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EFFECTS OF SEMEN SEXING AGENT (HEIFER-PLUSTM) IN DAIRY COW REPRODUCTION

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Abstract: *This study was conducted to investigate the use cryopreserved bovine sperm treated with Heifer-PlusTM Prefreeze kit and its effect on pregnancy rates calf sex ratio embryonic death, abortion, stillbirth and twinning rates in Holstein Friesian cows A total of 120 Holstein Friesian cows were enrolled in this study. Sixty cows were artificially inseminated (AI) whit cryopreserved bovine sperm treated with Heifer-PlusTM PREFREEZE kit (experimental group; EG) and 60 cows served as control group (CG).*

Findings showed that the AI of cows with bovine semen treated with Heifer PlusTM Prefreeze kit had slight increases in conception rate (5%) and in female and male ratio (18.5%). Use of cryopreserved bovine sperm treated with Heifer-PlusTM Prefreeze kit did not affected embryonic death, abortion, stillbirth and twinning ratios in Holstein Friesian cows. Further investigations are needed with larger numbers of tested dairy cows, which enable wide spread adoption of this technology.

Keywords: Heifer-Plus, artificial insemination, fetal sex, conception rate.

• Introduction

The use of sexed semen in dairy cattle production provides a number of benefits at farm level. In dairy farming there is a surplus production of unwanted male calves. Male dairy calves increase the risk of dystocia compared with female calves. Dystocia means difficult birth, and has adverse effects on the subsequent survival, health and performance of mothers and offsprings [1-4] (Dematowewa and Burger, 1997; Lombard et al. 2007; Tenhagen et al., 2007; Mee, 2008). Introduction of sexed semen into the breeding program can minimize the number of unwanted male dairy calves.

In mammals, sex is determined by genomes, where an XX chromosome combination determines a female and an XY chromosome combination determine a male. As sex chromosome differ significantly in length, a discrimination of X- and Y- chromosome bearing sperm populations is possible [5, 6] (Seidel, 2007; Siedel and Johnson, 1999). In cattle and X-chromosome bearing sperm contains 3.8% more DNA than a Y-chromosome bearing sperm, providing a feature that can be utilized the identify X- and Y-chromosome bearing sperm. A specialized type of cytometry called fluorescence-activated cell sorting was developed [7] (Garner et al., 2013).

This method is based on precise staining of the DNA of sperm with the nucleic acid-specific fluorophore, Hoechst 33342, to differential between the subpopulations of X- and Y- sperm.

The fluorescently stained sperm are than sex-sorted using a specialized high speed sorter, and collected into biologically supportive media prior to reconcentration and cryopreservation in numbers adequate for use in artificial insemination. The disadvantage of this method include sperm damage and increased embryonic death rate [8-10] (Türk et al., 2015; Balzani et al., 2021; Steel et al., 2020).

It has been claimed that bull semen treated with Heifers-PlusTM kit, marketed by a commercial company, increases the birth chance of female calves at least 20-25%and the pregnancy rates by at least 5-20% [8, 11] (Turk et al., 2015; Williams, 2007).

According to the manufacturer's claims Heifer-PlusTM (HP) is spermagenic agent, packaged in kit form. The agent is activated by adding bull sperm. The sexing process „stimulates" the fertility and motility of the X-chromosome bearing (female) sperm while „slowing" the fertility and motility of the Y-chromosome bearing (male) sperm. When inseminated the sperm are „sorted" in the reproductive tract. The results is more ova fertilized by the X-chromosome bearing sperm producing more heifer calves.

Ruemke et al., (2022) [12], reported that spermatozoa treated with Heifer-PlusTM consistently exhibited a high velocity and variable linearity using Computer Assisted Semen Analysis (CASA).

Steele et al., 2020, [10] investigated the effects of flow cytometric sorting spermatozoa on bovine sperm function and early embryonic development. Using CASA and Quantitative analyses they revealed highly significant alterations in both sperm motility, sperm morphokinetics and reduced percentage of hyperactivated sperm.

Hyperactivation is characterized by high-amplitude, asymmetrical flagellar bending (Suarez, 2008)[13] and it is important for the sperm to detach from the tubal sperm reservoir to migrate through the oviduct to reach the oocyte (Rodriguez-Martinez, 2007) [14], and allows the sperm to penetrate the cumulus oophorous (Ho and Suarez, 2001) [15].

Also, Steele et al., 2020 using time lapse video microscopy they were able to continuously monitor embryos derived from sexed and conventional semen from the zygote to the blastocyst stage. They reported that the use of sexed–sperm results in higher percentage of unfertilized oocytes and arrested zygotes. Arrested at zygote stage implies that after opposition of the male and female pronuclei subsequent mitotic events fail to take place. Higher percentage of arrested embryos results in decreased blastocyst rates and thus reduce pregnancy rates in vivo (Steele et al., 2020) [10].

Inaba et al., 2016, [16] reported that sperm of individual bulls are affected by sexing in different bull-dependent manner.

No one to our knowledge has explored the implications of using bovine sexed semen with Heifer-PlusTM PREFREEZE kit.

The objective of this study was to investigate the in vivo efficacy of cryopreserved bovine sperm treated with Heifer PlusTM PREFREEZE kit, and its effect on pregnancy rate, calf sex ratio, embryonic death, abortion, stillbirths and twinning rates in Holstein Friesian cows.

• Material and method

Animals and location

Animals involved in this study were from Agriculture Research and Development Station (ARDS) Simnic-Craiova (S-W Romania 44°19'0'' N, 23°48'0'' E). The experiment was performed in compliance with European Union Directive 86/609/EC on Holstein-Friesian cattle that belong to a long and large genetic improvement program.

A total of 110 Holstein-Friesian cows were enrolled in this study, with parity 1 to 4. All cows had normal estrus cycle, were: >50 days in milk, BCS ≥3, and free of postpartum disorders and uterine disease.

The animals were kept in closed barns in autumn and winter and grazed on green pasturage in spring and summer.

The selected cows have average to high genetic merit for kilograms of fat plus protein yield. The diet consisted of high energy ration based on some by-products and home grown components (forage maize, grazed grass, Lucerne, forage beet, maize silage, grains and beans). Additionally the rations was balanced with purchased minerals. Fresh drinking water was provided ad libitum.

Experimental procedure

The experimental cows (n=120) were selected between January to December 2020 and from February to September 2021. Holstein Friesian cows were randomly allocated to the experimental group (EG) of cows and to the control group (CG) of cows. The cows in EG (n=60) were inseminated by artificial insemination using cryopreserved bovine semen treated with Heifer PlusTM PREFREEZE (Semen Sexing Agent-Female) produced by SEMTEST CRAIOVA S.A. The cows in CG (n=60) were inseminated by A.I. using conventional semen (without sexing agent).

The cows in the control group were inseminated at the onset of standing estrus, and in the experimental group were inseminated at least 16 hours after the onset of standing estrus according to the manufacturer's instructions. In the table 1 the cows that are best suited for AI with sexed semen are indicated based on time since onset of standing estrus.

Table 1. Cows suited for AI, with sexed semen and conventional semen based on time since onset of standing estrus

First obs. standing heat	Hours since s. heat onset at 7AM	Suitable conv. sperm	Suitable for conv. semen	Hours since s. heat onset at 3 PM	Suitable for sexed semen	Suitable for conv. semen
06:00	1 hour	-	++	9	-	++++
10:00	21 hours	++++	++	5	-	+++
14:00	17 hours	++++	++++	1	-	++
18:00	13 hours	+++	++++	21	++++	++
22:00	9 hours	-	++++	**17**	++++	++++

If AI is being conducted twice a day, most cows will be at the optimum time for sexed semen either in the morning or in the evening (table 1).

Pregnancy was determined by uterine palpation per rectum on day 40 and confirmed on days 75 postservice. Embryonic death rate was calculated from day 40 to 75 of gestation. Calf sex was confirmed by parturition. Abortion rate after day 75 of gestation, stillbirth and twinning rate in both groups was also determined.

The data were entered into Microsoft Excel computer program 2007. Stata version 14 was used to summarize the data and descriptive statistics were used to express results.

The results are presented as mean ± SEM and values of $P \leq 0.05$ were considered as significant. Chi-square test was performed to determine the differences in pregnancy rates, female and male calf ratios, abortion rates, stillbirths and twinning rates between the two groups of cows.

• Results and discussions

The rates of pregnancy, abortion, stillbirth, sex ratio and twinning in EG of cows and CG of cows are presented in table 2.

Table 2. The mean (± ESM) regarding pregnancy rate (%), embryonic death rate (%) between days rate (%) stillbirth rate (%) embryonic death rate (%) from day 50 to 75 of gestation, abortion rate (%) after day 75 of gestation, female calf rate (%), male calf rate (%) and twinning rate (%)

Parameters	Holstein Friesian cows		Difference	Manufacturer of Heifer Plus™ claims
	EG (n/n) %	CG (n/n) %	EG – CG %	
Pregnancy rate % on day 40 of gestation	53.3 (32/60)	48.3 (29/60)	+ 5.0	**5-20%**
Embryonic death rate % between days 40 and 75 of gestation	3.1 (1/32)	3.4 (1/29)	+ 0.3	-
Abortion rate % after 75 days of gestation	3.2 (1/31)	3.5 (1/28)	- 0.3	-
Stillbirth rate %	0.0 (0/30)	0.0 (0/27)	0.0	-
Female calf rate %	66.7 (20/30)	48.2 (13/27)	+ 18.5	**20-25%**
Male calf rate %	33.3 (10/30)	51.8 (14/27)	- 18.5	-
Twinning rate %	**0.0 (0/30)**	**3.7 (1/27)**	**- 3.7**	-

The pregnancy rate in the EG of cows was 53.3%, and in the C.G. of cows 48.3% a difference of 5% between both groups of dairy cows (table 2).

There was one embryonic death between days 40 and 75 of gestation and one abortion after 75 days of gestation in each group of cows (table 2).

There was only one twining case in the control group of cows. No stillbirths were observed in the experimental and control groups.

The female calf rate in the E.G. of cows and in the C.G. of cows was 66.7% and 48.2%, respectively (table 2).

The male calf rate in the EG of cows and in the CG of cows was 33.3% and 51.8% respectively (table 2).

The commercial company of Heifer PlusTM kit claims that AI of cows with cryopreserved bovine semen treated with Heifer PlusTM PREFREEZE could result an increase in the pregnancy rates by at list at 5-20%. In this study the pregnancy rate increased only with 5%.

The same commercial company claims that AI of cows with semen treated with Heifer PlusTM could result an increases in the number of female calves born at the rate of 20-25%. In this study the female calf rate increased 18.5% in EG of cows compared with CG of cows.

In this study the changes in pregnancy rates, the ratio of female and male calves born, embryonic death, abortion twinning rates were examined to evaluate the vivo efficacy of Heifer PlusTM PREFREEZE kit in Holstein Friesian cows.

To the our knowledge a few studies have been published in the scientific journals regarding the effects of the Heifer PlusTM kits on pregnancy rate or on sex ratio. Previous studies have indicated the use of frozen-thawed semen treated with Heifer Plus+.

Gerard et al., (2008) [17] reported a lower rates of in vitro fertilized eggs (44.7% for Heifer PlusTM and 77.7% for control) as compared with control group.

Curry et al. (2009) [18] reported that the treatment of frozen thawed semen treated with Heifer PlusTM did not affected the number of embryos collected (4.76 in Heifer PlusTM group and 3.55 in control group in ovarian hiperstimulated).

Türk et al., (2015) [8] reported not statistically significant difference was found in pregnancy rates between Heifer PlusTM group (52%) and control group (56%).

In the studies of Gerard et al., (2008) and Curry et al., (2009) [17, 18] the female calf ratios in the Heifer PlusTM group were found to be 69% and 45.2% respectively. Türk et al., (2015) [8] reported that this ratios with Heifer PlusTM was 52%.

In the studies of the manufacturer of Heifer PlusTM kit (Williams, 2007) [11] the mean female calf ratio with Heifer PlusTM was 79%.

Chongsi et al., 2019, [19] reported a conception rate in Gaudali and F1 Gaudali-Simmental cattle, improved by an average of 4.13% in Heiferplus cows as compared to the control group.

In our study embryonic death rate between days 40 to 75 of gestation, Abortion rate, Stillbirth rate and Twinning rate were not affected by Heifer PlusTM PREFREEZE (table 2).

Türk et al., (2015) [8] reported only one embryonic death in the control group, while one embryonic death, one abortion and –two twinning cases were observed in the Heiferplus group.

Chongsi et al. 2019, [19] reported a abortion rate of 13.95% in HeiferPlus cows and 14.63% in the control group.

Sexed semen may facilitate faster, more profitable dairy herd expansion by increasing the number of dairy female replacements born.

Functional knowledge of the past-sorting alterations in sperm integrity and early embryonic development are pivotal in developing cost-effective remediation strategies to improve conception rates in vivo.

• Conclusions

Treatment of bovine semen with Heifer PlusTM slightly increased conception rate, and female and male calf ratios after A.I. The increase of CR, did reach the minimum percentage (5%) as claimed by the kit manufacturer. The increase in female and male calf ratio (18.5%) did not reach the 20-25% rates as claimed by the kit manufacturer.

Use of cryopreserved bovine sperm treated with Heifer PlusTM Prefreeze for A.I., did not affected embryonic death, abortion, stillbirth and twinning rates in dairy cows.

Further investigations are needed with larger numbers of tested Holstein Friesian dairy cows, which enable widespread adoption of this technology.

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