Cryobiology 73 (2016) 348–355

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Bovine epididymal spermatozoa: Resistance to cryopreservation and binding ability to oviductal cells

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ARTICLE INFO

Article history:
Received 17 April 2016
Received in revised form
8 September 2016
Accepted 28 September 2016
Available online 28 September 2016

Keywords:
Isthmus
Spermatozoa
Kinetics
Proteins

ABSTRACT

In this study we examined quality, longevity and ability of epididymal sperm (EP) to bind to oviduct explants (OE) after cooling and cryopreservation. Ejaculated (EJ) and EP sperm from seven bulls were evaluated before, during and after cryopreservation for total (TM), progressive motility (PM), sperm morphology, plasma membrane integrity (PMI) and acrosome integrity (ACI). For longevity, cryopreserved EP, EJ and a third group of cells in which EP spermatozoa were incubated with seminal plasm (SP) for 10 min after thawing (EPP group), were compared, and the groups were analyzed at 0, 3, 6, and 24 h for all parameters. Sperm from each group were co-incubated with OE for 30 min, 6 h, and 24 h for binding evaluation. Data were analyzed by the generalized linear models SAS 9.1 ($P < 0.05$). After cooling, EP displayed higher TM, higher PMI, and higher ACI ($P < 0.05$) than EJ. No differences were noted in the percentage of spermatozoa with PMI and AI between EJ and EP for fresh, cooled or cryopreserved sperm. However, a reduction in motility occurred in the EJ sperm after cooling, while in EP group such reduction occurred only after cryopreservation. At 6 h of incubation EP and EPP had higher PMI and ACI than EJ ($P < 0.05$). The number of spermatozoa bound to OE was similar $P > 0.05$) for all groups either at 30 min or 24 h. We conclude that EP are more resistant to cooling than EJ, and can bind to OE similarly to EJ.

1. Introduction

Genetic material from animals of economic interest, wild animals or endangered species may be lost at any time by unexpected death or by acquired reproductive failure. In these cases, most of the time, there is a loss of genetic material as well as an economic loss [51]. Therefore, efforts should be made to avoid the waste of that material, which can be achieved through the use of different assisted reproduction biotechnologies.

The recovery of spermatozoa from epididymides of dead animals is a feasible alternative to the preservation of male gametes and also to the maintenance of germplasm banks [36,26]. Those spermatozoa may be used for artificial insemination, *in vitro* embryo production, or for intracytoplasmic injection allowing the production of offspring from genetic material that otherwise would have been lost [38,39]. In fact the birth of offspring from Nellore (*Bos taurus indicus)* bulls using cryopreserved spermatozoa from the epididymis has already been reported [10].

Epididymal spermatozoa are stored in the cauda epididymis, and, at the time of ejaculation, they come in contact with fluids secreted by the accessory glands to form semen. The secretions of the accessory glands contain several factors, including ions, lipids, energy substrates, organic compounds and proteins [43,25], which are important for the survival and transport of spermatozoa in the female reproductive tract [4,25]. Furthermore, proteins present in seminal plasma (SP) are essential for cell membrane stability, formation of the isthmus reservoir, sperm capacitation and the

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http://dx.doi.org/10.1016/j.cryobiol.2016.09.170
0011-2240/

spermatozoa-oocyte interaction [19,40].

The capacitation process is defined by physiological changes that the sperm must undergo in the reproductive tract of the female, to become able to fertilize an oocyte [3]. This process involves the removal or change of substances that stabilize the sperm of the MP. Among the components related to the training process are albumin, sodium bicarbonate, and calcium (Ca2 +) [9,14,20]. Similar to BSP proteins, some of these molecules are already present in the SP and/or oviduct fluid, such as BSA, and may act as cholesterol acceptor from the sperm plasma membrane, promoting capacitation [52,54]. Heparin is also used for a similar purpose, and it appears to induce membrane cholesterol loss by interacting with SP BSP proteins [16,40,54], although through pathways that are still partially known, is know that SP proteins are fundamental for capacitation process.

Therefore, spermatozoa from the epididymis differ from those of the ejaculate, mainly because they have not come into contact with fluids from the accessory glands, which can affect their physiology, longevity and capacitation.

Spermatozoa from the epididymis can be used fresh, immediately after collection, or cooled at 5 °C, which ensures a temporary preservation by keeping them viable for a longer period of time [38,5]. A preferable alternative would be to cryopreserve those spermatozoa, making their use more applicable because they can be utilized at any time after the animal's death [38]. However, the success of cryopreservation depends on various factors such as method of collection, type of extender, source and quality of the sperm [1,35,53]. Regardless of the mentioned factors the process itself results in injury to the sperm cell, decreasing its fertility [8,9,58]. Although Stout [51] have reported that epididymal spermatozoa exhibit greater resistance to cryopreservation compared to ejaculated sperm, little is known about the behavior of these spermatozoa when exposed to a stress such as cooling or cryopreservation. Therefore, information on physiological differences between ejaculated and epididymal spermatozoa are very important for the efficient use of those gametes in assisted reproduction techniques. Therefore, aiming to have a better knowledge of their physiological behavior we compared epididymal and ejaculated sperm in regard to their quality, longevity and ability to bind to oviduct cells following cooling and cryopreservation.

2. Materials and methods

Unless otherwise indicated, the reagents used for this study were purchased from Sigma-Aldrich (St. Louis, MO, USA). All procedures with animals were approved by the Ethics Committee of Embrapa's Animal Genetic Resources and Biotechnology (Protocol CEUA – Cenargen 004/2013).

2.1. Animals

Ejaculated and epididymal spermatozoa from seven Gir bulls (*Bos taurus indicus*) aged between 36 and 40 months were used. Animals were raised in an extensive system, fed pasture (*Brachiara brizantha*), mineral salt and water *ad libitum*. Prior to the experiment, bulls were submitted to three andrological evaluations, and those males that showed a subjective total sperm motility ≥70% and a minimum of 70% of morphologically normal cells were selected.

2.2. Experimental design

2.2.1. Comparison between epididymal and ejaculated sperm regarding cooling and cryopreservation resistance

To determine changes in sperm function during cooling and cryopreservation, epididymal (EP) and ejaculated (EJ) sperm from the same bulls were evaluated. Fresh, (immediately after recovery), cooled (after being kept for 4 h at 4 °C) and frozen-thawed (after cryopreservation process). Spermatozoa were evaluated for total motility (TM), progressive motility (PM), sperm morphology, plasma membrane integrity (PMI) and acrosome integrity (ACI).

2.2.2. Longevity and viability of cryopreserved epididymal sperm

Then, longevity of epididymal spermatozoa after cryopreservation was characterized. For this evaluation, three groups were used: cryopreserved EP spermatozoa, cryopreserved EJ spermatozoa, and cryopreserved EP spermatozoa incubated with SP for 10 min at 39 °C after thawing (EPP group). Spermatozoa of all groups were subjected to a Percoll gradient [33] for selection. After Percoll, the pellet was resuspended and centrifuged for 5 min in SP-TALP medium, consisted of 99 mM NaCl, 3.1 mM KCl, 25 mM NaHCO3, 0.35 mM NaH2PO4, 10 mM HEPES, 2 mM CaCl2, 1.1 mM MgCl2, 21.6 mM sodium lactate, 1.1 mg/mL sodium pyruvate, 6 mg/mL BSA, and 1 mg/mL gentamycin (pH 7.4). The resultant pellet was resuspended in SP-TALP to produce a final concentration of 2×10^6 spermatozoa/mL. The spermatozoa were then incubated at 39 °C under a 5% CO_2 atmosphere for 0, 3, 6 and 24 h. At each time-point spermatozoa were evaluated for TM and PM, sperm morphology, PMI and ACI. The assessment at 24 h was performed to evaluate EP sperm viability after a long period of incubation when the EJ sperm was expected to be practically unfeasible. In addition, that evaluation was important to asses sperm pattern when the oviduct cells binding assay was carried out.

2.2.3. Binding of cryopreserved epididymal sperm to oviduct cell explants

Finally, the capacity of sperm to bind to oviduct cells was tested. Sperm from EJ, EP and EPP groups were incubated with oviduct cell explants (OE) for 30 min, 6 h or 24 h at 39 °C under 5% CO_2 in air. After incubation, the number of sperm bound per mm of each explant perimeter was assessed. For each bull, treatment, and incubation time, 30 OE were equally divided into three drops of SP-TALP, totaling 90 explants per drop.

2.2.4. Fresh and cryopreserved sperm binding to OE

To exclude that the results obtained were not influenced by the cryopreservation process, we repeated the binding assay using fresh and cryopreserved EP spermatozoa. To do this, testes were collected at a slaughter house, and the epididymal spermatozoa recovered were equally divided into two groups, fresh and cryopreserved. Using the same methodology described above, the binding capacity of fresh and cryopreserved EP spermatozoa were evaluated by the number of cells bound to a mm of OE.

2.3. Collection and cryopreservation of epididymal and ejaculated spermatozoa

One ejaculate from each Gir bull was collected by electroejaculation. Seven to fifteen days after semen collection, all sires were orchiectomized. Testes were kept for 2 h at 5 °C before being processed. Testes were cleaned with saline solution (NaCl 0.9%) and 70% alcohol, and sperm collection from the cauda epididymis was performed. The epididymis was thoroughly cleaned, and the superficial blood vessels of the cauda were punctured, so that most of the blood could be wiped off. Then, we extracted the sperm from the cauda by means of cuts performed with a scalpel, removing the white fluid coming out of the cut tubules with the aid of a blade [37]. After recovery, sperm from both groups were diluted in Tris-citrate-yolk-glycerol Dilutris® extender (SEMENCON - Agricultural Products Ltd., Porto Alegre, RS, Brazil), packaged at a concentration