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The Effect of Diffrent Factors on X- and Y Bearing Sperm in Bull Semen

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Abstract:

BACKGROUND: Newborn gender ratio is one of the most important factors in livestock industry. This ratio has affected on economic condition and controls sex-linked genetically transferred diseases. Offspring sex ratio is affected by such various factors. One of these factors is the Y to X bearing sperm ratio in normal semen that is called primary sex ratio, season and individual differences are important factors that can have an effect on primary sex ratio.

OBJECTIVES: The objective of this study was to investigate two main effective factors, Individual difference and season, on proportion of sperms containing Y-chromosomes to sperms containing X-chromosomes in bull semen.

METHODS: Semens were collected from 16 bulls during summer (Jul-Aug) and winter (Dec. to Jun). After sperm analysis, DNAs were extracted and Real-time PCR performed using three sets of primers to study SRY (Y-chromosome specific sequence), PLP (X-chromosome specific sequence) and PAR (Housekeeping) genes.

RESULTS: The results showed that the sex ratio of sperms in normal ejaculation was not equal in different bulls. Moreover, season can affect primary sex ratio. Y-bearing sperm increase in summer.

CONCLUSIONS: We concluded that the primary sex ratio is affected by individual differences and season. We also suggested using Northern blotting on PCR products to confirm results.

Keywords:

Individual difference, Real-time PCR, Season, Sex ratio, Sperm

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Introduction

Offspring sex ratio is an important statistic index in livestock industry (Delgado et al., 2014), which is expected to be 1:1 in large populations. But recent studies showed this proportion can vary significantly from expected rate (Chandler et al., 2007, Amadis et al., 2015).

In livestock industry, sex control has a special economic importance therefore causes and mechanisms of sex ratio are hot subjects for investigators (Seidel, 2003, Seidel, 2011, Miliaras et al., 2011).

Several hypotheses have been made to explain sex deviation in newborns. Cervical mucus, female reproductive tract secretions and metabolites present in the vaginal secretion and pH have been studied (Grant, 1994). Recently, Berry et al. (2011) proposed the following possibilities to explain the variations in the sex ratio: (1) sires may ejaculate unequal proportions of the X and Y-bearing spermatozoa; (2) differences may occur in the postejaculation and prefertilization (rates of capacitation and survival between the X- and Y-bearing spermatozoa); (3) fertilization rates may be different between the X- and Y-bearing spermatozoa; or (4) differences may occur in the rate of intrauterine embryonic losses at different stages of development. According to this hypothesis primary sex ratio of semen has critical effect on sex of newborn.

Male gametes have two different genotypes, therefore, change in proportion of X to Y bearing sperm can change secondary sex ratio (Dadpasand, 2012, Chandler, et al., 2007) and so can act as a control tool for offspring sex proportion. Theoretically, the proportion of X- to Y-chromosome bearing sperm in normal bull ejaculation is 1:1 but certain studies have shown that Y to X sperm proportion is not 1:1 (Colley et al., 2008). Environmental factors, inheritance, nutrition, season, diseases, level of Gonadotropins and steroid hormones, society and stress impress this ratio (Delgado at al., 2014).

Season has been statistically associated with secondary sex ratio (Lerchl et al., 1982). Hoseinzade (2012) reported a significant varity in male to female calving rate in different seasons. Roche et al (2006) and Berry and Cromie (2007) reported a significant effect of season on conception sex ratio. But some researchers did not report any relation between season and sex ratio (Yilmaz et al., 2012). Difference in reports may be due to difference of weather or increase in usage of sexed semen.

There are various methods for investigating the spermatozoa sex ratio, such as flow cytometry, fluorescence in situ hybridization (FISH) and quantitative polymerase chain reaction (real-time PCR) (Halnan and Watson 1982). Some advantages of Real-Time PCR are: the production is observable at any time during the reaction, the process can be stopped when necessary, the detection range is high and there exist quantitative (Edwards and Logan 2009).

The aim of this study was to clarify effects of season and individual difference on primary sex ratio in normal bull semen. Real-Time PCR technique was performed using three sets of primers to evaluate SRY (Y chromosome marker), PLP (X chromosome marker) and PAR (Housekeeping gene). ΔCt value was determined for each gene and statistically analyzed using SAS computer software.

Materials and Methods

Materials: All chemical materials used in DNA extraction were obtained from Merck Company (Darmstadt, Germany). Soils BioDyne (Cat. No: 08-24-0000S, Estonia) commercial kit was utilized to amplify the DNA fragments in qPCR.

Semen collection: Sixteen healthy male Holstein cattle, belonging to a commercial breeding center in Tehran-Iran, were used in this research. The cattle were provided with a standard, daily diet comprised of (49% barley, 18% corn, 11.9% soya, 19.35% bran, 0.5% salt, 0.6% minerals, and 0.15% multi-vitamins). Sperm samples were taken using an artificial vagina in the early morning in August and February. All samples were appraised by microscopic and macroscopic analysis where only good quality sperms were employed.

Sperm DNA extraction: Total DNA was extracted from samples using salting-out protocol (Kholghi et al, 2014). Quality and quantity of DNA were estimated by Nanodrop spectrophotometer and agarose-gel electrophoresis.

Target sequences and primers: Selected genes were PAR gene (Pseudoautosomal region) located at the end of the Y- and X-chromosome long arm ((NCBI number: AC234910.2), as housekeeping gene, SRY (Sex determining region) located at the end of the Y-chromosome short arm (NCBI number: EU581861.1) as Y-chromosome marker gene, and PLP (Proteolipid protein) located at the end of the X-chromosome long arm (NCBI number: AJ009913.1) as X-chromosome marker gene. Gene-specific primers were designed using Oligo 6 software (Molecular Biology Insights) and analyzed for secondary structures and dimer formation (Table 1).

Real-time PCR reaction: Real-time PCR was performed using standard protocols (Edwards et al, 2009). We employed GreenStar™ qPCR PreMix (Bioneer) in 20 µl volumes, including 10 µl PreMix, 100 ng sperm DNA and 5 pmol primers. The reaction was carried out in StepOne plus thermal cycler (Applied Biosystems) with the condition that was mentioned in Table 2.

Statistical analysis: The data obtained from the qPCR were analyzed according to the method outlined by Livak & Schmittgen (2001). The PAR gene served as a reference gene in order to estimate the initial Ct values. The mean Ct value was calculated for PAR and each of the two studied genes (PLP and SRY) and ΔCt value was determined for each gene using the following formula:

$$\Delta Ct = Ct_{(target\ gene)} - Ct_{(PAR)}$$

Following the calculation of ΔCt for all samples taken from cattle spermatozoids, the status of PLP and SRY genes relative to PAR was estimated using the following formula:

$$RE = 2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct\ (SRY) - \Delta Ct\ (PLP)}$$

All data were normalized statistically analyzed using SAS computer software version 9.1 (SAS Institute Inc., Cary, NC, USA). The normality of data was tested by a univariate procedure, after which the mean values were exposed to t-tests.

Results

Melting curve quantity assay: Product specificity was confirmed via melting curve plotting. Melting temperatures in both warm and cold seasons were 80.6, 77.07 and 81.9 for SRY gene, PLP gene and PAR gene, respectively.

Table 1. General properties of specific primer pairs in PAR (as reference gene), and PLP and SRY genes used for expression analysis.

Gene Name	Accession number	Sense primer sequence 5′→3′	Anti-sense primer sequence 5′→3′	Length (bp)	Tm (°C)
PLP	AF548366.1	GCCATCACATCTGAGACCAC	GACTCAGCATCTCGAAGCAA	197	59.8
SRY	EF583947.1	CTCAGACATCAGCAAGCAGC	GTAGTCTCTGTGCCTCCTCA	89	59.8
PAR	EF105407.1	GAGGGAGGGTGGATCATAGA	CCTCTGGGACCTTCAACAAT	90	59.8

Table2. Time and Temperature conditions of Real-time PCR.

Stage	phase	Time (Sec)	Temperature (◦c)	Cycle number
Holding	1	5	95	1
Cycling	Denaturation	10	95	
	Annealing	30	60	40
	Extension	30	72	
Melt curve	1	15	95	1
	2	60	60	1
	3	15	95	1

Table 3. The ratio of Y to X chromosome-bearing spermatozoids in different seasons. [a, b] Values with different superscripts within the same row differ significantly (P<0.05).

Gene	season	Y/X ratio (Mean±SD)	Max.	Min.
SRY /PLPgene	warm	1.83±0.5[a]	4.65	0.44
	Cold	1.07±0.42[b]	1.98	0.52

Table 4. The percentages of X- and Y-chromosome-bearing spermatozoids in different seasons. [a,b,c and d] Values with different superscripts within the same row differ significantly (P<0.05).

Gene	season	Percentage of X and Y (Mean±SD)	Max.	Min.
SRY gene	warm	59.2±15.2[a]	82	30
	Cold	49.6±9.7	66	34
PLP gene	warm	40.2±15.2[c]	70	18
	cold	50.3±9.6d	66	34

The three studied genes provided a single peak in the melting curve, implying the absence of primer-dimer formation during the reaction.

The average difference of Ct in SRY and the PLP related to the PAR (reference gene) was used to examine the ratio of X- and Y-chromosome bearing spermatozoids (Table 3). The Mean±SD of Y- to X-chromosome bearing sperm ratio was 1.83±0.5 (ranging from 4.65 to 0.44) in the warm season and 1.07±0.42 (ranging from 1.98 to 0.52) in the cold season. Table 4 illustrates percentages of each kind of sperm (Mean±SD, and the maximum and minimum). Under warm conditions, the percentage of Y-bearing sperm was 30-82% (Mean±SD = 59.2±15.2). X-bearing sperm percentages was 18-70% (Mean±SD = 40.2±15.2). In the cold season, meanwhile, the percentages of Y-bearing sperm fell between 34-66% (Mean±SD = 49.6±9.7). X-bearing sperm percentages varied between 34-66% (Mean±SD = 50.4± 9.6). In the warm season, 62.5% of the samples carried more Y-chromosome bearing sperm (the percentage of Y-bearing chromosomes was more than 52%) and 37.5% of the sam-

ples carried more X-chromosome bearing sperm (the percentage of X-bearing chromosome was more than 52%); on the other hand, none of the samples were the same ratio in Y to X bearing chromosome. Under cold conditions, 37.5% of the samples had more Y-chromosome bearing sperm (the percentage of Y-bearing chromosome was more than 52%), 43.75% of samples contained more X-chromosome bearing sperm (the percentage of X-bearing chromosome was more than 52%) and 18.75% of the samples were equal (the Y- and X-bearing chromosome sperm ranging between 48-52%). A significant difference was found between the seasons regarding the frequency of SRY- and PLP-carrying spermatozoids. Moreover, the X- and Y-chromosome bearing sperm ratio was affected by individual differences.

Discussion

There is strong evidence that the sex ratio at birth is partially determined by environmental and social factors (Pavic, 2014). Hypothetically, the X- or Y-bearing spermatozoa have equal chances to fertilize an oocyte, which results in a theoretical 1:1 sex ratio at the time of conception. However, this theoretical ratio is not actually observed at birth (Machado, et al, 2015). It has been reported that several factors associated with bull affect secondary sex ratio (Berry and Cromie 2007). And also male calf is significantly higher when AI was used compared with natural service (Khan et al, 2012)

The results of this study showed that heat stress augmented the Y to X-chromosome bearing sperm population. The effect of individual differences on sperm sex ratio was further observed in more than 90% of the samples (18.75% of samples collected in cold season were equal).

Previously, Checa et al. (2002) reported the average of 50.02 ± 2.79 for X-chromosome bearing sperms in semen by using Capillary electrophoresis technique. Colley et al. (2008) reported 44% of the sperm carrier X-chromosome, the used technique was Real-Time PCR. They reported that two of each six sperms were X-sperm and four were Y-sperm. PLP, SRY and Amilogenin genes were used in their experiment . Lobel et al. (1993) tested 98 human sperm samples. Their studied genes were ZFY (Y specific sequence) and ZFX (X specific sequence). They reported that 41.9-56.7% of the sperms contained Y chromosome in the semen. Another research has similarly reported 46.9-52.7% Y-sperms in each ejaculation (Mathews, et al 2005). Madrid et al (2003) reported bulls and ejaculates did not have any effect on the Y-CDBS percentage or on the sex ratio of embryos produced in vitro using these ejaculates. However, double swim-up sperm preparation method produced differences in Y-CDBS percentage in some of the sperm fractions. This suggested that there are intrinsic differences in capacitating of X- and Y-bearing spermatozoa that might be used to produce embryos of the desired sex with in vitro fertilization (Madrid-Bury et al, 2003). However, in two different studies on bull semen, it became clear that the Y-sperm population was between 24-84% in each ejaculation (Chandler et al 1998; Chandler et al 2002).The difference between previous studies may be due to the breeds of the employed bulls (Holstein and Galicia) or PCR technique. Chandler et al (2007) studies further investigated the correlation between the numbers of Y- and X-chromosome bearing sperms in

semen (primary sex ratio) with the sex proportion in offspring (secondary sex ratio). They concluded that the offspring population conforms to the primary sex ratio. Paul et al. (2003), using the RT-PCR and conventional PCR techniques, observed similar results in pig semen. Hence, the sperm sex ratio in most mammals was not 1:1. However, Amadis et al did not agree with these results. They believed that the primary and secondary sex ratio is equal and there was no correlation between primary and secondary sex ratio. Their data are in conflict with many researches and it may be due to the Real-Time PCR technique that did not use housekeeping gene.

Heat stress affects not only the sperm sex ratio in bulls, but also other reproductive performance. In Japan, Kadowa et al. (2012) studied reproductive index under heat stress. They found that heat stress induces hyperthermia, ensues death in certain cases, and reduces milk production, growth rate reproduction performance and other factors such as oocyte quality, embryo growth, irregular estrus and female sex hormone secretion. Afterwards, in warm seasons, reproductive changes, and perhaps the high survival resistance of Y-sperms to X-sperms, were thought to be some of the most important factors increasing male birth under warm conditions. A temperature of more than 39 °C in the female rectal temperature and a breathing rate of more than 60 times per minute reduce milk yield and fertility (Rensis, et al 2003). For each 0.5 °C rise in temperature, pregnancy rate decreases by 12%. Also, embryo growth is slower and more difficult during pregnancy under these conditions, while the number of male embryo increases (Dikmen et al, 2012). Rensis et al. (2003) investigated the effect of heat stress on fertility, its consequences and possible solutions. They reported that heat stress negatively affected the reproduction and pregnancy in cows. Their proposed solution was evaporative cooling for dry cows, which may not only improve milk production, but also the reproductive performance during summer. To improve fertility under heat stress, there exist certain procedures including embryo and genetic manipulation, and nutraceuticals and managerial change. Adverse effect of heat stress on male reproduction was determined by Perez-Crespo et al (2008). Number of spermatozoa, sperm viability, motility and progressive motility, sperm DNA integrity were tested by the TUNEL method. Spermatocytes under heat stress showed lower concentration and reduced viability and low motility. Even though DNA integrity of spermatozoa resulting from spermatocytes was also compromised by heat stress, the higher degree of DNA damage was found among spermatozoa. At last, heat shock effect on spermatozoa present in the epididymis at the time of thermal stress resulted in a sex ratio distortion. These findings point to a higher sensitivity of spermatocytes to heat exposure and also suggest a different response of X and Y chromosome-bearing spermatozoa to heat stress that warrants further investigation.

Conclusion: There is a possible correlation between primary sex ratio with male sex hormones and the secondary sex ratio is not only affected by primary sex ratio, but also the female physiology; animal body conditions influence X- and Y-chromosome bearing sperm options.

Considering the studies on the correlation between fertility and heat stress, it is concluded that female hormones are effective

in female physiology. Accordingly, there is a possible association between male and female sex hormones in bovines and, perhaps, all mammals with offspring sex ratio, a pertinent subject for future studies. We can propose to use northern blotting technique to confirm the results of experiment.

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مجله طب دامی ایران، ۱۳۹۷، دوره ۱۲، شماره ۳، ۲۲۶-۲۱۹

مطالعه تأثیر فاکتورهای مختلف بر نسبت جمعیتی اسپرم‌های واجد کروموزوم جنسی در منی گاو

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چکیده

زمینه مطالعه: جنسیت موالید یکی از مهمترین فاکتورها در اقتصاد دامداری صنعتی است زیرا در بهبود شرایط اقتصادی و بیماری‌های وابسته به ژن نقش دارد. جنسیت موالید به عوامل گوناگونی ربط دارد. یکی از این عوامل نسبت جنسیتی اسپرم‌های دام نر است. تنش گرمایی و تفاوت‌های فردی می‌توانند نسبت جنسیتی اسپرم را تحت تأثیر قرار دهند.

هدف: هدف از انجام این تحقیق، بررسی تأثیر دو فاکتور مهم، تفاوت‌های فردی و فصل، برنسبت اسپرم‌های واجد کروموزوم Y بر اسپرمهای واجد کروموزوم X در انزال تازه گاو بود.

مواد و روش کار: نمونه‌ها ی اسپرم از ۱۶ راس گاو هلشتاین در دو قصل گرم (مرداد ماه) و سرد (اذر ماه) اخذ شد. پس ارزیابی کمی و کیفی اسپرم‌ها، DNA استخراج و با استفاده از سه سری آغازگر برای تکثیر ژن SRY (بعنوان معرف کروموزوم Y)، PLP (معرف کروموزوم X) و PAR (ژن خانه دار) Real-Time PCR انجام شد. نتایج با استفاده از انالیز اماری مورد بررسی قرار گرفت.

نتایج: نتایج این تحقیق نشان داد که اولا نسبت جنسیتی در اسپرم یکسان و ۵۰-۵۰ نبوده و متأثر از تفاوت‌های فردی است و نیز تنش گرمایی باعث افزایش نسبت جنسیتی به نفع جنس نر می‌شود.

نتیجه‌گیری نهایی: چنین می‌توان نایجه گرفت که اولاً نسبت جنسیتی در جمعیت اسپرم انزالی یکسان نیست و متأثر از عوامل داخلی و خارجی متعدد است و نیز فصل می‌تواند منجر به افزایش تولد موالید نر گردد. به منظور تأیید نتایج بهتر است تکنیک Real-Time PCR بر روی محصول Northern blotting صورت پذیرد

واژه‌های کلیدی: تفاوت‌های فردی، Real-Time PCR، تنش گرمایی، نسبت جنسیتی، اسپرم

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