GnRHR gene polymorphism and its correlation with semen quality in Buffalo bulls (*Bubalus bubalis*)

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Article information

Abstract

Fresh semen from fifty bulls was evaluated for ejaculate volume, individual motility, concentration, lives sperm and abnormalities as well as acrosome integrity. Bulls were classified according to semen motility into two groups; good and poor. DNA was extracted from semen of both groups, then the PCR followed by single-strand conformation polymorphism (SSCP) techniques were performed for mutation detection in gonadotropin releasing hormone receptor (GnRHR) gene through multiple sequence alignment. The results showed that the percentages of sperm motility, sperm concentration and live sperm, were significantly higher in good than poor semen quality bulls. However, semen volume, percentage of acrosome integrity and abnormalities did not differ between good and poor. The PCR amplification of 240-bp fragment and the results of SSCP appeared a genetic polymorphism with two patterns. Pattern I was seen in all good bulls, with incidence of 100%. Pattern II appeared only in poor semen quality bulls with a frequency of 31.25%. The sequence analysis of the PCR product for the two patterns showed two single nucleotide polymorphisms (SNPs) as a transversion base substitution mutation at positions 20 (T/A) and 193 (A/T). The GnRHR gene could be used as a genetic marker related to semen quality in buffalo due to the good semen bulls had a unique pattern.

Introduction

Function of gonadotropin-releasing hormone (GnRH) is to motivate the synthesis and release of both follicle stimulating hormone and luteinizing hormone, which mange gametogenesis and steroidogenesis (1). The response of pituitary gonadotropes to GnRH associates with the concentricity of GnRH receptors (GnRHR) at the cell surface (2). GnRHR gene has effect on reproduction and the gene mutations influence sex hormone levels (3). It is outstanding that artificial insemination requires bulls with fantastic semen quality to transfer the characteristics to their offspring (4). Semen assessment has been utilized as a pointer of fertility (5,6). The motility, concentration and normal sperm rate have been commonly used as judging requirements for evaluation of semen quality (7). However, direct selection for semen quality is difficult due to their low heritabilities (8). In addition, reproductive traits are co-controlled by multiple genes (9). Selective breeding is essential for improvement the bull hereditary and candidate genes are used as markers for sperm quality after the development of molecular techniques. Few investigations on GnRHR polymorphisms gene as a marker for fertility and semen quality were recorded in cattle (10,11). It was a study in Chinese water buffalo recorded a significant correlation between ejaculate volume of the sperm and single nucleotide

polymorphisms (SNPs) in the GnRHR gene (12). Recently, another study in Chinese water buffalo by Wang *et al.* (13) showed a significant association between the SNPs of GnRH and sperm quality. Therefore, the authors recommended the working on additional animal breeds to confirm whether the GnRH gene is the major gene that can influence semen qualities. However, in Egyptian buffalo bull this relation has not been explored comparing to other buffalo breeds. To date, there is no significant correlation between GnRHR polymorphisms and sperm qualities in Egyptian buffalo bulls has been conducted. Along these lines, the current work intended to investigate the genetic polymorphism of GnRHR gene and its correlation with fresh semen quality in Egyptian buffalo bulls.

Material and methods

Bulls

Fifty buffalo bulls (*Bubalus bubalis*) aged 3-5 years were subjected to semen assessment and genetic characterization of GnRHR gene. These bulls belonged to a breeding station at Mahalet Mussa close to Sakha, Kafr el-Sheikh Governorate, Egypt. All buffalo bulls were exposed to the same management and nutrition programs. Semen ejaculates were collected weekly for a period of 6 weeks (replicates; n = 6). The bulls were classified according to fresh semen motility into two groups; the first had more than 60% motility (good; n=34) and the second had lower than 60% motility (poor; n=16). Libido evaluation was based on reaction time (in seconds) which was taken by the bulls from exposure to the teaser until mounting.

Semen evaluation

Ejaculates were collected by artificial vagina at 42°C. Each ejaculate was transferred to the laboratory rapidly and kept in a water bath at 37°C for performing evaluation tests. Volume of the ejaculates was assessed to nearest 0. 1 ml. Individual motility was expressed as the percentage of forward motile spermatozoa. Concentrations were recorded via Neubauerhaemocytometer. Live sperm and abnormalities percentages were evaluated using eosin-nigrosin stained smears (7). The percentage of acrosome integrity was assessed, by Giemsa staining (14).

DNA extraction from sperm cells

DNA was extracted chemically from fresh semen of all bulls as indicated by Weyrich (15) with some modifications. About fifty µl of semen was washed in 500 µl of 70% ethanol then were centrifuged at 10,000 ×g for 5 min and the supernatant was expelled. The former steps were repeated until the supernatant became clear, noticeable and a white pellet was obtained. About 500 µl lysis buffer (50 mM Tris-HCl, pH 8 (Merck, Germany); 10 mM EDTA (Sigma Aldrich, USA)*;* 100 mM NaCl (Modern lab, Egypt)*;* 1% SDS aqueous solution (ACROS Organics, Part of thermo Fisher Scientific, USA) was added to the sperm pellet. Additionally, 5 µl (0.5%) Triton-X100 (Bio-basic Canada Inc, Canada), 25µl Dithiothreitol1M (Research Product International, Illinois, Chicago, USA). and 50 µl proteinase K (20 mg/ml) were supplemented. The samples were mixed well and incubated overnight at 50°C in a thermo shaker. The tubes were centrifuged at 15,500 ×g for 10 min and the supernatant was transferred into another 1.5 ml tube. For DNA precipitation, 3M sodium acetate was added to the supernatant (about 1/10 Vol. of the supernatant) and mixed gently and cold absolute ethanol was added to the tubes (2 Vol. of the supernatant). DNA was precipitated at -20°C overnight and pelleted by centrifugation for 20 min at 15,500 ×g. After that, the supernatant was carefully removed by pipetting and the white pellet of DNA remained. DNA was washed by dispensing the pellet in 500 µl ethanol (75%). The samples were centrifuged for15,500 × gat 10 min then were dried until ethanol was disappeared. The DNA pellets were dissolved in 50 µl ddH$_2$O and concentration was estimated using NanoDrop1000 Thermo Scientific spectrophotometer (USA) then diluted to working concentration of 50 ng/µl for PCR.

PCR Reaction and DNA Amplification

The primer for amplification of 240-bp fragments of GnRHR gene was described by Milazzotto *et al.* (16) then Sosa *et al.* (17) with the following nucleotide sequence: F: AAACTACAACTGAATCAGTC and R: TAGAGAGAAATATCCATATA. Amplification reactions were done in 50 µl volume, containing 5.00 µl buffer 10x, 1.00 µl 2.5 mM (dNTPs mixture), 0.30 µl Taq polymerase (5 U/µl), 0.25µl primer, 3.00 µl 25 mM (MgCL$_2$), 35.20µl water (nuclease free water) and 5.00 µl DNA sample. The PCR program was; one cycle at 95°C for 4 min, and 35 cycles of the sequence: 95°C for 60 sec, 55°C for 30 sec and 72°C for 60 sec. Upon completion of the reaction, the products were exposed to electrophoresis in 2% agarose gel.

Single strand conformation polymorphism

PCR products of 7.00 µl were mixed with 8.00µl of denaturing solution (98% formamide; 20mM EDTA, pH 8.0; 0.05%xylene cyanol; 0.05%bromophenol blue). At this point, denaturation of the samples occurred by heating at 95°C for 8 min, then samples were put on ice for 8 min and loaded in 1x TBE buffer on to12% polyacrylamide gel consists of (37.5:1 acrylamide: bisacrylamide, 10 ml 1xTBE buffer (Trisbase, Boric acid, Na$_2$EDTA), 2.5 ml glycerol, 17.5 ml deionized water, 400µl APS solution (ammonium per sulfate) and 40µl of TEMED (N, N, N´, N´-Tetramethylethylenediamine). Electrophoresis was done at 4°C, 70V for 18h. Silver staining technique was utilized to visualize the separated DNA-fragments with high sensitivity on polyacrylamide gels as indicated by (18,19).

Sequence analysis

The PCR products of the two patterns were sequenced by the company of Macrogen Incorporation sequencing (Seoul, South Korea). The sequence data were analyzed and alignment was performed by NCBI/BLAST/blastn suite. Single nucleotide polymorphism was analyzed using BioEdit software, through pairwise sequence alignment of two different patterns.

Statistical analysis

Data were analyzed for effect of bulls semen motility (good vs. poor) on inspected semen parameters by Student t-test using SPSS (ver. 18) statistical software. Correlation coefficient between reaction time and fresh semen parameters was determined. Differences were assumed significant at $P<0.05$.

Results

Fresh semen characteristics in good and poor semen quality buffalo bulls are appeared in Table (1). The percentages of sperm motility ($P\leq0.001$), live sperm ($P\leq0.001$) and sperm concentration ($P\leq0.05$) were significantly higher in good than poor semen quality bulls. However, semen volume, percentage of acrosome integrity and abnormalities did not differ between good and poor. In our investigation, the reaction time (taken by the bulls from exposure to the teaser until mounting) of good group was 43.3 ± 1.4 sec with no significant from poor group (58.5 ± 3.3sec). Our results in table (2) detailed that reaction time had a significant negative correlation with live sperm volume (r=-0.55, $P\leq0.03$) in poor group and concentration (r=-0.64, $P\leq0.01$) in good group. Regardless semen quality, all bulls showed significant negative correlation with motility (r=-0.36, $P\leq0.05$), volume (r=-0.36, $P\leq0.04$) and concentration (r=-0.38, $P\leq0.03$).

In the present study, PCR-SSCP marker was used to demonstrate the polymorphism of GnRHR gene in Egyptian buffaloes bulls and their association with semen parameter. The PCR product was 240-bp fragment of GnRHR gene (Figure 1). Allele-frequency analysis of genetic polymorphism patterns of GnRHR gene locus in Table 3 and Figure 2 revealed that pattern I was the most common compared with patterns II (68.75% vs. 31.25%, respectively) in poor bulls. The findings of semen analysis were used as the phenotype information to classify the bulls into good and poor semen quality bulls. Accordingly, 34 animals were considered as good and 16 animals were found to be poor. Pattern I was seen in all 34 bulls, with an incidence of 100% in good bulls. Pattern II appeared in a frequency of 31. 25% of poor bulls.

The sequence alignment of 205 bp out of 240 bp of buffalo GnRHR gene with accession number (KY786096.1) sequence was performed, by BLAST and showed that the 205 bp segment possess identities at 99% (Figure 3). Moreover, the sequence of the same DNA segment was 99% identities with the accession number: GenBank: EU621854.1 (Figure 4). The DNA sequence of 205 bp of GnRHR amplified segment (240 bp) was determined. The sequence analysis of the PCR product for the two different patterns showed two SNPs as a transversion base substitution mutation at positions 20 (T/A) and 193 (A/T) as shown in Figure (5).

Table 1: Biophysical characteristics of fresh buffalo semen from good and poor semen quality bulls (Means±SE)

Bulls (No.)	Semen volume (mL)	Sperm concentration (10^9/ml)	Motility %	Live sperm %	Sperm abnormality %	Acrosome integrity %
Good (34)	2.7±0.3	1.5±0.1[*]	70.9±1.1[**]	78.9± 1.4[**]	17.7 ± 1.2	62.5± 3.4
Poor (16)	2.5±0.3	1.1±0.1	44.0±3.3	58.6± 3.4	19.1 ± 1.1	56.3 ±2.8

[*] = $P<0.05$ and [**] = $P< 0.001$ (t-Test).

Table 2: Correlation coefficient (r) between reaction time and semen parameters of good and poor semen quality bulls

Bulls Semen (n)	r	Motility	Volume	Live sperm	Abnormalities	Acrosome	Concentration
Good (34)	Reaction time	.055	-.447-	.163	-.307-	-.385-	-.640[**]
	P value	.841	.083	.546	.247	.141	.008
Poor (16)	Reaction time	.065	-.297-	-.547[*]	.034	.217	.235
	P value	.810	.264	.028	.902	.419	.381
Good+ Poor (50)	Reaction time	-.357[*]	-.358[*]	-.237-	-.097-	-.249-	-.379[*]
	P value	.045	.044	.191	.598	.170	.033

[*] = $P<0.05$ and [**] = $P<0.001$ (t-Test).

Table 3: Patterns and frequencies of GnRH receptors gene polymorphism in good and poor semen quality bulls

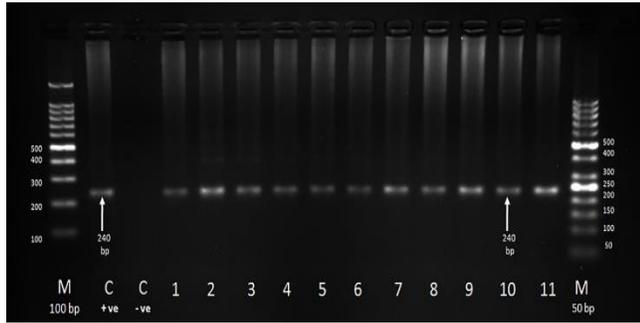| Bulls semen	Total number of animals	Pattern I		Pattern II	
		No	%	No	%
Good	34	34	100%	0	0%
Poor	16	11	68.75%	5	31.25%

Figure 1: 240-bp PCR product of buffalo GnRHR gene stained with ethidium bromide M: 50 and 100-bp ladder.

Figure 2: Two different SSCP patterns of GnRHR gene in tested Egyptian buffalo on 12 % silver stained-polyacrylamide gel. Lanes: 5-12: pattern I. Lanes: 1-4: pattern II.

Figure 3: Sequence analysis of 205 segment of Egyptian buffalo GnRHR amplified product compared to *Bubalus bubalis* breed Murrah gonadotropin-releasing hormone receptor (GNRHR) gene, complete cds. Sequence ID: KY786096. 1.

Figure 4: Sequence analysis of 205 segment of Egyptian buffalo GnRHR amplified product compared to *Bubalus bubalis* pituitary type-I gonadotropin releasing hormone receptor mRNA, complete cds. Sequence ID: EU621854. 1.

Figure 5: Pairwise sequence Alignment of two different patterns of GNRHR gene in Egyptian buffalo using BioEdit software, showing two transversion base substitution mutation.

Discussion

In the current study, the percentages of sperm motility, live sperm and sperm concentration were significantly higher in good than poor semen quality bulls. However, semen volume, percentage of acrosome integrity and abnormalities did not differ between good and poor. In accordance, many studies have recorded that bad sperm quality, leads to decrease the rate of blastocysts (20-22), and high rates of pregnancy failure (23). Moreover, (24) documented that fertility from the artificial insemination correlated with semen parameter**.** Motility is significant marker for semen quality as noteworthy connections between motility and both of membrane integrity and sperm abnormalities were reported (25). Likewise, correlations were present between sperm vitality and pregnancy rates (26).

In our investigation, the reaction time of good group was no significant from poor group. The two groups were scored as 3 for reaction time according to (27). The reaction time had a significant negative correlation with live sperm volume in poor group and concentration in good group. Regardless semen quality, all bulls showed significant negative correlation with motility, volume and concentration. In this regard, Moghaddam *et al.* (28) recorded that reaction time had a significant negative relationship (r=-0. 15, *P*≤0. 04)

384

with semen volume. Singh *et al.* (27) stated that head abnormalities had a positive significant correlation with reaction time. In buffalo, Anzar *et al.* (29), found that semen production was related to sexual behavior in low categories bulls (r=0. 84, *P*≤0. 005). The correlation between libido and seminal traits confirmed the needs for simultaneous selection for both libido and semen quality in the herd (30).

In the present study, PCR-SSCP marker was used to demonstrate the polymorphism of GnRHR gene in Egyptian buffaloes bulls and their association with semen parameter. Pattern I was seen in 100% in good bulls and pattern II appeared in a frequency of 31.25% of poor bulls. In this context, another study in dairy cattle indicated that GnRHR gene can be a possible marker for refinement the sperm quality, as bulls with GA or CT genotype should be selected for breeding (10). In pig, GnRHR has been utilized as a candidate gene for semen quality, as the association of GnRHR with motility, abnormal sperm rate, and plasma droplets rate was recorded (31). Kerekoppa *et al.* (32) confirmed high genetic polymorphism in GnRHR gene via PCR-SSCP technique in cattle.

In our study, the sequence analysis of the PCR product for the two different patterns showed two SNPs as a transversion base substitution mutation at positions 20 (T/A) and 193 (A/T). In accordance, the association between the sperm quality and SNPs in the GnRHR gene was recognized in Chinese buffalo (12). Additionally, Liron *et al.* (33) described bovine GnRHR polymorphisms in beef bulls and found eight SNPs of which five in the coding regions, might be responsible for the differences within bulls reach puberty. In our study, the presence of mutation in poor semen quality bulls may suggest it responsibility in lower motility. Interestingly, Sosa *et al.* (17) reported three nucleotide substitutions in GnRHR gene associated with ovarian inactivity in Egyptian buffaloes. So the genetic variation is needed for buffalo selection (34).

Conclusions

GnRHR gene in buffalo bulls is polymorphic with two SSCP patterns, the pattern I is characteristics for good semen quality bulls. These finding propose that GnRHR gene could be utilized as hopeful genetic marker for semen quality in buffalo bulls.

Acknowledgements

Conflicts of interest

None.

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تعدد الأشكال الجينية لمستقبلات الهرمون المنظم لعمل المناسل وعلاقته بجودة السائل المنوي في طلائق الجاموس

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الخلاصة

تم تقييم عدد خمسون طلوقة لحجم القذفة، الحركة الفردية، تركيز وحيوية وتشوهات الحيامن وكذلك سلامة الاكروزوم. وتم تقسيم الطلائق على حسب نسبة حركة الحيوانات المنوية إلى جيدة أو ضعيفة. تم عزل الحمض النووي من الحيوانات المنوية وعمل اختبار تفاعل البلمرة المتسلسل. كما تم عمل تعدد الأشكال الجيني وتحليل التتابع النيوكليتيدى لنتائج اختبار تفاعل البلمرة المتسلسل. أظهر تفاعل البلمرة المتسلسل لجزء ٢٤٠ قاعدة نيتروجينية ونتائج تعدد الأشكال الجيني بنمطين. لوحظ النمط الأول في جميع الطلائق الجيدة بنسبة ١٠٠ %. وظهر النمط الثاني فقط في الطلائق ذات جودة السائل المنوي الرديئة وبتكرار ٣١,٢٥%، كما أظهر تحليل التتابع النيوكليتيدى لنتائج تفاعل البلمرة المتسلسل للنمطين تعدد أشكال النيوكليوتيدات المفردة كطفرة استبدال قاعدة في الموضع ٢٠ (T/A) و ١٩٣ (A/T). والخلاصة انه يمكن استخدام جين الهرمون المنظم لعمل المناسل كعلامة وراثية تتعلق بجودة السائل المنوي في طلائق الجاموس المصري نظراً للنمط الفريد في الحيوانات المنوية الجيدة.