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Research Article

Blood Testosterone Level Affects Sex Ratio of Bull Semen

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Abstract

Sex ratio has a direct impact on livestock economy and controling sex-linked genetically diseases. Offspring sex ratio is affected by such various factors. One of these factors is the Y/X-hromosome bearing sperm ratio in fertile specimen. This study was conducted to explore the effect of testosterone concentrations of blood and semen on the relative frequency of Y/X -chromosome bearing sperm in Holstein bovine semen. Blood and semen testosterone level were measured by ELISA technique. Quantitative real-time PCR was performed to estimate the ratio between sperm with Proteolipid Protein (PLP) and the Sex-Related Y (SRY) genes, locating on non-homologous regions of X and Y chromosomes. Blood and semen samples of 26 Holstein bovines were taken simultaneously. DNA was extracted from semen sampls and real-time PCR was performed to amplify the fragments of 90, 89, and 79 base pairs (bp) for PLP, SRY and PAR (as reference) genes, respectively. Wide variation was shown in Y- and X - chromosome bearing sperm, ranging between 18-82%. The least mean square of Y-bearing sperm (1.23±0.15) was significantly higher than that of X-bearing sperm (0.71±0.02). The correlation coefficients of SRY and PLP with blood and semen concentration of testosterone were 0.38, 0.47, -0.67 and -0.60, respectively. The results demonstrated that higher testosterone levels are probably associated with a higher proportion of Y- bearing sperm. A significant positive correlation ($P<0.05$) was detected between the age of cattle and the ratio of Y-bearing sperm. The testosterone concentration of blood and semen was positively correlated to the cattle age ($P<0.05$). The results may provide insights into the effects of paternal testosterone on sex ratio of sperm transferred to females.

Keywords: Real-time PCR, Semen, Sex ratio, Testosterone

Introduction

Offspring sex ratio is an important statistic index defined as the proportion of males to females newborn[1], which expected to be 1:1 in populations. But recent researches have shown this proportion can vary significantly from the expected rate [2,3]. In the livestock industry, sex preselection of offspring has a special economic importance, therefore causes and mechanisms of sex selection are hot subjects for investigators[4-6]. From 1970 onwards, factors affecting sex ratio have been studied and role of breed and genetic, season, nutrition, age and weight of parent, gestation periods, male ejaculation frequency, time of insemination, different movement speed of the X- and Y-chromosome bearing sperm were studied[7-10]. These factors may be affect cervical mucus, metabolites and female reproductive tract secretion and vaginal pH [11]. Ability of father to bias offspring sex ratio has been dismissed given the expectation of an equal proportion of Y/X-chromosome bearing sperm during ejaculation. This expectation has been recently refuted[12]. Gomendio[13] reported a strongly sexually dimorphic species and a classic example for large variance across males in reproductive success-to show that fathers can bias sex ratio at birth. More fertile fathers produce more sons and less fertile produce more daughters. Saragusty [14] shown that variation in the ratio of Y/ X-chromosome bearing sperm in the ejaculation associates with variation in the sex of the offspring produced. Analysis of large dataset was shown males with higher

reproductive success have a higher proportion of male offspring, and also such sex ratio bias is adaptive[15].

It has been reported that the concentration of testosterone plays a key role in offspring sex ratios in different mammals, as there was a positive correlation between high concentration of testosterone and bias in sex ratio of offspring toward males[16,17]. Recent advances in molecular genetics has been resulted in the development of a variety of techniques (such as real-time PCR, fluorescent in-situ hybridization (FISH), and flow cytometry) for accurate estimation of X- and Y-chromosome bearing sperm. Among these techniques, real-time PCR provides an easy-to-use context for estimation of the copy number of genes [18]. The sex-related Y gene (SRY), which is located on the short arm of Y chromosome close to the centromere, has been frequently used as a marker for detection of Y-chromosome bearing sperm. SRY involves in initiation of transcription, processing of mRNA, participation in spermatogenesis, motility of sperms, interaction between sperm and ovum, and testosterone production[19-21]. SRY can affect the viability of Y chromosome and its role in population ratio of X and Y chromosome-bearing sperm suggested to examined[22]. The proteolipid protein (PLP) gene, on the other hand, is routinely used for detection of X-chromosome bearing sperm. PLP locates on non-homologous region of X chromosome and is expressed in all nervous and non-nervous tissues. The increasing expression of PLP under different physiological conditions has been shown to stimulate apoptosis process [23].

Despite the high frequency of studies on the role of testosterone on offspring sex ratio in different mammals, the potential effect of testosterone concentration of bovine in the ratio of his Y/X-chromosome-bearing sperm has been poorly studied. In this research, The effect of blood and semen testosterone concentration on the viability and proportion of Y/X -chromosome bearing sperm in male Holstein bovine was Studied.

Materials and Methods

Materials

Soils Bio Dyne kit (Cat. No: 08-24-0000S) was used to amplify DNA fragments in qPCR.

Sampling

Twenty-six healthy male Holstein bovine were used in this study. Semen samples were taken using artificial vagina during early morning. Simultaneously, blood samples were collected from the caudal vein using gel-clot activator tubes. Rectal temperature of all bovines were taken immediately after blood sampling.

Blood and semen testosterone concentration

Serum and semen testosterone level were measured by AccuBind ELISA commercial kit (Monobind Inc. Lake Forest, USA, Cat. No: 3725-300). Immediately after blood collection, all tubes were puted in incubator 37˚C for 10 minutes to promotion clot formation. All tubes were centrifuged at3000 × g for 10 minute. Serums were collected and transfer to 2ml microtube and freezed at -70 untile hormone misurment. 0.5cc of semen samples were transfer to 1.5 ml microtube and freezed at -70 until hormone misurment.

Sperm concentration and viability

Collected semen was analyzed macroscopic and microscopic. Volume, color, density, contamination, concentration, viability ratio and motility were evaluated, high quality semen was used in this study.

DNA extraction, Primer design and qPCR

Total DNAs were extracted from sperm using salting-out protocol [24]. All chemical materials used for DNA extraction were obtained from Merck (Darmstadt, Germany). Concentration and purity of extracted DNAs were estimated by Nanodrop spectrophotometry absorption ratios at 260 nm and 260/280 nm respectively. The quality of extracted DNA was assessed by electrophoresis at 1% agarose-gel containing Ethidium Bromide. PAR gene was used as reference gene for normalization of expression data obtained from qPCR. The nucleotide sequences of genes, SRY (NCBI number: EU581861.1) and PLP (NCBI number: AJ009913.1) and PAR (NCBI number: AC234910.2) were obtained from NCBI (GenBank, National Center for Biotechnology Information). Primer pairs were designed using primer3Plus software and were shown in Table 1. The specificity of designed primers were evaluated using PrimerBLAST software of NCBI database.

Table 1: General properties of specific primer pairs for PLP, SRY and PAR genes.

Gene Name	Accession number	Sense primer sequence 5'→3'	Anti-sense primer sequence 5'→3'	Length (bp)	Tm(°C)
PLP	AF548366.1	GCCATCACATCTGAGACCAC	GACTCAGCATCTCGAAGCAA	97	59.8
SRY	EF583947.1	CTCAGACATCAGCAAGCAGC	GTAGTCTCTGTGCCTCCTCA	89	59.8
PAR	EF105407.1	GAGGGAGGGTGGATCATAGA	CCTCTGGGACCTTCAACAAT	90	59.8

Quantitative PCR was performed using SYBR Green super mix. The reactions consisted of 4µl SYBR Green PCR Master Mix (SYBR biopars, GUASNR, Iran), 0.5µl of each specific forward and reverse primers, 1µl of DNA, and 14 µl nuclease free water to a final volume of 20µl.

Statistical analysis

The data obtained from qPCR were analyzed according to the method of Livak&Schmittgen [25]. 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 in each sample using following formula:

ΔCt = Ct (target gene) - Ct (reference gene)

After calculation of ΔCt for all samples, the expression status of PLP and SRY genes relative to PAR was estimated using the following formula:Copy number of chromosome (X- or Y-chromosome) = 2-ΔΔCt = 2-(ΔCt (target gene) - ΔCt (PAR)

Finally, the ratio of PLP and SRY was considered as the ratio of X and Y chromosomes, respectively.All data were statistically analyzed using SAS computer software version 9.1 (SAS Institute Inc., Cary, NC, USA). The normality of data was tested by univariate procedure, and then the mean values were exposed to t-tests. Additionally, Pearson's correlation coefficients between the ratio of PLP/SRY with some biological traits including blood and semen testosterone, semen concentration, age, rectum temperature, and viability of sperm were calculated using Corr in SAS software.

Results

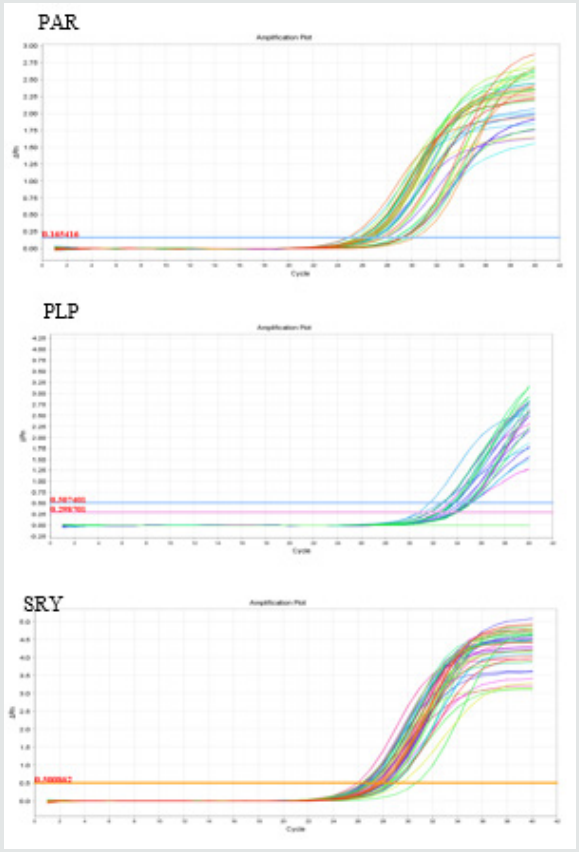

Figure 1: Amplification plots of PLP, SRY, and PAR genes.

Electrophoresis of the extracted DNA had acceptable quality. Additionally, the least mean square values for the quantity of the extracted DNA were determined as 813.27±114.88 ng/µl, respectively. The amplification plots of PLP, SRY, and PAR genes plotted by Step One software (v.2.1) have been shown in Figure 1.The three studied genes provided a single peak in the melting curve. This implies on absence of primer-dimer formation during the reaction (Figure 2). The blood and semen testosterone concentrations are shown in Table 2. Wide variation was found in testosterone concentration of both blood and semen samples. The blood testosterone concentrations were reported ranging between 6- 12.5 ng/ml (Mean±SD: 9.45± 2.39 ng/ml) and also semen testosterone concentrations were reported ranging between 0.32- 4ng/ml (Mean±SD: 2.07± 1.59 ng/ml). There was a positive correlation between blood and semen testosterone concentration.A significant difference was found in the frequency of SRY- and PLP-carrying sperm of the sperm samples among the studied. The Y/X-chromosome bearing sperm was 1.75±0.44 (Mean±SD), ranging between 0.44-4.65 (Table 3). Y- Chromosome bearing sperm percentage was 53.7±12.5% (Mean±SD), Ranging between 18-82%. These data show a large variety in X and Y- chromosome bearing sperm population in different bovine semen.

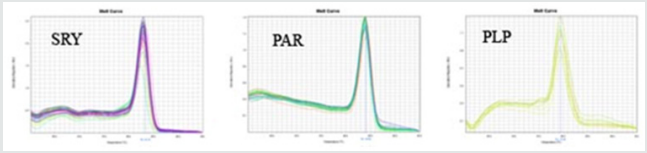

Figure 2: Melting curves related to amplified products of SRY, PAR, and PLP genes.

Table 2: The testosterone concentration of blood and semen in Holstein bovine.

Number of cattle	Blood testosterone *ng / ml*	Semen testosterone *ng / ml*
1	11.4	3.6
2	7.5	0.8
3	12	4.2
4	6	0.32
5	6.5	0.3
6	7.2	0.9
7	12.5	4
8	6.5	0.32
:	:	:
26	11.6	3.2
Average	9.45±2.39	2.07±1.59

Table 3: Compare mean $2^{-\Delta cT}$ SRY and PLP-carrying Sperm.

	SRY-carrying Spermatozoid	PLP-carrying Sperm	Y/X
Least square means	1.23[a]	0.71[b]	1.75
Standard error	0.15	0.02	0.44

[a,b]Values with different superscripts within the same row differ significantly (p<0.05).

A strong correlation (0.98) was found between testosterone concentration of blood and semen, meaning that cattle with higher blood testosterone content have also higher testosterone concentrations in their semen (Table 4). Furthermore, the correlation coefficients of testosterone concentration of blood and semen and the ratio of SRY-carrying sperm were 0.38 and 0.47, respectively, meaning that bovine with higher testosterone concentrations have significantly higher proportion of Y-chromosome bearing sperm than those with lower blood and semen testosterone contents. Additionally, the correlation coefficients of testosterone concentration of blood and semen with the ratio of PLP-carrying sperm were -0.67 and -0.60, respectively (Table 4).

A significant positive correlation (0.63, P<0.05) was detected between the age of bovine and the ratio of SRY-carrying sperm (Table 4). However, the correlation between age and the ratio of PLP-carrying sperm was not statistically significant (-0.26, P>0.05) (Table 5). Testosterone concentration of blood and semen was positively correlated with age of bovine(0.72 and 0.79 respectively, P<0.05). Rectum temperature was positively correlated to blood testosterone concentration (0.41, P<0.05), but not correlated with the ratio of SRY- and PLP-carrying sperm (-0.20, P>0.05) (Table 5). The results of correlation analyses between different biological characteristics of Holstein bovine have been summarized in Table 5. The correlation of rectum temperature with population of sperm and sperm stimulation was 0.05 and -0.1, respectively, which were not statistically significant (p>0.05).

Table 4: The correlation coefficient between *PLP* gene, *SRY* gene, blood testosterone, semen testosterone, age and rectum temperature among Holstein bovine.

Type variable	Blood testosterone	Semen testosterone	Age	Rectum temperature
SRY gene	0.38*	0.47**	0.63**	-0.20ns
PLP gene	-0.67**	-0.60**	-0.26ns	-0.20ns
Blood testosterone		0.98**	0.72**	0.41*
Semen testosterone			0.79**	-0.30ns
Age				-0.04ns

*P < 0.05; **P< 0.01; and ns: non-significant (P > 0.05).

Table 5: The correlation coefficient between blood testosterone, semen testosterone, age, volume of semen, population and viability of sperm among Holstein bovine

Type variable	Semen testosterone	Age	Volume of semen	population of sperm	Sperm viability
Blood testosterone	0.98	0.70**	0.49**	0.62**	-0.56**
Semen testosterone		0.77**	0.45**	0.67**	-0.64**
Age			0.39*	0.35 ns	-0.60**
Volume of semen				0.33 ns	-0.17 ns
Population of sperm					-0.54**

*P< 0.05; **P< 0.01; and ns: non-significant (P > 0.05).

Discussion

There is a strong evidence of effect of environment and social factors on sex ratio [26]. Several factors have been reported in bovine that affect secondary sex ratio and also male calf is significantly higher when Artificial Insemination (AI) used compared to natural service [27,28]. Checa [29] reported that 50.02 ± 2.79% of sperm bearing X-chromosome, but another study found that 44% of the sperm carrier X-chromosome[30]. Lobel[31]examined 98 human semen. They reported that 41.9-56.7% of the sperms bearing Y chromosome. Another research reported similar data, 46.9-52.7% Y-sperms in each ejaculation [32]. Madrid-Bury [33] reported neither bovines nor semen didn't have effect on Y-chromosome bearing sperm percent or sex ratio of embryos produced *in vitro* but the method of sperm preparation affected the primary sex ratio. However, double swim-up sperm preparation method produced differences in %Y- chromosome bearing sperm in some of sperm fractions. They suggested that there are intrinsic differences in capacitating of X and Y-bearing sperm that might be used to produce

embryos of the desired sex in laboratory production of embryo. However in two different studies on bovine semen, it became clear that Y-chromosome bearing sperm population was between 24-84% in each ejaculation[34,35]. The difference between previous studies may be due to the breeds of the employed bulls (Holstein and Galicia) or PCR techniques.

Wide variation was found in testosterone concentration in both blood and semen samples. This variation may be related to some factors such as individual difference, breed, and age. Although the main source of testosterone in male is testicular tissue, the concentration of semen testosterone in all studied animals was much lower than blood. Lower levels of testosterone in semen is due to transfer testosterone from blood in semen. Laydig cells produce and release testosterone in blood, and semen testosterone comes from blood[36].

Recently, studies have shown that offspring sex ratio is significantly influenced by maternal dominance, a characteristic which has been shown to be linked to testosterone in mothers[37,38]. Testosterone has been suggested to play an important role in the viability of germ cells, probably by regulating a specific pathway for apoptosis [39,40]. A lot of studies have been carried out to verify the role of testosterone in alteration of sex ratio in different mammals and our results were in agreement with these investigations. Helle reported that a 1pg/ml increase in serum testosterone content of rats could lead to 19% biased in sex ratio of offspring toward males.

They also showed that mothers with higher testosterone produced more male offspring than those with low testosterone content. James demonstrated that increase in blood testosterone level in males can bias the sex ratio of offspring toward males. Similarly, Shargal found that female ibexes (Capra Nubiana) with higher fecal testosterone produced more male offspring. Grant and Irwin and Grant focused on follicular testosterone, instead of conventional serum and fecal testosterone, and demonstrated that ova, developing in follicular fluid with high levels of testosterone, were subsequently more likely to be fertilized by Y-chromosome-bearing spermatozoa, probably due to the differential stimulation or viability. These authors proposed that there might be a critical time, in which the follicular testosterone level affects the molecular composition of zonapellucida and alters the susceptibility of oocyte to be fertilized by a Y-bearing spermatozoon. According to García-Herreros when the average testosterone level of bovine follicular liquid exceeded 32.12 ng/ml, the probability of male birth increased, while higher proportion of female birth was observed when the follicular testosterone was around 23.98 ng/ml.

The important role of SRY in the viability of Y chromosome, stimulation of apoptosis signaling pathway by PLP (due to lipoprotein synthesis) and increasing of testosterone level, seems to trigger the apoptosis of the X-chromosome bearing sperm during the early stages of spermatogenesis. Additionally, some other biological parameters of male Holstein cattle were found to be influenced by testosterone concentration. Although, this is the first study on the effects of paternal testosterone on offspring sex ratio, a relatively large volume of studies on different mammalians have demonstrated that mothers with higher blood, follicle or fecal testosterone produce significantly more male offspring than females with lower testosterone levels.

It has been reported that in terms of association between the weather change and secondary sex ratio, by increasing of 1°Centigrades above the average value in the temperature of weather during the week before fertilization, the probability of male calf birth will increase about 1 % [41]. Evaporation rate has the same effect on the birth of male calf. In addition, it has been expressed that the birth of male and female in the hot and cold weather is increased, respectively [42]. Perez-crespo[43,44] reported that the birth of male can be increased by increasing the temperature of environment and scrotum.

Conclusion

The results of the current study revealed a difference in percentages of Y and X -chromosome bearing sperm. A positive correlation between the frequency of SRY and testosterone was shown. Therefore, investigating the molecular mechanism involved in the effects of paternal testosterone on the ratio of its X/Y-chromosome bearing sperm may be an interesting subject for future studies.

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