Influence of semen collection method on sperm cryoresistance in small ruminants

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Abstract

The semen recovery for cryopreservation is a key step in conservation programs of small ruminants. The semen collection in these species is mainly by artificial vagina (AV), being electroejaculation (EE) an alternative when males are not trained to AV. In this work, it has been studied the response to sperm cryopreservation depending on the method of semen collection in two species of small ruminants, sheep and goat. Semen from Blanca-Celtibérica bucks and Manchega rams was collected by AV and EE at same time. For both species, sperm samples were diluted with the same extender and frozen over nitrogen vapors. At thawing, sperm motility by CASA, and viability, membrane stability, mitochondrial activity and chromatin stability by flow cytometry, were assessed. The collection method influenced on the sperm quality at thawing in bucks, whereas no differences between both methods were observed in rams. Thus, sperm motility was higher for samples collected by AV in relation to EE samples in bucks. For this same species, parameters evaluated by flow cytometry also resulted in worse sperm quality for samples collected by EE compared to those obtained by AV and, those from rams obtained by both methods. Results obtained suggest that ovine and caprine spermatozoa have different response to the cryopreservation process depending on the semen collection method used. The EE procedure could lead to changes in the composition of the ejaculate in bucks that would cause spermatozoa to be more susceptible to the cryopreservation process whereas this procedure would have had no effect on ram spermatozoa.

Keywords: artificial vagina, electroejaculation, buck, ram, sperm cryopreservation

Introduction

Artificial insemination (AI) in small ruminants is mostly performed using fresh semen since fertility is very low using frozen semen. However, the semen cryopreservation is an important tool for improving breeding or conservation programs in different species. For this reason, in recent years, numerous studies have been carried in order to improve the semen cryopreservation protocols in these species.

It should be highlighted that the first step in the semen cryopreservation is the recovery of sperm samples. For small ruminants, semen collection methods of wider use are the artificial vagina (AV) and electroejaculation (EE). The collection by AV is the preferred method (Leboeuf et al., 2000), but this techniques requires a previous training period (Wulster-Radcliffe et al., 2001). On the other hand, the EE is an alternative when males are not trained to AV, being a viable method of repeatedly collecting ejaculates from individual specimens without causing death (Jiménez-Rabadán et al., 2012, 2013, 2015). Differences on semen quality after freezing have been observed between collection methods depending of species. Marco-Jiménez et al. (2005) reported that spermatozoa collected by EE from rams were more resistant to cryodamage than when obtained by AV, whereas other studies (Jiménez-Rabadán et al., 2012) found higher sperm quality after thawing for samples collected by AV from bucks. It has been described that the EE procedure changes the secretory function of one or more accessory glands modifying the composition of the seminal plasma (Marco-Jiménez et al., 2008). Moreover, proteins contained in the seminal plasma may vary in composition and concentration with the collection procedure. Some of these proteins have been related with an important function for preventing the cold-shock damage (Barrios et al.,

2005) and therefore, modifications in their composition could influence on the cryoresistance of sperm samples.

Small ruminants as a whole include sheep and goat as well as other wild species. Focusing on domestic ones, sheep and goats have been considered together and similar reproductive technologies have been applied to them. Thus, for example, most of semen cryopreservation protocols used for caprine are extrapolated from ovine (Chemineau et al., 1991; Parkinson, 2009; Shipley et al., 2007), having both many similarities in extenders, cryoprotectants and cooling rates (Salamon and Maxwell, 1995, 2000). In spite of that, some differences could exist between species included in the same group and the response of spermatozoa to the cryopreservation procedure could vary.

Although there are research work about the effect of the semen collection method on sperm quality at thawing in bucks (Jiménez-Rabadán et al., 2012) and rams (Álvarez et al., 2012; Marco-Jiménez et al., 2005) separately, they are not comparable since semen collection techniques and cryopreservation protocols differed between studies.

With this background, the objective of the present work was to examine, using the same protocols and freezing extenders, how the method of semen collection influences the sperm cryopreservation in two phylogenetically close-related species of small ruminants. The results obtained could help us to increase the understanding of changes that occur during the cryopreservation procedure due to the collection method used which could determine the freezability of sperm samples. Thus, modifications in cryopreservation protocols could be performed according to the collection method used depending on species.

Material and methods

Animals and reagents

Animal handling was performed in accordance with Spanish Animal Protection Regulation, RD 53/2013, which conforms to European Union Regulation 2010/63. Seven males of Blanca-Celtibérica goat breed (age > 1.5 years) and five males of Manchega sheep breed (age > 1.5 years) were used. All males were maintained and managed at the Regional Center of Animal Selection and Reproduction (CERSYRA) located in Valdepeñas (Spain). The thawing procedure was conducted at laboratories from SaBio group in Albacete (Spain).

Chemicals were of reagent grade and purchased from Sigma (Madrid, Spain). Biladyl[®] was purchased from Minitüb (Tiefenbach, Alemania). Fluorescence probes were purchased from Invitrogen (Barcelona, Spain), except for propidium iodide (PI) which was purchased from Sigma. Chromatographically purified acridine orange (AO) was purchased from Polysciences Inc. (Warrington, PA, USA).

Semen collection

For each species and male, the collection of ejaculates was performed using AV and EE, both on the same day and using the same protocols, as a way to make results comparable between both species and collection methods. Prior to carry out this study, males were trained to conduct collections by AV and ejaculates were routinely obtained once per week. A total of 2 semen collections (replicates) for each method of semen collection were made in this study. Semen was firstly collected by AV and after a 2 h-interval EE procedure was performed. The EE collection was carried out using the protocol previously described by our group (Jiménez-Rabadán et al., 2012, 2015). Males were anesthetized with xylazine (0.2mg/kg Rompun[®] 2% i.m.; Bayer S.A., Barcelona, Spain), the rectum was cleaned of faeces and the prepucial area was shaved and washed with physiologic saline serum. A three electrode probe connected to a power source that allowed voltage and amperage control was used (P.kT. Electronics,

Boring, OR, USA). Probe diameter, probe length and electrode length were 3.2, 35.0 and 6.6 cm, respectively. The EE regime consisted of consecutive series of 5-sec pulses of similar voltage, each separated by 5-sec break. Each series consisted of a total of four pulses. The initial voltage was 1 V which was increased in each series until a maximum of 5 V. Urine contamination was tested and ejaculates with urine were rejected.

Evaluation of ejaculates

Immediately after the collection of ejaculates, the volume of ejaculate (measured in a conical graduated tube) and sperm concentration (by spectrophotometer) were evaluated. Spermatozoa total number (STN) was calculated with the volume and concentration (Volume $\times$ Concentration). The proportion of motile spermatozoa (SM) was evaluated subjectively in aliquots of semen diluted (1:200) in a phosphate buffer saline (PBS) and incubated for 5 min at 37 °C, using a phase-contrast microscope ($\times$ 100).

Semen cryopreservation

Semen from each male and species was diluted in a commercial extender, Biladyl[®] (20% clarified egg yolk) by the two-step dilution method. First, non-glycerolated fraction was added at 30 °C and the diluted semen was cooled to 5 °C for 2 h. Then, it was further diluted (v:v) with fraction containing glycerol. The diluted sperm samples were then held at 5 °C for 2 h more before freezing. At the end of this time, the diluted semen was loaded into 0.25 mL plastic straws and frozen. Straws were frozen over nitrogen vapours for 10 min, by placing the straws 4 cm above the surface of nitrogen liquid. The straws were subsequently plunged into liquid nitrogen and stored. Sperm samples were frozen to a final concentration of 140 to 200 $\times 10^6$ spermatozoa/mL. Both, cryopreservation protocol and freezing extender are usually used in goat and sheep in the conservation program carried out by CERSYRA. In addition, this methodology has

been showed to be the best in our lab for goat (Jiménez-Rabadán et al., 2012) and ram (data not published).

Sperm evaluation after thawing

Thawing was performed by placing the straws in a water bath at 37 °C for 30 sec, emptying the contents into dry tubes and incubating for 5 min at the same temperature. One straw from each male, method of collection and replicate was thawed and analyzed for both species.

Frozen-thawed spermatozoa were evaluated for motility and velocity by Computer Assisted Semen Analysis (CASA) using the Sperm Class Analyzer software (SCA[®] 2002, Microptic, Barcelona, Spain). Sperm samples were diluted (1:1) with TALP-HEPES medium (87 mM NaCl, 3.1 mM KCl, 2 mM CaCl₂, 0.4 mM MgCl₂, 0.3 mM NaH₂PO₄, 40 mM HEPES, 21.6 mM sodium lactate, 1 mM sodium pyruvate, 50 µg/mL kanamycin, 10 µg/mL phenol red, and 6 mg/mL bovine serum albumin (BSA) (pH 7.5)) and 5 µL were put on a Makler chamber recording the characteristics of sperm motility. The following motility parameters were assessed: total motile spermatozoa (TM, %), progressive motile spermatozoa (PM, %), velocity according to the straight path (VSL, µm/s), linearity index (LIN, %) and amplitude of lateral displacement (ALH, µm).

Also, aliquots of thawed semen were used to conduct flow cytometry analyses. We assessed the membrane stability with YO-PRO-1, the viability with PI and the mitochondrial activity with Mitotracker Deep Red (Martínez-Pastor et al., 2008). A staining solution using TALP-HEPES was prepared by adding 50 nM YO-PRO-1 (stock: 100 µM in DMSO) and 15 µM propidium iodide (stock: 7.5 mM in milli-Q water). Twenty µL of sample were diluted in 0.5 mL of staining solution in polypropylene tubes for flow cytometry and were kept in dark for 15 min. Mitochondrial activity of the samples was evaluated with 0.1 µM YO-PRO-1 and 100

nM Mitotracker Deep Red solution (stock: 1 mM in DMSO) in TALP-HEPES. Sperm samples were diluted to 1×10^6 spermatozoa/mL, and 300 μ L were transferred to a polypropylene tube and left in dark for 30 min. After incubation times, sperm samples were analyzed using a Cytomics FC500 flow cytometer (Beckman coulter, Inc. USA). YO-PRO-1-/PI- spermatozoa were considered as intact spermatozoa (indicating live spermatozoa with intact plasmalemma) and Mitotracker+/YO-PRO-1- as viable spermatozoa with active mitochondria.

Chromatin stability was assessed by using the SCSA[®] (Sperm Chromatin Structure Assay) technique (SCSA[®] Diagnostics, Inc., Brookings, SD, USA) (Evenson and Jost, 2000). This technique is based on the susceptibility of the sperm DNA to acid-induced denaturation *in situ* and the metachromatic staining AO. This stain fluoresces green when combined with double-stranded DNA, and red when combined with single-stranded DNA (denaturated). Thawed spermatozoa were diluted with TNE buffer to 2×10^6 cells/mL. Samples were flash frozen in LN₂ and stored at -80 °C until analysis. We calculated the DNA fragmentation Index (DFI) for each spermatozoon as the ratio of red fluorescence respect to total fluorescence (red + green). High values of DFI indicate chromatin abnormalities. The %DFI was calculated as the percentage of spermatozoa with DFI > 25.

Statistical analysis

The statistical analysis was performed using the R statistical package (R Development Core Team 2014). Normality of data was verified previous to data analysis and a significant level of 0.05 was assumed. To study the effects of species and collection method on characteristics of ejaculates and sperm quality at thawing, a mixed effects model with male as random effect was conducted, and multiple comparison analyses were performed using the Bonferroni adjustment.

Results

Effect of semen collection method on characteristics of ejaculates

For both species, ejaculates collected by EE had greater volume than those obtained by AV, although only significant differences ($P \leq 0.05$) were found between collection methods for bucks (Figure 1). Higher sperm concentration ($P \leq 0.05$) was observed for samples collected by AV for both species. However, goat samples had lower ($P \leq 0.05$) concentration than sheep ones for each collection method (Figure 1). Samples collected by EE from bucks had the lowest ($P \leq 0.05$) STN (Figure 1) and no differences ($P > 0.05$) were found for SM between collection method and species (Figure 1).

Effect of semen collection method on sperm quality after thawing

The semen collection method influenced ($P \leq 0.05$) on sperm motility at thawing in Blanca-Celtibérica bucks, whereas no differences ($P > 0.05$) were found between both collection methods in Manchega rams (Figure 2). Thus, results showed no differences ($P > 0.05$) on percentages of total motile and progressive motile spermatozoa for ram samples collected by both methods and these values were higher ($P \leq 0.05$) than those obtained for buck samples collected by AV, followed by electroejaculated samples respectively. There were no differences ($P > 0.05$) for velocity parameters between species and collection methods. Flow cytometry analyses showed lower ($P \leq 0.05$) proportion of intact spermatozoa, percentage of viable spermatozoa with active mitochondria and DNA intact for buck sperm samples obtained by EE in relation to those collected by AV in the same species. There were no differences ($P > 0.05$) for these

parameters in ram semen collected by both methods and they were similar to those observed for buck semen obtained by AV (Figure 3).

Discussion

Semen cryopreservation linked to other reproductive techniques as artificial insemination is an important tool in breeding and conservation programs. However, cryopreservation process produces serious detrimental changes in sperm function (Watson 1981). It is known that the first step in the semen freezing is the recovery of sperm samples and suggestions about an effect of the method collection on seminal parameters have been made based mainly on the differences in seminal plasma composition (Jiménez-Rabadán et al., 2012; Ledesma et al., 2014; Marco-Jiménez et al., 2008). Although some studies have evaluated the effect of the semen collection method on the sperm quality at thawing in goat and sheep separately, none of them has evaluated this effect on both species jointly using the same cryopreservation protocol and freezing extender. Therefore, we planned this study in order to evaluate how the semen collection method affects resistance of spermatozoa to the cryopreservation process in two species of small ruminants handled in the same way. Results showed that the collection method had similar influence on semen characteristics evaluated in fresh ejaculates for both species. Thus, ejaculates obtained by EE had higher volume and lower concentration than those collected by AV. These results agree with other authors who compared these collection methods in bucks (Austin et al., 1968; Jiménez-Rabadán et al., 2012; Memon et al., 1986) and rams (Marco-Jiménez et al., 2005; Mattner and Volgmayr, 1962). A higher volume for samples collected by EE could be due to contribution of accessory sex glands because

of electrical stimuli as was suggested by Mattner and Volgmayr (1962). Nevertheless, no differences on fresh sperm motility were observed between collection methods for both species agreeing with other authors (Álvarez et al., 2012; Jiménez-Rabadán et al., 2012; Marco-Jiménez et al., 2005).

Focusing on sperm quality after the cryopreservation procedure, it was observed how the collection method had a significant effect on the resistance of spermatozoa to cryopreservation in bucks, whereas no effect was found in rams. In this way, buck samples showed lower sperm quality after thawing when were collected by EE showing lower percentages of motility, intact spermatozoa and viable spermatozoa with active mitochondria and higher percentages of spermatozoa with fragmented DNA. It is known the negative effect during buck sperm cryopreservation due to the interaction between the egg yolk coagulating enzyme (EYCE) presents in seminal plasma and the freezing extender. As consequence, lysolecithin is produced and this substance it has been demonstrated to have a toxic effect on buck spermatozoa (Iritani and Nishikawa, 1963) which may result in sperm damage and finally, cell death. Results obtained in the present study agree with a previous work carried out by our group (Jiménez-Rabadán et al., 2012), which showed better sperm quality at thawing for buck samples collected by AV. Moreover, individual variability has been observed (not showed data) in the resistance to cryopreservation for electroejaculated samples. Thus, some males showed frequently low values of sperm motility because of freezing extender was coagulated. However, the same males displayed acceptable values of sperm quality when samples were collected by AV.

On the other hand, no differences between collection methods on sperm parameters at thawing were observed for ram samples. Moreover, ram samples collected by both methods showed higher sperm motility than buck samples. It could be due to the

negative effect of EYCE did not happen in this species since ram seminal plasma did not contain this enzyme. Marco-Jiménez et al. (2005) observed that ram semen collected by EE showed higher number of stable and functional spermatozoa at thawing, mainly related to viability and acrosome integrity, than samples obtained by AV. In the same line, other authors (Ledesma et al., 2015) have suggested that sperm cells obtained by electrical stimulation were, by themselves, more resistant to cryodamage than sperm collected by AV. Contrary, lower values of motility for electroejaculated samples and similar values for the percentage of normal acrosomes and viability for both collection methods were observed by Álvarez et al. (2012) in this species. Differences between studies could be produced by differences on sperm cryopreservation protocol used and therefore, the effect of collection method could be not compared among works.

We consider that the negative effect of electroejaculation method on buck thawed sperm could be due to the presence of EYCE in seminal plasma in this specie, which could be increased in samples collected by EE. Since rams do not have this enzyme in seminal plasma is less probable that the cryopreserved sperm samples were damaged when the electroejaculation was used.

Conclusion

Results obtained in this study showed how the method of semen collection may influence the resistance of spermatozoa to cryopreservation depending on the small ruminant species. Here, semen from Blanca-Celtibérica bucks collected by EE showed lower sperm quality at thawing. We hypothesized that electrostimulation of accessory glands during EE procedure resulted in changes on seminal plasma composition modifying the amount of EYCE. Finds observed in this work suggest that species close-related such as ovine and caprine have different response to the cryopreservation

process depending on the semen collection method used. Although freezing protocols and extenders have been similar for both species up to date, this trend should be changed using specific procedures taking in account the species as well as the collection method performed. Therefore, on one hand it is recommended to use different cryopreservation protocols and extenders in bucks depending on semen collection method used since characteristics of ejaculates may vary determined by the method used through the effect on the secretion of sex accessory glands. On the other hand, both semen collection methods (AV and EE) could be performed equally for sperm cryopreservation in rams due to sperm quality at thawing is unaffected by the collection method in this species. Hence, future work should aim to further study on improvements in cryopreservation protocols and extenders for buck semen collected by EE and to increase the knowledge of mechanism affecting to sperm cryopreservation related to the semen collection method such as modifications in seminal plasma composition.

Acknowledgements

This research was supported by INIA (RZ2008-00009-CO2). The authors thank AGRACE, their farmers and CERSYRA staff for their collaboration in the collection of the samples. Manuel Ramón is supported by the DOC-INIA program.

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381 **Figure 1.** Effect of semen collection method on characteristics of ejaculates in
382 Manchega sheep and Blanca-Celtibérica goat
383 **Figure 2.** Effects of sperm collection method on motility parameters after thawing in
384 Manchega sheep and Blanca-Celtibérica goat.
385 **Figure 3.** Effects of sperm collection method on parameters assessed by flow cytometry
386 after thawing in Manchega sheep and Blanca-Celtibérica goat.
387

389 Data are mean $\pm$ standard error of mean (s.e.m.). Different letters show significant
390 differences ($P \leq 0.05$). ●: artificial vagina; Δ: electroejaculation; STN: spermatozoa
391 total number; SM: sperm motility.

392
393

395 Data are mean $\pm$ standard error of mean (s.e.m.). Different letters show significant
396 differences ($P \leq 0.05$). ●: artificial vagina; Δ: electroejaculation; VSL: velocity according
397 to the straight path; LIN: linearity index; ALH: amplitude of lateral head displacement.
398

399 Figure 3. Jiménez-Rabadán et al.

400 Data are mean $\pm$ standard error of mean (s.e.m.). Different letters show significant
 401 differences ($P \leq 0.05$). ●: artificial vagina; Δ: electroejaculation; YO-PRO-1-/PI-: intact
 402 spermatozoa; Mitotracker+/YO-PRO-1-: viable spermatozoa with active mitochondria;
 403 DFI: DNA fragmentation index.

404

***Conflict of Interest Statement**

CONFLICT OF INTERESTS: NONE