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EFFECT OF SEMINAL PH VALUE AT INSEMINATION ON SEX RATIO OF BOVINE EMBRYOS

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ABSTRACT

The objective of this study was to evaluate the effect of changes in seminal pH value at insemination on sex ratio of bovine embryos and confirm embryo sexing using Polymerase Chain Reaction (PCR). Eighteen Friesian cows superovulated with 2500 IU of PMSG and divided into three groups were used in this study. Cows in the first (G1), second (G2) and third (G3) group were artificially inseminated by diluted semen with pH values of 6.9 (control), 5.4 (acidic) and 8.4 (alkaline), respectively. Results showed insignificant ($P \geq 0.05$) effect of seminal pH value on ovulatory response and quality of recovered embryos. Change of seminal pH value to acidosis in (G2) produced higher ($P<0.01$) number of females (19/32, 59.4%) than males (13/32, 40.6%). Change of seminal pH value to alkaline (G3) produced higher ($P<0.01$) number of males (61.8%) than females (38.2%). However, sex ratio of cows inseminated with control semen (pH=6.9, G1) was 51.9% males: 48.2% females.

In conclusion, change of seminal pH value to alkaline tended to produce more males, while acidic semen tended to produce more females**.** Based on these results motility of spermatozoa bearing Y-chromosome may be affected by the acidic condition of the semen, while X-chromosome had an opposite trend.
Keywords: Superovulation, seminal pH value, embryo sexing, PCR.

INTRODUCTION

Embryo transfer (ET) technology is high importance in modern cattle breeding programs. Implementation of embryo sex determination techniques holds great potential for genetic improvement in cattle herds and market demand satisfaction (Lopatarova *et al.,* 2010).

The techniques for sex determination of bovine embryos have evolved from karyotyping of older preimplantation embryos (Hare *et al.,* 1976), but the current technique of DNA amplification by polymerase chain reaction (PCR) has improved the accuracy, efficiency, and speed of embryo sex detection (Herr and Reed, 1991).

Many reports have suggested that diet, particularly one enriched with either saturated or unsaturated fatty acids, can alter serum steroid concentrations in a variety of species, including rodents, animals, and humans (Hilakivi-Clarke *et al.,* 1996; Woods *et al.*, 1996; Hilakivi-Clarke *et al.,* 1997 and Dorgan *et al.,* 2003).

Sperm of one sex might have differential motility or make their way more directly to the oocyte than the other depending on the conditions prevailing in the reproductive tract of the impregnated female, e.g., state of cervical mucus, nutrient/energy status of tract secretions, vaginal pH relative to the precise time at which copulation occurred in relation to estrus (Pratt *et al*., 1987 and Martin 1997).

Shettles (1970) noted that at the time of ovulation the cervical mucus was mostly alkaline, of lowest viscosity, and conductive to sperm penetration

and survival. However, more acidic conditions which prevail during the remainder of the menstrual cycle, except for a day or so before and after ovulation, permit only the more fit sperm to survive. Although both X- and Y-bearing spermatozoa survive longer in the slightly alkaline environment found at ovulation, the smaller, faster, Y-sperm is more likely to reach the ovum first and fertilize it. Since the Y-sperm is more labile under acidic condition, the slower would survive to fertilize the ovum.

In addition, Wakim (1972) demonstrated an altered sex ratio in the offspring of rabbit in relation to the pH of the vagina at the time of mating. When the cervical pH value was 6.5 to 7.3, there was a predominance of female. There was difference in sex ratio with a pH value from 7.3 to 7.5. Values of pH from 7.5 to 8.3 produced more males. However, Unterberger (1932) suggested that a very alkaline seminal fluid favor males and a very acid vagina would favor females.

No reports are available on the effect of pH value of seminal fluid on sex ratio of bovine embryos.

The current study was planned to evaluate the effect of pH value of extended semen on sex ratio of bovine embryos.

MATERIALS AND METHODS

This work was carried out at Sakha and El-Karada Experimental Stations and International Livestock Management Training Center (ILMT), Sakha, belonging to Animal Production Research Institute (APRI) during the period from January 2009 to July 2010.

Experimental design:

Eighteen sexually mature Holstein Friesian cows and one fertile Friesian bull were used in this study. All cows were at 60-120 days postpartum, 2-3 parities and having displayed at least two estrous cycles of normal duration.

The experimental cows (n=18) were divided into three groups (6 cows in each). Cow in the first (G1), second (G2) and third (G3) groups were artificially inseminated by diluted semen with pH value of 6.9 (control), 5.4 (acidic) and 8.4 (alkaline), respectively.

Semen preparation:

Semen was collected with an artificial vagina from one Frisian bull and extended in tris-egg yolk extender (3.025 g tris, hydroxymethyl amino methane), 1.675 g citric acid, 0.75 g glucose, 15 ml egg yolk, 0.005 g streptomycin, 0.25 g lincospectin and completed with bi-distilled water up to 100 ml and considered as a control semen, pH= 6.9.

The control diluted semen was divided into three portions. The 1^{st} without any adjustment, while the 2^{nd} and 3^{rd} portions were adjusted to specific pH value of 5.4 and 8.4, respectively, using 0.1 N of citric acid and sodium hydroxide.

The diluted semen with different pH values was left for 20-30 minutes at 5 ^{o}C and packaged in 0.25-ml French straws (20 $X10^{6}$ spermatozoa/straw) thereafter to be used for insemination.

Superovulation:

Cows in all groups were injected with 2 ml $PGF_2\alpha$ (Estrumate, containing 263 µg of cloprostenol sodium BP, Vet., equivalent to 250 µg of cloprostenol, Friesoythe, Germany) to bring them in heat to start the estrous cycle. Based on the beginning of the oestrous cycle, superovulation was induced by i.m. injection of 2500 IU of PMSG/cow (Folligon, Intervit International BV. Boxmeer - Holland) on day 10 of the second synchronized estrus. After 48 h, each cow was received an i.m. injection of $PGF_2\alpha$ analogue (2 ml Estrumate, Coopers Animal Health LTD, Berkhamsted–England). Each ml of Estrumate contained 263 µg of Cloporostenol sodium equivalent to 250 µg Cloporostenol. Cows come in heat within 48 h from Estrumate injection were artificially inseminated twice at 12 h-interval (Ravindranatha and Reddy 1999).

Embryo recovery and classification:

Embryos were recovered on day 7 post-insemination by flushing the uterine horns with Dulbeco's phosphate-buffered saline (D-PBS solution, Nutricell-Brazil) supplemented with 1% of fetal calf serum (FCS, Sigma. Co.), 100 µg/ml streptomycin and 100 IU/ml penicillin, through a Foley catheter. The pH value of the medium was adjusted at 7.3-7.4 and osmolarity of 280-300 mOsmol/kg. The medium was filtrated by 0.22-µm millipore filter. The ovulatory response in term of total number of corpora lutea and embryos were recorded for each group.

The recovered embryos were classified according to their quality as, excellent, good, fair and poor (Reddy, 1994). Only, excellent and good embryos were used in this study.

Embryo biopsy:

Microsurgical intervention was carried out with the Lica micromanipulator (Leica, Germany). From the embryos specified for fresh transfer, a small portion (5-10%) of each embryo was cut off by ranszonal incision using a microsurgical blade as described by (Lopatarova et al., 2007).

No holding pipette was used during cutting, only DPBS without proteins was employed for fixation of embryos. During the analysis, the treated embryos were cultured in 4-well dishes containing 1 ml of PBS with 10% FCS per well at room temperature (20–25°C).

Sex determination:

Extraction of DNA:

DNA was extracted from the biopsies from the embryos. Commercial kits were used for DNA extraction from the embryo biopsies (Macherey-Nagel, Nucleospin tissue kit, 740952.50) according to the manufacturer's instructions (Canada).

Primer design:

The primers were designed to be specific for the bovine Y chromosome and bovine G3PDH genes. The sequences of the S4b primers were forward: (5'-CAAGTGCTGCAGGATGTGGAG-3' and Reverse: 5'-GAGTGAGATTTCTGGATCATATGGC-3') (Kageyama *et al.,* 2004).

Polymerase chain reaction (PCR):

The PCR mixture consisted of 3 µl of template DNA, 0.4 µM each Y-chromosome primer and 15 µl of PCR master mix (Fermentas) in a total volume of 25 µl. An amount of 25 µl of mineral oil was added to prevent evaporation. Amplification was performed in a Thermocycler (Biometra). The reaction condition consisted of initial denaturizing at 95 ^{o}C for 15 min, followed by 15 cycles of 97 ^{o}C for 8 s, 50 ^{o}C for 25 s, at 72 ^{o}C for 15 s, and 30 cycles of shuttle PCR at 98 ^{o}C for 8 s and at 66 ^{o}C for 20 s, followed by incubation for 5 min at 72 ^{o}C (Hirayama *et al.,* 2004).

After PCR, the amplification products were electrophoresed on a 2% agarose gel (Pharmacia LKB Biotechnology, Uppsala), stained with ethidium bromide (10 mg/ml in distilled water) and evaluated using ultraviolet light. Using the bovine control primers in the reaction, the presence of two bands (one male–specific and one autosomal bovine specific) is interpreted as a male embryos, when the male–specific band is absent and the bovine specific band visible, the embryo is considered as a female, the lack of the two bands indicates the absence of embryonic DNA in the sample.

Statistical analyses:

Data were statistically analyzed according to Snedecor and Cochran (1982) using computer programme of SAS (1987). The significant differences were determined according to Duncan's Multiple Range Test (1955).

RESULTS

Results in Table (1) show no significant effect of seminal pH value on number of corpora lutea (CLs)/cow and total number of recovered embryos/cow. The recovery rate of embryos was significantly (P<0.05) higher in G2 (87.1%) than in G1 (74.4%). While the difference in recovery rate between G2 and G3 was insignificant.

Table (1): Means and standard errors of ovulatory response of superovulated cows in experimental groups.

Item	Seminal pH value		
	G1 (pH=6.9)	G2 (pH=5.4)	G3 (pH=8.4)
Number of corpore lutea/cow	7.8±0.58	7.7±0.84	8.3±0.96
Number of recovered embryos/cow	5.8±0.44	6.7±0.73	6.8±0.90
Recovery rate (%)	74.4±0.51^{b}	87.1±0.78^{a}	81.9±0.94^{ab}
Number of embryos/cow (based on quality):			
Excellent	1.8±0.13	2.1±0.13	2.0±0.33
Good	1.5±0.19	1.8±0.23	2.2±0.31
Fair	1.6±0.32	1.7±0.45	1.7±0.37
Poor	0.9±0.13	1.1±0.19	0.9±0.13

a and b: Means denoted within the same row with different superscripts are significantly different at (P< 0.05).

On the other hand, the effect of seminal pH value on embryo quality in term of number of excellent, good, fair and poor embryos/cow was not significant.

Results presented in in Table (2) show that the sexing of 93 bovine embryos by PCR analysis resulted in identification of 48 males (51.6 %) and 45 female (48.4%) embryos for all groups. Cows in G2 produced significantly (P<0.01) lower male embryos (13/32, 40.6%) than those in G3 (21/34, 61.7%). An opposite trend was occurred for female embryos of both groups. However, the differences in males or females produced by cows in G1 did not differ significantly than that in each of G2 and G3.

Table (2): Effect seminal pH value on sex ratio of bovine embryos in different experimental groups.

Group	Total number of embryos	Male embryos		Female embryos	
		n	%	n	%
G1(pH=6.9)	27	14	51.85^{ab}	13	48.15^{ab}
G2 (pH=5.4)	32	13	40.63^{b}	19	59.38^{a}
G3 (pH=8.4)	34	21	61.76^{a}	13	38.24^{b}

a and b: Means denoted within the same column with different superscripts are significantly different at (P<0.05).

DISCUSSION

The commercial PCR-sexing protocols for bovine embryos involve the use of Y-specific primers in co-amplification of bovine control primers, reporting 90-95% of efficiency and 93-98% of accuracy (Nibart et al., 1997; Roschlau et al., 1997; Shea, 1999; Thibier and Nibart, 1995 and Zoheir and Allam, 2010) according to biopsy procedure.

In the first, ovulatory response and embryo quality were not influenced by the change in seminal pH value in different experimental groups. As expected the present results regard the control semen was normal for sex ratio of male and female embryos (51.85: 48.15). However, the obtained results for G2 and G3 indicated that the change in pH value of extended semen to alkaline (G3) produced more males than females, while, the acidic semen (G2) produced more females than males, the difference was highly significantly higher.

These results may reflect that spermatozoa bearing Y-chromosome may be affected by acidosis condition of the surrounding medium, while, those bearing X-chromosome may be affected by alkaline conditions. In this respect, Pratt et al. (2004) studied the relationship between sex ratio and vaginal pH at certain times of mating. They found significant negative correlation between vaginal pH and sex ratio of subsequent litters when matings occurred during midestrus. They concluded that fluctuations in vaginal pH may differentially affect longevity, motility, or fertilizing capacity of X- and Y-sperm and/or may reflect other physiological changes in the female which occur over the receptive period.

In the other species, Emmens (1960) treated rabbit semen with lactic acid or sodium bicarbonate prior to artificial insemination. He obtained no offspring at PH from 3.8 to 5.1, 29 males and 35 females at pH from 5.6 to 6.2 and 20 males and 23 females at pH from 7.9 to 9.2. Using other parameters for determination sex ratio, Avery et al. (1989) examined sex ratio of sixty four bovine embryos from superovulated and inseminated cows. The

embryos were cultured for 12 hours, during which their cleavage rates were judged every second hour, followed by allocation into the fast, intermediate or slow group. Sex was determined by karyotyping substantiated the sex of twenty nine of the embryos. They recorded seven males and two females in the fast group, three males and eight females in the intermediate group, and one male and eight females in the slow group.

King et al. (1992) reported significant difference in the sex ratio of morulae bisected and cultured either *in vitro* or *in vivo* for 24-30 h, probably due to that the environmental condition around bisected embryos resulted in a specific loss of female embryos. Keefer et al. (1994) report a higher male to female ratio in bovine embryos biopsied for sexing by PCR analysis.

Tominaga (2004) found that the rate of male embryos recovered 7 days after insemination was influenced by sires. Avery et al. (1989) determined sex ratio of bovine embryos using karyotyping after flushing and cultured for 12 h, embryos divided into three categories according to developmental stage (slow, intermediate and fast). They found that slow and intermediate of developmental stage produced more number of females, while, the fast developmental stage produced more number of males.

In conclusion, results obtained from this study showed that the changes in pH of diluted semen at the time of insemination to alkaline (pH=8.4) tended to produce more males, while, acidic semen (pH=5.4) tended to produce more females than males in superovulated cows. These results indicated that the efficiency and accuracy of the sexing procedures by PCR are compatible with commercial use of sexing in embryo transfer programs in dairy cattle.

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تأثير درجة حموضة السائل المنوى أثناء التلقيح على النسبة الجنسية لأجنة الابقار

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تهدف هذه الدراسة الى تقدير مدى تأثير التغير في درجة حموضة السائل المنوي على النسبة الجنسية لاجنة الأبقار والتأكد من جنس الأجنة باستخدام PCR.

تم عمل برنامج التبويض المتعدد لعدد 18 بقرة فريزيان من محطتي سخا والقرضا باستخدام 2500 وحدة دولية من هرمون الفرس الحامل PMSG ،تم تقسيم الأبقار إلى ثلاث مجموعات ،المجموعة الأولى كنترول،تم تلقيحها بسائل منوي درجة حموضته (6.9)، والمجموعة الثانية تم تلقيحها بسائل منوي درجة حموضته (5.4) حامضى ، والمجموعة الثالثة تم تلقيحها بسائل منوي درجة حموضته (8.4) قاعدي

أظهرت النتائج أن تأثير درجة حموضه السائل المنوى فى المجاميع المختلفة على الاستجابة المبيضية وجودة الأجنة المتحصل عليها غير معنوى. كما أوضحت النتائج أن التغير في درجة حموضة السائل المنوي إلى حامضى (في المجموعة الثانية) نتج عنها عدد من الإناث أعلى من الذكور (59.38 و 40.63% للذكور والإناث، على الترتيب) بينما نتج عن التغير في درجة حموضة السائل المنوي إلى القلوي (المجموعة الثالثة) عدد من الذكور أعلى من الإناث (61.76 و38.24% لكل من الذكور والإناث، على الترتيب) ،بينما نتج التلقيح باستخدام السائل المنوي في المجموعة الأولى عن عدد متساوى تقريبا من الذكور والإناث(51.85 و 48.15% للذكور والإناث، على الترتيب) ، هذه النتائج تعكس تأثر الحيوانات المنويه الحامله لكروموسوم Y بالحموضة، وتأثر تلك التى تحمل الكر وموسوم X بالقلوية.

نستخلص من ذلك أن، التغير في درجة حموضة السائل المنوي إلى القلوية نتج عنه عدد أكبر من الذكور عن الإناث، بينما كان التغير إلى الحامضية في السائل المنوي نتج عنه عدد أكبر من الإناث عن الذكور. أيضا أظهرت النتائج أنه يمكن استخدام الـ PCR كتقنية لتجنيس أجنة الأبقار بنجاح.

قام بتحكيم البحث

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