PREGNANCY RATES IN HEIFERS INSEMINATED WITH SEMEN OF NELORE BULLS SEXED BY FLOW CYTOMETRY

ADRIANA MENDES DUARTE,[1] ELMO GOMES DINIZ,[2] MARA REGINA BUENO MATTOS NASCIMENTO,[2] MARCELO TAVARES[3] E ROSSANA VILELA RESENDE FRANCO[4]

1. Médica veterinária, Ms em Ciências Veterinárias.
2. Faculdade de Medicina Veterinária, Universidade Federal de Uberlândia, Av. Ceará, s/nº, Bloco 2T, Campus Umuarama. CEP:38400-902, Uberlândia, MG. E-mail: maran@umuarama.ufu.br
3. Faculdade de Matemática, Universidade Federal de Uberlândia, Núcleo de Estudos Estatísticos e Biométricos, Av. João Naves de Ávila, 2121 Campus Santa Mônica, CEP: 38408-100, Uberlândia, MG
4. Goyaike Brasil Agropecuária Ltda. BR 050 Km 196, Caixa Postal 4046, CEP: 38020-300 Delta, MG

ABSTRACT

The flow-sorting sperm technology is considered as the most promising technique for sexing semen, due to the separation accuracy of around 90%. Thus, the objective of this study was to determinate the effect of Articial Insemination (AI) with semen of Nelore bulls sexed by flow cytometry on the pregnancy rate and its accuracy. Throughout the study, 427 Nelore heifers were inseminated with only one dose of semen as follows: 241 heifers were treated with flow-sexed sperm (3×10^6 total flow-sorting X-chromosome bearing sperm/dose) and 181 were treated with unsexed sperm (10×10^6 total sperm/dose). The heifers were inseminated after detection of natural heat or after synchronization treatment. The results obtained for pregnancy rates did not indicate differences between flow-sexed and unsexed sperm and the accuracy in the sex determination presented high levels and reliability (92.4%).

KEY-WORDS: Bovine, Nelore, spermatozoa, sexing.

RESUMO

TAXA DE PRENHEZ EM NOVILHAS INSEMINADAS COM SÊMEN DE TOUROS DA RAÇA NELORE SEXADOS POR CITOMETRIA DE FLUXO

A citometria de fluxo é considerada a técnica de sexagem de sêmen mais promissora, em virtude de a acuidade de separação estar em torno de 90%. Dessa forma, o presente estudo objetivou determinar o efeito da IA com sêmen de touros Nelores sexados por citometria de fluxo sobre a taxa de prenhez, bem como sua acurácia. Foram inseminadas 427 novilhas da raça Nelore utilizando uma única dose de sêmen por animal, sendo que em 241 novilhas utilizou-se o sêmen sexado por citometria de fluxo com concentrações de 3×10^6 espermatozóides portadores do cromossomo X/dose e em 186 utilizou-se o sêmen não sexado com concentrações mínimas de 10×10^6 espermatozóides viáveis/dose. As inseminações ocorreram depois da detecção de cio natural ou após tratamento para sincronização. Os resultados dos índices de prenhez não indicaram diferenças entre o sêmen sexado e o não-sexado e a acurácia na determinação do sexo apresentou níveis elevados de confiabilidade (92,4%).

PALAVRAS-CHAVES: Bovino, espermatozóide, Nelore, sexagem.

INTRODUCTION

For several decades, one has searched for scientific basis that would allow the use of spermatozoa in order to provide descendants of the desired sex; in other words, the separation of gametes according to the presence of X or Y chromosome in its genome.

The optimization and widespread use of Artificial Insemination and Embryo Transfer techniques performed in animal genetic improvement programs, as well as the demand for more efficient production systems have led to the dissemination of sex sorting techniques in spermatozoa and pre-implanted embryos in farm animals (HOSSEPIAN, 1998)

Many sex-sorting methods have been developed and tested. Almost all the researches from the beginning of 1920 to 1980 were aimed at spermatozoa sexing using physical separation methods; however, these methods did not present any effective results (JOHNSON, 1995).

Flow cytometry has been considered as the most promising sexing sperm technique due to the separation accuracy of around 90% and because it is based on the identification of differences in the amount of chromatin between X or Y chromosome bearing spermatozoa (PINKEL et al., 1982), which is 3.8% in bovines.

In most experiments recently carried out, the pregnancy rates with frozen sexed semen were between 70% and 90% of those obtained with frozen unsexed semen, which presented 7 to 20 times more spermatozoa per dose (SEIDEL et al., 1999; SCHENK, 2000; MAXWELL et al., 2004). Several reports on pregnancy rates using sexed and unsexed (conventional) semen have shown that the former method presented results considerably lower when compared with the latter one (SEIDEL & GARNER, 2002). On the other hand, other authors have found no significant differences between both methods when the pregnancy rates in inseminated heifers were compared (SEIDEL et al., 1996; BROGLIATTI et al., 2001).

Brazil has the world's greatest commercial cattle, presenting high genetic and economic value animals, which characterizes it as a country with high potential to absorb flow sorting sperm technology. However, there are no reports on the use of this technology on zebu breeds in the country up to this moment.

Thus, the objective of this work was to determinate the effect of artificial insemination with sperm of Nelore bulls sexed by flow cytometry on the pregnancy rate of heifers as well as its accuracy.

MATERIAL AND METHODS

Animals

The sperm sexing was conducted at the of Goyaike Brasil Agropecuária Ltda laboratory located at Uberaba, Minas Gerais, Brazil. Eight adult Nelore bulls kept in a regular semen production system have been used in this study.

Evaluation, strain and semen preparation

The semen collected through artificial vagina was evaluated regarding its progressive motility in an optical microscope (Coleman), using samples presenting motility rate above 60% only. A photometer was used to measure the sperm concentration and only those presenting concentration above 1.0 x 10^6 spermatozoa/mL were used. For the morphological evaluation, a phase contrast microscope was used. The imperfections were classified according to BLOM (1973) and only samples presenting total imperfections below 25% were used. The semen was kept in an acclimatized room (20º C) for eight hours. After this period, the samples were prepared by diluting an aliquot of this semen in a staining buffered medium, resulting in a concentration of 200 x 10^6 spermatozoa/mL. Hoechst 33342 was added, which is a stain that permeates through the cell membrane and binds selectively to the DNA A-T basis. Then, these samples were incubated at 35ºC for 45 minutes. After this period, the samples were diluted in a sorting medium containing 100 x 10^6 spermatozoa/mL added of food coloring agents (FD&C40), which action is to quench the Hoechst fluorescence of spermatozoa that have damaged membranes in order to be removed during the dead –cell gating sorting process.

Spermatozoa separation through DNA content via flow cytometry

The spermatozoa were processed through flow cytometer (Moflo SX, Cytomation, Inc.). These cells were submitted to the sorting medium, where two optical detectors perpendicularly placed measured the fluorescence intensity resulting from Hoechst excitation under UV light from the laser. The detectors transferred the information into a computer in order to be processed. The droplets, generated by the stream rupture, were submitted to an electric field where opposite charges caused the deflection of the selected spermatozoa, which were collected in tubes containing catch fluid. For this experiment, only X chromosome bearing spermatozoa were separated.

Semen processing and freezing

The sexed sperms were kept at 4ºC for at least one and a half hour. Later, the sperms were centrifuged in a refrigerated centrifuge (Cientec – CT 6000R) for 15 minutes at 4ºC with 600 G rotation. The supernatant was discarded and the pellet re-diluted in a freezing synthetic extender (Bioxcell – IMV, France), placed into 0.25 mL French straws with spermatozoa concentration of 3 x 10^6 per straw and frozen using a computerized freezer (Digitcool 5300 – IMV, France). Concurrently, part of the *in natura* semen from each ejaculation was conventionally diluted and processed to be used as control in the Artificial Insemination, again placed into 0.5 mL French straws with minimum

Post-melting evaluation of the samples

One straw of each lot was melted at 35ºC during 30 seconds, evaluated regarding the post-melting motility and submitted to a sort reanalysis afterwards. To do so, the sperm tails were removed by sonication and additional Hoechst 33342 was added, incubated for 20 minutes at 35ºC and once again submitted to the flow cytometer. Separate analysis of each aliquot produced a histogram that suits a double gaussian curve to determine the proportions of X and Y populations. Only the semen presenting 85% of purity of X chromosome bearing spermatozoa and motility rate above 35% was released.

Experimental design

Nelore heifers originated from herds of five Brazilian states: Mato Grosso(MT), Mato Grosso do Sul (MS), Minas Gerais (MG), Rondônia (RO), Acre (AC) were inseminated: 241 with sexed semen and 186 with unsexed semen (control). Only one intrauterine insemination was performed. The animals were divided into two groups:

1) synchronized heifers (MT, MS, MG, RO) with two doses of prostaglandinF2 (Cloprostenol 2 mL im) within a 12-day interval and inseminated 12 hours after estrus behavior detection. From these, 180 were inseminated with sexed semen and 103 with unsexed semen;

2) non-synchronized heifers (AC), using natural estrus. From these, 61 were inseminated with sexed semen and 83 with unsexed semen.

Thirty days after insemination, a pregnancy diagnostic and a fetal sexing 60 days after were performed, both by means of ultrasound.

The binomial test was used for to compare the pregnancy and female fetus percentages in the artificial insemination with sexed semen and unsexed semen with a significance level of 0.05% (TRIOLA, 1999).

RESULTS

The results of the pregnancy rates and female percentage obtained with sexed semen and unsexed semen in synchronized and non-synchronized females are shown in Table 1 and their significance in Tables 2, 3, 4, 5. A difference ($p<0.05$) in the pregnancy rates between synchronized and non-synchronized groups was observed not only when sexed semen was used but also when unsexed semen was used, as well as between the non-synchronized heifers and the total heifers of the sample (Table 2). In relation to the female percentage in the groups, the results showed differences ($p<0.05$) when sexed semen was used comparing synchronized and non-synchronized animals and between synchronized animals and the total number of animals. (Table 3). When artificial inseminations performed with sexed and unsexed semen were compared, no di-

fferences in the pregnancy rates were observed in relation to the groups and the total number of animals (Table 4). Differences ($p<0.05$) in the artificial inseminations were verified for both types of semen in synchronized groups, non-synchronized groups and total, when compared to the proportion of females (Table 5). The results of the pregnancy rates did not indicate differences between sexed and unsexed semen in the total of animals, 51.4 and 58.6 % respectively. The accuracy in the sex determination presented reliability levels of 92.4% (Table 1).

TABLE 1. Pregnancy rates (%P), female percentage (%F) and pregnancy percentage in sexed and unsexed semen (%P sex/unsex) of Nelore bulls between synchronized heifers, non-synchronized heifers and the total of animals.

Groups	Treatment	**N**	%F	%P	% P sex/ unsex
Synchronized	Sexed	180	97.50	46.10	93.13
	Unsexed	103	46.15	49.50	
Non synchronized	Sexed	61	86.80	67.21	96.20
	Unsexed	83	49.09	69.80	
Total	Sexed	241	92.40	51.40	87.70
	Unsexed	186	48.50	58.60	

TABLE 2. Comparison of pregnancy rates (%P) in synchronized, non-synchronized and total in AI with sexed and unsexed semen from Nelore bulls.

	Significances	
Comparison % P between groups	Unsexed	Sexed
Synchronized x Non-synchronized	0.0020	0.0014
Synchronized x Total	0.0681	0.1405
Non-synchronized x Total	0.0288	0.0103

*Figures in bold are statistically different.

TABLE 3. Comparison of female percentage (%F) in synchronized, non-synchronized and total in AI with sexed and unsexed semen from Nelore bulls.

	Significances	
Comparison % F between groups	Unsexed	Sexed
Synchronized x Non-synchronized	0.3469	0.0086
Synchronized x Total	0.3537	0.0068
Non-synchronized x Total	0.4638	0.1146

*Figures in bold are statistically different.

TABLE 4. Comparison of pregnancy rates (%P) between AI with sexed and unsexed semen from Nelore bulls in different groups and in the total of animals.

	Significances		
Comparison %P between sexed and unsexed	Synchronized	Non-synchronized	Total
Sexed x unsexed	0.2908	0.3701	0.0684

*Figures in bold are statistically different.

TABLE 5. Comparison of female percentage (%F) between AI with sexed and unsexed semen from Nelore bulls in different groups and in the total of animals.

Comparison %F between sexed and unsexed	Significances		
	Synchronized	Non-synchronized	Total
Sexed x unsexed	0.0000	0.0000	0.0000

*Figures in bold are statistically different.

DISCUSSION

Most studies on the fertility of inseminated females with sexed semen are misleading due to the use of lower dose concentrations per insemination comparing to unsexed semen doses; however, the lower spermatic concentration was not a limiting factor in the present experiment. These results are in agreement with those found by JOHNSON (2000) and GARNER (2001), who reported that artificial insemination with a lower number of spermatozoa in bovines presents effective results and with SEIDEL et al. (1996) and BROGLIATTI et al. (2001), once there were no considerable differences between artificial inseminations using sexed or unsexed semen with concentrations 3.0×10^6 and 10×10^6 of spermatozoa per straw. Nevertheless, these results are not in agreement with those obtained by SEIDEL and GARNER (2002), who found pregnancy rates of 53% and 66% with sexed and unsexed semen, respectively. Moreover, in other studies (JOHNSON et al., 1989; JOHNSON, 1991; CRAN et al., 1993; HOLLINSHEAD et al., 2002a; HOLLINSHEAD et al., 2002b; MAXWELL et al., 2003) lower pregnancy rates were reported after artificial insemination with sexed semen when compared to unsexed semen. ANDERSSON et al. (2004) also found considerable reduction on the pregnancy rates when low doses of sexed semen were used in artificial insemination. CRAN et al. (1995) reported that this technique reduces the spermatozoa fecundation capacity, what was not corroborated in this experiment. The results found here also confirm those obtained by SEIDEL et al. (1999a) and SCHENK (2000), who reported that in most experiments recently performed, the pregnancy rates with frozen sexed semen were between 70 and 90% in relation to those obtained with frozen unsexed semen and MAXWELL et al. (2004), who reported that in some experiments, pregnancy rates obtained with unsexed semen were considerably higher than those obtained with low doses of sexed semen.

The sex accuracy determined by ultrasound in heifers inseminated with sexed semen ranged from 85% to 100% of females and although considerable differences were found between farms in groups and in the total of animals, the results are in agreement with results obtained by HOSSEPIAN (1998), who reported that flow cytometry is a promising technique due to its high separation accuracy and also confirms studies of JOHNSON et al. (1999), AMANN (1999) and SEIDEL (2003), who reported that sexing technology produces sexed offspring with accuracy of 85- 95% for X or Y chromosome bearing spermatozoa.

The differences between pregnancy rates of synchronized and non-synchronized heifers and between these and the total of animals occurred probably due to the period when the artificial inseminations were performed, once heifers from farms located in MT, MS and MG were at the end of the breeding season, which is known for its lower fertility. In states where heifers were synchronized, the pregnancy rates were numerically lower than those obtained with the non-synchronized heifers in AC and the synchronized heifers in RO, although it does not represent a significant difference. These results may be explained by different periods of the breeding seasons, because of climate differences.

Therefore, it was demonstrated that the pregnancy rates with sexed semen flow cytome-

try were the same as those obtained with unsexed semen in the evaluation groups, indicating that in the present experiment, sexed semen of Nelore bulls were indicated for the use in normal field conditions and did not compromise fertility, and that the purity or accuracy of spermatozoa sexed with this technique presented high reliability levels.

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