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Use of Cooled Bull Semen as a Strategy to Increase the Prenancy Rate in Fixed-Time Artificial Insemination Prgrams-Case Report

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ABSTRACT

Semen cryopreservation is still considered suboptimal due to lower fertility when compared to fresh semen. The reasons for the loss of fertility are various and related to irreversible damage caused to the cells during the freeze-thaw process. An alternative to conventional cryopreservation represents the use of chilled bull semen, preventing the damage associated with freezing, thereby guaranteeing greater sperm viability. The aim of this study was to describe the use of cooled bull semen as a strategy to increase the pregnancy for Fixed-Time Artificial Insemination (FTAI) of Nellore (*Bos indicus*) cows. One ejaculate of a select Nellore bull obtained by electroejaculation was used; the semen sample was fractioned into two aliquots: one diluted in Botu-Bov® extender containing 6.4% glycerol for cryopreservation (BB-F, frozen group) and one diluted in the same extender, free from cryoprotectants and used for cooling (BB-C, cooled semen group). The samples in the BB-C group were chilled to 5°C using an isothermic box and maintained for 24 h prior to use. A total of 349 lactating Nellore cows (70-90 days after birth) were synchronized by the insertion of a progesterone releasing device (1.0 g) and estradiol benzoate (2.0 mg i.m.) on a random day of the estrous cycle (Day 0); FTAI was performed 44-48 h after the removal of the device. The pregnancy rates were 45.71 and 61.49% ($P<0.05$), respectively, for the cryopreserved or chilled bovine semen groups. In conclusion, the use of bull semen cooled for 24 h represents an alternative to conventionally cryopreserved semen, as determined by the increase the pregnancy per artificial insemination in bovine herds.

Keywords: Bull, Cooled Semen, Artificial Insemination, Pregnancy, Reproduction

1. INTRODUCTION

Several studies involving various animal species indicated that cryopreserved sperm have a shorter half-life in the female reproductive tract in comparison to fresh semen (Curry, 2000) and that the reduction in the number or quality of the sperm used for insemination results in an exponential decrease in animal fertility (Watson, 2000).

Studies conducted in Australia and New Zealand have indicated the possibility of the use and commercialization of bovine semen in liquid form under refrigeration (Verberckmoes *et al*., 2005). The main advantages related to the use of liquid-stored semen are a higher viability than cryopreserved semen, the possibility of using reduced insemination doses, the optimization of high-merit bulls, the lower cost of storage and the practicality for use in artificial insemination (Vishwanath

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and Shannon, 2000) thus eliminating the use of cryobiology containers, the need for electricity in cattle sheds and semen-thawing devices.

Fresh extended semen is advantageous over conventional frozen semen because of its greater longevity in the female reproductive tract, ensuring higher rates of fertilization, even in the case of asynchronous ovulation at the time of insemination (Bucher *et al*., 2009). However, even for cooled semen, there is a significant decrease in sperm motility (Verberckmoes *et al*., 2005) and the fertility of bovine spermatozoa during storage (Vishwanath and Shannon, 2000). For ovine semen, there is an inverse association between fertility and the duration of cooling, with a significant decrease in conception rates associated with the use of semen samples refrigerated for more than 24 h (O'Hara *et al*., 2010).

As a significant decrease in the conception rates of cows inseminated with bovine semen cooled for 48 h compared with conventionally cryopreserved semen was observed in previous studies (Crespilho *et al*., 2009), the aim of the present case report was to test whether bull semen cooled for only 24 h enhances the pregnancy Per Artificial Insemination (P/AI) in cows synchronized for FTAI.

1. MATERIALS AND METHODS

2.1. Semen Collection and Procedures

For this study one Nellore bull (*Bos taurus indicus*), aged 8 years, with known fertility history was selected from a Semen Collection and Processing Center (SCPC) in Brazil; two semen samples were obtained by electroejaculation. The ejaculates were pooled and were immediately subjectively evaluated for sperm motility and vigor using light microscopy. The sperm concentration was estimated using a Neubauer counting chamber.

The semen sample was divided into two equal aliquots: one diluted in cryopreservation extender Bov-Botu® (Botupharma, Botucatu, Brazil) containing 6.4% of the cryoprotectant glycerol (BB-F group); and one diluted in the same extender, free of cryoprotectants and cooled (BB-R group).

After filling 0.5 mL French straws (IMV® Technologies, L'Aigle Cedex, France) using a final concentration of 30×10^6 total sperm/straw, the samples were subjected to two types of processing. In the control group (BB-F), the samples were cooled in digital refrigerator (Mini-Tube® Tiefenbach, Germany) at a constant temperature of 5°C for 4 h, placed 5 cm from the surface of liquid Nitrogen (N_2) in a 40 L polystyrene box for 20 minutes and cryopreserved by direct immersion in the N_2. The samples in the BB-R group were subjected to passive cooling to a temperature of 5°C in an isothermal Botutainer® box (Botupharma, Botucatu, Brazil) and were maintained under this condition until use after 24 h of storage.

All of the semen samples were evaluated prior to use for artificial insemination; the minimum standards of quality for inclusion in the experiment were total motility >60% and total sperm defects ≤20%.

2.2. Synchronization Protocol for FTAI

The samples in each group were used for the fixed-time artificial insemination of 349 Nellore or cross-bred Nellore cows that were multiparous and lactating (60-90 days postpartum) and maintained exclusively under *Brachiaria decumbens* pasture, with free access to mineral salts and water. The synchronization protocol followed for the FTAI was performed during the Southern Hemisphere summer period (February, 2010). All of the cows were maintained at the same commercial farm in Mato Grosso do Sul, Brazil (21°53'09''S latitude; 54°09'21''O longitude) and the inseminations were performed in two random replicates.

On a random day of the estrous cycle (D0), all of the females received 2.0 mg of estradiol benzoate (Ric-Be®, Tecnopec, Sao Paulo, Brazil) and a new intravaginal progesterone device (Primer®, Tecnopec, Sao Paulo, Brazil). On D8, the implants were removed and 10 mg of FSH (Folltropin®, Bioniche Animal Health, Belleville, Canada), 0.375 mg of D-cloprostenol (Prolise®, Tecnopec, Sao Paulo, Brazil) and 1.0 mg of estradiol benzoate (Ric-Be®, Tecnopec, Sao Paulo, Brazil) were administered. The FTAI was performed 44-48 h after the removal of the progesterone device (D10).

Pregnancy diagnoses were performed by ultrasound (Aloka SSD-500, 5 MHz probe, Tokyo, Japan) 60 days after the FTAI. The cows were considered pregnant when an embryonic vesicle was present and a fetal heartbeat was detected.

Table 1. Effect of the semen preservation method (BB-R, semen cooled for 24 hours; BB-C, frozen-thawed semen) on the pregnancy rates of Nellore cows subjected to FTAI

Groups	n	Pregnancy rate % (n)	Adjusted OR (95% CI)[a]	P
BB-R	174	61,49[a] (107)	Reference[b]	
BB-C	175	45,71[b] (80)	0.537 (0.349-0.826)	0.0047

Different letters in the same column indicate statistical differences between the treatments (P<0.05). [a]OR= odds ratio; CI = confidence interval. [b]Reference point (i.e., "1.0"); the odds ratio is higher or lower than this value

2.3. Statistical Analysis

A binomial distribution was assumed for the categorical response variable. The P/AI was analyzed using the GLIMMIX procedure of SAS, with the cows as a random effect. The variables that were initially included in the model were the treatments (cooled or frozen-thawed semen), Body Condition Score (BSC) on the first day of the synchronization protocol (1 to 5 scale, where 1 = emaciated and 5 = obese), according to Ayres *et al*. (2009), breeding group (two replicates) and their interactions. For the final logistic regression model, the variables were removed through backward elimination based on the Wald statistics criterion when P>0.20 and only the semen treatment was included.

3. RESULTS

The BCS on the first day of the synchronization protocol (P = 0.4367), insemination replicate (P = 0.4518) and their interactions were not associated with the P/AI after the FTAI. The conception rates observed after AI using the cooled for 24 h or frozen-thawed semen were 61,49% (107/174) and 45,71% (80/175), respectively (P <0.005, **Table 1**).

4. DISCUSSION

In general, the use and production of bovine semen in liquid form can only be justified by the increase in conception rates when compared with frozen semen (Bucher *et al*., 2009), as the low longevity limits the wide dissemination and maintenance of semen for long periods of time.

Although a small number of viable bull spermatozoa are sufficient to achieve fertilization (Holt, 2000), the possible failure of sperm to contact the female oocyte after AI can represent one of most important sources of failure in the fertilization process (Hawk, 1986).

The ultimate objective of artificial insemination is for viable spermatozoa to reach one or more female gametes (Hunter, 2003). Although the quantity of spermatozoa reaching the site of fertilization is reduced in relation to the number of sperm cells inseminated (Januskauskas and Zilinskas, 2002) it can be assumed that cooled bovine semen ensures a greater number of intact accessory spermatozoa capable of fertilization in comparison to frozen semen, therefore justifying to the use of cooled semen.

Garcia *et al*. (1999) reported similar findings, noting that the use of bovine semen cooled for 8 h promotes an increase in the conception rates of heifers subjected to FTAI (P<0.05) when compared with sperm samples refrigerated for 32 h or frozen-thawed; the results were attributed to a greater availability and quality of the bovine semen stored in liquid form for a short period of time.

Bucher *et al*., (2009) obtained similar pregnancy results by comparing bovine semen stored in liquid form for 24 h at a concentration of 3×10^6 total sperm to frozen-thawed semen at 20×10^6 spermatozoa/straw, demonstrating that even an 85% reduction in the insemination dose can attain acceptable pregnancies that may be offset by the higher sperm quality. In this sense, one can conclude that the use of bovine semen in liquid form achieves a similar result to that found by increasing the concentration of frozen thawed semen, leading to a "compensatory effect" that ensures a greater number of viable cells at the fertilization site.

One of the main factors limiting the widespread use of semen in liquid form is the inevitable drop in sperm viability during storage. Previous studies involving evaluations of bull (Crespilho *et al*., 2009; Bartlett and Demark, 1962), ram (O'Hara *et al*., 2010) pig (Johnson *et al*., 2000), stallion (Webb *et al*., 2009) and bird (Zaniboni and Cerolini, 2009) semen in liquid form showed similar results, indicating that the critical period for semen storage in liquid is approximately 48 h, with a significant decrease in sperm viability and fertility for samples stored for longer periods of time.

Although the decline in the quality of fresh or chilled semen are directly influenced by such factors as the maintenance temperature, the compounds

present in the extenders, the sperm concentration and the variability between different bulls (Vishwanath and Shannon, 2000) it is plausible to assume that the maintenance of bovine semen in liquid form under refrigeration probably has a maximum efficiency in terms of viability when stored for up to 24 h in conventional egg yolk-based extenders.

5. CONCLUSION

Cooling semen for 24 h can represents an effective strategy to increase the P/AI in fixed-timed artificial insemination programs and represents a highly viable alternative to conventional frozen-thawed bull semen in terms of both economic and biological comparisons.

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