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Quality and fertility of frozen ovine spermatozoa from epididymides stored at room temperature (18–25 °C) for up to 48 h post mortem

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ABSTRACT

This study investigates the effect of time of storage of epididymides at room temperature and the addition of 20% of seminal plasma to the cryopreservation extender, on post thaw quality and fertility of ovine spermatozoa collected from the cauda epididymis. Spermatic kinetics, integrity and the stability of plasma membrane, damage to the acrosome and fertility following laparoscopic artificial insemination were evaluated in samples collected in an artificial vagina (AV) and from epididymides stored at room temperature for zero (G0), six (G6), twelve (G12), twenty-four (G24) and forty-eight (G48) hours post mortem. There were no significant differences in spermatic parameters between the methods of sample collection, except for progressive motility and velocity according to the straight path(VSL). G48 samples had significant lower total motility(TM), progressive motility(PM), kinetic parameters, viability and acrosomal integrity. Pregnancy rate after insemination was similar for samples collected using AV, and the G0, G6, G12 and G24 samples. In conclusion, ovine epididymides can be exposed to room temperature, for up to 24 h post mortem, with no effect on viability and fertility of cryopreserved seminal samples. The addition of seminal plasma to the cryopreservation extender had no effect on spermatozoa quality nor fertility.

1. Introduction

In modern livestock breeding the preservation of gametes after the death of a genetically valuable animal can be important. Besides that the preservation of gametes post mortem is an important tool to form a genetic bank for wild animals, especially for endangered species [1]. Authors have report recovery of viable spermatozoa from the cauda epididymis of sheep [1], goats [2], bulls [3], stallions [4], dogs and cats [5] and red deer [6] when the epididymides were kept at 5 °C. However, the maximum period for which epididymis can be kept at room temperature post mortem and viable spermatozoa can still be collected for freezing whilst maintaining their fertilization potential is not well understood. Spermatozoa from cauda epididymides are susceptible to environmental temperature variation [1,7–9]. Some researchers have investigated the maximum sperm viability period when cauda epididymides were kept refrigerated at 5 °C [3,10]; and also it has studied the effect of extender osmolarity and glycerol concentration on post-thawing quality of epididymal spermatozoa [11]. However, in reality, most dead livestock and wild animals are discovered after lying at ambient temperatures for some time.

It has been reported that some components of seminal plasma can prevent or reverse damage caused by temperature variation in ovine spermatozoa [12]. Spermatozoa collected from epididymides lack the protection of seminal plasma. Addition of seminal plasma to spermatozoa collected from epididymides seems to stimulate motility initially [13] and have beneficial effects after thawing [14]. When seminal plasma is added to swine epididymal spermatozoa extender after thawing some authors observed an increase in gestation rate and litter size [15]. To the authors' knowledge there are no reports of the fertility potential of ovine epididymal spermatozoa cryopreserved with the addition of seminal plasma.

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The objectives of this study were: 1. To evaluate the viability and fertility of ovine epididymal spermatozoa after thawing when epididymides were kept at room temperature (18–25 °C) for 48 h after death using as control semen collected with an artificial vagina; 2. To evaluate the addition of 20% seminal plasma to the cryopreservation extender of ovine semen collected with an AV or from the cauda epididymis.

2. Materials and methods

2.1. Experiment design

This project was approved by the Ethics Committee on Animal Use (CEUA) of the agricultural sciences sector of the Federal University of Paraná – Brazil, protocol number 52/2016.

Ten cross breed rams, aged between 24 and 48 months, were used. The animals remained in an intensive system, with access to pasture during the day and were confined overnight. They received food supplementation with corn, soybean meal and a mixture of minerals and water *ad libidum*. Inclusion criteria were the rams that, although semen collection were performed in non-breeding season, presented the following parameters in fresh semen subjective evaluation: TM higher than 80%, PM higher than 70%, morphological defects lower than 15% and membrane functionality (hiposmotic swelling test) higher than 90%.

With the use of a sheep as a dummy, semen collection was performed using the artificial vagina (AV) method. The internal temperature of the artificial vagina was 40 °C–43 °C. AV collections were performed twice a week for two months in summer (January and February), and the rams were subsequently slaughtered. A section was made in the region of the spermatic funiculum for excision of the scrotum, testis and epididymis. Scrotum, testis and epididymis were transported to the laboratory in a styrofoam box at room temperature (18–25 °C). In the laboratory scrotum, testis and epididymis were removed from the styrofoam box and allocated in empty Becker dishes maintained on room temperature and covered with paper towel until the end of the period of exposure to room temperature.

Scrotum, testis and epididymis were randomly divided into five groups, corresponding to the period of exposure to room temperature (18–25 °C): zero hours (G0), 6 h (G6), 12 h (G12), 24 h (G24) and 48 h (G48). Each group consisted of four epididymides from four different rams.

2.2. Post mortem spermatozoa recovery

After the period of exposure to room temperature a scalpel blade was used to incise the scrotal skin and extract testes and epididymides [16]. Testes and epididymides were washed with 0.09% sodium chloride solution warmed to 35 °C. The tail of the epididymis was divided into warmed petri dishes and maintained at 35 °C. Using anatomical tweezers, scissors and a scalpel blade, superficial blood vessels were dissected to minimize blood contamination of semen. The cauda epididymis was sectioned and light pressure was applied to expel semen from the tubules [1]. After the first cut the role tissue of cauda epididymis was sliced to maximize spermatozoa recovery. The cauda epididymis was then lavaged with 2 mL of control media (CM) warmed to 35 °C. After 5 min a pipette was used to collect the diluted semen from the Petri dish and the sample was placed in a conical tube (Falcon BD) [17].

2.3. Cryopreservation

The control medium (CM) used in the cryopreservation process consisted of 75 mL mother solution (200 mL distilled water, 1.4 g glycine, 2.97 g sodium citrate, 3 g fructose, 0.004 g amikacin), 15 mL skim milk, 5 g egg yolk and 4.6 distilled water and 4% glycerol [18]. The SP medium was formed with addition of 20% seminal plasma to the CM. The seminal plasma was from the same rams from which semen had been collected using an artificial vagina in summer (January and February). The seminal plasma was obtained, and extender with seminal plasma was produced following the methodology of López-Pérez and Pérez-Clariget [19].

Dose dilution with CM and SP extenders was performed to obtain a concentration of 400 million spermatozoa per mL. Inseminating doses were packed in 0.25 mL French straws with 100 million spermatozoa per dose. The doses were frozen in automated equipment (TK 3000®, TK Congelações, Brazil) with a standard ovine freezing curve "S3P2" that decreased from ambient temperature to 5 °C in ratio of 0.5 °C per minute, straws were stabilized at 5 °C for 2 h, after that the temperature decreased 15 °C per minute until −80 °C and in ratio of 10 °C per minute until −120 °C then straws were submerged at liquid nitrogen. Samples were thawed in a water bath at 40 °C for 20s. Sperm quality parameters were evaluated immediately after thawing.

2.4. Spermatozoa evaluation

2.4.1. Fresh analysis

Before freezing analysis were performed in a smaller laboratory next to slaughterhouse and far from the main laboratory with CASA system. TM and PM were evaluated by optical microscopy(Coleman, N 107, Brazil). A drop of semen diluted to concentration of 400×10^6 sperm per mL on CM or SP was deposited between slide and cover slip warmed at 37 °C and examined at 400× magnification. All the analysis were performed by the same person. Sperm morphology was assessed by a differential count of 200 cells on slides prepared with swabs of semen diluted and stained according to the method of Cerovsky (Cerovsky, 1976).

2.4.2. Post thaw motility parameters

Motility was assessed using a computer-assisted sperm analysis system (CASA) (Hamilton Thorn Motility Analyser – HTMA –IVOS 12 – Hamilton Research – Beverly, MA, USA). For evaluation of the sperm kinematic, 30 μL thawed sperm was diluted in 300 μL CM and warmed to 37 °C. Then, 6 μL of diluted semen was deposited into the Makler chamber (Makler Counting Chamber – Selfi-Medical, Haifa, Israel). One frame of the chamber was chosen by the examiner and two other frames were randomly chosen by the equipment and analyzed for total motility (TM,%), progressive motility (PM,%), velocity according to the smoothed path (VAP, μm/s), velocity according to the straight path (VSL, m/s), velocity according to the actual path (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), head beat-cross frequency (BCF, Hz), straightness (STR, %) and linearity index (LIN, %). The setup used was based on the manufacturer's recommendations, (HAMILTON THORNE SETUP FOR IVOS-12.3). The cell size was 5 pixels, cell intensity 55, VAP 75 μ/s, STR 80%, VAP cutoff 21.9 μ/s, VSL cutoff 6.0 μ/s, minimum static intensity gates 0.25, maximum static intensity gates 1.5, minimum static size gates 0.6 and maximum static size gates 8.0, minimum elongation gates 0 and maximum elongation gates 95. Magnification calibration was of 1.95, video source frequency of 60 Hz, 2400 light intensity, low photometer 73 and high photometer 125.

2.4.3. Flow cytometry post thaw

Flow cytometry sperm evaluation was performed in a BD LSR Fortessa (Becton Dickinson, Mountain View, CA, USA) equipped with lasers: blue 488-nm, 100 mW, red 640-nm, 40 mW and violet 405-nm, 100 mW. After analysis, the data were evaluated by