

A field study to compare post-thaw sperm progressive motility and pregnancy rate using Brahman bull semen frozen in milk based extender containing egg yolk or soybean lipids extract

Fernando Perea,^a Roberto A. Palomares,^{b,c} Maria S. Ferrer,^c Rumualdo González,^{d,e} Andrzej T. Palasz,^f Jormaryory Rosales,^a H. Hector Nava,^d Alejandro Hoyos-Jaramillo,^b Eleazar Soto-Belloso^{d,e}

^aDepartamento de Ciencias Agrarias. Universidad de los Andes, Trujillo, Venezuela; ^bDepartments of Population Health and ^cLarge Animal Medicine, College of Veterinary Medicine University of Georgia, Athens GA; ^dFacultad de Ciencias Veterinarias, Universidad del Zulia, Maracaibo, Venezuela; ^eVenezolana de Inseminación Artificial y Transplante de Embriones (VIATECA), Villa del Rosario, Venezuela; ^fDepartamento de Reproducción Animal. INIA, Madrid, Spain

Abstract

The objective of this study was to compare post-thaw sperm progressive motility and pregnancy rate using Brahman bull semen frozen in milk based extender containing egg yolk or soybean lipids extract. Three semen ejaculates from two Brahman bulls were divided into three aliquots for dilution with one of three milk-based semen extenders: complete egg yolk (CE), processed egg yolk (PE) or soybean extract (SE) before freezing. Mean post-thaw progressive motility was not different between soybean extract and processed egg yolk treatments, except for two time points (0.5 and 1 h) when soybean extract was superior to processed egg yolk ($P < 0.01$). Post-thaw progressive motility was consistently lower in frozen semen in complete egg yolk during the 2.5-h incubation period compared to processed egg yolk and soybean extract ($P < 0.05$). Pregnancy rate was not significantly different among extenders, with values of 35.7, 40.1 and 40.6 % for complete egg yolk, processed egg yolk and soybean extract, respectively. In conclusion, the use of semen extenders containing processed egg yolk or soybean extract resulted in higher post-thaw sperm progressive motility than complete egg yolk based extender. Pregnancy rates using Brahman bull frozen semen containing complete egg yolk, processed egg yolk or soybean extract, did not differ. Procedures to obtain the processed egg yolk seem to diminish the detrimental compounds of egg yolk. The use of soybean extract represents a promising alternative for milk-based bull semen extender and provides additional benefits reducing the potential risk for microbial contamination.

Keywords: Semen extenders, egg yolk, soybean extract, sperm progressive motility, pregnancy rate

Introduction

The quality of frozen semen used for artificial insemination (AI) is a critical point that affects cattle reproductive performance and profitability of the farms.^{1,2} The cryopreservation process has been shown to reduce sperm viability and impair sperm function. It is commonly accepted that a substantial number of sperm are damaged during cryopreservation.³ Cold shock, osmotic stress, ice crystal formation and oxidative damage are considered main sources of sperm cryoinjury.⁴ Therefore, the freezing protocol and selection of semen extender are key steps to maintain optimal quality of frozen semen.

Egg yolk has been used as an extender component extensively. It has been categorized as a biological compound that supports sperm viability and fertilization capacity of frozen bull semen.⁵ It is considered the most effective agent to protect sperm against cold shock during cryopreservation.⁶ Egg yolk and particularly the low-density lipoprotein fraction (LDL) are reported to have a cryoprotectant effect, since it prevents damage of membrane phospholipids, thus increasing the sperm tolerance to cold shock.^{6,7} In addition, it has been suggested that egg yolk lipoproteins bind bovine seminal plasma (BSP) proteins,⁸ which contain factors that are detrimental for sperm fertilization ability. The effect of BSP on sperm function is time and concentration dependent; therefore, continuous exposure of sperm to BSP may damage the cell membrane by increasing cholesterol efflux.^{6,9} However, the precise mechanism through which LDL protects the spermatozoa from cold shock remains unclear.

Previous studies have reported that the beneficial effects of LDL during the cryopreservation process depend on lipoprotein concentration. Moussa et al¹⁰ reported that the optimal concentration of

LDL in semen extender is 8%, and that an increase to 10% may impair post-thaw sperm function. Other components in egg yolk, such as high-density lipoprotein (HDL) affect cell respiratory activity and sperm motility.^{3,11-13} Moreover, the use of non-pasteurized egg yolk in semen extender represents a risk of bacterial contamination,^{14,15} in addition to the lack of consistency in the individual quality standards due to differences inherent to egg yolk composition depending on the storage period after laying.¹⁶

The use of chemically defined semen extenders (not originated from animal tissues) containing only the protecting fraction of egg yolk (e.g. LDL) could be a valuable alternative for the AI industry.¹⁷ Another alternative is the use of extenders containing soybean extract in substitution for egg yolk. Semen extenders based on soybean extract provide a higher consistency of the components and eliminate the risk for bacterial contamination.¹⁶ In previous reports, soybean extenders have shown higher post-thaw motility compared to egg yolk based diluent.^{2,17} Some studies have reported a higher non-return rate after using semen that contains a soybean extract based extender.¹⁸ However, other studies did not show a positive effect of soybean extract on reproductive performance.^{15,19-21}

In the present study we hypothesized that the use of semen extenders containing egg yolk or soybean extract will result in different post-thaw sperm progressive motility and pregnancy rate after artificial insemination using semen from Brahman bulls. The aim of this study was to compare post-thaw sperm progressive motility and pregnancy rate using Brahman bull semen frozen in milk based extender containing egg yolk or soybean lipids extract.

Materials and methods

Semen collection and processing

Semen samples were obtained from two Brahman bulls (Bulls 34 and 56), which were housed on an AI center, located in the County Rosario de Perija, Zulia State, Venezuela, under dry tropical forest conditions (mean rainfall: 1147 mL/year; temperature: 27.9°C; relative humidity: 74 %, and wind velocity: 4.6 Km/h). Bulls had four days of sexual rest between collections. Semen was collected using artificial vagina (AV) and a steer for sexual stimulation. The inner rubber liner of the AV was filled with warm water (45°C). A small amount of non-spermicidal lubricant was applied to the inner liner before the collection was performed. After 3-4 false mounts for sexual stimulation, the penis was grasped through the sheath and directed to the opening of the lubricated AV. Immediately after collection, the semen samples were assessed for volume, sperm concentration and percentage of sperm progressive motility and percentage of morphologically normal sperm by an experienced technician according to the guidance by the Society for Theriogenology (SFT).²² Volume was assessed using a 10 mL tube. Individual sperm progressive motility was visually evaluated using a phase contrast microscope (Olympus, Bx20) under 400x magnification. Sperm concentration was determined using a spectrophotometer following instructions from the manufacturer (SpermaCue, Minitube, Germany). Ejaculates with >75% progressive motility, >85% morphologically normal sperm, and > 750 million sperm/ mL were used for this study. The research protocols used in this study were reviewed and approved by the scientific and animal care committee of the Consejo de Desarrollo Científico, Humanístico, Tecnológico y de las Artes (CDCHTA), Universidad de los Andes.

Three semen ejaculates from each bull that met the minimum requirements for cryopreservation, were divided into three aliquots for dilution with the experimental semen extenders: complete egg yolk (CE), processed egg yolk (PE) or soybean extract (SE). Each extended semen aliquot was frozen using a conventional two-step freezing protocol. Briefly, a solution A (without glycerol) which was maintained at 37°C, was added and then refrigerated at 5°C for 60 minutes (equilibration period). After that, pre-cooled solution B (containing 14% glycerol) was added at a 1:1 (v:v) ratio in four fractions at 15 minute intervals at 5°C (within a refrigerator) to reach a final glycerol concentration of 7%. The solutions A and B were added to obtain a final sperm concentration of 30×10^6 spermatozoa/mL. Extended semen was packed in pre-cooled 0.54 mL straws, so that each straw contained 15×10^6 spermatozoa. Straws were frozen in liquid nitrogen vapors at -120°C for 5 minutes and then plunged in liquid nitrogen at -196°C. Straws were stored at -196 C until use.

Extender preparation

Semen was frozen in three different low-fat milk (1%) based extenders containing 10 mg/mL of fructose and supplemented with: 8% of whole egg yolk (CE extender), 8% processed egg yolk (PE extender), and 10% (7.3 mg/mL) of phospholipids of soybean-origin rich in phosphatidylcholine (Bioniche Inc., Beleville, Ontario, Canada; SE extender).

Egg yolks from fresh chicken eggs were carefully separated from albumin and then homogenized by blending and refrigerated at 7°C. Egg yolk was not further processed for the CE extender. For the PE extender, egg yolk was diluted 1:1 (v:v) in an isotonic saline solution (0.9% sodium chloride; w/v). After homogenization, egg yolk solution was centrifuged at 3,000 rpm for one hour at 5°C to remove the egg yolk granules. The resulting supernatant was transferred to a new tube and again centrifuged at 3,000 rpm for one hour at 5°C. The new supernatant fraction was then used to prepare the PE extender. All three extenders were supplemented with 1000 IU of penicillin, 1 mg/mL of streptomycin and 150 µg/mL of lincomycin.

Post-thaw progressive sperm motility evaluation

Seventy two hours after freezing, six straws per extender [one of each extender (CE, PE, and SE), ejaculate (three different ejaculates) and bull (two bulls, 34 and 56)] were individually thawed in a water bath at 37°C for 30 seconds. The thawed semen samples were transferred into 1 ml Eppendorf tubes and incubated in a water bath at 37°C for 2.5 h. Semen from each straw was evaluated twice at 0, 0.5, 1.0, 1.5, 2.0 and 2.5 hours after thawing to determine the progressive sperm motility visually using a phase-contrast microscope (400x, Olympus, Bx20) equipped with a heating plate (37°C). Sperm progressive motility was determined as the percentage of sperm crossing the microscopic field linearly, according to the SFT guidance.²² One experienced technician, who was blinded to the treatments (semen extenders) evaluated all samples.

Field study. Location and cattle management

Six hundred and sixteen multiparous crossbred Brahman cows were included in this study. Cows were artificially inseminated on detected estrus as described below. Each cow was inseminated once with frozen semen using one of the three semen extenders. Allocation to extender treatment was done randomly. The number of cows allocated into each treatment was distributed as follows: CE= 207 cows; PE= 212 cows; SE= 197 cows. Only cows that received their first artificial insemination (first service cows) were considered in this study. Artificial inseminations were performed during all year round. Seasons were classified according to the accumulative rainfall; dry: December-March (rainfall: 131.6 mL; temperature: 28°C; relative humidity: 71.5 %; wind velocity: 6.0 km/h); intermediate: April-July (rainfall: 637.1 mL; temperature: 28.6°C; relative humidity: 73.6 %; wind velocity: 5.3 km/h); humid: August-November (rainfall: 881.3 mL; temperature: 28°C; relative humidity: 77.1 %; wind velocity: 4.5 km/h). Cows were housed in a commercial farm located in Machiques de Perijá County, Zulia State, Venezuela; in an area classified as sub-humid tropical forest (mean daily temperature: 28.3°C, relative humidity: 65-80% and mean rainfall: 1650 mL/year), characterized by a bimodal rainfall pattern with two high rain peaks in May and October. Cows were milked by hand twice daily. After each milking, they were suckled by their calf for 1 h, until the calf was weaned at seven months of age. Daily average milk production of the cows was 5.4 kg/day. Cows grazed German grass (*Echinochloa polystachya*). Water and mineral supplementation were offered ad libitum and during the dry season, cows received supplementation (2 kg/cow) of 50% Guinea hay, 30% chicken litter, 10% corn flour, and 10% molasses-urea mix (16% crude protein). The herd health program was based on diagnostic tests, periodic deworming, vaccinations and biosecurity measures to prevent common diseases that affect the herds at the Maracaibo Lake basin in Venezuela, including brucellosis, leptospirosis, infectious bovine rhinotracheitis, bovine viral diarrhea virus, campylobacteriosis, and clostridial diseases. Additionally, it also included the prevention of mastitis through cleaning and disinfection protocols during milking.

Estrus detection, artificial insemination and pregnancy diagnosis

Estrus detection was done by visual observation of estrous behavior for a period of 1 h each morning (06:00–07:00) and afternoon (18:00–19:00) and with the help of teaser bulls that were surgically altered with resection of both epididymis tails and penile deviation, so that intromission could not occur. Cows were artificially inseminated by two experienced technicians (A and B) 12 h after first detected in estrus according to the AM-PM insemination rule. Pregnancy diagnosis was performed by an experienced veterinarian by palpation per rectum 45 to 60 d after service. After calving, the voluntary waiting period was 30 days. Cows were inseminated after 30 days postpartum if uterine involution was completed and there were no signs of any ovarian or uterine pathology. Pregnancy rate was calculated as the number of pregnant cows 45 to 60 days after AI divided by the number of cows inseminated, multiplied by 100.

Statistical analysis

Data were analyzed using the Statistical Analysis System (SAS, version 9.2). Analysis of variance was performed to compare post-thaw progressive sperm motility among groups for each day. Differences among means were compared by the LSD procedure of SAS. Pregnancy rate was analyzed using Proc Logistic and differences were detected using Chi-square test from SAS. The following explanatory variables were considered: semen extender (CE, PE and SE), bull (34, 56), inseminator (A, B) and season (dry, intermediate and humid).

Results

Post-thaw sperm progressive motility

Mean post-thaw sperm progressive motility was not different between SE and PE treatments, except for two time points (0.5 and 1 h) when SE was superior to PE ($P < 0.01$; Figure 1). Post-thaw progressive motility was consistently lower in semen frozen in CE at all time points during the 2.5-h incubation period compared to PE and SE ($P < 0.01$ from 0 to 1.5 hours and $P < 0.05$ at 2 and 2.5 hours; Figure 1). Moreover, the decline in sperm motility after 2.5 hours of incubation was more pronounced in semen with CE (26.6 %) compared to PE (20 %) and SE (13.4 %; Figure 1). Consequently, after 2.5 h incubation sperm motility was greater in SE (56.6 %, $P < 0.01$) and PE (48.3 %, $P < 0.05$) than in CE (35.0 %) extender (Figure 1). The effect of semen extender on sperm motility was observed in both bulls. No differences were found between bulls (Figure 2).

Pregnancy rate

Pregnancy rate was not significantly different among extenders with values of 35.7 % (74/207), 40.1 % (85/212) and 40.6 % (80/197) for CE, PE and SE, respectively. There was no significant effect of bull or the interaction bull x treatment on pregnancy rate ($P > 0.05$; Table 1). Inseminator B had a higher pregnancy rate than inseminator A (44.5 vs 33.2 % $P < 0.01$; Table 1). For inseminator B, pregnancy rate tended to be greater when using semen containing PE (48.6 % $P \leq 0.05$) and SE (50.0 % $P \leq 0.02$) compared to CE (35.9 %; Table 1). In contrast, pregnancy rate did not differ among extenders for inseminator A. For all treatments, cows inseminated during the dry season had a higher pregnancy rate than those inseminated during the humid season, (46 vs 28 %, respectively; $P < 0.01$; Table 2). There was no significant effect of the interaction season x treatment on pregnancy rate.

Discussion

Under the conditions of this study, post-thaw sperm progressive motility was significantly higher for extenders containing processed egg yolk or soybean compared to extender containing complete egg yolk. Previous reports have demonstrated that deleterious components in egg yolk can negatively affect bull sperm motility.^{4,23} Phospholipids, lecithin and lipoprotein fractions contained in egg yolk might form yolk granules that impair sperm movement, resulting in lower post-thaw sperm progressive motility when semen is evaluated *in vitro*.^{11,13} In the present study, dilution and processing of egg yolk apparently

decreased the concentration of harmful components or yolk granules that mechanically altered *in vitro* motility patterns. However, it appears that the remaining LDL present in the processed egg yolk extender provided appropriate conditions to improve post-thaw sperm motility, as previously reported.¹⁰ The positive effects of LDL on sperm motility might be associated with sequestration of proteins in seminal plasma^{6,8} or adhesion to the sperm membrane.^{24,25}

In the present study, the use of soybean based extender resulted in an improved post-thaw sperm motility of Brahman bull semen when compared to the extender containing complete egg yolk. These results might imply a great potential of chemically defined semen extenders with vegetal additives as an alternative to substitute semen extenders of animal origin which are associated with biosafety issues.^{14,15}

In a previous study, substitution of soy milk based extender for egg yolk based extender for bovine semen cryopreservation did not result in significant differences in post-thaw motility, viability, membrane integrity, acrosome integrity and cryo-capacitation.²⁶ Further, Aires et al¹⁸ reported similar sperm-zona pellucida binding capacity and progesterone-induced acrosome reaction for egg yolk and soybean extenders. However, in that study post-thaw sperm motility was significantly lower when semen was extended in the tris-egg yolk diluent.

In the present study pregnancy rate was not affected by the semen extenders used for cryopreservation. The results indicate that frozen semen extended with soybean or egg yolk based extenders had similar fertilization ability *in vivo*, resulting in similar pregnancy outcomes in crossbred Brahman cows under tropical conditions. In spite of the observed *in vitro* differences in mean motility among extenders, post-thaw motility was acceptable under commercial standards with all treatments. Moreover, if the lowered post-thaw motility were associated with mechanical interference of yolk granules on motility, this effect could be counteracted during dilution of semen with female genital tract secretions. This might have possibly contributed to the lack of difference in fertility. Similar to the results of the present study, a field insemination trial using frozen semen from Holstein bulls revealed that extenders containing egg yolk or soybean extract showed similar non-return rate at 56 days after AI.²⁷ In contrast, another field study reported a higher pregnancy outcome for semen extended in soybean lecithin based extender compared to that for the egg yolk extender.¹⁸

The results presented here also contrasts with previous studies performed by Van Watgtendonk et al.¹⁷ where semen extended with a commercial soybean extract-containing extender resulted in a significant reduction in the fertility (56-day non-return, estimated conception and calving rates) of bovine frozen semen. In subsequent trials, the authors observed that the sperm motility and survival were significantly lower for semen frozen in a soybean-based extender than in egg yolk-based extender.^{17,27} Comparable results have been previously reported where progressive motility of semen containing the soybean-based extender, Biociphos® was lower compared to a tris-egg yolk diluent.^{2,19,21} Additionally, in the study performed by Thun et al,² the use of a soybean-based extender resulted in increased proportion of secondary sperm defects and decreased osmotic resistance. Amirat et al²³ observed that both extenders containing egg yolk or soybean were relatively non-toxic to cells during the first hour of incubation. However, acrosome and plasma membrane alterations occurred after 4 h of incubation in semen diluted with soybean-based extender.

The differences observed between the results of the current trial and previous studies could be associated with individual variation, differences in the method to assess progressive motility (visual vs computerized), breed of bulls (*B. taurus* vs *B. indicus*) and frequency of semen collections. A major limitation of this study is the low number of bulls included. In the present trial, for all semen extenders pregnancy rate was higher during the dry season compared to the humid season. Previous studies to evaluate the monthly variation of fertility (n= 8,308 AI) and estrus frequency in crossbred dual-purpose cows (n= 2,960 cows) in three agroecological areas of the South American tropics revealed that the pregnancy rate of first serviced cows was the highest during the cooler and drier months of the year (December- April), while the lowest fertility was obtained during the months of highest rainfall and humidity (August- November).^{28,29}

Conclusions

The use of semen extenders containing processed egg yolk or soybean extract resulted in higher post-thaw sperm progressive motility than complete egg yolk based extender. Pregnancy rates after artificial insemination of crossbred Brahman cows using frozen semen containing complete egg yolk, processed egg yolk or soybean did not differ. Procedures to obtain the processed egg yolk are easy and inexpensive and seem to be able to diminish the detrimental compounds of egg yolk. The use of soybean extract represents a promising alternative for milk-based bull semen extender and provides additional benefits reducing the potential risk for microbial contamination.

Conflicts of interest

The authors of this article have no conflicts of interest.

Acknowledgements

The authors thank Consejo de Desarrollo Científico, Humanístico, Tecnológico y de las Artes (CDCHTA), Universidad de los Andes, Venezuela (Project NURR-C-321-02-03-F) for financial support. The authors extend their gratitude to Venezolana de Inseminación Artificial y Transplante de Embriones (VIATECA) for providing the facilities, bulls and equipment to conduct this research. Thanks to Agropecuaria Santa Ana farm for providing support on experimental units, animal husbandry, facilities and records during this study.

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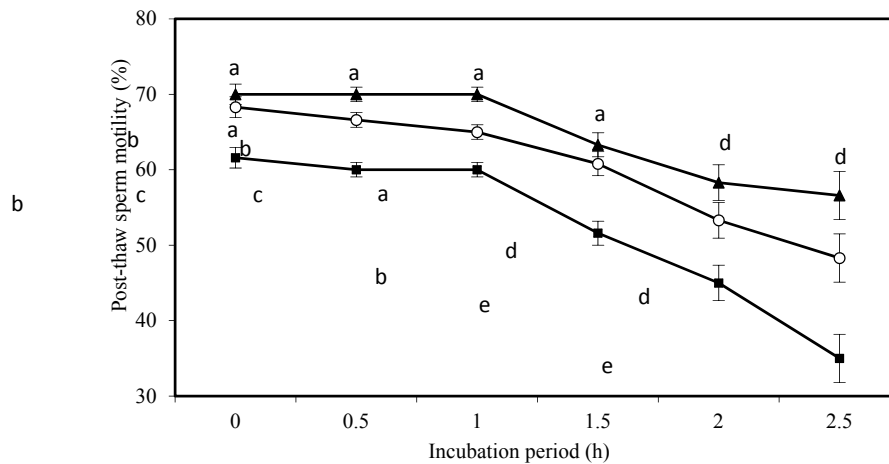


Figure 1. Post-thaw sperm motility during 2.5 hour incubation period of Brahman bull semen frozen with complete egg yolk- (■), processes egg yolk- (○) or soybean (▲) extract-containing diluent. Values are mean ± SEM of 6 ejaculates (three ejaculates × two bulls). ^{a,b,c} $P < 0.01$; ^{d,e} $P < 0.05$.

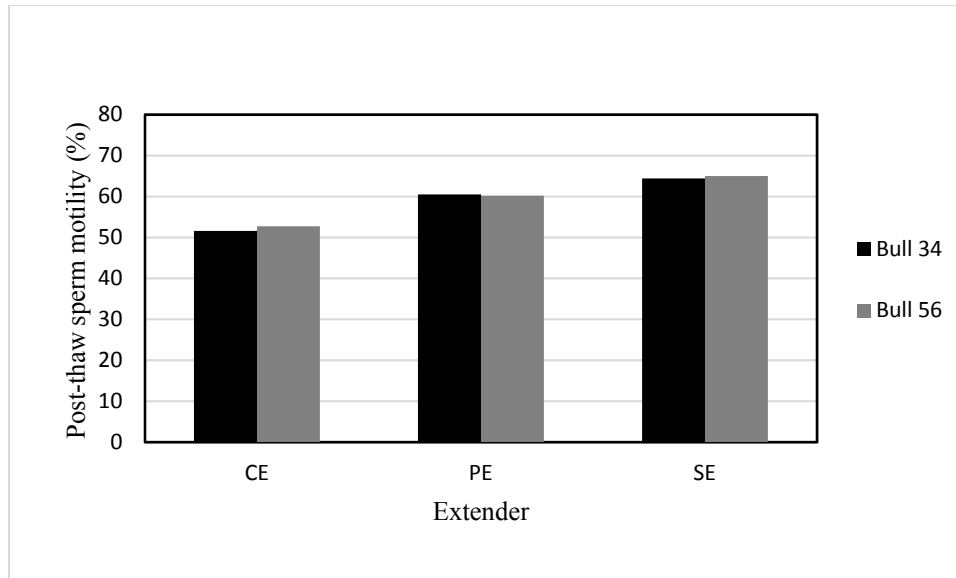


Figure 2. Average of post-thaw sperm progressive motility during 2.5 h of incubation of semen from two Brahman bulls frozen in milk-based extender containing complete egg yolk (CE), processed egg yolk (PE) or soybean extract (SE). No significant differences between bulls.

Variable	Extender						Overall
	CE		PE		SE		
	n	%	n	%	n	%	
Bull							
34	40/109	36.7 ^{a,1}	34/96	35.4 ^{a,1}	39/99	39.4 ^{a,1}	37.8 ¹
56	34/98	34.7 ^{a,1}	51/116	43.9 ^{a,1}	41/98	41.8 ^{a,1}	40.4 ¹
Inseminator							
A	32/92	34.8 ^{a,1}	32/96	33.3 ^{a,1}	32/101	31.7 ^{a,2}	33.2 ²
B	41/114	35.9 ^{a,1}	59/109	48.6 ^{b,2}	48/96	50.0 ^{b,c,3}	44.5 ³
Overall	74/207	35.7 ^a	85/212	40.1 ^a	80/197	40.6 ^a	

Values with different superscript letters in the same row differ significantly: ^{a,b} $P \leq 0.05$; ^{a,c} $P \leq 0.02$

Values with different superscript numbers in same column differ significantly: ^{1,2} $P \leq 0.02$; ^{2,3} $P = 0.01$;

Table 1. Effects of bull and inseminator on pregnancy rate in cows inseminated with semen frozen in milk-based extenders containing complete egg yolk (CE), processes egg yolk (PE) or soybean extract (SE). Values with different superscript letters in the same row differ significantly: ^{a,b} $P \leq 0.05$; ^{a,c} $P \leq 0.02$. Values with different superscript numbers in same column differ significantly: ^{1,2} $P \leq 0.02$; ^{2,3} $P = 0.01$;

Season	Extender						Overall
	CE		PE		SE		
	n	%	N	%	n	%	
Dry	46/107	43.0 ^{a,1}	44/95	46.3 ^{a,1}	43/87	49.4 ^{a,1}	46.0 ³
Intermediate	16/49	32.6 ^{a,1,2}	25/60	41.6 ^{a,1,2}	18/50	31.4 ^{a,1,2}	37.1 ⁴
Humid	12/51	23.5 ^{a,2}	16/57	28.1 ^{a,2}	19/60	31.6 ^{a,2}	28.0 ⁵

Values with different superscript letters in same row differ significantly ^{a,b} $P = 0.05$

Values with different superscript numbers in same column differ significantly: ^{1,2} $P = 0.05$; ^{3,4} $P = 0.06$; ^{4,5} $P = 0.06$; ^{3,5} $P = 0.001$

Table 2. Effects of season on pregnancy rate in cows inseminated with semen frozen in milk-based extender containing complete egg yolk (CE), processed egg yolk (CE) or soybean extract (SE). Values with different superscript letters in same row differ significantly ^{a,b} $P = 0.05$. Values with different superscript numbers in same column differ significantly: ^{1,2} $P = 0.05$; ^{3,4} $P = 0.06$; ^{4,5} $P = 0.06$; ^{3,5} $P = 0.001$

