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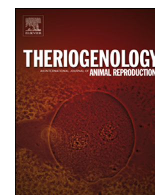

Effect of seminal plasma removal before cryopreservation of bovine semen obtained by electroejaculation on semen quality and *in vitro* fertility

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A B S T R A C T

Cryopreservation of bull semen is a common biotechnology procedure in cattle breeding. However, when the ejaculate is obtained by electroejaculation, wide variation is observed in the sperm/seminal plasma (SP) ratio that can affect the freezability of semen in this species. The removal of SP may improve the quality of frozen bull semen. The objective of this study was to evaluate the effect of SP removal from the ejaculate on the cryopreservation of semen from 38 Nellore bulls collected by electroejaculation. After collection, the ejaculate was divided into three aliquots: (1) control (N) diluted to a concentration of 60×10^6 spermatozoa/mL and frozen with SP; (2) centrifugation (C) at $\times 600g$ for 10 minutes and the pellet resuspended and frozen at the same concentration as N; and (3) filtration (F) through SpermFilter and sperm recovered and frozen at the same concentration as N. After thawing, sperm kinetics, plasma and acrosome membrane integrity, mitochondrial membrane potential, oxidative stress, and *in vitro* fertility were evaluated. Statistical analysis was performed using the SAS 9.2 package, and differences were considered significant when $P < 0.05$. Higher average path velocity and straight-line velocity were observed in the groups submitted to SP removal compared to the control group ($P < 0.01$). In contrast, filtered samples exhibited higher beat cross frequency, straightness, and linearity compared to the other groups. Plasma membrane integrity was reduced when SP was removed, but lower oxidative stress was observed in groups C and F ($34.91 \pm 2.95\%$ and $31.63 \pm 2.95\%$, respectively) compared to group N ($57.39 \pm 2.95\%$). However, the percentage of hatched blastocysts was similar in the N and F groups ($21.22 \pm 1.05\%$ and $24.00 \pm 1.05\%$, respectively) and higher compared to group C ($18.83 \pm 1.05\%$). In conclusion, removal of SP by centrifugation for bull semen

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freezing reduced the rate of *in vitro*–produced embryos, whereas filtration of prefrozen semen was found to be an efficient alternative in terms of semen freezability and *in vitro* production of bovine embryos.

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1. Introduction

Artificial insemination (AI) is the first generation of animal reproduction biotechnology that had a major impact on animal breeding. By means of AI, a single ejaculate of a bull is used for the insemination of various females, which could not have been possible without sperm cryopreservation [1]. Although bull semen has been cryopreserved for AI for over half a century [2], many of the protocols used are still empirical, with the number of spermatozoa that do not survive the freezing process being considerable and those that do survive being affected structurally or functionally after thawing [3]. In general, many of the strategies tested to achieve successful cryopreservation do not involve the spermatozoon itself but the medium in which it is stored [4].

The fluid that is added to spermatozoa during epididymal transport and ejaculation is called seminal plasma (SP) and is produced by the epididymis and accessory sex glands [5]. Despite several reports of its beneficial effect on spermatozoa, SP has also been described to cause sperm damage [6], an effect that is intimately related to the duration of contact between sperm and SP [7]. During natural mating, spermatozoa remain in contact with SP for a relatively short time, whereas they can stay in contact for hours, or even a day, with diluted SP during semen processing for cryopreservation [5]. Furthermore, in bulls reared in the field, semen is collected by electroejaculation. The ejaculates obtained by this method differ in terms of the quantity and quality of SP from those obtained using artificial vagina, a fact that considerably alters the SP/sperm ratio in semen [8,9].

The composition of SP shows high individual variability in Nellore bulls [10], a fact that results in different responses of the animals to sperm cryopreservation [11,12]. However, the main components of SP (on a weight basis) are proteins, which are considered the predominant modulator of sperm function [13]. Some proteins participate in the regulation of cholesterol and phospholipid efflux from the sperm membrane. This process is concentration and time dependent, and continuous exposure of spermatozoa to SP may therefore damage their membrane [6,14]. This fact can render the spermatozoon more vulnerable to cryopreservation [7] or cause premature post-thaw capacitation, affecting the process of fertilization [15]. In bulls, relative abundance of SP proteins that are positively associated with low fertility has been reported [16–18].

The technique generally used for the removal of SP is centrifugation; however, its intensity and duration can negatively interfere with sperm motility and plasma membrane integrity [19]. Another easily applied separation method is the SpermFilter (Ceafepe Tecnologia Veterinária Ind., Sorocaba, São Paulo, Brazil) proposed by Alvarenga et al. [20]. This method consists of a synthetic hydrophilic membrane filter with a pore size that permits the passage of SP, while retaining only spermatozoa. In stallions, this filter has been shown to be as efficient as centrifugation in the removal of SP and has the advantage of greater practicality and a lower rate of sperm loss [19].

In view of the aforementioned considerations, it is believed that the cryopreservation of bull ejaculates obtained by electroejaculation may be compromised by the deleterious action of SP and that techniques of SP removal improve the quality of cryopreserved sperm in cattle. Centrifugation is commonly used for this purpose but can cause substantial cell injury, and filtration emerges as an alternative to obtain better results. Because no study has so far applied filtration to SP removal in bulls, the objective of the present study was to evaluate the effect of the removal of SP from the ejaculate by centrifugation and filtration on the cryopreservation of bull semen.

2. Material and methods

2.1. Ethics committee

The study was approved by the Ethics Committee on Animal Use of FCAV/UNESP, Jaboticabal, on June 6, 2014 (Protocol No. 009713/14).

2.2. Semen collection

Thirty-eight Nellore (*Bos taurus indicus*) bulls aged 2 to 6 years belonging to Centro APTA Bovinos de Corte, a research unit of the Instituto de Zootecnia, Sertãozinho, São Paulo, Brazil, were used. The bulls were maintained on pasture, with water and mineral salt available *ad libitum*, and evaluated regarding the normality of andrologic parameters before the study according to the recommendations of the Brazilian College of Animal Reproduction (mass motility: $\geq$3; sperm motility: $\geq$60%; concentration: 350 million spermatozoa/mL; total number of spermatozoa in the ejaculate: 3 to 5 billion; morphologically normal spermatozoa: $\geq$70%; major sperm defects: $\leq$10%; minor sperm defects: $\leq$20%; major individual defects: $\leq$5%; minor individual defects: $\leq$10%) [21]. Only one ejaculate was collected per bull with an Autojac electroejaculator (Neovet, Uberaba, Brazil) in the manual mode, totaling 38 ejaculates.

2.3. Experimental groups

After collection, each ejaculate was divided equally into three aliquots to compare three techniques performed before sperm cryopreservation. The control group (group N) was submitted to conventional cryopreservation without the separation of SP. The semen sample was diluted conventionally to a final concentration of 60×10^6 spermatozoa/mL in BotuBov diluent (BotuPharma, Botucatu, Brazil) containing 7% glycerol as cryoprotectant. In the centrifugation group (group C), SP was separated by

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