IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS)
e-ISSN: 2319-2380, p-ISSN: 2319-2372. Volume 12, Issue 10 Ser. I (October 2019), PP 28-33
www.iosrjournals.org

Evaluationof the Cryopreservation and fecundating capacity of Bullfighting Bull Semen Obtained by Post-Mortem Collection

J. P. R. Pacheco Lima[1,2]; H. Moreira da Silva[1]andF. Moreira da Silva[1,2]*
[1] –*AgrarianDevelopmentServicesof Terceira Island, Vinha Brava, 9700 Angra do Heroísmo. Portugal*
[2] -*University of the Azores, Faculty of Agrarian Sciences and Environment, CITA-A (Research Centre for Agricultural and Environmental Sciences and Technology of the Azores), Department of Animal Reproduction, Angra do Heroismo 9700-042, Portugal*

Abstract: *Testicles of six bullfightingadult animals from "Brava dos Açores" breed were recovered at the slaughterhouse. Theepididymis tail was dissected; sperm was collected and evaluated for volume, cell concentration, and by flow cytometry for membrane and acrosomal integrity assessment measuring FITC-PNA and PI fluorescence, respectively.Semen was then frozen in 0.25 ml French straws and the same sperm assessments were performed after thawing. Sperm potential ability of fertilization was tested in vitro and in vivo. For in vitro fertilization, ovaries (n=210) were collected at the same slaughterhouse,cumulus–oocyte complexes (n=1134) were aspirated,matured for 24 h, fertilized in vitro and on day 7, embryos were classified.Frozen semen was also used to inseminate heifers.*

After collecting, sperm viability was86.5±4.2%, decreasing to64.5±5.0% after cryopreservation. By FCM, the percentage of sperm with intact plasmatic membrane and acrosome before and after thawing was respectively 90.7 ± 2.9% and 90.8 ± 1.9% (p>0.05). Concerning the fertilization rate, 64.1±3.9%, of the matured oocytes became fertilized with no significant differencesbetweenbulls. For in vivo fertilization, no statistical differences were found among bulls.

In conclusion, results clearly demonstrated that fertilizing ability of the bullfighting epididymal spermatozoa can be obtained after being cryopreserved.

Key Words: *Sperm viability; Brava dos Açores; bullfight breed.*

Date of submission: 18-09-2019 Date of acceptance: 03-10-2019

I. Introduction

The fighting bull on Terceira Island is the result of the introduction of fighting breed bovine from Portugal and Spain mainland (Bruges, 1997). These animals have suffered rapid evolutionary changesdifferentiating them from their ancestors. This sub-population presents self-characteristics ideal for *Rope Bullfight,* an ethnographic entertainment common on this Island. The importation of this animals since 1910, led Terceira Island to become one of the most important center of production of this kind of cattle in Portugal, fact that is cultural, historical and socio-economic important for Azores Autonomic Region.The reduced number of these animals makes imperative the safeguarding of this genetic heritage (Lima and Vieira, 2014) together with the current difficulties of importing breeding animals from the Iberian Peninsula due to different diseases and particularly bovine paratuberculosis(Lucas, 2012). The traditional method of exploitation of this bovine breed is extensive, which retains most of the traditions of previous centuries. The handling of these animals is very complicated due to the special idiosyncrasy of the specimens of the breed, characterized by their innate aggressiveness making any manipulation of the man a specialized and difficult task(Buxadé, 1996). The usual breeding system is the natural mating in complete freedom, although artificial insemination (IA) is increasing, promoting more and more the cryopreservation of semen of high performant animals.

The aim of the present study is to evaluate the fertilization potential of the semen recovered from the epididymal cauda of bullfighting bulls from "Brava dos Açores" breed before and after cryopreservation, accessed by flow cytometry as well as by in vitro and in vivo fertilization.

II. Materials and Methods

Testicles of six bulls from "Brava dos Açores" breed,aged from three to eight years(4.9 ± 2.8)and numbered from A to F,were collectedin the slaughterhouse of Terceira Island, transported to the University of the Azores where epididymiswere dissected, their tail isolated, and sperm collected and diluted in Tris medium at room temperature, according to Cormier and collaborators (Cormier et al., 1997).After extraction, semen of each bull was divided in three different aliquots and processed individually. For the concentration, semen was diluted 1/100 in water, and evaluated in a Neubauer chamber. 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).

Subsequently,plasma membrane integrity, percentage of live spermatozoa and acrosomal integrity were evaluated by flow cytometry (FCM) (Dickinson concentration, San Jose, California USA), collecting the data of 10,000 sperm per sample, further analyzing the results by using the CellQuest^{TM}software (Pro version 4.0.2). Through inspection of light scatter data, non-spermatic events ("debris") were discarded.

For plasma membrane integrity and live spermatozoa evaluation, a double fluorescent labeling: SYBR-14 and PI markers were used as previously described (Olivera, 2009; Garner et al., 1997). Briefly, the staining solution was prepared by adding 5 µL of a 2mM solution of PI (in water) and 2µL of a solution of 0.1 mM of SYBR-14 to 2 ml of phosphate buffer saline (PBS). Before analyzing, 295µl of staining solution were added to 5µ of semen with a concentration of 10^6 cells/mL.SYBR-14 fluorescence was detected using a FL1 530/30 nm band-pass filter and PI fluorescence detected using a long-pass FL3 650 nm filter. Then, graphic regions were defined from which the relative proportions of the subpopulations of spermatozoa were calculated. The population with low percentage of SYBR-14 staining and high PI staining, signifying dead spermatozoa. The other region indicates other non-interested particles that are not sperm cells. Figure 1 shows an example of a dot plot, representing how these regions were defined, showing the population of spermatozoa with a high percentage of staining with SYBR-14 and low percentage of staining with PI (live spermatozoa).

Acrosomal integrity was performed by staining the samples incubated with PI (Olivera, 2009) and FITC-PSA as a marker of the acrosome state (Casey et al., 1993; Hossain et al., 2011). Prior to the evaluation, 500 µ semen, 30 FITC-PSA (0.1 mg / ml) and 2µ PI (2 mg / ml) were added and incubated for 10 min at 37 °C. From the conjugation of the two fluorochromes, four sperm subpopulations were identified (Franco et al., 2013): alive with intact acrosome, without staining at PI and FITC-PSA; living with damaged acrosome, without PI staining and FITC-PSA staining; dead with intact acrosome, PI staining and no staining to FITC-PSA and dead with damaged acrosome, PI staining and FITC-PSA. Figure 2 shows typical output for this assessment.

In the meantime, semen straws were filled by vacuum and sealed with PVA, the thermal equilibrium was made at 5°C for 2 h and then placed in a programmed freezing chamber (IceCube 14S; SyLab, Austria), and the temperature dropped to -10 ° C at -4 ° C / min and then to - 145 ° C at -40 ° C / min. The straws were then plunged in liquid nitrogen and stored. For thawing, three straws of each bull were immersed in a water bath at 37ºC and by phase contrastmicroscope and by flow cytometry as afore described.

For in vitro fertilization, ovaries(n=750)wereobtainedatthe sameabattoirfromadultcattle,trimmedofadheringtissueandtransportedtothelaboratoryinDulbecco'sphosphatebuffe redsaline(DPBS),attemperatureangingfrom35to37ºCwithin2hourspost-slaughtering. Cumulus oocyte complexes (COCs) were washed twice in TCM-199mediumsupplementedwith2%fetalbovineserum(FBS),0.3mg/mlglutamineand50mg/mlgentamicinandthenwa shedtwiceinmaturationmediumsupplementedwith10%FBS,5µg/ml of FSH-LH, 1µg/ml estradiol-17ß, 0.15 mg/ml glutamine, 22 µg/ml Na-pyruvate, 50 µg/ml gentamicin. Then, COCs were transferred tosterilePetridishescontaining100µlofmaturationmediumsupplementedwithcortisol,previouslydilutedinethanolat0 (control) 50, 150 and 250 µM final concentration (10-15 oocytes/ droplet)at in an incubator (Lab line instrument Inc. USA) at 38.5 ºCfor22-24hourswithsaturatedhumidityand5%ofCO2,formaturation. Afterthis time,oocytesdesignatedtoIVFwereselected under the stereomicroscope, transferred into a Petri dish containingmaturationmediumandwashedtwiceinIVFmedium.Afterthawing,semenwaswashedtwicebycentrifugeat 320xgfor5minuteseach,innon-capacitatingHEPES-bufferedmedium(NCM)spermwashingmedium(5mleachtime).Afterremovingthe supernatant, sperm pellet was homogenized by pipetting in the remainingNCMSpermwashmedium(0.25-0.5ml)foradjustingthespermconcentrationandmotility.A volume of 20µl of the semen (11.5 x 10^6) was uploaded on a slide, covered with a cover slip and the motility of the sperms was checked under an inverted microscope. Each bull was used five times for IVF, representing 125 oocytes per bull. To adjust the concentration, fiveµl from the semen were added to 95 µl distilled water in an Eppendorf tube, and then exposedtodirectlightuntilthesemendied.Oocytesandspermswerethenco-culturedin500µlofIVF. Tyroide'sAlbuminLactate Pyruvate(TALP)for22-24hoursat38.5ºCintheincubatorwithsaturated humidity and 5%

of CO2. Twenty-four hours from the fertilization, presumptive zygotes were transferred to a glass tube containing 1 ml of washing medium and vortexed to remove granulosa cells attachedthem.Thenthe presumptive embryoswerecheckedunderthemicroscope,washedtwiceinculture medium and cultured for 7 days, being then evaluated after this time.

For AI, 18adult and pubescent heifers in a considered good body condition (Rae et al., 1993) with a score of 2.5 to 3.5 were used. Estrus synchronization was accomplished with CIRD (INC) as previously described by Cutaia et al. (2004), the estrous behavior was detected by visual observation, the AI performed, and the diagnosis of pregnancy was made by ultrasound at 60 days.

Statistical analysis was performed by simple ANOVA and the differences $P < 0.05$ were considered significant.

III. Results

Figure 1 shows a typical example of a dot plot, representing the populations of spermatozoa with a high percentage of staining with SYBR-14 and low percentage of staining with PI (live spermatozoa-R1). The population with low percentage of SYBR-14 staining and high PI, signifying dead spermatozoa appears as R2. The R3 region indicates other non-interested particles (debris) that are not sperm cells.

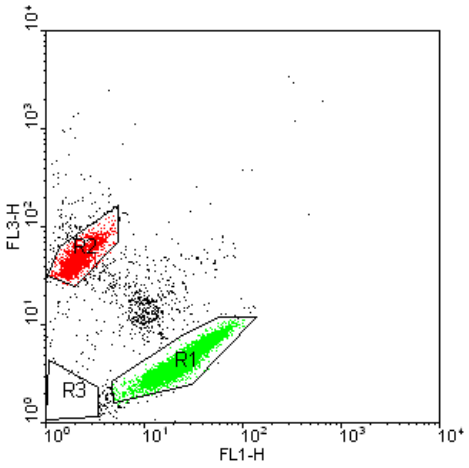

Figure 1: Typical plot showing the flow cytometry distribution of events and the fluorescence intensities of SYBR-14 (FL1-H) and propidium iodide (PI) (FL3-A).
R1 (live sperm), R2 (dead spermatozoa) and R3 (other events).

From the conjugation of the two fluorochromes, four subpopulations of spermatozoa were identified as described by Maxwell and Johnson (1997): live spermatozoa with intact acrosome, without PT staining and FITC-PSA (R2); live spermatozoa with damaged acrosome, without PI staining and FITC-PSA staining (R4); dead spermatozoa with intact acrosome, PI staining and no FITC-PSA staining (R3); spermatozoa deaths with damaged acrosome, PI staining and FITC-PSA (R1) (Figure 2).

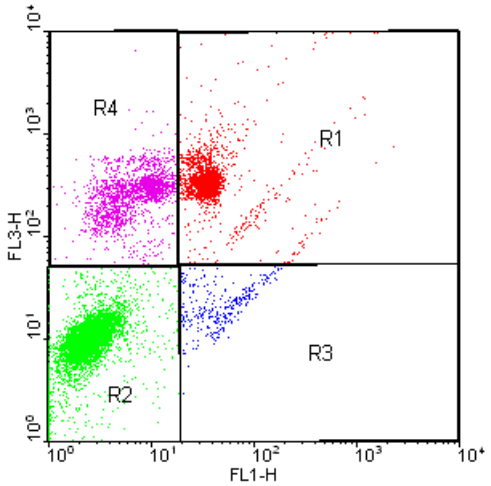

Figure 2: Output obtained by flow cytometry representing live spermatozoa with intact acrosome, without PT staining and FITC-PSA (R2); live spermatozoa with damaged acrosome, without PI staining and FITC-PSA

staining (R4); dead spermatozoa with intact acrosome, PI staining and no FITC-PSA staining (R3); spermatozoa deaths with damaged acrosome, PI staining and FITC-PSA (R1).

For progressive sperm motility, referring sperm that are swimming in a mostly straight line or largecircles, it was observed that, on average, bull E presented the best motility results (60%), followed by bull B(50%). Bulls A, C and D showed a motility ranging from 35 to 45% and bull F, 20% (Figure 3). On average,sperm viability was 56.8% ± 3.41. Although the values were statistically different ($P < 0.05$), this evidence was not observed in the other parameters used to evaluate semen fertility such pregnancy rates or in vitro fertilization results. Regarding sperm viability, observed by phase contrastmicroscopy, bulls B, C and E present about 70%, whose values were higher than the other bulls ($P < 0.05$) (Figure 4), which also did not affect AI success or the in vitro fertilizing rate. No statistical differences were observed for the percentage of droplets found in the alive sperm cells of which6.6% did not have protoplasmic droplet, 69.2 and 24.5% had distal and proximal droplets, respectively.

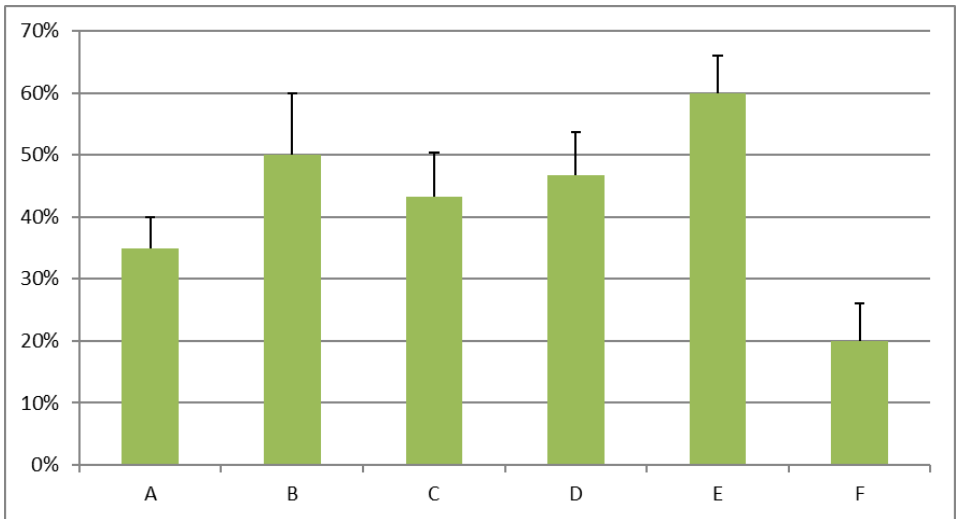

Figure 3: Percentage of progressive sperm motility, referring sperm that are swimming in a mostly straight lineor large circles. Each column represents the mean ± SEM of three aliquots made for each recover.

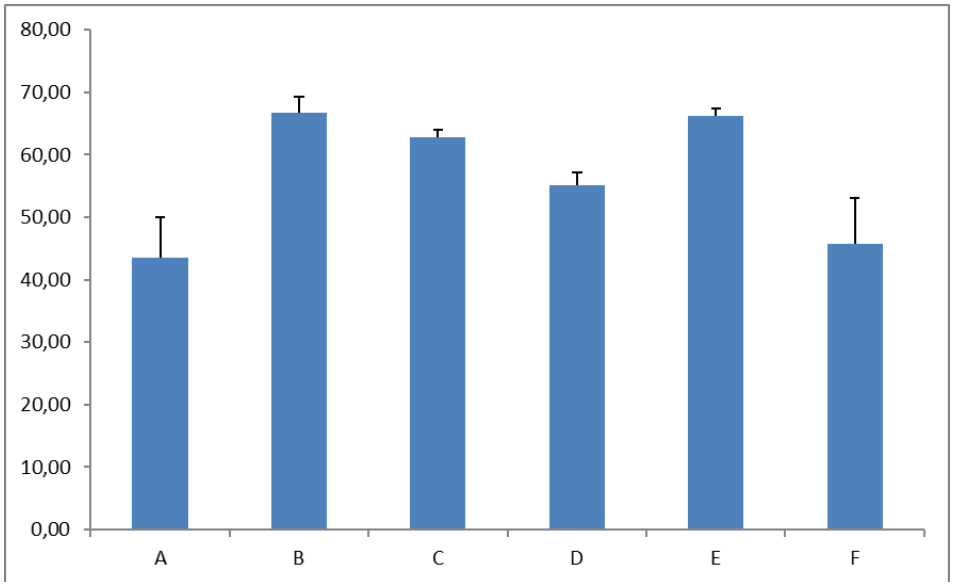

Figure 4: Percentage of sperm cell's viability of the different bulls. Values represent the mean ± SEM of three Aliquotsmade for each recover.

Concerning the acrosomal integrity, bulls B, C, D, E present 40-50% of the smears without any damages, superior to bulls A and F (20%) $P < 0.05$. The percentage of intact spermatozoa is consistent with the

results found for viability, since these two factors of analysis had similar results. The percentage of spermatozoa with intact plasma membrane and damaged acrosome was less than 10%, with no significant differences between bulls. In the AI results, the gestation rate was 50% and there were no differences among the bulls.

For in vitro fertilizing results, no statistical difference has been observed among bulls or among bullfighting animals. On average, concerning maturation rate, 94.1 ± 1.1% the oocytes were considered as matured. The percentage of cleavage embryos calculated from the oocytes located for maturation was (79.1 ± 2.7%) andfrom these, the embryonic development (number of embryos developed and classified as morula, early blastocyst and blastocyst stages) it has been observed a quite good result (34.4 ± 1.4 % while the others blocked at the stage of 2 up to 16 cells.

Relating the AI results, besides the number of females were very low,any statistical difference among the bulls was observed, and at 60 days after AI, 50% of the females remained pregnant.

IV. Discussion

As known, normal ejaculation contains billions of spermatozoa, so dilution is necessary to increase the number of doses to use. Much of the effort to preserve semen was fostered by the growing interest in preserving endangered species and preserving sperm cells from animals of high genetic value. However, in special situations such when the male accidentally dead or must be slaughtered for reasons of urgency, collection of semen cannot be performed by conventional methods. In the present study spermatozoa was collected from the epididymis after slaughtering bull from the Bullfight breed called "Raça Brava dos Açores" being their potential ability to fertilize being evaluated by flow cytometry in vitro and in vivo fertilization. According to Fernandéz-Santos et al. (2009), the epididyms have adequate conditions to prolong sperm survival, since the tail of the epididymis provides optimal environmental conditions for the storage of the gametes under physiological conditions. Nevertheless, cryopreservation of spermatozoa collected from epididyms causes a decrease in total sperm motility and in the percentage of sperm cells with intact membranes and acrosomes (Martins et al., 2007),affecting this way the fertility rate by damage to the acrosomal structures (Critser et al., 1987). Defrosting the semen is also a key step to the survival, mobility and fertilizing ability of cryopreserved semen. Rapid thawing of the doses is preferred to avoid the possibility of recrystallization of water molecules in the straw, which may be detrimental to the plasma membrane integrity (PMI) (Papa et al., 2008). Based on the collected data, two groups of results were differentiated: the first corresponds to those obtained from the microscopic analysis and the second corresponds to the spermatozoa analysis performed by flow cytometry. From the results obtained by flow cytometry after semen evaluation, it was possible to identify the individualized spermatozoa of smaller size particles, after which the green and red fluorescence was identified only from the spermatozoa population. Regarding motility and quality of movement, it was verified in this study that the percentage of sperm motility did not affect the AI success rate. In fact, we can check for the different results, for example, with bulls E and F. Bull semen E, which presented 60% motility, presents a negative result in the first IA while the bull semen F, which has the lowest percentage of motility, presents a positive result to AI.

The sperm motility is estimated subjectively by the visual evaluation of the cells under conventional optical microscopy, representing the main laboratory analysis used by the semen collection and processing centers, although thelarge variability on methods and criteria employed. Despite the importance of the analysis in the context of seminal evaluation, the relationship between sperm motility and fertility remains contradictory, suffering a great divergence between the different researches that indicate variable correlation indexes between 0.15 and 0.83 (Rodríguez-Martínez, 2013). Recently data published by Morado et al. (2015) postulated that besides routine laboratory tests (progressive motility, vigor, vitality and acrosome integrity) can be used to evaluate semen quality, although their usefulness to predict semen fertility is limited mainly due to the complexity of sperm and the fertilization process, some studies report that in vitro Fertilization (IVF) developed with frozen semen from bulls of high fertility yielded higher cleavage and blastocyst formation rates (Zhang et al., 1997).For these authors rates of cleavage and blastocyst formation are significantly related to the nonreturn rate ($r = 0.59$, $P < 0.001$; $r = 0.35$, $P < 0.05$, respectively), showing that this parameter can also be an useful tool to identify bull's fertility. Nevertheless, all these techniques depend mainly on the experience of the technician conducting the tests, can be considered the main responsible for the low precision in terms of test repeatability, evidencing the great variability of published data. In this sense, variations of 30 to 60% are reported in the estimation of sperm motility for the same ejaculate when evaluated subjectively (Sozanska et al., 2005; Verstegen, 2002).Despite the great controversy regarding the correlation of sperm movement patterns with in vivo fertility indexes, significant differences in movement patterns between spermatozoa reaching high and low fertilization rates are observed (Verstegen, 2002). In this work, the low number of inseminations performed may be the basis of these results since a larger number of AI would be necessary to make this aspect explicit.

The evaluation of spermatic movement, even with the advent and use of computer-assisted sperm analysis, provides an inconsistent correlation with the indices of animal design when used alone, since sperm

motility represents only one of several essential prerequisites for sperm to conclude biological function represented by oocyte fertilization (Graham and Mocé 2005).

In the present study, for the sperm viability, it can be stated that the analyzed bulls present considerable viability values. In this way, we are facing leads to a high rate of spermatozoa with whole plasma membrane and acrosome, and lower rates of spermatozoa with intact plasma membrane and a damaged acrosome. In addition, most spermatozoa with damaged plasma membrane have intact acrosome. All these factors resulted, however, in favor of a good AI success rate.

Fertility reduction, associated with AI and cryopreserved semen, has been attributed to processes that occur during semen freezing, where about 10 to 50% of ejaculate spermatozoa do not withstand this process and die (Watson, 2000). The analysis of sperm viability by microscopy is a subjective method in which it is necessary to use some time in individualized sperm count (Chaveiro et al., 2007). In this way the viability and integrity of the plasma and acrosomal membranes were evaluated by flow cytometry.The addition of PI to FITC-PSA allows the simultaneous determination of the viability and integrity of sperm acrosome, as the iodinated propidium emits red fluorescence and FITC-PSA emits green fluorescence (Harrison et al., 1993).We can observe, in this work, that motility, viability and acrosome are factors that are interconnected taking into account the quality of the semen. The semen analyzed has a high percentage of motility, and the viability and percentage of spermatozoa with acrosome and whole plasma membrane is also high, and vice versa, if the motility rate is low, since these factors are closely linked. In these conditions we observed that the conservation of semen made in this work allows the obtaining of doses of quality semen of any of the bulls. Approximately 50% of intact spermatozoa (E bull) are achieved, while the lowest rates are at the level of 20% for bull B. The percentage of viable spermatozoa in a seminal sample can be defined by the number of cells which exhibit plasma membrane integrity and stability (Graham et al., 1990). PMI is a prerequisite for the occurrence of physiological events related to the fertilization process, which include sperm capacitation, zona pellucida, acrosomal reaction and fusion of the gametes, promoting fertilization (Vazquez et al., 1993).Regarding the results of the AI that only the bull C failed, to generate a gestation during this work. However, the small number of AIs performed and the various factors that determine the success rate of AI may have conditioned these results.

The analysis of sperm viability by microscopy is a method in which it is necessary to use some time in individualized sperm count (Moreira da Silva et al., 2006). In this way the viability and integrity of the plasma and acrosomal membranes were evaluated by flow cytometry.The addition of PI to FITC-PSA allows the simultaneous determination of the viability and integrity of sperm acrosome, as the iodinated propidium emits red fluorescence and FITC-PSA emits green fluorescence (Harrison et al., 1993).

We can observe, in this work, that motility, viability and acrosome are factors that are interconnected taking into account the quality of the semen. The semen analyzed has a high percentage of motility, and the viability and percentage of spermatozoa with acrosome and whole plasma membrane is also high, and vice versa, if the motility rate is low, since these factors are closely linked. In these conditions we observed that the conservation of semen made in this work allows the obtaining of doses of quality semen of any of the bulls. Approximately 50% of intact spermatozoa (E bull) are achieved, while the lowest rates are at the level of 20% for bull B. The percentage of viable spermatozoa in a seminal sample can be defined by the number of cells which exhibit plasma membrane integrity and stability (Graham, 2001).PMI is a prerequisite for the occurrence of physiological events related to the fertilization process, which include sperm capacitation, zona pellucida, acrosomal reaction and fusion of the gametes, promoting fertilization (Vazquez et al., 1993).

Regarding the results of the AI that only the bull C failed, to generate a gestation during this work. However, the small number of AIs performed and the various factors that determine the success rate of AI may have conditioned these results.

V. Conclusion

A method of cryopreservation was developed for sperm salvaged from the cauda epididymis and vas deferens of a local bullfight breed testes, demonstrating that cryopreservation of epididymal bullfight sperm can be performed and that despitesome damage, spermatozoa retain their functional ability to fertilize in vivo and in vitro. The use of these techniquesallows the maintenance of the genetic potential of the fighting bulls, as their sperm, harvested from the epididymis, remain functionally competent after thawing.

Acknowledgments

This project was financed in 85% by FEDER and in 15% with regional funds through the ProgramaOperacional Açores 2020 (Operational Program Azores 2020), in scope of the project «BEMAP-ET - ACORES-01-0145-FEDER-000026.

References

[1]. Bruges, A. (1997). O Toiro na Ilha Terceira-Breve nota sobre a sua aparição e evolução. Festa na Ilha, 1: 6-7.
[2]. Buxadé, CC. (1996). Zootecnia-Bases de Producion animal: produciones equinas y de ganado de lidia. Ediciones Mundi-Prensa. Madrid, 247-260.
[3]. Casey, PJ.; Hillman, RB; Robertson, KR; Yudin, AI; Liu, IKM; Drobins, EZ. (1993). Validation of an acrosomal stain for equine sperm that differentiates between living and dead sperm. JournalofAndrology, 14: 289-297.
[4]. Chaveiro, A; Santos, P; Da Silva, F (2007). Assessment of sperm apoptosis in cryopreserved bull semen after swim-up treatment: A flow cytometric study. Reproduction in Domestic Animals, 42(1): 17-21.
[5]. Cormier, N.; Sirard, MA; Bayley, JL. (1997). Premature capacitation of bovine spermatozoa is initiated by cryopreservation. Andrology Journal, 18: 461-468.
[6]. 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.
[7]. Cutaia L, Chesta P, Morebo D, Aviles M, Bertero F, Bó GA (2004). Effect of time of prostaglandin F2 and estradiol benzoate administration on follicular wave emergence and ovulation in beef cattle treated with progesterone vaginal devices. In: Abstracts of the 15th International Congress of Animal Reproduction, 2004, Porto Seguro, Brazil. Porto Seguro: ICAR. 111.
[8]. Fernandéz-Santos, MR; Dominguez-Rebolledo, AE; Esteso, MC; Garde, JJ and Martinez-Pastor, F (2009). Refrigerated storage of red deer epididymal spermatozoa in the epididymis, diluted and with vitamin c supplementation. Reproduction in Domestic Animals, 44(2): 212-220.
[9]. Franco, J, Chaveiro, A, Gois A and Moreira da Silva, F (2013). Effects of alpha-topherol and ascorbic acid on equine semen quality after cryopreservation. Journal of Equine Veterinary Science, 33(10): 787-793.
[10]. Garner, DL; Thomas, CA; Joerg, HW; Dejanette, JM and Marshall, CE. (1997). Fluorometric assessment of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biology of Reproduction, 57: 1401-1406.
[11]. Graham, J (2001). Assessment of sperm quality: a flow cytometric approach. AnimReprod Sci., 68: 239–47.
[12]. Graham. J and Mocé E (2005). Fertility evaluation of frozen/thawed semen. Theriogenology,64: 492–504.
[13]. Graham, JK, Kunze EY, Hammerstedt R (1990). Analysis of sperm cell viability, acrossomal integrity, and mitochondrial function using flow cytometry. Biology of Reproduction, 43: 55-63.
[14]. Harrison, RAP, Asworth PJC and Miller NGA (1993). Rapid effects of bicabornate/CO2 on boar spermatozoa detec by merocyanine, a probe of lipid packing. Journal of Reproduction and Fertility - Abstract Series, 12-14.
[15]. Hossain, MS; Johannisson, A; Siqueira, AP; Wallgren, M and Rodriguez-Martinez, H (2011). Spermatozoa in the sperm-peak-fraction of the boar ejaculate show a lower flow of Ca2+ under capacitation conditions post-thaw which might account for their higher membrane stability after cryopreservation. Animal ReproductionScience, 128: 37-44.
[16]. Lima, JP and Vieira, JE (2014). Ilha Terceira: O Lugar Atlântico do Toiro. Bluedições, Ilha Terceira. 53-73.
[17]. Lucas, AV (2012). Origem e evolução do toiro de lide em Portugal. Contra-Barreira. Revista de Equitação e Tauromaquia, 26: 26-27.
[18]. Martins, CF; Rumpf, R; Pereira, DC andDode, MN (2007).Cryopreservationof epididymal bovinespermatozoafromdead animal andits use in vitro embryoproduction. Animal Reproduction Science, 101:326-331.
[19]. Maxwell, WM and Johnson, LA (1997). Membrane status of boar spermatozoa after cooling or cryopreservation. Theriogenology, 48: 209-219.
[20]. 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.
[21]. Morado, S; Pereyra, V; Breininger, E; Sara, R andCetica P (2015). Study of Sperm Evaluation Parameters to Estimate Cryopreserved Bovine Semen Fertility: Austin J. Vet. Sci. &Anim. Husb.,2(1): 1005.
[22]. Olivera, JNA (2009). Manual de Evaluación de Semenen Bovinos. Facultad de Medicina Veterinaria e Zootecnia, UniversidadVeracruzana. Veracruz. 7-25
[23]. Papa, FO; Melo, CM; Fioratti, EG; Dell'aqua, JA Jr; Zahn, FS and Alvarenga,MA (2008). Freezing of stallion epididymal sperm. Anim. Reprod. Sci., 107: 293-301.
[24]. Rae, DO., WE. Kunkle, PJ. Chenoweth, RS. Sand, and T. Tran. 1993. Relationship of parity and body condition score to pregnancy rates in Florida beef cattle. Theriogenology 39:1143.
[25]. Rodríguez-Martínez, H (2013). Semen evaluation techniques and their relationship with fertility. Anim. Reprod. 10:148–159.
[26]. Sozanska, A; Kolwas, K; Galas, J; Blocki, N and Czyzewski, A (2005). Simple optical method of qualitative assessment of sperm motility: preliminary results. Proc. SPIE 5959, Medical Imaging, 59590U (23 September).
[27]. Vazquez, JM; Martinez, E; Roca, J; Coy, P and Pastor LM (1993). Acrossome reaction of boar spermatozoa in homologous in vitro fertilization. Molecular Reproduction and Development, 36: 84-88.
[28]. Verstegen, J; Iguer-ouada, M and Onclin, K (2002). Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology, 57: 149–179.
[29]. Watson P(2000). The causes of reduced fertility with cryopreserved semen. AnimReprod Sci., 60(61): 481–92.
[30]. Zhang, BR; Larsson, B; Lundeheim, N and Rodriguez-Martinez, H. (1997). Relationship between embryo development in vitro and 56-day non return rates of cows inseminated with frozen-thawed semen from dairy bulls. Theriogenology, 48: 221-231.

J. P. R. Pacheco Lima. " Evaluation of the Cryopreservationandfecundatingcapacity of bullfightingbullsemenobtainedbypost-mortemcollection. "IOSR Journal of Agricultureand Veterinary Science (IOSR-JAVS) 12.10 (2019): PP- 28-33.