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Effects of semen sexing kits (Heiferplus™ and Bullplus™) supplemented to frozen-thawed bull semen on pregnancy rates, foetal sex ratios and selected reproductive parameters in cows

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ABSTRACT: It has been claimed that artificial insemination (AI) of cows with frozen-thawed semen treated with commercially produced kits, Heiferplus™ (HP, in favour of female gender) and Bullplus™ (BP, in favour of male gender), increases the birth chance of calves with desired sex ratio by at least 20–25% and pregnancy rates by at least 5–20%. Hence, this study was conducted to investigate the efficacy of HP and BP kits as combined with AI on the pregnancy rates, foetal sex ratios and some reproductive parameters in cows. For this, a total of 200 cows (100 Holsteins and 100 Simmentals) from three to five years old were used. Fifty Holstein and 50 Simmental cows served as controls. The other half of Holstein and Simmental cows was artificially inseminated with frozen-thawed semen treated with HP and BP, respectively. Findings showed that the AI of cows with frozen-thawed semen treated with HP had no significant effect on the pregnancy rate [52.0% (26/50) in HP group; 56.0% (28/50) in control group], female calf ratio [52.0% (13/25) in HP group; 44.4% (12/27) in control group], embryonic death, abortion, stillbirth, twinning and gestation length as compared to the control group. Similarly, AI of cows with frozen-thawed semen treated with BP did not lead to any significant effect on the pregnancy rate [64.0% (32/50) in BP group; 58.0% (29/50) in control group], male calf ratio [53.1% (17/32) in BP group; 39.3% (11/28) in control group] and other reproductive parameters as compared to the control group. In conclusion, HP and BP treatments of semen used in the AI provided only slight, non-significant increases in female (7.6%) and male (13.8%) calf ratios, respectively.

Keywords: Heiferplus; Bullplus; foetal sex; pregnancy rate; cow

Recently, sex pre-selection before fertilisation has been attempted in the field of reproductive biotechnology. The sex of foetuses is determined by whether a sperm, which performs fertilisation, carries an X or Y chromosome (Seidel 1999; Seidel and Johnson 1999). The optimal method for determining foetal sex is to separate X and Y chromosome-bearing spermatozoa before fertilisation, and to inseminate the females with the desired (sexed) semen portion. For this purpose, X and Y chromosome-bearing spermatozoa can be separated with a 85–90% success rate using flow cytometric sorting. However, the disadvantages of this method include sperm damage due to the separation procedure, increased embryonic death rate due to the usage of DNA dyes and the high financial cost of the method, which has limited its worldwide availability in field conditions (Seidel 2007).

It has been claimed that insemination of cows with frozen-thawed bull semen treated with Heiferplus™ (HP) and Bullplus™ (BP) kits, marketed by a commercial company, increases the birth chance of calves with desired sex ratio by at least 20–25% and the pregnancy rate by at least 5–20%. Although the ingredients are undisclosed, the manufacturer, quoted on the internet, cites HP efficacy in both ovarian hyperstimulated and naturally ovulating cattle (Williams 2007). According to the manufac-

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turer's claims, HP stimulates the X chromosome-bearing sperm (female) while concurrently slowing the motility of Y chromosome-bearing sperm (male), and when inseminated, the female sperm swims faster than the male sperm and therefore reaches the egg first. The result is that more eggs are fertilised by female sperm, leading to more heifer calves without deterioration of fertility. Similarly, the company further claims that BP enhances the male calf ratio in a relatively similar process, but that the male sperm is stimulated instead of the female sperm resulting in more bull calves then heifers and greatly increasing the revenue generated from bull calf sales (Reidhead 2007). To the best of our knowledge, only a single study has been published in a scientific journal (Curry et al. 2009) along with two conference proceedings (Gerard et al. 2008; Sassone et al. 2009) regarding the efficacy of HP, while for the efficacy of BP, only a single *in vitro* study has been presented at a conference (Gerard et al. 2008). The results of the few studies are controversial regarding the effects of the HP kit on the sex ratio (Gerard et al. 2008; Curry et al. 2009). Further, there is no *in vivo* study published in any scientific journal about the efficacy of the BP kit. This study was therefore conducted to investigate the *in vivo* efficacy of both HP and BP kits on pregnancy rate, foetal sex ratio, embryonic death, abortion, stillbirth, twinning and gestation length in Holstein and Simmental cows, respectively.

MATERIAL AND METHODS

Animals and location. This study was conducted in Elazig province of Turkey located at a latitude of 38°40'N. A total of 200 cows (including 100 Holsteins and 100 Simmentals) from three to five years old were used in the present study. The cows were selected both from clinically healthy animals admitted to the Firat University (Animal Hospital, Unit of Reproduction and AI) for insemination, and also from small and large farms in the province. All cows had normal oestrous cycle, were free from reproductive disorders and were at least on Day 60 *postpartum*. According to the available knowledge, animal owners had applied similar maintenance and feeding programmes to the lactating cows. The animals were grazed on green pasturage in spring and summer and kept in closed barns in autumn and winter. The animals were fed on barley, bran, sugar beet pulp and hay when kept in closed barns. Fresh drinking water was provided *ad libitum*.

Experimental protocol. Following the approval of the experimental protocol by the Firat University Animal Experimentation Local Ethics Committee (Elazig, Turkey), Holstein cows were divided into two equal groups, 50 animals each.

(1) Control group (n = 50): Cows in this group were inseminated by AI using frozen-thawed semen, with no additive.

(2) HP group (n = 50): Cows in this group were inseminated by AI using frozen-thawed semen treated with the HP (Heiferplus™, Bovine semen sexing agent-FEMALE, lyophilised vial for 0.25 ml straw, Emlab Genetics, Arcola, IL, USA) kit.

Similarly, Simmental cows were also divided into two equal groups, 50 animals each.

(1) Control group (n = 50): Cows in this group were inseminated by AI using frozen-thawed semen, with no additive.

(2) BP group (n = 50): Cows in this group were inseminated by AI using frozen-thawed semen treated with the BP (Bullplus™, Bovine semen sexing agent-MALE, lyophilised vial for 0.25 mL straw, Emlab Genetics, Arcola, IL, USA) kit.

Treatment of frozen-thawed bull semen with HP and BP kits. The male or female calf ratios obtained from the same breed but from different sires may differ even if they have undergone the same maintenance and feeding programmes. Therefore, 0.25 ml straws, produced from the ejaculate of one bull for Holstein and one bull for Simmental groups, were used to minimise the likelihood of individual increases/decreases in female or male calf ratios arising from the use of different bulls. Both kits were stored at –20 °C in a deep freezer until used. Straws were treated with kits according to the manufacturer's instructions. Firstly, kit vial and frozen semen were warmed to 35–38 °C within the same water bath. Straw was cut at a 60° angle using sharp scissors. The cut-end of straw was inserted through the rubber septum into the kit vial. The semen within the straw was then added into the vial by grasping both the vial and straw within the palm of the hand, and they were vigorously shaken downwards three or four times. Semen was gently mixed with the content of the vial. To transfer the enriched semen from the vial back into the straw, the vial and straw were grasped in an inverted position and vigorously shaken again downwards three to four times. Semen was incubated in a water bath

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at 35–38 °C for 15–20 min. Following the removal of the straw from the water bath, it was dried and cut routinely. Then, the straw was uploaded into the insemination catheter.

Insemination of animals. The recto-vaginal AI technique was used for the insemination of animals. Control groups were inseminated at the onset of standing oestrus. However, the animals in HP and BP groups were inseminated at least 16 h after the onset of standing oestrus according to the manufacturer's instructions. In addition, the approval of animal owners was taken before the AI of their animals for the use frozen-thawed semen treated with HP or BP in accordance with the suggestion of the Local Ethics Committee.

Determination of pregnancy, foetal sex and some reproductive parameters. Pregnancy was determined by trans-rectal ultrasonography using B-Mode Real-Time ultrasound with a 7.5 MHz rectal probe (Falco Vet, Pie Medical, Maastricht, Netherlands) on Day 23 after the AI by detecting the embryonic heartbeat. Foetal sex was determined on Day 75 after the AI by ultrasonography and was confirmed by parturition. Embryonic death rate between Days 23 and 75 of gestation, abortion rate after day 75 of gestation, stillbirth and twinning rates in all groups were also determined and recorded accordingly.

Data analysis. The SPSS programme (Version 21.0; Chicago, IL, USA) was used for data analysis. Data are presented as mean ± SEM, and a value of $P < 0.05$ was considered as significant. Firstly, the Shapiro-Wilk Normality test was used for all parameters to determine whether the raw data showed normal distribution or not. According to the Shapiro-Wilk Normality test, the raw data of all parameters had normal distribution. The differences in gestation length were compared using an independent samples-*t*-test. Chi-square test was performed to determine the differences in pregnancy rates, female and male calf ratios, and other reproductive parameters.

RESULTS

HP effect on pregnancy rate, foetal sex ratio and other reproductive parameters

The rates of pregnancy, embryonic death, abortion, stillbirth, female/male sex, twinning, and gestation length in HP and corresponding control groups are presented in Table 1. No significant difference was observed between control [56.0% (28/50)] and HP [52.0% (26/50)] groups for the pregnancy rate. Also, there was no significant effect on female calf ratio in cows inseminated with frozen-thawed semen

Table 1. Some reproductive outcomes in Heiferplus (HP)- and Bullplus (BP)-treated groups and their corresponding controls. Data are expressed as mean ± SEM

Parameters	Holstein cows			Simmental cows		
	control	HP	*P*-value	control	BP	*P*-value
Pregnancy rate (%) on Day 23 of gestation	56.0 (28/50)	52.0 (26/50)	0.688	58.0 (29/50)	64.0 (32/50)	0.539
Embryonic death rate (%) between Days 23 and 75 of gestation	3.6 (1/28)	3.9 (1/26)	1.000	3.5 (1/29)	0.0 (0/32)	0.960
Abortion rate (%) between Day 75 to gestation	0.0 (0/27)	4.0 (1/25)	0.969	0.0 (0/28)	3.1 (1/32)	1.000
Stillbirth rate (%)	0.0 (0/27)	0.0 (0/24)		0.0 (0/28)	0.0 (0/31)	
Female calf rate (%)	44.4 (12/27)	52.0 (13/25)	0.586	60.7 (17/28)	46.9 (15/32)	0.284
Male calf rate (%)	55.6 (15/27)	48.0 (12/25)	0.586	39.3 (11/28)	53.1 (17/32)	0.284
Twinning rate (%)	0.0 (0/27)	8.0 (2/25)	0.437	3.6 (1/28)	6.3 (2/32)	1.000
Gestation length (days)						
bearing female calf	281.2 ± 1.6 (n = 12)	280.1 ± 1.2 (n = 13)	0.603	280.6 ± 1.6 (n = 17)	279.8 ± 1.5 (n = 15)	0.721
bearing male calf	280.8 ± 1.3 (n = 15)	277.2 ± 1.5 (n = 11)	0.072	286.7 ± 2.0 (n = 11)	287.4 ± 1.6 (n = 16)	0.785
total	281.0 ± 1.0 (n = 27)	278.6 ± 1.0 (n = 24)	0.100	283.0 ± 1.4 (n = 28)	283.9 ± 1.3 (n = 31)	0.647

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treated with HP [52.0% (13/25)] compared to the control group [44.4% (12/27)]. There was only one embryonic death in the control group, while one embryonic death, one abortion and two twinning cases were observed in the HP group. No stillbirths were observed in the control and HP groups.

BP effect on pregnancy rate, foetal sex ratio and other reproductive parameters

The rates of pregnancy, embryonic death, abortion, stillbirth, female/male sex, twinning, and gestation length in BP and corresponding control groups are presented in Table 1. Treatment of frozen-thawed semen with BP [64.0% (32/50)] did not lead to any significant effect on the pregnancy rate in comparison to the control group [58.0% (29/50)]. There was only one embryonic death and one twinning case in the control group, while one abortion and two twinnings were observed in the BP group. No stillbirths were observed in either the control or BP groups. Control cows had 60.7% (17/28) female and 39.3% (11/28) male calves. The AI of cows with frozen-thawed semen treated with BP resulted in 46.9% (15/32) female and 53.1% (17/32) male calves. Although cows in the BP group had a numerically greater number of male calves (a 13.8% increase) than the control cows, the Chi-square analysis showed no statistically significant difference between the control and BP groups.

DISCUSSION

Currently, a commercial company collectively claims that;(i) the AI of cows with frozen-thawed semen treated with HP and BP could result in increases in the number of female and male calves born, respectively, at the rate of 20–25%, (ii) the use of HP and BP is very easy (practical) in field conditions, and (iii) the costs are relatively cheap ($6.50 per vial for HP and $10.00 per vial for BP). However, there have been important differences between the results of the manufacturer (Williams 2007) and the findings of several studies, as well as between the studies regarding the efficacy of the HP (Gerard et al. 2008; Curry et al. 2009) kit on the sex ratio. Although the *in vitro* efficacy of the BP kit has been reported in conference proceedings, there has as of yet been no *in vivo* study published in any scientific journal regarding the efficacy of the BP kit, according to our knowledge. Therefore, in this study, the changes in pregnancy rates, the ratio of female and male calves, embryonic death, abortion, twinning rates and gestation lengths were examined to evaluate the *in vivo* efficacy of HP in Holstein and BP in Simmental cows, respectively. In addition, to minimise bull-specific effects on the sex ratios, the straws produced from only a single ejaculate of one bull for both Holstein and Simmental cows were used in this study.

It has been reported that (Gerard et al. 2008) the use of frozen-thawed semen treated with HP and BP led to lower rates of *in vitro* fertilised eggs (44.7% for HP, 54.0% for BP and 77.7% for control) as compared to the control group. However, Curry et al. (2009) have reported that the treatment of frozen-thawed semen with HP did not affect the number of embryos collected (4.76 in HP group, 3.55 in control group) in ovarian hyperstimulated, or the pregnancy rates (54.5% in HP group, 48.0% in control group) in single-ovulating cows. Besides, the manufacturer has also claimed that these kits provide an increase in the pregnancy rates by at least at 5–20%. In this study, no statistically significant difference was found in pregnancy rates between the HP group [52.0% (26/50)] and corresponding control [56.0% (28/50)], and between the BP group [64.0% (32/50)] and corresponding control [58.0% (29/50)]. These results are in agreement with the findings of Curry et al. (2009).

In the studies (involving controls) of Curry et al. (2009) and Gerard et al. (2008), the female calf ratios in the HP group were found to be 45.2% and 69.0%, respectively. In the present study, this ratio with HP was 52.0%. In terms of BP effect on male calf ratio, Gerard et al. (2008) found the male embryo rate to be 39.9% in the BP group, while the male calf rate with BP was 53.1% in this study. However, in the six consecutive studies of the manufacturer (Williams 2007), that used no control animals and no individual bull data, the mean female calf ratio with HP was determined to be 79% (54/68). If a 50% female/male calf ratio is the theoretical consensus (optimal female/male ratio) in the bovine population with large numbers, the results of the present study show that HP and BP did not in actual fact affect the female (52.0%) and male (53.1%) calf ratios, respectively. However, when compared to corresponding controls, HP and BP provided slight, non-significant increases in female (7.6%)

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and male (13.8%) calf ratios, respectively. Yet, these increases did not reach the 20–25% rates in desired sex ratios, as claimed by the kit manufacturer. In this respect, we consider that the exclusion of control animals and individual bull data in the studies made by the manufacturer might be responsible for the contradiction between our results and the manufacturer's findings.

In conclusion, the present results suggest that treatment of semen with BP, but not with HP, numerically increases the percentage of pregnancy at the rates reported by the manufacturer (5–20%). Treatment of frozen-thawed semen with HP and BP provided only slight, non-significant increases in female (7.6%) and male (13.8%) calf ratios after AI, respectively. These increases did not reach the 20–25% rates in desired sex ratios, as claimed by the kit manufacturer. As the number of animals used in this study was limited, further investigations are needed in bovine populations with larger numbers of tested cows.

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