The Viability of Cryopreserved Spermatozoa Recovered From The CAUDA EPIDIDYMIS OF EQUINES and The Time of Collection Post-Orchiectomy

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ABSTRACT— ***The experiment was conducted using 20 epididymides recovered from ten stallions with the objective of correlating the viability of cryopreserved spermatozoa derived from the cauda epididymis of these animals and the time of collection of the spermatozoa post-orchiectomy. After submitting the stallions to bilateral orchiectomy, the spermatozoa were flushed from each cauda epididymis and subsequently cryopreserved for further evaluation. The spermatozoa were classified into two groups (G): G1-*** *cryopreserved ejaculated spermatozoa* ***(pre-orchiectomy); and G2- cryopreserved spermatozoa recovered from the cauda epididymis (post-orchiectomy). The following parameters were evaluated, using the double stain Tripan-blue/Giemsa to determine: defects in the acrosome and the number of live and dead spermatozoa. The results obtained demonstrated that the cryopreserved sperm from ejaculated semen samples corresponded to 65% of live spermatozoa with acrosomes. This was significantly higher (p<0,01) when compared to cryopreserved spermatozoa recovered from the cauda epididymis (45%), zero hours post-orchiectomy. The rate of live spermatozoa with acrosomes declined rapidly 24-hours post-orchiectomy in relation to prior periods. Thirty-six (36) hours post-orchiectomy the rate of live spermatozoa was significantly lower (p <0.05) than at 24 hours, but represented 17% of live sperm cells with acrosomes. This study demonstrates that it is possible to recover viable spermatic cells and freezing them 36 hours post-orchiectomy. Therefore, the data suggests that it is possible to transfer the methodology employed in this study to be utilized in genetically valuable stallions in the event of sudden death so as to conserve their semen for future use.***

Keywords— cryopreservation, epididymis, spermatozoa, stallion, trypan blue/giemsa

1. INTRODUCTION

The recuperation of viable spermatozoa from the epididymides post mortem is an important technique in obtaining genetic reserves from valuable animals or endangered species. With an increase in equine artificial insemination (AI), the process of dilution, cryopreservation and thawing of semen in the presence or absence of seminal plasma could minimize the loss of genetic material when a genetically valuable animal dies, especially when there is a lack of previously cryopreserved genetic material [01].

According to Watson (1995)[02], the process of freezing and thawing spermatozoa could damage its motility and fertilization capacity due to alterations in the plasma membrane. These changes in the plasma membrane could be due to the dilution of the spermatozoa that may remove proteins, hence changing the composition of the sperm samples [03; 04; 05]. The proteins when removed from the plasma membrane may modify the composition of the lipids, thus preventing the occurrence of the acrosome reaction. The stability of lipids in the plasma membrane is a prerequisite for maintaining the functionality of the sperm [02], and it may be due to this, that frozen semen as a shorter fertile life in the female genital tract.

The aim of the present study therefore was to correlate the progressive decline in equine spermatozoa viability parameters post-orchiectomy with the time of collection of the spermatozoa from the cauda epididymis conserved at room temperature at 22°C and the spermatozoa cryopreservation after.

2. MATERIALS AND METHODS

2.1 Animals

Ten stallions of various breeds: 1 Lusitano, 2 Ponies, 2 Crioulos, 2 crossbred English Thoroughbreds, 2 Appaloosas

and 1 Mangalarga, aged 24-90 months were included in the present study. Immediately after elective orchiectomy with local anaesthesia, a total of 20 (testes) epididymides were recovered. The animals used in the experiment were housed in individual stalls at the Veterinary Hospital of the Federal University of Paraná and fed with a commercial concentrate containing 13% crude protein, 10% total fibre, 1.5% Ca and 0.5% P, Coast-Cross hay and water ad libitum throughout the experiment.

2.2 Collection of semen samples by artificial vagina

Semen samples were collected twice a week via an artificial vagina and then subjected to semen analysis (spermogram). Afterwards, one of the samples was frozen to assess the freezability of the semen of each stallion. This procedure was done 63 days prior orchiectomy, with the objective of determining the fertility capacity of each stallion. After this period, the stallions were randomly chosen and submitted to bilateral orchiectomy.

2.3 Collection of spermatozoa from the cauda epididymis

One week after the final collection of semen via an artificial vagina, the stallions were subjected to bilateral orchiectomy to obtain the epididymides. Immediately after elective castration, the testes and epididymides were kept at a temperature of 22ºC in a Styrofoam box, in individual plastic wrappings, without any other form of conservation before the start of activities. The following sequence of activities were done in chronological order: dissection and washing the epididymis, followed by the evaluation and cryopreservation of the spermatozoa obeying the following time intervals: 0, 6, 12, 18, 24, 30 and 36 hours. The collection technique used in the recovery of the spermatozoa from the cauda epididymis was as described by Granemann et al. (2006)[06], where the cauda epididymis were isolated from each testis. The external surface of the cauda epididymis was washed with pre-heated saline solution (37°C) to remove any traces of blood. The connective tissue surrounding the cauda epididymis was carefully dissected and removed, avoiding the rupture of adjacent blood vessels and the epididymal duct. As such, the complex of coiled tubes of the epididymis and the epididymal duct were unravelled to its full-length.

Then the epididymal duct was sectioned transversely into three parts to facilitate the flushing and collection of the spermatozoa. Each segment was cut in 20 to 40cm, depending on the size of the cauda epididymis. To facilitate the flushing of the cauda epididymis, the ends of the segments were clamped (Kelly clamps) to avoid the loss of spermatozoa. Afterwards, the segments were maintained in a vertical position on a filter attached to a pre-heated (37°C) collecting cup. The clamp at the lower end of the segment was removed and the cryodiluent was injected into the epididymal lumen, immediately below the remaining upper clamp using a syringe and needle gauge 0.45 X 13. This resulted in the spermatozoa being carried by the diluent to the lower end of the segment to be recovered into the collecting cup below. The flushing of each cauda epididymis was done using 10 ml of cryodiluent (Cryo-Botu® - Botucatu, SP) pre-heated at 37°C. The spermatozoa recovered from the cauda epididymis were then subjected to cryopreservation.

2.4 Sperm Cryopreservation

The freezing protocol of the spermatozoa during the period pre-and post-orchiectomy (considered as 2 groups) of the stallions was identical. An hour after the collection of the spermatozoa, standard time for both groups, they were diluted, loaded and identified in 0.5 ml straws. The loaded straws were kept at a temperature of 5°C for 20 minutes, after which they were cooled in liquid nitrogen vapour to -120°C for 15 minutes. The straws were subsequently immersed in liquid nitrogen at –196°C for at least 24 hours before thawing. The straws were thawed in a water-bath at 37°C for 30 seconds.

2.5 Assessment of the acrosomes with the double stain Trypan blue and Giemsa

According to Dias (2002) [07] 20 µl aliquot of the thawed semen was transferred into a 1,5 ml tube containing 20µl of 4% Tryan blue stain (Sigma-Aldrich). The samples were kept in a water bath at 37°C for 20 minutes. After this time, 1.0 ml of distilled water was added to the sample, which was centrifuged at 700 g for 5 minutes to remove the excess stain. Each sample suffered three consecutive washings in order to remove the excess cryodiluent. After the removal of the supernatant from each sample, three smears per sample were made. These were then air dried and fixed in methanol for 5 minutes and dried again. Subsequently, the smears were immersed into Giemsa stain (Merck, Germany) (1:10) for 18 to 20 hours. The smears were then removed from the stain and the following parameters were evaluated: alterations of the acrosome, as well as the percentage of live and dead spermatozoa. The smears were examined using a phase contrast microscope at x1000 magnification, and 100 sperm cells were observed per slide.

The spermatozoa were classified as follows: dead spermatozoa with intact acrosomes: blue or purple head with pink acrosomes; dead spermatozoa with reactive acrosomes: blue or purple head with pale acrosomes; live spermatozoa with intact acrosomes: pink head with pink acrosomes, live spermatozoa with reactive acrosomes: pink or white head with pale acrosomes. Data were analysed by chi-square test, analysis of variance and the Tukey test.

3. RESULTS AND DISCUSSION

Table 1 shows the viability of the spermatozoa collected at 6-hour intervals from the cauda epididymis of the stallions and kept at room temperature for up to 36 hours post-orchiectomy and subsequently cryopreserved. The percentage of live spermatozoa with acrosomes 24 hours post-orchiectomy declined rapidly. This decline was significantly lower ($p<0,05$) 30 and 36 hours post-orchiectomy, however results obtained showed that after this time, 17% of live spermatozoa with acrosomes still existed.

Table 1- Parameters of the spermatozoa viability recovered from the cauda epididymis of equines post-orchiectomy, in relation to the time of collection and cryopreservation (n=20 epididymides) (2008)

Hours Post-orchiectomy	Sample number (n)	Alive with acrosomes %	Alive without acrosomes %	Dead with acrosomes%	Dead without acrosomes%
0	2	45,3 [a]	13,0 [a]	2,0 [a]	40,5 [a]
6	3	39,0 [a]	9,0 [b]	3,0 [a]	49,0 [a]
12	3	38,3 [a]	6,6 [b]	2,6 [a]	52,3 [a]
18	3	37,3 [a]	8,3 [b]	2,0 [a]	52,3 [a]
24	3	33,6 [a b]	3,3 [c]	2,6 [a]	60,3 [ab]
30	3	25,3 [b]	3,6 [c]	2,0 [a]	69,0 [b]
36	3	17,0 [c]	3,6 [c]	1,3 [a]	77,6 [b]

[a b c] Different letters in the same column indicate that the values are significantly different by the Tukey test ($p<0.05$).

In similar studies in equines by Muradás et al. (2006)[08], no significant difference was found between the spermatic assessment parameters such as: total motility, progressive motility and vigor of the epididymal spermatozoa up to 24-hours post-orchiectomy corroborating the findings of the present study.

In a study in African bovids Herrick et al. (2004)[09], reported epididymal spermatozoa can be recovered post mortem and be successfully cryopreserved. Further these authors stated that the proportion of viable spermatozoa with intact acrosomes was not altered in the cryopreserved spermatozoa when compared with sperm collected from the epididymides. Thus, this data supports the results obtained in this study. However, it should be noted that these authors worked only with spermatozoa collected and cryopreserved immediately after the death of animals.

Bruemmer et al. (2002)[10], observed that there were no significant differences in the motility of epididymal spermatozoa from stallions assessed immediately (zero-hour) and 24-hours post- orchiectomy, provided that the epididymis was kept cool at 5°C. James et al. (2002)[11] demonstrated that viable epididymal spermatozoa from stallions could be collected up to 96 hours post mortem provided that they are kept cool at 4ºC. In a study published by Kato et al. (2002)[12], demonstrated the viability of epididymal spermatozoa kept at room temperature and collected from the cauda epididymis of rats. Kato et al. (2002)[12], assessed sperm motility and acrosome integrity: 1, 3 and 5 hours post-orchiectomy. The study showed that there was a significant decrease in sperm motility after 5 hours, but there was no significant change in acrosome integrity.

In the present study, Figure 1 shows the percentage of live spermatozoa with acrosomes that are capable of fertilization. This fertilization capacity is reduced when the storage time of the spermatozoa at room temperature increases and as such, increases the percentage of dead spermatozoa without acrosomes.

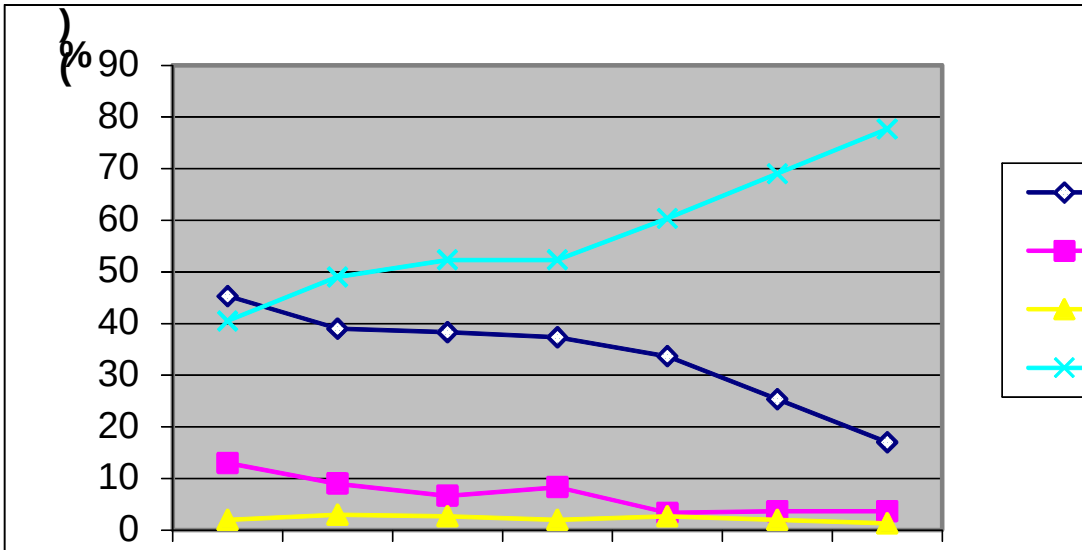

Figure 1 – variations in viability parameters of spermatozoa collected from equine cauda epididymis and cryopreserved, in relation to time (hours) post-orchiectomy.

- Alive with acrosome
- Alive without acrosome
- Dead with acrosome
- Dead without acrosome

In the present study, there was a significant decrease in the percentage of live spermatozoa with acrosomes kept at room temperature for 24-hours and subsequently frozen. In research studies by Nizan'ski et al. (2005)[13], with spermatozoa of domestic cats, confirmed results similar to the present study. Lessard et al. (1999)[14] demonstrated in studies that bovine spermatozoa with 25kDa protein could loss up 70% of its protein content when frozen, affecting the spermatozoa-oocyte bond and consequently the rate of fertilization.

According to Watson (1995)[02], the fertility and motility capacity of frozen-thawed spermatozoa is reduced due to alteration of its plasma membrane. These alterations may be due to the washing or dilutions of the spermatozoa that can remove proteins, thus altering the composition of the sperm sample, as cited in studies by Hammerstedt et al. (1990)[03]; Collin and Bailey (1999)[04]; Roncolleta et al. (1999)[05]. The proteins when removed can modify the configuration of the lipids in the plasma membrane, thus impeding the sperm acrosome reaction. The stability of lipids in the plasma membrane is a prerequisite for maintaining the function of spermatozoa [02], and it is due to this that frozen semen as a shorter fertile life in the female genital tract.

Table 2 shows the percentage of live spermatozoa with acrosomes from the cryopreserved ejaculate samples were significantly higher ($p < 0.01$) 65%, than the samples cryopreserved from the epididymides at zero-hour post-orchiectomy (45,3%). However, according to the norms of the Brazilian Society for Reproduction (CBRA,1998)[15], 45,3% of viable spermatozoa from cryopreserved sperm samples in horses is acceptable.

Table 2- Comparison between cryopreserved ejaculated spermatozoa pre-orchiectomy and spermatozoa recovered from the cauda epididymis at zero hours post-orchiectomy, in relation to the percentage of live spermatozoa with acrosome. (n= 10 ejaculates and n= 2 epididymes).

Spermatozoa	Number of samples (n)	Alive with acrosome %
Ejaculate	10	65,0 [a]
0 hours post-orchiectomy	2	45,3 [b]

[a b] Different letters in the same column indicate that the values differ by the Chi-square test ($p < 0.01$).

In an experiment at goats, Blash et al. (2000)[16], demonstrated that in addition to being possible a recovery post mortem of epididymal spermatozoa and have them successfully cryopreserved, they showed higher values in acrosome integrity, percentage of live spermatozoa and had a greater fertilization capacity "in vivo" and "in vitro", when compared to other spermatozoa collected from the ejaculate of live animals. In other studies supporting this theory, Kikuchi et al (1998)[17], assessed boar spermatozoa and concluded that frozen epididymal spermatozoa were more fertile and more resistant to freezing stress than sperm frozen from normal ejaculate, similar results were confirmed in a study Blash et al. (2000)[16].

Observations made during the present study, makes it possible to state that the recovery of semen from the cauda epididymis is a viable, practical and feasible method. In accordance with the present study, [18], cited that the aim of cryopreservation of spermatozoa is not only useful in equine reproduction, but as well in other domestic animals [19,20, 21,22,23,24].

In conclusion the percentage of live spermatozoa with acrosomes declined rapidly 24 hours post-orchiectomy, being significantly lower ($p<0.05$) 30 and 36 hours afterwards. It has also been shown the possibility of the existence of viable spermatic cells at room temperature up to 36 hours post-orchiectomy with 17% viable cells post-thawing. The samples of cryopreserved ejaculated spermatozoa represented 65% of live spermatozoa with acrosome, which was significantly higher ($p<0,01$) when compared with spermatozoa recovered from the cauda epididymes (45%) zero hour post-orchiectomy and subsequently cryopreserved.

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