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Effect of glycerol on the viability and fertility of cooled bovine semen

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ABSTRACT

The aim of the present study was to compare the viability and fertility of bovine semen diluted in Botu-Bov (BB) commercial extender with and without the cryoprotectant glycerol then cooled at 5 °C for 24 hours in the Botu-Flex passive cooling system and of semen diluted in BB with glycerol then frozen. One ejaculate of 30 Nelore *Bos taurus indicus* bulls between 24 and 30 months of age was used for *in vitro* analysis. Sperm kinetics and cell viability were analyzed using computer-assisted sperm analysis and flow cytometry, respectively. Three Nelore bulls approximately 30 month old were used for *in vivo* test using fixed-time artificial insemination for the fertility analysis. The ejaculates were divided into three experimental groups: semen in BB extender with 7% glycerol cooled at 5 °C for 24 hours (cooled semen with cryoprotectant), semen in BB without glycerol cooled at 5 °C for 24 hours (cooled semen without cryoprotectant), and semen diluted in BB with 7% glycerol then subsequently frozen rather than cooled (frozen semen). For the fertility analysis, 762 Nelore cows (*B taurus indicus*) were randomly inseminated using fixed-time artificial insemination. For the groups corresponding to cooled semen with cryoprotectant, cooled semen without cryoprotectant, and frozen semen, 278, 268, and 216 cows were inseminated, respectively, and the resulting conception rates were 51% a, 44%ab and 41%b ($P < 0.05$), respectively. In conclusion, the fertility rates improved, when samples were cooled with glycerol at 5 °C for 24 hours compared with the frozen samples.

1. Introduction

Among the available reproductive biotechnologies, artificial insemination (AI) is an efficient and important technique for the genetic improvement of herds. Before the development of cryopreservation and thawing processes for semen storage, AI was only performed with fresh and cooled semen samples. With the discovery of cryoprotectant agents and advances in the production of extenders, the use of fresh or cooled bovine semen was gradually abandoned, given the many advantages of cryopreservation [1].

Compared with fresh semen with similar numbers of motile spermatozoa, cryopreserved semen generally yields lower fertility rates. This reduction in fertility can be attributed to changes in temperature during the freezing process as well as osmotic and toxic stress owing to exposure to the cryoprotectant and the formation of ice crystals [2]. The main advantage of the

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process of cooling semen is that it prevents damage associated with freezing, thereby ensuring greater sperm viability and allowing for reduced sperm concentrations per insemination dose and consistent use of genetically superior breeding animals [3,4].

According to Crespilho et al. [3], cooled bovine semen is assumed to ensure a greater number of intact, accessory spermatozoa capable of fertilization compared with frozen semen, which justifies the use of cooled semen. The successful preservation of semen in liquid form under refrigerated conditions depends on the handling procedures and the extenders used for sperm preservation [5].

Although the cryoprotectant properties of glycerol were first described 70 years ago, their effects on spermatozoa remain unclear [6]. Some authors have proposed that glycerol interacts with bovine spermatozoa and is metabolized and converted into an extra source of energy that is subsequently used during the fertilization process [7,8]. Alternatively, the protective mechanism of the glycerol molecule may reflect the binding of the hydrogen atoms of its hydroxyl groups to oxygen atoms of the sperm membrane phosphate groups, thus promoting membrane stabilization during the cryopreservation process [9]. In a previous study, the use of glycerol significantly decreased the damage to ram semen caused by 120 hours of refrigeration in tris-based egg yolk media, thus preserving total motility and plasma and acrosomal integrity. The addition of glycerol has proven to be a good alternative for preserving the viability of refrigerated semen [10].

Thus, the objectives of the present study were to evaluate the use of cryoprotectant glycerol for cooled bovine sperm in terms of sperm parameters assessed *in vitro* and fertility rates in fixed-time AI (FTAI) programs. This study also tested the hypothesis that the use of semen subjected to refrigeration for 24 hours in the presence of glycerol may increase the pregnancy ratios for beef cows in FTAI programs synchronized with a progesterone and estrogen-based protocol.

2. Materials and Methods

2.1. Experiment 1: In vitro analysis of sperm characteristics of fresh, cooled, and frozen–thawed bovine semen

2.1.1. Animals

Experiment 1 was performed at the College of Veterinary Medicine and Animal Science of São Paulo State University in Botucatu, São Paulo, Brazil from October 2011 to April 2012. We used 30 Nellore *Bos taurus indicus* bulls between 24 and 30 months of age belonging to Braido Farm in São Paulo for the *in vitro* analysis.

2.1.2. Semen processing

Semen samples were obtained using electroejaculation and subjectively evaluated at the time of collection for total motility, vigor, and sperm concentration. Cell morphology was subsequently analyzed using differential interference contrast microscopy. The bulls were only considered suitable for the experiment if their ejaculates had at least 80% motile cells, a vigor of 3 (on a scale of 1–5), a sperm concentration exceeding 2 billion per ejaculate, and major and minor defect percentages of less than 20% and 10%, respectively. After the initial screening, each ejaculate was split into four aliquots corresponding with 1) fresh (no extender), 2) Botu-Bov extender (BB; Botupharma Ltda., Botucatu, São Paulo, Brazil) with 7% glycerol cryoprotectant under cooling conditions, 3) BB without cryoprotectant under cooling conditions, and 4) BB with 7% cryoprotectant under freezing conditions. For all the groups, the final sperm concentration was fixed at 40×10^6 sperm/mL, and the samples were packed into 0.5-mL straws (IMV Technologies, L'Aigle Cedex, France). Thereafter, samples from groups 2 and 3 were placed in a Botu-Flex (Botupharma Ltda., Botucatu, São Paulo, Brazil) passive cooling container for 24 hours. The samples were cooled to 5 °C at a rate of −0.05 °C/min. Sample 4 was frozen using a previously described and validated methodology with the same extender [12].

2.1.3. Sperm analysis

The fresh and cooled semen samples were evaluated upon arrival at the laboratory and 24 hours after the cooling process. For the frozen semen, the samples were evaluated after thawing at 37 °C for 30 seconds. Each sample was deposited in a 1.5-mL plastic microcentrifuge tube and prewarmed in a dry bath at 37 °C for 10 minutes before the analysis as described herein.

2.1.3.1. Sperm kinetics. Five fields for each sample were selected for computer-assisted sperm analysis (HTM-IVOS 12, Hamilton Thorne Research, Beverly, MA, USA). The following parameters were analyzed: Total motility (%), progressive motility (PM [%]), average path velocity (μm/s), straight-line velocity (μm/s), curvilinear velocity (VCL [μm/s]), straightness (STR [%]), linearity (LIN; %), amplitude of lateral head displacement (ALH [μm]), tail beat frequency (TBF [Hz]), and percentage of sperm with rapid movement (%). The computer-assisted sperm analysis setup used in this experiment is summarized in Table 1.

2.1.3.2. Flow cytometry. A Becton Dickinson LSRFortessa cell analyzer (Becton Dickinson, Mountain View, CA, USA) equipped with a 488-nm blue (100 mW) laser, a 640-nm red (40 mW) laser, and a 405-nm violet (100 mW) laser was used for the flow cytometry analysis. The data were

Table 1
Setup for computer-assisted sperm analysis of bovine sperm kinetics.

Parameters	Set value
No. of frames	30
Minimum contrast	60 pixels
Minimum cell size	6 pixels
Contrast for the cell	60 pixels
Linearity	70%
Minimum VAP mean	<40 μm/s
Minimum VAP for progressive cells	<30 μm/s
Minimum VSL for slow cells	<20 μm/s
Size of static heads	0.30–7.89
Intensity of static heads	0.41–1.19
Elongation of static heads	96–0
Magnification	1.95
Temperature	37 °C

Abbreviations: VAP, average path velocity; VSL, straight-line velocity.