WV, Pickard RP. Role of reproductive technologies and genetic resource banks in animal conservation. Rev Reprod 1999;4:143–150.2.
[2] Yoshida M. Conservation of sperm: current status and new trends. Anim Reprod Sci 2000;60/61: 349–55.
[3] Sanchez A, Sanchez M. Razas ovinas Españolas. Ministerio de agricultura, pesca y alimentación. Madrid: Publicaciones de extensión agraria, Ministerio de agricultuta, pesca y alimentación; 1986. p. 773–82.
[4] Salamon S, Evans G, Maxwell WMC. Inseminación artificial de ovejas y cabras. Editorial ACRIBIA, S.A. Zaragoza (España); 1990. p. 87–95.
[5] Gunn RMC. Artificial production of seminal ejaculation and the characters of the spermatozoa therein. Council Sci Ind Res Bull 1936;94.
[6] Nichols GD, Edgar DG. A transistorised rectal probe for ejaculating rams. N Z Vet J 1964;12:145–6.
[7] Dziuk SP, Graham FE, Donker DJ, Marion BG, Peterson EW. Some observations in collection of semen from bulls, goats boars and rams by electrical stimulation. Vet Med 1954;69:455–8.
[8] Brady DE, Gildow EM. Characteristics of ram semen as influenced by method of collection. In: Proc 32nd Annu Mtg Am Soc Anim Prod. 1939.p. 2500–254.
[9] Terrill C. Comparison of ram semen collection obtained by three different methods for artificial insemination. In: Proc Eerd Annu Mtg Am Soc Anim Prod. 1940.p. 2010–207.
[10] Mattner PE, Voglmayr JK. A comparasion of ram semen collection by the artificial vagina and by electroejaculation. Aust J Exp Agric Anim Husb 1962;2:78–81.
[11] Wulster-Radcliffe MC, Williams MA, Stellflug JN, Lewis GS. Technical note: artificial vagina vs. a vaginal collection vial for collecting semen from rams. J Anim Sci 2001;79:2964–7.
[12] Foote RH. The history of artificial insemination: selected notes and notables. J Anim Sci 2002;80(electronic suppl 2).

[13] Fiser PS, Hansen C, Underhill L, Marcus GJ. Thermal stress test to ass ess the viability of cryopreserved boar sperm. Cryobiology 1991;28:454–9.
[14] Salamon S, Maxwell WMC. Frozen storage of ram semen I. Processing, freezing, thawing and fertility after cervical insemination. Anim Reprod Sci 1995;37:185–249.
[15] Hammerstedt RH, Graham JK, Nolan JP. Cryopreservation of mammalian sperm: what we ask them to survive. J Androl 1990;11:73–88.
[16] de Leeuw AM, den Daas JH, Woelders H. The fix vital stain method. Simultaneous determination of viability and acrosomal status of bovine spermatozoa. J Androl 1991;12:112.
[17] Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod Fertil Dev 1995;7:871–91.
[18] Watson PF. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 2000;60/61:481–92.
[19] Gillan L, Evans G, Maxwell WM. Capacitation status and fertility of fresh and frozen–thawed ram spermatozoa. Reprod Fertil Dev 1997;9:481–7.
[20] Shannon P, Vishwanath R. The effect o optimal and suboptimal concentration of spermatozoa on the fertility of fresh semen and frozen bovine semen and theoretical model to explain the fertility differences. Anim Reprod Sci 1995;39:1–10.
[21] Marti JI, Cebrian-Perez JA, Muino-Blanco T. Assessment of the acrosomal status of ram spermatozoa by RCA lectin-binding and partition in an aqueous two-phase system. J Androl 2000;4:541–8.
[22] Gillan L, Evans G, Maxwell WM. Flow cytometric evaluation of sperm parameters in relation to fertility potential. Theriogenology 2005;63:445–57.
[23] Graham JK. Assessment of sperm quality: a flow cytometric approach. Anim Reprod Sci 2001;68:239–47.
[24] Verstegen J, Iguer-Ouada M, Onclin K. Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology 2002;57:149–79. Review.
[25] Pursel VG, Johnson LA. Glutaraldehyde fixation of boar spermatozoa for acrosome evaluation. Theriogenology 1974;1:63–8.
[26] Quinn PJ, White IG. Variation in semen cations in relation to semen quality and methods of collection. Fertil Steril 1966;17:815–25.
[27] Pineda MH, Dooley MP, Hembrough FB. Retrograde flow of spermatozoa into the urinary bladder of rams. Am J Vet Res 1987;48:562–8.
[28] Pineda MH, Dooley MP. Effect of method of seminal collection on the retrograde flow of spermatozoa into the urinary bladder of rams. Am J Vet Res 1991;52:307–13.
[29] Hulet CV, Foote WC, Blackwell RL. Effects of natural and electrical ejaculation on predicting fertility in the ram. J Anim Sci 1964;23:418–24.
[30] Lightfoot RJ. The effect of mating on semen characteristics. Aust J Agric Res 1968;19:1043–57.
[31] Thundathil J, Gil J, Januskauskas A, Larsson B, Soderquist L, Mapletoft R, et al. Relationship between the proportion of capacitated spermatozoa present in frozen–thawed bull semen and fertility with artificial insemination. Int J Androl 1999;22:366–73.
[32] Quinn PJ, Salamon S, White IG. The effect of cold shock and deep-freezing on ram spermatozoa collected by electrical ejaculation and by an artificial vagina. Aust J Agric Res 1968;119:128.
[33] Yanagimachi R. Fertility of mammalian spermatozoa: its development and relativity. Zygote 1994;371–2 [review].
[34] Barrios B, Perez-Pe R, Gallego M, Tato A, Osada J, Muino-Blanco T, et al. Seminal plasma proteins revert the cold-shock damage on ram sperm membrane. Biol Reprod 2000;63:1531–7.
[35] Harrison RAP. Sperm plasma membrane characteristics and boar semen fertility. J Reprod Fertil 1997; 52:271–83.