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Short communication

Cryopreservation of epididymal bovine spermatozoa from dead animals and its uses *in vitro* embryo production

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Abstract

The present study aimed to evaluate viability and *in vitro* fertilizing ability of cryopreserved epididymal spermatozoa obtained from dead animals. To collect spermatozoa, epididymides from three males (Bulls A1, A2 and A3) were collected at a local slaughterhouse. As a reference ejaculate from a bull with known in vitro fertility, was used. Sperm characteristics (motility, chromatin and acrosome integrity) were evaluated before and after cryopreservation. Then, frozen spermatozoa from all animals were used for *in vitro* fertilization. Cleavage and blastocyst rates at 48 h (day 2) and 168 h (day 7) post *in vitro* insemination, for bull A1 (82.1 and 38.6%) and A2 (80.7 and 33.8%) were similar ($P > 0.05$) to the reference bull (88.9 and 57.2%). Bull A3 had the lesser cleavage (42.0%) and blastocyst (26.1%) rates. The results showed that epididymal spermatozoa from dead animals can be successfully cryopreserved and used *in vitro* production of embryos.

Keywords: Bovine; Cryopreservation; Epididymal sperm; Genes

1. Introduction

Genetic material either from animals of economical interest or from wildlife can be lost anytime by unexpected death of the animal. In this case, efforts can occur to avoid the total lost of that genetic material, which can be achieved by using reproductive assisted techniques.

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One possibility to preserve the germplasm from dead males is the recovery of spermatozoa from the epididymis. This *post-mortem* procedure has been considered as a very important tool for enhanced utilization of germplasm from valuable animals or animals in risk of extinction (Kaabi et al., 2003).

Although the use of fresh spermatozoa recovered from the epididymis is an alternative procedure, cryopreservation of spermatozoa allows a more efficient and economical way to use that material, because it can be utilized anytime not just after death of an animal.

Based in the importance of that procedure for bovine production and conservation, the present study was designed to evaluate the viability and fertilizing ability of cryopreserved epididymal spermatozoa obtained from dead animals.

2. Materials and methods

2.1. Epididymides collection and transportation

Testis of crossbreed bulls were collected at the abattoir 2 h after the death of the animal, and then epididymides were separated from the testis. Isolated epididymides were placed in 50 mL tubes containing Tyrode's albumin lactate pyruvate medium (TALP) (Parrish et al., 1995) and were transported to the laboratory at room temperature. The time between the removal of the epididymis and arrival at laboratory was 5 h.

2.2. Recovery and cryopreservation of spermatozoa from the epididymis

In the laboratory, the epididymides were washed with alcohol 70%. 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 placed in a 15 mL tube and diluted with egg yolk Tris glycerol extender as described by Cormier et al. (1997). Then, they were loaded into 0.25 mL straws in a total concentration of 20×10^6 cells and equilibrated for 4 h at 5 °C. After the equilibration period the straws were cooled down to a temperature between −80 and −120 °C, in which they were kept for 20 min. Finally the straws were immersed in liquid nitrogen and stored until their use.

2.3. Sperm evaluation

All sperm samples were evaluated for motility, concentration, morphology, acrosome and chromatin integrity. The percentage of motile spermatozoa was assessed subjectively on a warmed glass-slide immediately after sperm recovery and dilution with cryopreservation medium. Concentration was determined in a hemocytometer in a 1:200 dilution and results are presented as sperm cell/mL. Sperm morphology was evaluated using a phase contrast microscope in 1000× magnification according to Barth and Oko (1989). A total of 200 cells were counted and the results are expressed in percentage.

For acrosome integrity evaluation, spermatozoa were washed twice in TALP medium through centrifugation at 700 × *g* for 6 min. The *pellet* was resuspended with 0.5 mL of TALP and three smears from each sample were made. The smears were fixed in methanol for 5 min, air-dried and stained overnight with Giemsa at 10%. Presence of acrosome was assessed in bright field microscope in 1000× magnification. A total of 200 cells were analyzed and the results are express as percentage.

Chromatin integrity was determined using the acridine orange staining procedure. Three smears were prepared for each sample, air-dried and fixed overnight in Carnoy solution (methanol:acetic acid, 3:1). Then, the slides were air-dried and stained for 5 min with a solution containing acridine orange stain (0.1 mg/mL), citric acid (0.1 M) and di-sodium phosphate (0.3 M). The slides were analyzed in the same day using an epifluorecense microcope (450/530 nm). For all samples 200 cells were examined in a 100× magnification. Only spermatozoa stained green were considered to have a normal chromatin.

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 of 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 and into four groups and transferred to a 200 μL drop of fertilization medium. Motile spermatozoa from all animals were obtained by the Percoll (Amersham Biosciences AB, Uppsala, Sweden) gradient 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 SOF acid, supplemented with 2.77 mM of myo-inositol and 5% FCS in a humidified atmosphere of 5% of CO_2 in air at 39 °C. Embryos were evaluated on day 2 post-insemination for cleavage and days 6, 7 and 8 for blastocyst rates.

2.5. Statistical analysis

Analysis of data was performed using Sigma Stat for Windows version 3.11 (Systat software, Inc.). One-way repeated measures ANOVA was used to compare the effects of different treatments. The data are given as mean values ± S.D. When ANOVA revealed a significant effect, the treatments were compared by Tukey test. A difference of $P < 0.05$ was considered significant.

3. Results

3.1. Sperm evaluation

Motility of fresh sperm varied between 70 and 80% for epididymal samples and it was 90% for the reference (ejaculated semen). After cryopreservation motility for epididymal spermatozoa of bull A3 did not change (70%). However, when comparing before and after cryopreservation a decrease in motility was observed for animal A1 (80 and 60%), A2 (80 and 70%) and reference bull (90 and 80%). The mean of sperm concentration was 440×10^6 sperm cells/each epididymis and 800×10^6 sperm cell/mL for ejaculated semen.

Cryopreserved spermatozoa from ejaculated semen had less morphological abnormalities (23%). In contrast, a greater percentage of abnormal cells was observed in spermatozoa recov-

Table 1
Cleavage (day 2) and blastocyst rates at days 6, 7 and 8 of embryos produced *in vitro* using *in vitro* matured oocytes fertilized with semen from a reference bull and three bulls (A1, A2, A3) that had the epididymal spermatozoa recovered after death of the animal

Animals	No. of oocytes	Cleavage (%)	Blastocysts day 6 (%)	Blastocysts day 7 (%)	Blastocysts day 8 (%)
Reference	144	128 (88.9) a	41 (28.5) a	78 (54.2) a	83 (57.6) a
A 1	140	115 (82.1) a	27 (19.3) a	54 (38.6) ab	61 (43.6) ab
A 2	145	117 (80.7) a	29 (20.0) a	49 (33.8) ab	55 (37.9) ab
A 3	135	77 (57.0) a	22 (16.3) a	29 (21.5) b	34 (25.2) b

Within a column, percentages with different letters differ significantly, $p < 0.05$.

ery from epididymus from all bulls, being 54, 48 and 40% for A1, A2 and A3, respectively. As expected, after centrifugation in Percoll gradient, a reduction in the total percentage of abnormal cells occurred not only in the reference samples (12%) but also for epididymal samples (22.0, 20.5 and 22.0% for A1, A2 and A3).

The percentage of sperm cells with an intact acrosome was not significantly decreased after cryopreservation (97.0 and 88.0%) in the reference sample. The same pattern was not observed for spermatozoa from epididymis, in which a decrease ($P < 0.05$) in percentage of sperm cells with an intact acrosome after cryopreservation was observed for bull A1 (87.0 and 66.1%), A2 (92.0 and 68.0%) and A3 (90.0 and 60.0%).

No differences were observed in chromatin integrity of spermatozoa from reference, A1, A2 and A3 bulls before (0, 1, 3 and 2%, respectively) and after (0, 2, 5 and 2%, respectively) cryopreservation.

3.2. Embryo development

Blastocyst rate was similar ($P > 0.05$) for all groups on D6 of culture. However, at days 7 and 8 blastocyst production was less ($P < 0.05$) for bull A3 than for the other bulls (Table 1).

4. Discussion

The recovery of the epididymal spermatozoa from dead animals, their 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. Actually, the importance of these technologies can be confirmed by the cattle breeding and selection programs, which have demonstrated that the introduction of beef and dairy breeds in certain parts of the world has led to the near extinction of native breeds; for instance: in West Africa (Rege, 1999), Madagascar (FAO, 2000) and Brazil (Mariante and Egito, 2002). This results have dire consequences, because the available native gene pool is precious and irreplaceable to generate crossbred cattle that are adapted to local climate conditions, poor quality feed and endemic diseases (Jabbar and Diedhiou, 2003; Mariante and Egito, 2002; Scherf, 2000).

Because it is difficult, if not impossible, to anticipate the death of an animal, having the techniques of recovery and cryopreservation of epididymal spermatozoa well established is an important strategy. In the present study, recovery of spermatozoa from epididymis 7 h after animal's death did not affect any of the sperm characteristics studied. Indeed, sufficient amounts of sperm cells for cryopreservation and formation of a germplasm bank were obtained from all animals.

A significant reduction in motility after cryopreservation was not observed in the present study. However, acrosome integrity, especially from epididymal spermatozoa, was altered by cryopreservation. It has been reported that cooled and re-warmed sperm cells show changes resembling the capacitation process (Watson, 2000), which could explain alterations in acrosome status.

Regardless if the spermatozoa were from ejaculate or from an epididymis, nuclear chromatin was very stable and no changes were detected after cryopreservation. One reason for this finding could be the sperm chromatin structure itself, because in cattle, sperm DNA packaging in very compact due to the greater percentage of DNA-protamines as compared with many other species, which protects chromatin integrity.

All samples obtained from epididymis, had more than 50% of the cells showing morphological abnormalities, mainly distal cytoplasmic droplet and tail abnormalities (data not showed). This result was expected because the distal droplet is mainly lost at ejaculation when spermatozoa come in contact with seminal vesicle fluid, which has a droplet-removing effect (Barth and Oko, 1989). Total abnormalities were drastically reduced after centrifugation in a Percoll gradient, providing a population of cells with enhanced quality for use in IVF.

After passage through a Percoll gradient epididymal sperm from animal A1 and A2 still had a lesser percentage of cells with an intact acrosome and some morphological abnormalities, even though embryo production at day 7 was similar to the ejaculated sperm. For those bulls the embryo production was between 33 and 54%, which is similar to the results obtained in the majority of IVF laboratories. Only epididymal spermatozoa from bull A3 had a lesser blastocyst rate, when compared to the other animals. However, A3 also had the greatest percentage of cells with morphological abnormalities and acrosome damage after passing through the Percoll gradient. This finding could explain the lesser blastocyst production (21.48%) obtained for this animal, however for a sample from an animal that is in risk of extinction, 21% is a reasonable rate for embryo production. If these embryos are transferred to recipients, it would be possible to develop offspring, avoiding the loss of this germplasm.

In conclusion, the present study showed the potential for utilization of spermatozoa from the epididymis of dead animals for preservation of valuable males.

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