Evaluation of frozen thawed cauda epididymal sperms and *in vitro* fertilizing potential of bovine sperm collected from the cauda epididymal

Chaveiro, A.[1*]; Cerqueira, C.[2]; Silva, J.[3]; Franco, J.[4] and Moreira da Silva, F.[1]

[1]Department of Agrarian Sciences, Centre for Agricultural Research and Technology of the Azores (CITA-A), University of Azores, 9700-042 Angra do Heroísmo, Portugal; [2]BSc Student, Centre for Agricultural Research and Technology of the Azores (CITA-A), University of Azores, 9700-042 Angra do Heroísmo, Portugal; [3]Graduated from Centre for Agricultural Research and Technology of the Azores (CITA-A), University of Azores, 9700-042 Angra do Heroísmo, Portugal; [4]MVSc, Department of Agrarian Sciences, Centre for Agricultural Research and Technology of the Azores (CITA-A), University of Azores, 9700-042 Angra do Heroísmo, Portugal

[*]***Correspondence:*** *A. Chaveiro, Department of Agrarian Sciences, Centre for Agricultural Research and Technology of the Azores(CITA-A), University of Azores, 9700-042 Angra do Heroísmo, Portugal. E-mail: antoniochaveiro@uac.pt*

(Received 30 Jun 2014; revised version 3 Mar 2015; accepted 14 Mar 2015)

Summary

In the present study, the fertilizing potential of semen recovered from slaughtered bulls epididymis was evaluated after cryopreservation, by conventional techniques and flow cytometry methods. The cauda epididymal was dissected and sperm were recovered and evaluated for volume, sperm concentration, and membrane and acrosome integrity using a flow cytometer. Sperm fertility potential was tested by *in vitro* fertilization (IVF). For each bull, three trials of IVF were performed. Before freezing, on average, the sperm concentration was 216 ± 27.5 × 10^6 sperm/ml. Sperm viability averaged 86.5 ± 4%. The mean percentage of sperm with intact plasma membrane and acrosome before and after cryopreservation was 90.7 ± 2.9% and 90.8 ± 1.9% ($P \geq 0.05$), respectively. The fertilization rate using frozen/thawed epididymal semen averaged 64.1 ± 3.9% fertilization with no significant differences between bulls ($P > 0.05$). For the bull considered as control, the fertilization rate was 72.2 ± 4.5%, differing significantly ($P > 0.05$) from the frozen/thawed epididymal semen's fertilization rate. In conclusion, it is possible to use *in vitro* techniques with cryopreserved spermatozoa obtained from bull's epididymis using a controlled rate freezing method with a predetermined freezing curve, and with assessment of sperm's viability by conventional techniques and flow cytometry methods, together with the fertilizing ability of cryopreserved epididymal spermatozoa.

Key words: Bovine, Cryopreservation, Epididymis, IVF, Semen

Introduction

The development of appropriate techniques for the preservation and storage of semen enables better utilization of high-value livestock animals. Technology has advanced in a way that makes the cryopreservation of semen through recovery of epididymal sperm possible, enabling the reproduction of elite bulls that can accidentally die. Freezing epididymal sperm samples has been performed in different species: dogs (Martins *et al*., 2007b), cats (Axner *et al*., 2004), equine (Barker and Gandier, 1957) bulls (Martins *et al*., 2007a) and bucks (Turri *et al*., 2014), with the aim to develop techniques suitable for storage of genetic material from these animals. To determine the quality and decay of sperm stored in epididymis post-mortem, some studies have been carried out in species such as mice (Sankai *et al*., 2001), boar (Kikuchi *et al*., 1998), dog (Yu and Leibo, 2002), some African wild species (Killian *et al*., 2000; Lubbe *et al*., 2000), mouflon (Pérez *et al*., 1995) and Iberian red deer (Soler *et al*., 2003).

The recovery of the epididymal spermatozoa from dead animals, the cryopreservation and subsequent *in vitro* fertilization (IVF) are useful tools to rescue genetic material that otherwise would be lost, either from highly productive animals or from endangered species (Martins *et al*., 2007b).

The fortuitous discovery of glycerol as an effective cryoprotective agent (Polge, 1949) introduced a completely new system of bull semen storage, a method which is widely in practice today. The cryopreserved bovine semen generally provides lower fertility rates compared to fresh semen. When comparisons were made based on a similar number of motile sperm, the results of fertility of the frozen semen are inferior to those obtained using fresh semen (Watson, 2000). The main factors involved in the decrease of fertility temperature are changes during the biotechnological process, the toxic and osmotic stress depicted by exposure to cryoprotectants and the formation and dissolution of extracellular ice crystals (Watson, 2000). Cryo-preservation is stressful for sperm, affecting membrane integrity, acrosomal and mitochondria (Januskauskas *et al*., 2003).

Concerning epididymal sperm characteristics, there seems to be a difference in sperm movement

characteristics between ejaculated and epididymal semen. Cauda epididymal sperm is less motile than ejaculated semen. In comparison to ejaculated semen, epididymal semen has a lower velocity, and its movement shows less straightness and linearity. Moreover, the quality of the semen from paired caudae epididymides of a bull is not fully comparable, except for the parameters of linearity and percentage of live spermatozoa (Goovaerts *et al*., 2006). Sperm quality and the potential effects of retrieval and storage protocols on it, are traditionally assessed by the evaluation of morphological parameters. The desire to assess sperm quality in a more objective way is reflected by the increased use of flow cytometry techniques (Hossain *et al*., 2011b). The present study aimed to evaluate the effect of cryopreservation of bovine semen taken from the cauda epididymal using a controlled rate freezing method with a predetermined freezing curve, and further the assessment of sperm's viability by conventional techniques and using flow cytometry methods, together with the fertilizing ability of cryopreserved epididymal spermatozoa.

Materials and Methods

Unless stated otherwise, chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA).

To develop this study we used spermatozoa collected from epididymides of Holstein Friesian bulls (n=7), ranging in age from 2 to 5 years. Testes with attached epididymis were obtained from the bulls slaughtered at the local abattoir (IAMA, Angra do Heroismo). Immediately after removal, the testis were placed into plastic bags with sterile isotonic saline solution at room temperature (19-22°C) and transferred to the laboratory within 2 h. In the laboratory the epididymides were dissected and separated from the testis, as described by Yu and Leibo (2002), with some modifications. Briefly, each cauda epididymis was dissected free, rinsed with 0.9% saline and placed into a 35 mm Petri dish. Caudae epididymides were held with forceps, and multiple incisions were made in the tubuli with abistoury. The semen was aspirated into a glass pipette and transferred to a 15 ml tube with 4 ml of Trisegg-yolk medium, (van Wagtendonk-de Leeuw *et al*., 2000). Each suspension of spermatozoa was filtered with a metal tamis and then transferred into a 15 ml plastic tube, with a final volume of filtered semen of approximately 4 ml.

Sperm motility, morphology and concentration

The sperm progressive motility was determined by Phase-Contrast microscopy (×200), on a warm stage at 37°C. Spermatozoa were assessed for a percentage of motile spermatozoa with a scale of 0-100%. In parallel, the percentage of sperm carrying cytoplasmic droplets (proximal, medial and distal) was also assessed on a fixed sample (Melo *et al*., 2005).

Sperm concentrations of the original suspensions were determined in a hemocytometer (Neubauer Improved, Marienfeld, Germany) (Atiq *et al*., 2011) and results are presented as sperm cells/ml.

Membrane integrity-sperm's viability

The integrity of sperm plasma membrane was assessed by a method modified from that described by Garner and Johnson (1995) using the fluorescent double stain. Briefly, the percentage live sperm was assessed by flow cytometry, using SYBR14 (Molecular Probes, Eugene, OR, USA) and propidium iodide (PI). Before measurement, a staining solution was prepared by adding 5 µL of a 2 mM solution of PI (in water) and 2 µL of a 100 mM solution of SYBR-14 to 2 ml of PBS. Prior to measurement 295 µL of the staining solution was added to 5 µL of semen (final concentration of 1×10^6 cells/ml). After 10 min incubation at 37°C, two-colour flow cytometry was performed using a FacsCalibur flow cytometry equipped with a 15 mW argon Laser (Becton and Dickinson, San Jose, CA, USA), collecting fluorescent data in a logarithmic mode and forward and side light scatter data in a linear mode from 10,000 events per sample, at a rate of 400-600 events/s. Fluorescence of SYBR-14 was detected using the FL1 530/30 nm "band-pass" filter, and fluorescence of PI was detected using the FL3 650 nm "long-pass" filter. Data were analyzed using CellQuest™ (software Pro version 4.0.2). Through inspection of light scatter data, non-sperm events ("debri") were gated out. Then regions were defined from which the relative proportion of the sperm subpopulations was calculated. Figure 1 shows an example of a dot plot, depicting the way these regions were defined, and showing the population of sperm with high SYBR 14 and low PI staining (R1; live sperm), the population of sperm with low SYBR 14 and high PI staining (R2; dead sperm) and population of sperm that is found in between the two populations with intermediary PI and SYBR14 staining (moribund sperm). Non-sperm events that are not gated out on the basis of their light scatter characteristics do not show significant fluorescence and appear as separate region (R3).

Acrosome integrity

The evaluation of the viability and the acrosomal status was examined by staining the incubated samples with PI as a marker for cell viability (Garner *et al*., 1986) and Pisumsativum agglutinin conjugated to fluorescein isothiocyanate (FITC-PSA) as a marker for acrosomal status (Casey *et al*., 1993; Hossain *et al*., 2011a), as described by Rathi *et al*. (2001). Briefly, the FITC-PSA conjugate was added to a working solution of 0.1 mg/ml in PBS without BSA, and PI was dissolved in PBS to make up a working solution of 2 mg/ml.

Prior to measurement, 30 µL FITC-PSA (0.1 mg/ml) and 2 µL PI (2 mg/ml) were added to 500 µL of semen. After incubation for 10 min at 37°C, the proportions of FITC-PSA bound and PI-stained sperm cells were quantified by two-color flow cytometry using a FacsCalibur (Becton and Dickinson, San Jose, California, USA).

For the conjugation of fluorochromes, four subpopulations of sperm were identified, as described by

Maxwell and Johnson (1997) live sperm with intact acrosome, unstained PI and unstained FITC-PSA (LL); live sperm with damaged acrosome, unstained PI and stained FITC-PSA (LR); dead sperm with intact acrosome, stained PI and unstained FITC-PSA (UL); dead sperm with damaged acrosome, stained PI and stained FITC-PSA (UR).

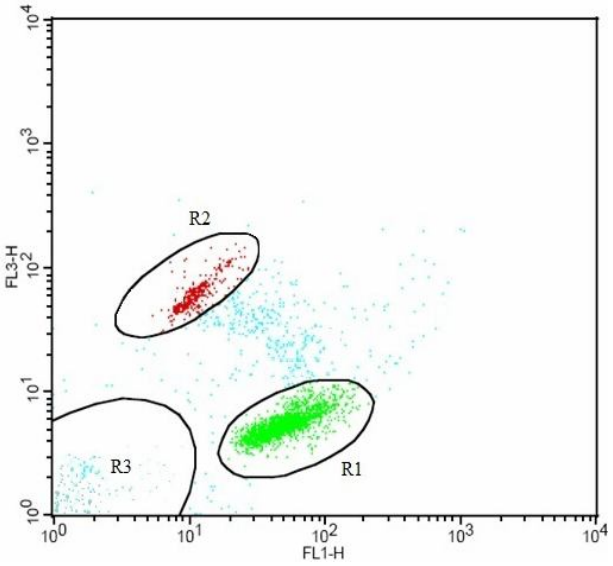

Fig. 1: Flow cytometric analysis of frozen-thawed bull sperm. Typical example of a dot-plot depicting the distribution of flow cytometric "events" as functions of their fluorescence intensities of SYBR-14 (FL1-H) and propidium iodide (PI) (FL3-H). Region R1 (live spem), R2 (dead sperm) and R3 (non sperm events) are defined as shown

Cryopreservation and thawing of semen

The cauda epididymal sperm cells from each bull were diluted slowly to a final concentration of 50×10^6 spz/ml, using Tris egg-yolk medium (van Wagtendonk-de Leeuw *et al*., 2000). Semen was processed as previously described by Chaveiro *et al*. (2006). Briefly, the diluted semen was packed in 0.25 ml "french" straws (i.d.=1.6 mm, IMV. L'Aigle, France), at room temperature, and closed with a plug of polyvinyalcohol. The straws were placed horizontally in a Styrofoam box, in a place at 5°C for 2 h (slow cooling and equilibration period). Then, the straws were frozen in horizontal position in a programmable freezer (IceCube 14S; SyLab, Austria). The freezer was preset at 5°C. After placing the rack of straws inside the freezing chamber, it was cooled at a rate of -4°C/min to -10°C. Then, the straws were cooled from -10°C to -145°C at a rate of -40°C/min, and subsequently plunged into liquid nitrogen and stored for at least 48 h before thawing.

Thawing was achieved by immersingthestraws for 20 s in a waterbath set at 37°C. Immediately after thawing the semen was processed for assessment as previously described.

Oocyte collection and *in vitro* fertilization with cauda epididymal semen

Ovaries were obtained at a local abattoir from adult animals, trimmed of adhering tissue and transported to the laboratory in Dulbecco's phosphate buffered saline (DPBS) at temperature ranging from 34 to 37°C within 2 h postslaughtering.

All collected oocytes were counted and only the good quality cumulus-oocyte complexes (COCs) based on their morphological appearance, covered by at least four layers of compacted cumulus cells and evenly granulated ooplasm, were used, as described by Santos *et al*. (2008). COCs were then washed twice in TCM-199 medium supplemented with 2% FBS (fetal bovine serum), 0.3 mg/ml glutamine and 50 µg/ml gentamycin and 20 µg/ml of nystatin, and matured in TCM-199 supplemented with 10% FBS, 5 µg/ml of FSH-LH (Stimufol, Belgium), 1 µg/ml estradiol-17, 0.15 mg/ml glutamine, 22 µg/ml Na-pyruvate and 50 µg/ml gentamycin and 20 µg/ml of nystatin. After 24 h of maturation under 5% CO_2 in a humidified atmosphere at 38.5°C, oocytes were placed in fertilization TALP medium. Briefly, thawed epididymal sperm from each bull (3 replicates per bull) was washed three times by centrifugation, twice in sperm-TALP medium (4 ml each time) and finally washed in IVF-TALP medium supplemented with 10 µg/ml heparin, 6 mg/ml bovine serum albumin (BSA, essentially fatty acid free), 22 µg/ml Na-pyruvate and 50 µg/ml gentamycin and 20 µg/ml of nystatin. After removing the supernatant, sperm pellet was homogenized with 0.25-0.5 ml of IVF TALP to a sperm concentration of 1×10^6 sperm/ml. In parallel, frozen/thawed ejaculated semen from a commercial available bull, used in our laboratory for IVF studies, with high fertility was used as control. Oocytes and sperm were co-cultured in 50 µL of fertilization medium (10-15 oocytes/droplet) for 22-24 h at 38.5°C in 5% CO_2 in air. Presumptive zygotes were denuded by vortexing, washed and cultured in TCM-199 with Hepes supplemented with 3 mg/ml BSA (Fr. V), 22 g/ml Na-pyruvate, 10 L/ml NEAA (MEM, non-essential aminoacids), 20 µL/ml EAA (BME, essential aminoacids) and 50 µg/ml gentamycin and 20 µg/ml of nystatin in incubator at 38.5°C in 5% CO_2 in air. Cleavage rate was determined after 3 days of fertilization (day 0) and the embryonic development was evaluated 8 days (day 8) post insemination.

Statistical analysis

For statistical analysis One-Way ANOVA was performed in order to evaluate the effects of different treatments. When ANOVA revealed a significant effect, the treatments were compared by Tukey test. A difference of $P<0.05$ was considered significant. All analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, USA).

Results

The mean sperm concentration obtained was 216 ± 27.5×10^6 sperm/ml (Table 1). On average, about 5.4 ± 2.0% of the spermatozoa had proximal cytoplasmatic droplets, and about 33.9 ± 12.8% had median cytoplasmatic droplets (Table 1). As expected the

Table 1: Sperm characteristics, percentage of spermatozoa collected from epididymides of Holstein Friesian bulls (n=7)

Sperm characteristics	Bulls							Mean±SE
	A	B	C	D	E	F	G	
Sperm concentration (× 10^6 cells/ml)	325	165	225	150	250	125	275	216 ± 27.5
% Proximal cytoplasmatic droplets	1.5	2.6	15.6	6.7	1.5	0.3	1.7	5.4 ± 2.0
% Median cytoplasmatic droplets	73.8	81.8	51.1	80.0	16.0	3.25	15.4	33.9 ± 12.8

Table 2: Mean percentage (±SE) of total motility, sperm viability and acrosome integrity of bull epididymal spermatozoa before and after freezing

Sperm characteristics	Before freezing (%)	After freezing (%)
Total progressive motility	78.1 ± 3.9[a]	56.9 ± 4.8[b]
Viable sperm	86.5 ± 4.2[a]	64.5 ± 5[b]
Sperm alive with intact acrosome	90.7 ± 2.9[a]	90.8 ± 1.9[a]
Sperm alive with damage acrosome	0.7 ± 0.2[a]	2.4 ± 0.8[a]

[a, b] Values within the same row with different letters ([a, b]) differ significantly (P<0.05)

Table 3: Cleavage rate and subsequent *in vitro* rates of embryo development using frozen/thawed bovine spermatozoa recovered from epididymides

Treatments	No. of oocytes	Maturation rate (%) (No.)	Cleavage rate (%) (No.)	Rates of embryos development (%)			
				2-cell stage	4-cell stage	Morula	Blastocyst
				(Day 3)		(Day 8)	
Epidydimal sperm	336	94.9±1.5[a] (318)	80.2±2.7[a] (255)	44.5±2.9[a] (113)	26.0±6.5[a] (66)	23.5±3.6[a] (42)	21.8±2.7[a] (39)
Ejaculated sperm-control	288	95.2±0.9[a] (274)	85.4±0.9[b] (234)	46.1±1.5[a] (107)	27.5±3.6[a] (64)	24.3±4.5[a] (41)	22.1±2.8[a] (38)

[a, b] Values within the same column with different letters ([a, b]) differ significantly (P<0.05)

cryopreservation had a detrimental effect on sperm's quality, (Table 2). Epididymal sperm progressive motility evaluation obtained by phase contrast microscopy showed an average of 78.1 ± 3.9%. Upon freezing there was a significant loss (P<0.05) of progressive motility, lowering the value of motility to 56.9 ± 4.8% (P<0.05) (Table 2). Cryopreservation of fresh semen also affected the percentage of viable spermatozoa assessed by flow cytometry. Fresh semen presented an average viability of 86.5 ± 4.2%, while the frozen semen showed a lower (P<0.05) viability value of 64.5 ± 5%, with a decrease of 24.9% on sperm's viability. The average percentage of sperm with intact plasma membrane and intact acrosome before and after thawing was 90.7 ± 2.9% and 90.8 ± 1.9% (P≥0.05), respectively. Prior to freezing, the percentage of live sperm with damaged acrosome was 0.7 ± 0.2%, increasing to 2.4 ± 1.9% after cryopreservation (P≥0.05) (Table 2).

Fertilization rate of *in vitro* produced embryos using frozen/thawed epididymal sperm, showed an average of 64.1 ± 3.9% fertilization with no significant differences between bulls (P≥0.05). For the bull considered as control, the fertilization rate (72.2 ± 4.5%) was significantly higher (P<0.05) when compared to the fertilizing rate obtained from frozen/thawed epididymal sperm.

The results of using frozen/thawed epididymal sperm for embryo *in vitro* production are shown in Table 3. As it can be observed, the maturation rate, assessed by cumulus oocyte expansion, showed no significant difference (P≥0.05) between the epididymal group and the control, (94.9 ± 1.5% and 95.2 ± 0.9%, respectively). The percentage of cleaved embryos was significantly different (P<0.05) between the two groups, with 80.3 ± 2.7% and 85.4 ± 0.9% for epidydimal and control groups, respectively. Early embryonic developmental rates from 2-cell and 4-cell stages were not significantly different between groups. At morula and blastocyst stage, although there were no significant differences (P≥0.05) between epidydimal and control groups, epidydimal showed somewhat lower morula and blastocysts rates in comparison with the control.

Discussion

This study evaluated the effects of cryopreservation on epididymal bovine spermatozoa. Progressive motility, viability, sperm's membrane, acrosome integrity and epididymal sperm's fertilization potential were all evaluated after sperm freezing. Salamon and Maxwell (1995) reported that cryopreservation process causes ultrastructural damage, functional, physical and biochemical in spermatozoa. This damage causes changes in membrane fluidity with increased volume and rupture of the plasma and acrosomal membranes (Watson, 1995; Holt, 2000). These modifications lead to decrease in the fertilization rate, since the integrity and functionality of the plasma membrane and acrosomal are necessary for fertilization and subsequent embryo development. The recovery of the epididymal spermatozoa from dead animals, the cryopreservation and subsequent IVF are useful tools to rescue genetic material that otherwise would be lost, either from highly

productive animals or from endangered species (Martins *et al*., 2007b). Despite the well known effects of cryopreservation on bovine sperm cells (Watson, 2000), *in vitro* embryos were obtained from recovered spermatozoa from the cauda epididymal of dead animals (Martins *et al*., 2007b). Martin *et al*. (2007a) concluded that spermatozoa can be obtained from epididymis of either a highly productive animal or of an animal from an endangered species, which can be used for the production of viable embryos.

According to Fernandez-Santos *et al*. (2009), epididymides have adequate conditions to prolong sperm survival, because the cauda epididymal provide the optimal environment for gamete storage in physiological conditions. Cryopreservation of spermatozoa recovered in this study from epididymides, caused a decreased in total motility and percentage of cells with intact membranes and intact acrosomes when compared to ejaculated sperm. Critser *et al*. (1987) stated that, cryopreservation process is responsible for damage in the sperm cells and has an effect of sperm motility and fertilization rate, with considerable focus on the damage to the acrosomal structures. In other species, reports indicate that, spermatozoa recovered from epididymides are less tolerant to freezing/thawing process than ejaculated spermatozoa (Zomborszky *et al*., 1999), which is in agreement with our findings, where despite the high percentage of live sperm with intact acrosome after freezing (90.8 ± 1.9) there was a significant decrease ($P<0.05$) in the total number of motile and viable sperm cells, especially in the sperm recovered from the epididymis.

Although IVF techniques have been widely studied in cattle, few reports were found (Martins *et al*., 2007b) regarding the evaluation of *in vitro* fertilizing potential of epididymal spermatozoa from dead animals. Concerning IVF results, fertilization rate of *in vitro* produced embryos using frozen/thawed epididymal sperm showed no significant differences between animals. In terms of cleavage rate, the values are similar to those obtained by Martins *et al*. (2007b), however, our blastocyst rate was somewhat lower when using semen obtained from epididymis *versus* the ejaculated semen from the control bull. While the quality of the frozen semen obtained from the epididymis was lower, the embryo's development rates were not significantly different between treatments, proving that it is possible the use frozen semen obtained from the bull's epididymis with significant changes of IVF produced embryos.

In conclusion it is possible to use *in vitro* techniques by cryopreserved spermatozoa obtained from bull's epididymis using a controlled rate freezing method with a predetermined freezing curve, and with assessment of sperm's viability by conventional techniques and flow cytometry methods, together with the fertilizing ability of cryopreserved epididymal spermatozoa.

References

Atiq, N; Ullah, N; Andrabi, SMH and Akhter, S (2011). Comparison of photometer with improved neubauer hemocytometer and makler counting chamber for sperm concentration measurement in cattle. Pak. Vet. J., 31: 83-84.

Axner, E; Hermansson, U and Linde-Forsberg, C (2004). The effect of Equex STM paste and sperm morphology on post-thaw survival of cat epididymal spermatozoa. Anim. Reprod. Sci., 84: 179-191.

Barker, CA and Gandier, JC (1957). Pregnancy in a mare resulting from frozen epididymal spermatozoa. Canadian J. Comp. Med. Vet. Sci., 21: 47-51.

Casey, PJ; Hillman, RB; Robertson, KR; Yudin, AI; Liu, IKM and Drobnis, EZ (1993). Validation of an acrosomal stain for equine sperm that differentiates between living and dead sperm. J. Androl., 14: 289-297.

Chaveiro, A; Machado, L; Frijters, A; Engel, B and Woelders, H (2006). Improvement of parameters of freezing medium and freezing protocol for bull sperm using two osmotic supports. Theriogenology. 65: 1875-1890.

Critser, JK; Arneson, BW; Aaker, DV; Husebenda, AR and Ball, GD (1987). Cryopreservation of human-spermatozoa. 2. Postthaw chronology of motility and of zona-free hamster ova penetration. Fertil. Steril., 47: 980-984.

Fernández-Santos, MR; Martínez-Pastor, F; Matias, D; Domínguez-Rebolledo, AE; Esteso, MC; Montoro, V and Garde, JJ (2009). Effects of long-term chilled storage of red deer epididymides on DNA integrity and motility of thawed spermatozoa. Anim. Reprod. Sci., 111: 93-104.

Garner, DL and Johnson, LA (1995). Viability assessment of mammalian sperm using Sybr-14 and propidium iodide. Biol. Reprod., 53: 276-284.

Garner, DL; Pinkel, D; Johnson, LA and Pace, MM (1986). Assessment of spermatozoal function using dual fluorescent staining and flow cytometric analyses. Biol. Reprod., 34: 127-138.

Goovaerts, IG; Hoflack, GG; Van Soom, A; Dewulf, J; Nichi, M; de Kruif, A and Bols, PE (2006). Evaluation of epididymal semen quality using the Hamilton-Thorne analyser indicates variation between the two caudae epididymides of the same bull. Theriogenology. 66: 323-330.

Hossain, MS; Johannisson, A; Siqueira, AP; Wallgren, M and Rodriguez-Martinez, H (2011a). Spermatozoa in the sperm-peak-fraction of the boar ejaculate show a lower flow of Ca^{2+} under capacitation conditions post-thaw which might account for their higher membrane stability after cryopreservation. Anim. Reprod. Sci., 128: 37-44.

Hossain, MS; Johannisson, A; Wallgren, M; Nagy, S; Siqueira, AP and Rodriguez-Martinez, H (2011b). Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art. Asian J. Androl., 13: 406-419.

Januskauskas, A; Johannisson, A and Rodriguez-Martinez, H (2003). Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. Theriogenology. 60: 743-758.

Kikuchi, K; Nagai, T; Kashiwazaki, N; Ikeda, H; Noguchi, J; Shimada, A; Soloy, E and Kaneko, H (1998). Cryopreservation and ensuing *in vitro* fertilization ability of boar spermatozoa from epididymides stored at 4 degrees C. Theriogenology. 50: 615-623.

Killian, I; Lubbe, K; Bartels, P; Friedmann, Y and Denniston, R (2000). Evaluating epididymal sperm of African wild ruminants: longevity when stored at 4°C and viability following cryopreservation. Theriogenology. 53: 336 (abst.).

Lubbe, K; Bartels, P; Kilian, I; Friedmann, Y and Godke, RA (2000). Comparing motility and morphology of horse,

zebra and rhinoceros epididymal spermatozoa when cryopreserved with two different cryodiuents or stored at 4°C. Theriogenology. 53: 338 (abst.).

Martins, CF; Bao, SN; Dode, MN; Correa, GA and Rumpf, R (2007a). Effects of freeze-drying on cytology, ultrastructure, DNA fragmentation, and fertilizing ability of bovine sperm. Theriogenology. 67: 1307-1315.

Martins, CF; Rumpf, R; Pereira, DC and Dode, MN (2007b). Cryopreservation of epididymal bovine spermatozoa from dead animals and its uses *in vitro* embryo production. Anim. Reprod. Sci., 101: 326-331.

Melo, MIV; Henry, M and Beker, ARCL (2005). Hypoosmotic test to predict viability of equine chilled semen in different extenders. Arq. Bras. Med. Vet. Zoo., 57: 757-763.

Pérez, S; Aguado, M; Ayllon, E; Garrido, D; Montoro, V and Garde, J (1995). Live birth of hybrid (O. musimon X Q. aries) lambs following intrauterine insemination in domestic sheep with mouflon semen obtained 40 hours postmortem. Theriogenology. 43: 218 (abst.).

Rathi, R; Colenbrander, B; Bevers, MM and Gadella, BM (2001). Evaluation of *in vitro* capacitation of stallion spermatozoa. Biol. Reprod., 65: 462-470.

Salamon, S and Maxwell, WMC (1995). Frozen storage of ram semen. 2. Causes of low fertility after cervical insemination and methods of improvement. Anim. Reprod. Sci., 38: 1-36.

Sankai, T; Tsuchiya, H and Ogonuki, N (2001). Short-term nonfrozen storage of mouse epididymal spermatozoa. Theriogenology. 55: 1759-1768.

Santos, P; Chaveiro, A; Simoes, N and Moreira da Silva, F (2008). Bovine oocyte quality in relation to ultrastructural characteristics of zona pellucida, polyspermic penetration and developmental competence. Reprod. Domest. Anim., 43: 685-689.

Soler, AJ; Perez-Guzman, MD and Garde, JJ (2003). Storage of red deer epididymides for four days at 5 degrees C: effects on sperm motility, viability, and morphological integrity. J. Exp. Zool. Part A, Comp. Exp. Biol., 295: 188-199.

Turri, F; Madeddu, M; Gliozzi, TM; Gandini, G and Pizzi, F (2014). Effect of testicle postmortem storage on goat frozen-thawed epididymal sperm quality as a tool to improve genebanking in local breeds. Anim.: Int. J. Anim. Biosci., 8: 440-447.

van Wagtendonk-de Leeuw, AM; Haring, RM; Kaal-Lansbergen, LM and den Daas, JH (2000). Fertility results using bovine semen cryopreserved with extenders based on egg yolk and soy bean extract. Theriogenology. 54: 57-67.

Watson, PF (2000). The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci., 60-61: 481-492.

Yu, I and Leibo, SP (2002). Recovery of motile, membrane-intact spermatozoa from canine epididymides stored for 8 days at 4 degrees C. Theriogenology. 57: 1179-1190.

Zomborszky, Z; Zubor, T; Toth, J and Horn, P (1999). Sperm collection from shot red deer stags (Cervus elaphus) and the utilisation of sperm frozen and subsequently thawed. Acta Vet. Hung., 47: 263-270.