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Recovery, cryopreservation and fertilization potential of bovine spermatozoa obtained from epididymides stored at 5 °C by different periods of time

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ABSTRACT

The objetive of the present study was to evaluate the effect of the interval between animal's death and sperm recovery on the freezability and fertilizing ability of spermatozoa from bull epididymides stored for different periods of time. Testis from 25 bulls were collected at the abattoir 2 h after the slaughter. In the laboratory spermatozoa from one epididymis were recovered and analysed for motility. The remaining epididymis was stored for 24 h (G24), 48 h (G48) and 72 h (G72) at 5 °C. At the end of each time period, spermatozoa were recuperated and cryopreserved in Tris-egg yolk and glycerol. Pre-freeze and post-thaw sperm samples were taken to assess total and progressive motility, concentration, membrane integrity and acrosome integrity. For evaluation of fertilizing ability, in each time period five straws of each bull were thawed, pooled and used for *in vitro* embryo production. The results showed that after 48 h of storage there was a decline in total motility, which did not change until 72 h. Progressive motility, plasma membrane and acrosome integrity were not affected by any of the storage periods. Conversely, all sperm parameters, except progressive motility, were reduced after cryopreservation. Embryo production was less ($P < 0.05$) in the treatments than in the reference group. However, there was no differences ($P > 0.05$) in blastoycst rate among experimental groups. Considering all the embryos produced by epididymal spermatozoa a greater proportion of female embryos was

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observed, which was similar to the reference embryos. The shift observed on sex ratio toward female for those two groups was also observed when they were compared with the expected 1:1 ratio ($P < 0.05$). The results showed the possibility to produced *in vitro* embryos using cryopreseved spermatozoa from epididymides and stored for long period of time at 5 °C. These procedures became an important tool for animal preservation when the sperm cells cannot be cryopreserved immediately after the animal's death.

1. Introduction

After the death of an animal, spermatozoa in males and oocytes in females remain alive for a period of time. If those gametes are recovered and used to produce embryos by assisted reproductive techniques it is possible to have progeny even after animals are dead (Songsasen et al., 1998).

In this context, recovery of epididymal spermatozoa from dead animals, cryopreservation and subsequent utilization for 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., 2007).

However, one of the difficulties to the application of assisted reproductive technologies (ART) for the preservation of biodiversity is the recovery of gametes which are still viable to be cryopreserved, especially for animals physically separated from the laboratory by long distances. In countries with continental dimensions, when a genetically important animal dies unexpectedly, most of the time there is no technician and/or equipment available to recuperate and cryopreserve the germplasm in a short time frame. Therefore, it is important to determine the proper procedure to handle and transport male germplasm to guarantee germ cell viability until these arrive at the laboratory to be appropriately cryopreserved.

Previous studies have indicated in various species that storage of epididymis at 5 °C could be a suitable way of preserving sperm motility and fertilizing ability for several days (An et al., 1999; Jishage et al., 1997; Kikuchi et al., 1998; Kishikawa et al., 1999; Yu and Leibo, 2002; Martínez-Pastor et al., 2005; Fernández-Santos et al., in press). However, few studies had focussed on the *in vitro* fertilizing potential of cryopreserved spermatozoa obtained from refrigerated epididymis (Kikuchi et al., 1998), especially in cattle in which, to our knowledge no reports have been published using that approach.

Therefore, the present study aims to simulate the transportation of bull testis for long periods of time at 5 °C and evaluate the freezability and *in vitro* fertilizing ability of spermatozoa from the bull epididymis.

2. Materials and methods

2.1. Testis collection and transportation

Testis from crossbreed bulls were collected at the abattoir 2 h after slaughter. In the laboratory spermatozoa from one epididymis, of each bull, were recovered and analyzed for viability. To simulate the time of transportation before sperm recovery and cryopreservation, the contra-lateral epididymis–testicle complexes were stored for 0 h (G0), 24 h (G24), 48 h (G48) and 72 h (G72) at 5 °C.

2.2. Sperm evaluation

Sperm samples from all groups were evaluated for total motility, progressive motility, concentration, morphology, acrosome and membrane integrity. Total motility (0–100%) and progressive motility (0–5; 0, no movement; 5, progressive rapid and linear movement) were subjectively assessed under a light microscope.

Concentration was determined in a hemocytometer in a 1:200 dilution and results are presented as sperm cells/mL. Sperm morphology was evaluated using a phase contrast microscope at 1000×

magnification according to Barth and Oko (1989). A total of 200 cells were counted and the results are expressed in percentage.

The evaluation of sperm membrane and acrosome integrity was performed by optical microscopy as described by Didion et al. (1989) with slight modifications. A sample of 20 μL from each treatment suspension was incubated with 20 μL of trypan blue stain (0.2%) at 37 °C for 10 min, and then centrifuged twice at 700 × *g* for 6 min. The pellet was re-suspended with 0.5 mL of Tyrode's albumin lactate pyruvate medium (TALP) (Parrish et al., 1995), and three smears were made from each sample. The smears were fixed with methanol for 5 min, dried and stained overnight with Giemsa (10%). Slides were evaluated by counting 200 cells in bright field microscopy and the results were expressed as percentage of live cells with intact acrosome.

2.3. Cryopreservation of spermatozoa from the refrigerated epididymis

In the laboratory, the epididymides were separated from the testicles and washed with 70% alcohol. Various incisions in the tail of epididymis were performed and then, by pressing that region manually the spermatozoa were released and collected. The recovered spermatozoa were evaluated and placed in a 15 mL tube and diluted with Tris-egg yolk and glycerol extender as described by Cormier et al. (1997). Then, they were loaded into 0.25 mL straws in a total concentration of 15 × 10 cells and equilibrated for 4 h at 5 °C. After the equilibration period the straws were cooled to a temperature between −80 and −120 °C, in which they were kept for 20 min. The straws were subsequently immersed at liquid nitrogen and stored until use.

2.4. In vitro maturation, fertilization and embryo culture

Ovaries from crossbreed cows (*Bos indicus* × *Bos taurus*) were collected at a commercial slaughterhouse. Cumulus oocyte complexes (COC) were aspirated from 2 to 8 mm diameter follicles, after selection the COC were washed and transferred to a 200 μL drop maturation medium under silicone oil and incubated for 22 h at 39 °C in 5% of CO_2 in air. The maturation medium consisted of TCM-199 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS (v:v), 24 IU/mL of LH, 10 μg/mL of FSH and antibiotics (100 IU/mL of penicillin and 50 μg/mL of streptomycin), with 5% CO_2 in air at 39 °C. After maturation period COC were randomly distributed into four groups and transferred to a 200 μL drop of fertilization medium. For *in vitro* insemination of each treatment (G24, G48 and G72 h) straws of five different bulls were thawed and mixed to compose a pool of spermatozoa. Motile spermatozoa from each pool were obtained by washing method and were added into the fertilization drop in a final concentration of 1×10^6 spermatozoa/mL. Spermatozoa and oocytes were co-incubated for 18–20 h at 39 °C with 5% CO_2 in air. In the reference group, COC were inseminated with frozen semen with known *in vitro* fertility. Presumptive zygotes, from each group were washed in embryo culture medium and transfer to the culture drops. In all experiments, embryo culture took place in SOFaaci, supplemented with 2.77 mM of myo-inositol and 5% FCS in a humidified atmosphere 5% of CO_2 in air at 39 °C. Embryos were evaluated on day 2 post-insemination for cleavage and days 6, 7, 8 and 9 for blastocyst rates.

2.5. Embryo sexing procedure

Embryo sex identifications were determined by PCR, using two different pair of primers. The first primer was specific to a region of the Y chromosome, while the second pair of primers was specific to a bovine autosomal gene. The primer sequences and fragment size for each gene are shown in Table 1. Initially embryos were exposed for 5 min to a lysis solution containing 1 μL PCR buffer 10×, 0.75 μL proteinase K (20 mg/mL—Invitrogen) in 6.25 μL of water, followed by Proteinase K inactivation at a 95 °C for 5 min. Amplifications were performed by adding to each sample tube a PCR mix containing 20 nM of each pair of primers, 800 μM dNTPs, PCR buffer 1× and 1 U Platinum Taq DNA Polimerase® (Invitrogen). Samples were submitted to 40 cycles of 94 °C (20 seg), 57 °C (30 seg) 72 °C (30 seg), followed by a final extension at 72 °C for 30 min. Amplified PCR products were visualized in 2% agarose gel stained with ethidium bromide. When two bands corresponding to the specific sequences were

Table 1
Sequences of the primers used for sexing (S: sense; A: antisense) and size (pb) of the amplified fragments.

Gene	Primer	Fragment	Reference
Chromosome Y	S: 5′-CCTCCCCTTCAAACGCCCGGAATCATT-3′ A: 5′-GGCCATAGTCAGGATCTT-3′	210 pb	Bondioli et al. (1989)
Autosomic	S: 5′-CCCATCACCATCTTCCAGG-3′ A: 5′-AGTGAGCTTCCCGTTCAGC-3′	280 pb	Ellis et al. (1988)

visible the embryo was considered as male, while only one band corresponding to the autosomal gene was present the embryos was considered female.

2.6. Statistical analysis

One-way ANOVA was used to compare the sperm data between the treatments. When ANOVA revealed a significant effect, the treatments were compared by *t*-test using Sigma Stat for Windows Version 3.11 (Systat Software, Inc., Richmond, CA, USA). The embryo development and the sex ratios between the treatments were performed by Chi-square test. An analysis was also performed to compare sex ratios of each treatment with the expected 1:1 ratio. A difference of $P < 0.05$ was considered significant for all data.

2.7. Experimental design

Testes from 25 crossbreed bulls were collected at abattoir 2 h after the slaughter. In the laboratory spermatozoa from one epididymis, of each bull, were recovered and analysed for morphology, total and progressive motility. The contra-lateral epididymis–testicular complexes were stored at 5 °C for 0 ($n = 6$) 24 ($n = 6$), 48 ($n = 6$) and 72 h ($n = 7$). At the end of each storage period, spermatozoa were recuperated and evaluated for total and progressive motility, concentration, acrosome integrity and membrane integrity. Then, the spermatozoa from all treatments were cryopreserved in Tris-egg yolk and glycerol, a sample from each bull was thawed at 35 °C for 30 s in a water bath and evaluated for the same variables performed for the pre-freeze analysis.

To evaluate *in vitro* fertilizing potential straws from five bulls were pooled to minimize the bull effect. Pooled sperm was used for *in vitro* fertilization of *in vitro* matured oocytes and six replicates were performed. For this experiment, epididymal sperm stored at 5 °C for 24, 48 and 72 h were used and considered as the experimental groups G24, G48 and G72, respectively. The fertilizing potential of 0 h storage epididymal sperm was not included because it has already been evaluated in our laboratory (Martins et al., 2007).

Cleavage rate was evaluated at D2 and blastocyst rate at D6, D7, D8 and D9 post insemination. Semen from a bull of known *in vitro* fertility, which has been the laboratory bull of reference for *in vitro* embryo production, was used as the control. To evaluate if the condition (time) under which the epididymis is handled could cause a shift in sex ratio of *in vitro* produced embryos, the sex of all embryos was determined by PCR.

3. Results

The mean sperm concentration obtained per epididymis was 432 ± 77 cell/mL, which provided a mean of 31 straws per epididymis. The percentage of total abnormal spermatozoa was 36.68%. As expected, the most frequent abnormality was the presence of cytoplasmic droplets (26.62%).

After 24 h of storage at 5 °C no change in total motility was observed when compared to the reference group values. However, a decrease occurred at 48 h of storage, which remained unchanged until 72 h. In contrast, progressive motility, acrosome and plasma membrane status did not undergo significant changes during storage period (Table 2).

Cryopreservation had a detrimental effect on sperm quality, except for progressive motility which showed similar values before freezing and after thaw (Table 2). Although cryopreservation caused

Table 2
Mean (S.D.) total motility, progressive status and membrane and acrosome integrity of bull epididymal spermatozoa frozen after 0, 24, 48 and 72 h of epidimydes storage at 5 °C.

Variables	Epididimydes Refrigerated at 5 °C			
	0 h (n = 6)	24 h (n = 6)	48 h (n = 6)	72 h (n = 7)
Total motility before freezing	78.1 ± 10.2^{aA}	67.8 ± 7.5^{abA}	60.4 ± 11.7^{bcA}	56.6 ± 10.3^{cA}
Total motility after thaw	51.6 ± 11.6^{aB}	45.0 ± 20.2^{abB}	40.0 ± 18.2^{abB}	24.1 ± 13.5^{bB}
Sperm progressive status (0–5) before freezing	3.2 ± 0.5^{aA}	2.9 ± 0.6^{aA}	3.1 ± 0.3^{aA}	2.8 ± 0.8^{aA}
Sperm progressive status (0–5) after thaw	2.6 ± 0.5^{aA}	2.7 ± 0.4^{aA}	2.7 ± 0.5^{aA}	2.6 ± 0.5^{aA}
Sperm alive with intact acrosome before freezing	75.5 ± 4.0^{aA}	72.3 ± 17.5^{aA}	70.7 ± 12.4^{aA}	64.0 ± 15.4^{aA}
Sperm alive with intact acrosome after thaw	64.0 ± 10.5^{aB}	36.2 ± 14.5^{bB}	39.5 ± 22.0^{bB}	38.2 ± 13.0^{bB}

Within a row, values without common superscript letters (a–c) were different (P < 0.05). Within a column, values without common superscript letters (A and B) were different (P < 0.05), considering correlates variables.

Table 3
Cleavage (day 2) and blastocyst rates at days 6, 7, 8 and 9 of embryos produced *in vitro* using frozen spermatozoa recovery from epididymides refrigerated for 24, 48, and 72 h at 5 °C.

Treatments	Number of oocytes	Cleavage	D6	D7	D8	D9	Total of blastocyst
G24	175	85 (48.50%)^{a}	8 (4.57%)^{a}	8 (4.57%)^{a}	5 (2.85%)^{a}	2 (1.14%)^{a}	23 (13.14%)^{a}
G48	175	75 (42.80%)^{a}	1 (0.57%)^{a}	2 (1.14%)^{a}	5 (2.85%)^{a}	3 (1.71%)^{a}	11 (6.28%)^{a}
G72	172	67 (38.95%)^{a}	4 (2.32%)^{a}	7 (4.06%)^{a}	3 (1.74%)^{a}	1 (0.58%)^{a}	15 (8.7%)^{a}
Reference	174	129 (74.13%)^{b}	30 (17.24%)^{b}	30 (17.24%)^{b}	26 (14.94%)^{b}	2 (1.14%)^{a}	88 (50.57%)^{b}

Within a column, percentages with different letters (a and b) differ, P < 0.05.

Table 4
Proportion of male and female embryos produced *in vitro* using frozen spermatozoa recovery from epididymides refrigerated for 24, 48 and 72 h at 5 °C and reference (sperm from ejaculate).

Treatments	Number of embryos	Male	Female
G24	23	10 (43.5%)^{a}	13 (56.5%)^{a}
G48	10	2 (20%)^{a}	8(80%)^{bA}
G72	14	7 (50%)^{a}	7 (50%)^{a}
Sum of treatments	47	19 (40.4%)^{a}	28 (59.6%)^{bA}
Reference	86	35 (40.7%)^{a}	51 (59.3%)^{bA}

Within a row, values without common superscript letters (a and b) were different (P < 0.05). In female column, values with the letter 'A' attached differ (P < 0.05) from the expected 1:1 sex ratio.

a decreased in total motility, the 72 h group was affected to a greater extent with less post-thaw motility than other groups. Acrosome and plasma membrane integrity were affected, not only by cryopreservation, but also by the period of time that the epididymides were stored. The post-thaw samples from G24, G48 and G72 had a lesser percentage of sperm alive with intact acrosome compared to G0 (Table 2).

In vitro embryos were produced in all treatment groups, however, use of epididymal sperm that had been stored and then cryopreserved resulted in a reduction in cleavage and blastocyst rates (Table 3). The time of storage did not affect fertilizing ability, because cleavage and blastocyst formation using epididymal sperm from groups G24, G48 and G72 did not differ significantly (Table 3).

When sex ratios were compared among treatments the proportion of female embryos was greater with treatment G48 (Table 4). The shift observed in sex ratio toward females for these groups was also observed when compared with the expected 1:1 ratio (P < 0.05).

4. Discussion

In the present study, storage of epididymides attached to counterpart testis at 5 °C can decrease sperm metabolism and maintain viability, which then allows for transport to the laboratory so that adequate manipulation and cryopreservation can occur. However, refrigeration followed by cryopreser-

vation can compromise fertilization potential of spermatozoa. With all the structural compromising that occurs as a result of refrigeration and cryopreservation, *in vitro* embryos were nevertheless produced with all treatments in the present study. These results indicate that spermatozoa from refrigerated epididymides after death of either a highly productive animal or of an animal from an endangered species can be used for production of embryos.

According to Fernández-Santos et al. (in press), epididymides have adequate conditions to prolong sperm survival, because the cauda epididymis provide the optimal environment for gamete storage in physiological conditions. Although the cauda epididymis offers good conditions to keep sperm viability for an extended period, when long periods are needed until sperm recovery several studies agree that refrigeration is necessary to minimize compromising of sperm integrity (Yu and Leibo, 2002; Martínez-Pastor et al., 2005; Fernández-Santos et al., in press).

The results of the present study clearly showed that refrigeration of bull epididymides resulted in sustained sperm viability, however, total motility decreased after 48 h of storage, being the only variable affected. This result is in agreement with previous reports, which found in other species that total motility is the variable most affected by time of storage (Sankai et al., 2001; Yu and Leibo, 2002; Hishinuma et al., 2003; Kaabi et al., 2003; Martínez-Pastor et al., 2005). Epididymal environment provides the most ideal condition for sperm survival, however, refrigeration at 5 °C is necessary to diminish energy wastage and extend sperm lifespan.

Progressive motility, membrane and acrosome status were not affected by 3 days of storage at 5 °C. These results suggest that storage conditions (temperature and epididymal environment) can preserve bull spermatozoa viability by delaying epididymal degeneration processes. Martínez-Pastor et al. (2005) also found that membrane and acrosomal integrity of epididymal spermatozoa from wild ruminants was enhanced in postmortem conditions if cooling of the epididymides occurred. However, inconsistent with results from the present study, the previous study provided evidence that progressive motility, instead of total motility, was affected to the greatest extent by refrigeration processes.

Cryopreservation of spermatozoa recovered from epididymides, independent of storage time, caused a decrease in total motility and percentage of sperm cells with intact membranes and acrosomes, but no changes were detected in progressive motility. Cryopreservation processes are known as being damaging to the sperm cells, and can have an effect on sperm motility and fertilization rate due to compromising the integrity of acrosomal structures (Critser et al., 1987; Wakayama and Yanagimachi, 1998). Reports in other species indicate spermatozoa from epididymides are less tolerant to cryopreservation than spermatozoa from an ejaculate (Krzywinski, 1981; Zomborszky et al., 1999). In the present study, spermatozoa maintained in testes without blood flow and refrigerated during storage, suffered more damage when temperature decreased to −196 °C. In contrast to results in the present study, cryopreservation only affected motility and viability of epididymal sperm after long periods of storage in *Cervus nippon* (Hishinuma et al., 2003) and *Cervus elaphus hispanicus* (Fernández-Santos et al., in press).

Although IVF techniques have been widely studied in cattle, no reports were found regarding the evaluation of *in vitro* fertilizing potential of epididymal spermatozoa from dead animals stored at 5 °C. Previously, fertilizing ability of bull epididymal spermatozoa that were cryopreserved shortly after death of the animals (Martins et al., 2007) was demonstrated. In the present study, sperm from all treatments involving different storage periods produced fewer embryos than the reference group, which confirms that storage and cryopreservation damaged germ cell integrity. Treatment in which sperm were stored for 24 h produced 52.1% and 34.7% more embryos than G48 and G72, respectively, however the increase in blastocyst rate was not different. Even though production was less in all treatments, it was possible to produce embryos from sperm stored for up to 72 h after death of the animal.

In normal ejaculates, proportion of X and Y-bearing sperm are about equal (Madrid-Bury et al., 2003; Checa et al., 2002). Therefore, differences in sex ratios of offspring might be due to treatment manipulations that differentially affect X and Y bearing sperm. The shift in sex ratio can be attributed to events that occur before fertilization that favor selection of Y- or X-chromosome-bearing spermatozoa or events that occur after fertilization, such as culture conditions or a combination of both (Kochhar et al., 2001, 2003; Lechniak et al., 2003). In the present study, sex of the embryos was used to determine if storage of the epididymides could affect population of Y- or X-chromosome-bearing

spermatozoa. Cooling the epididymides to 5 °C, followed by cryopreservation did not affect proportion of male:female embryos. The only difference was for group G48, however, there were very few sexed embryos for this group. Thus, when embryos of all treatments were considered together percentage of male and female embryos did not differ from the reference group. In both groups, the shift observed in sex ratio toward more females was also observed when compared with the expected 1:1 ratio ($P < 0.05$). Several studies with cattle embryos produced *in vitro* have shown that sex ratio may differ from 1:1 and according to the laboratory conditions (Lonergan et al., 1999; Gutierrez-Adan et al., 1999, 2001). The majority of studies showed that male embryos have a selective survival advantage over female embryos under *in vitro* conditions (Peippo et al., 2001; Alomar et al., 2008). Therefore, the greater proportion of female embryos observed in the present study could be attributed to culture system and/or sperm preparation technique, which differed from the typical percoll or swim up technique in that spermatozoa were washed only before IVF.

In conclusion, although there were few embryos produced with all treatments it was possible to use *in vitro* techniques to produce embryos using cryopreserved spermatozoa obtained from bull epididymides and stored for long periods of time at 5 °C. If animals were of high genetic value or from a species in risk of extinction, the association of storage and transportation of the epididymides, followed by sperm cryopreservation and use in IVF provides for an avenue through which multiplication of such important germplasm can occur. These procedures can also be optimized for cattle and adapted and used for wild animals. These techniques are important tools for animal preservation when sperm cells cannot be cryopreserved immediately after an animal's death.

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