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In vitro and *in vivo* fertilization potential of cryopreserved spermatozoa from bull epididymides stored for up to 30 hours at ambient temperature (18 °C–20 °C)

Melina Andrea Formighieri Bertol [a,b,*], Romildo Romualdo Weiss [a,b], Luiz Ernandes Kozicki [c], Ana Claudia Machinski Rangel de Abreu [b], João Filipi Scheffer Pereira [c], Jonathan Jesus da Silva [c]

[a] *Department of Veterinary Medicine, Federal University of Paraná, Funcionários Street, 1540, 80035-050 Curitiba, Paraná, Brazil*
[b] *Department of Technology, Postgraduate studies in Bioprocess Engineering and Biotechnology, Human and Animal Health, Federal University of Paraná, Curitiba, Parana, Brazil*
[c] *Department of Veterinary Medicine, Postgraduate studies in Animal Science, Pontifical Catholic University of Paraná, Curitiba, Paraná, Brazil*

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ABSTRACT

The aims of this study were to compare the viability and *in vivo* and *in vitro* fertilization potential post-thaw sperm collected at different times postorchiectomy from bull epididymides (EP) at 18 °C to 20 °C, with those of semen collected by electroejaculation (EJ) from the same bulls. Semen samples were collected by EJ from 10 Zebu bulls and cryopreserved. A week later 20 epididymides from these bulls were obtained by orchiectomy and randomly divided into five groups (G) to be maintained at ambient temperature for 6, 12, 18, 24, and 30 hours before sperm recovery by retrograde flow. The sperm were cryopreserved, and post-thaw parameters were determined by both computer-assisted sperm analysis and morphologic analysis. *In vitro* fertilization of oocytes was performed to assess the cleavage rate, blastocyst rate, total number of cells, and hatching rate of embryos. The G30 sperm samples were also used for fixed time artificial insemination (FTAI) of Zebu heifers (n = 10). The results of post-thaw sperm viability showed that total and progressive motility and plasma membrane integrity were lower in sperm in which cryopreservation was delayed for 30 hours, showing a negative correlation of these parameters with delay before cryopreservation. In all groups, it was possible to obtain viable embryos, and embryos from G6 samples had more cells than the other groups. The greatest embryo production rates were observed in G6, G12 and G18 (27.2 to 32.2%) and it was significantly lower in G24 and G30 samples. For EJ, many individual variations were observed in embryo production potential between bulls. G30 samples, with only 5.2% of post-thaw progressive motility, were able to fertilize and produced a pregnancy. To the authors' knowledge, this is the first time *in vitro* embryos up to 8 days of development and a pregnancy after FTAI have been produced with sperm from bull epididymides that had been stored at 18 °C to 20 °C for up to 30 hours.

1. Introduction

The recovery, preservation, and use of epididymal sperm are essential tools to preserve genetic stocks of valuable domestic or wild animals [1–3] under adverse conditions [4] and also as an alternative source of gametes in cases of human infertility [5,6]. Previous studies have already demonstrated the viability of bovine spermatozoa collected directly from the tail of epididymis [7,8], but in most cases, the gametes were obtained immediately after slaughter or

* Corresponding author. Tel.: 55 41 3350-5623; fax: 55 41 3361-3695.
E-mail address: melbertol@hotmail.com (M.A.F. Bertol).

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castration, or from epididymides that had been refrigerated at 5 °C for long periods [9,10]. Few studies [11,12] have reproduced the real and more frequent situation, of the need for gamete utilization, i.e., accident, death, or inability to obtain spermatozoa in the conventional way, when structures are exposed to ambient temperature before preservation.

Cryopreservation is the most effective method for long-term preservation of genetic material from valuable breeding individuals. The protocols and diluents used for cryopreservation of conventional bovine semen are well established, but when working with sperm extracted directly from the epididymis many challenges remain. The spermatozoa retrieved from the tail of epididymis have special features, such as the absence of seminal plasma and large numbers of distal cytoplasmic droplets, which necessitate special handling, both for cryopreservation and IVF [9,13]. Although it is a relatively new practice, good results have been achieved in cryopreservation of bovine epididymal spermatozoa using TRIS-based diluents containing egg yolk, glycerol, and citric acid [1,8,10,14].

After cryopreservation, the gametes can be used in biotechnologies such as artificial insemination and IVF. The *in vitro* production of embryos is an indispensable biotechnology in mass propagation of genetic material because the number of embryos produced is far greater than those produced *in vivo*, and it allows genetic material from subfertile females, of high genetic value, high livestock production, and at different ages or reproductive status or even after death to be used [15]. Although production of *in vitro* embryos using cryopreserved spermatozoa obtained from bovine EP and stored at 5 °C for long period of time [9] has previously been demonstrated, there are no reports of the fertilization potential of gametes retrieved from EP kept at average ambient field temperatures. In this context, the aims of this study were: to cryopreserve and assess the post-thaw viability of recovered sperm from the EP of zebu bulls that had been kept at 18 °C to 20 °C, and to assess potential for IVF *in vitro* embryo production by evaluation of the cleavage rate, number of blastocysts, hatching rate, and the number of embryo cells after fertilization with sperm from the EP and by electroejaculation (EJ) of the same bulls and also to determine the *in vivo* potential of fertilization in fixed time artificial insemination (FTAI) of heifers.

2. Material and methods

Animals in this study were used in accordance with all necessary recommendations and guidelines and approval of the Ethics Committee on Animal Use (CEUA-SCA/UFPR, number 017/2013) was obtained. All *in vitro* procedures were approved by the Ethics Committee on Animal Use (CEUA/PUCPR, number 894/2014).

2.1. Animal selection and ejaculate samples

Pure Zebu bulls (*Bos taurus indicus*) of the Tabapuã breed (n = 10) with an average age of 63,1 ± 23 months and weight 560 ± 100,3 kg, from a beef cattle farm (25°37′0.4.4″south, 52°48′58.9″west, and at 505 m above the sea level) were selected on the basis of a general clinical examination and a breeding soundness examination. All animals were healthy and free of any signs of disease. The bulls were kept in an extensive grazing system, with grass (*Cynodon spp*) divided into paddocks and access to shelter, water, and mineral salt *ad libitum*. Ejaculate samples were collected in the spring by EJ, as this was the usual technique for semen collection for bulls. The electroejaculation device (TK 800) was introduced into the rectum, and repeated electrical stimulation with direct current from zero to 780 mA for 3 seconds with 3-second interval was applied until semen emission occurred into a sterile graduated tube. Prioritization was given to minimizing discomfort. Two ejaculations were performed on each bull at 3-day intervals to remove sperm stored in the epididymis, and a third ejaculate was collected a further 3 days later for inclusion in the study. The semen samples were evaluated for: subjective analysis of motility (0%–100%)–the average score during microscopic examination by two different evaluators, and sperm concentration, morphology, and acrosomal defects. The sperm concentration was determined using a hemocytometer 1:100 dilution (semen:-buffered saline-formalin solution) and morphology assessed on smears stained with Congo red.

2.2. Obtaining testis and epididymis samples

A week after obtaining the ejaculate, bilateral orchiectomy was performed under local anesthesia with 2% lidocaine without epinephrine. Skin and deeper tissue layers were incised, and blood vessels and spermatic cord were clamped. The testes and epididymis were removed and immediately taken to the laboratory at the farm and randomly divided into five groups (n = 4). Each group was maintained at 18 °C to 20 °C for a variable period of time 6, 12, 18, 24, and 30 hours (G6, G12, G18, G24, and G30). The temperature of 18 °C to 20 °C was chosen, as it is close to the average annual temperature in region where the study was conducted. Sperm was recovered from the epidymides by retrograde flow [16]. Each cauda epididymis was washed with 20 mL of a Botu-Turbo-skimmed milk diluent (Botupharma) warmed to 37 °C with its osmolarity specific for bovids (20 mL of distilled water to 100 mL of medium). After sperm harvest, samples were subjectively evaluated as described for ejaculate samples in Section 2.1. The total motility ranged from 67.5% to 41.25% for G6 and G30, respectively, and the concentration of sperm per mL ranged from 426.8×10^6 (G12) to 101.8×10^6 (G30). The percentage of morphologic defects and acrosome integrity was considered normal for epididymal sperm. A final volume of 20 mL (recovery medium and gametes) from each sample was centrifuged at 600 × *g* for 10 minutes to separate washing diluent and other contaminants such as blood and dirt.

2.3. Cryopreservation

After centrifugation, the supernatant was discarded, and the pellet resuspended with the extender Botu-bov (Botupharma) consisting of Tris-egg yolk, and 7% glycerol as cryoprotectant. The pre-freezing parameters of motility and the total cell number were assessed to verify the effects