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Identification of SNPs in Intron 3 of Osteopontin Gene in Egyptian Buffalo Bulls with High and Low Quality Semen

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THIS study investigated the genetic polymorphism of osteopontin (OPN) gene in Egyptian buffalo bulls in a trial for association with the quality of fresh semen. A total of 228 fresh semen ejaculates were collected from 57 buffalo bulls. Checking the fertility potential, the ejaculates were evaluated for volume, individual motility, live sperm, sperm abnormalities and concentration. The bulls' semen was grouped according to the individual motility into high (>60%, n=47) and low (<60%, n=10) quality. A fragment of 250 bp from intron 3 of OPN gene was amplified by PCR then genotyped by restriction fragment length polymorphism, single strand conformation polymorphism and DNA sequencing. The high-quality semen was significantly increased in individual motility % ($p<0.0001$), live sperm % ($p<0.0001$) and sperm concentration ($p<0.05$) than the low quality. Also, the monomorphic pattern of OPN gene in both groups of bulls was noticed. However, five mutations were discovered including three nucleotide insertions (T 44, A49 and A62) and two nucleotide substitutions (G11>C and A108>G) when comparing the sequence with that of buffalo in the GenBank. In conclusion, OPN gene might have no genetic variation and so it is not associated with semen quality in Egyptian buffalo bulls. Future studies with a larger number of populations on different regions of OPN gene are recommended.

Keywords: Buffalo bull, Semen evaluation, Osteopontin (OPN) gene, Polymorphism.

Introduction

The bull fertility has been traditionally evaluated in the laboratory by using semen quality parameters such as sperm motility, viability and morphology [1-3]. Artificial insemination (AI) is an essential technique for bovine reproduction, and it greatly improves breeding efficiency [4]. The success of artificial insemination depends to a great extent on good quality semen which can be used as a physiological indicator of bull fertility [4].

The quality of the sperm is determined by a number of characteristics, including sperm motility, viability, and abnormal sperm % as well as some genetic factors linked to the bull [5]. Semen production characteristic, including volume and concentrations of sperm, have moderate heritability (from 0.15 to 0.30), whereas semen quality traits, such as motility and percentage of abnormal sperm, have high heritability (close to 0.60) as demonstrated by Druet et al. [5] suggesting genetic selection may be recommended. In this

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context, different studies have been performed on the genetic basis of semen traits and components as molecular markers of bull fertility [6-8].

It is of great importance to include introns of the genes in genetic analysis reports. Introns are essential components for the basic process of gene expression. Because the most important sequences that regulate gene expression are situated within introns [9]. Certain introns may be the principal structure directing the expression of some highly expressed genes in the genome, leading the gene to be greatly activated which could maximize protein production in biotechnological and therapeutic applications [9]. Other introns, on the other hand, have direct or indirect negative impacts on gene expression and so inhibit the expression of the gene [10]. Another function of introns for the cell is that the physical presence of introns in the genome enhances cell survival under starving conditions by increasing the repression of ribosomal protein genes downstream of nutrient-sensing pathways [11].

Osteopontin is an arginine-glycine-aspartate (RGD) containing glycoprotein that is involved in different biological processes, various tissues and body fluids including seminal plasma of the bulls and milk [12]. OPN is considered a fertility protein and has great importance in reproduction. It is found in higher quantities in the bovine seminal plasma of high fertility than low fertility bulls [12]. It binds to sperm during ejaculation and is so carried to the site of fertilization [13] to facilitate capacitation and viability of bovine sperm [14]. Also, OPN is involved in removing the outer acrosomal membrane in the capacitation process of bovine sperm [15]. Moreover, it plays a role in the sperm-oocyte interaction and fertilization events through OPN-integrin complexes present on the surface of both sperm and oocyte [16]. In vitro fertilization studies, treatment of bovine sperm or oocytes with purified OPN protein enhances the rate of fertilization and embryonic development [17].

The bovine OPN gene is located on bovine chromosome 6 (BTA6) close to qualitative trait loci (QTL) affecting milk production traits [18]. Its size is about 9 kb and consists of an upstream regulatory region and 7 exons with the included introns [18, 19]. The coding regions are exons 2, 3, 4, 5, 6 and 7 that encode a protein of 278 amino acids [18, 19]. Besides, the buffalo OPN gene was characterized by Tantia et al [20] that encodes a protein of 280 amino acids. About six SNPs (five in introns and one in the upstream region) were observed in buffalo OPN different from cattle OPN gene [20] that were also observed by Dubey et al [21].

Various studies in cattle and buffalo concerned with the association of osteopontin gene polymorphisms with milk production traits [22-24], somatic cell count in milk [25], lactation persistency [26] and body weight [27] making it an excellent candidate gene for selection by molecular markers. However, few researchers investigated the associations of polymorphisms in the osteopontin gene with semen production traits in buffaloes [28] and cattle [29, 30]. As a result, the aim of the current study was to determine mutations and polymorphisms in a fragment of intron 3 of osteopontin gene in a trial to associate these polymorphisms with semen quality as a buffalo fertility marker for the first time in Egyptian buffalo bulls.

Materials and Methods

Animals and samples

The current investigation was performed on 228 ejaculates collected from 57 river buffalo bulls (Bubalus bubalis) with the same ages (3-4 years). The bulls raised in a breeding station at Sakha, Kafr el-Sheikh Governorate, Egypt. Four ejaculates were collected and evaluated for each bull through the period from October 2018 till April 2019. Semen samples were collected by a sterile artificial vagina (adapted to a temperature at 42 ^{o}C) and kept at 37^{o}C in a water bath to analyze the quality of fresh semen immediately after collection. All animals were subjected to semen evaluation, as a field fertility index, and genetic characterization of the osteopontin gene by the PCR-restriction fragment length polymorphism (PCR-RFLP) as well as PCR-single strand conformation polymorphism (PCR-SSCP) and DNA sequencing.

Semen evaluation

Using the standard techniques, fresh semen was directly evaluated for ejaculate volume, individual motility %, live sperm %, sperm concentration and sperm abnormalities %. After evaluation, the rest of each ejaculate was stored at -20 ^{o}C for DNA extraction. Semen of the buffalo bulls was grouped, following the initial evaluation, into high and low quality semen according to individual motility; the first group had more than 60% motility (high; n=47) and the second group had lower than 60% motility (low; n=10) according to Vale et al [31].

DNA isolation from sperm cells

DNA was extracted from the buffalo bulls' semen according to Hasanain et al. [32]. DNA pellets were dissolved in 50 µl water free of nucleases. The quality and concentration of DNA were evaluated by NanoDrop1000 Thermo Scientific spectrophotometer. A working concentration of 50 ng/µl of DNA was prepared which is suitable for PCR reaction.

Polymerase chain reaction (PCR) and DNA amplification

The DNA fragment (250 bp) from intron 5 of OPN gene was amplified through polymerase chain reaction [33]. The mixture of PCR was 12.5 µl of PCR master mix (composed of 0.1 U/µlTaq polymerase, 500 µM of dNTP each, 20 µM of Tris-Hcl (pH 8.3), 100 mM of KCl, 3 mM of MgCl and stabilizer), 2.0 µl of forward primer (10 pM/µl), 2.0 µl of reverse primer (10 pM/µl), 2.0 µl of DNA (50 ng/µl) and nuclease free water till 25 µl.

The primers were selected by primer3 software version 4.1.0 (http://primer3.ut.ee/) based on OPN gene sequence of *Bubalus bubalis* on GenBank (accession number: DQ899755.1). The sequences of the primers were, forward: GCCTGCTATGGTTCTAGTGC and reverse: GCCAACTCTCCAATACGTGA. The amplification conditions for PCR were initial denaturation at 95 °C for 5 minutes. then 32 cycles of denaturation at 95 °C for 45 seconds, annealing at 57 °C for 1 minute and extension at 72°C for 1 minute then a final extension at 72°C for 5 minutes. The PCR products were electrophoresed with a DNA molecular size marker at 100 V in 1.5 percent agarose gel containing 0.5 µg/mL red safe in 1X TBE buffer. The gels were visualized using UV transilluminator.

PCR-Restriction fragment length polymorphism (PCR-RFLP).

The fragment of 250 bp of OPN gene was genotyped initially using PCR-Restriction fragment length polymorphism (PCR-RFLP) technique [34]. The BsgI restriction enzyme (New England Biolabs Inc, U.K.) was identified through the online tool NEBcutter (http://nc2.neb.com /NEB cutter2). The reaction was performed according to the manufacturer's instructions (product R0559S). The PCR-RFLP products were electrophoresed in 1.5 % agarose gel stained with 0.5 µg/ml ethidium bromide. The gels were visualized by a UV transilluminator.

PCR-single strand conformation polymorphism (PCR-SSCP)

The amplified PCR fragment of OPN gene was further demonstrated for polymorphism using PCR-single strand conformation polymorphism (PCR-SSCP) technique [35]. A cocktail of 3 µl of PCR products, 20 µl denaturing solution (95% formamide, 10 mMNaOH, 0.05% xylene cyanol, 0.05% bromophenol blue and 20 mM EDTA) and 17 µl distilled water was prepared. Then heat denatured at 95°C for 10 minutes followed by 15 minutes of fast chilling on an ice block. Then the cocktail was run on a 10% non-denaturing PAGE (acrylamide and bis-acrylamide ratio, 29:1) gel using 0.7X TBE buffer in vertical electrophoresis unit (Cleaver, UK) for 16 hrs (28mA and 180 V). The gels were stained by 0.5 µg/ml ethidium bromide.

Nucleotide Sequencing

Thermo Scientific GeneJET PCR Purification Kit (#K0701) was used to purify the amplified fragments according to the manufacturer's instructions. The purified PCR products for eight samples were sequenced by using an automated DNA Sequencer by Macrogen Inc (Seoul, South Korea). CodonCode Aligner and BioEdit softwares were used for the alignment of sequence data to detect any SNPs. Also, sequence data were further aligned using NCBI/BLAST/blastn suite for comparing the observed sequences with the reference sequences in the GenBank.

Statistical analysis

SPSS version 25 was used to conduct the statistical analysis. The results of the sperm analysis were presented as a mean SE. Semen parameters (Mean ± SE) in high and low quality semen of the buffalo bulls were compared by Student's t-test at least $P<0.05$.

Results

Semen evaluation

Our findings in Table 1 showed fresh semen parameters (Mean ± SE) in high (188 ejaculates from 47 bulls) and low (40 ejaculates from 10 bulls) quality semen collected from Egyptian buffalo bulls. The individual motility and live sperm % were significantly ($p<0.0001$) increased in high quality than low quality semen. Sperm concentration (Billion/ml) was significantly ($p<0.05$) different in high quality from low quality semen. Meanwhile, the ejaculate volume and sperm abnormalities % did not show significant differences between high and low quality semen.

Genetic polymorphism of osteopontin gene

All the tested buffalo bulls gave the specific PCR product at the expected size (250 bp) targeted in the OPN gene (Fig.1). When RFLP technique was carried out on the 250 bp PCR fragment, all buffalo bulls were monomorphic with no genetic variation as PCR products were not digested by BsgI restriction enzyme in all animals (Fig.2). When the SSCP technique was performed on the PCR product of OPN gene, no polymorphism was detected and all buffalo bulls had the same pattern (Fig.3).

A total of eight samples were successfully sequenced for the amplified PCR product (250 bp) of OPN gene. By alignment of the sequence data using BioEdit software, no genetic variation was detected giving 100% similarity among the resulted sequences (Fig.4). The sequence alignment of the amplified fragment of OPN gene, using NCBI/BLAST/blastn suite, with the reference sequence of osteopontin gene for *Bubalus bubalis* in the GenBank (Sequence ID: DQ899755.1) showed 98 % identity (Fig.5). Our findings discovered five different mutations when the sequence was

TABLE 1. Semen parameters values (Mean ± SE) in high and low quality semen of Egyptian buffalo bulls

Semen quality (No. of ejaculates)	Ejaculate volume (ml)	Individual motility (%)	(%) Live sperm	Sperm concentration (ml/10^9)	Sperm abnormalities (%)
High (188)	2.9±0.1	75.4±0.9***	81.3±1.0***	1.41±0.07*	18.11
Low (40)	2.9±0.1	48.3±1.4	61.3±2.1	1.18±0.06	19.31

* = P<0.05 and ***= P< 0.0001

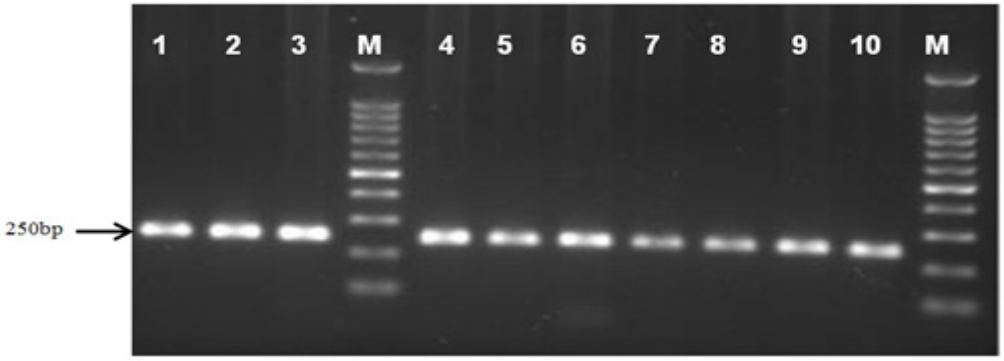

Fig.1. PCR product of OPN gene (250 bp) visualized on 1.5% agarose gel. M= 100 bp. Molecular marker Lanes 1-10 resemble PCR products

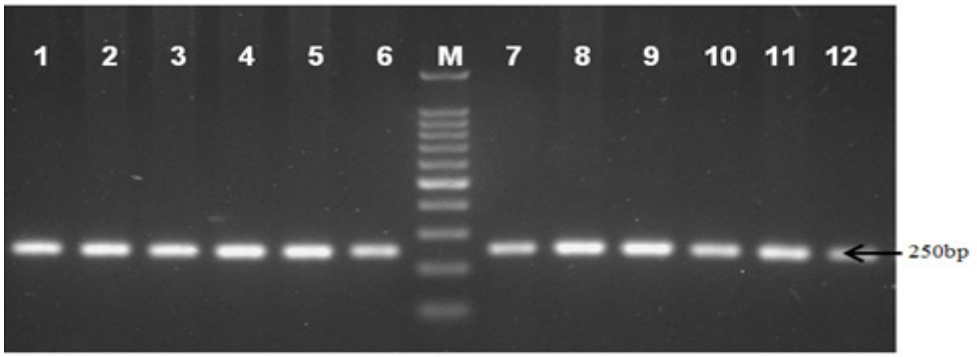

Fig.2. BsgI PCR-RFLP products of OPN gene in 1.5% agarose gel. M= 100 bp Molecular marker. Lanes 1-12 are monomorphic AA Genotype (250bp).

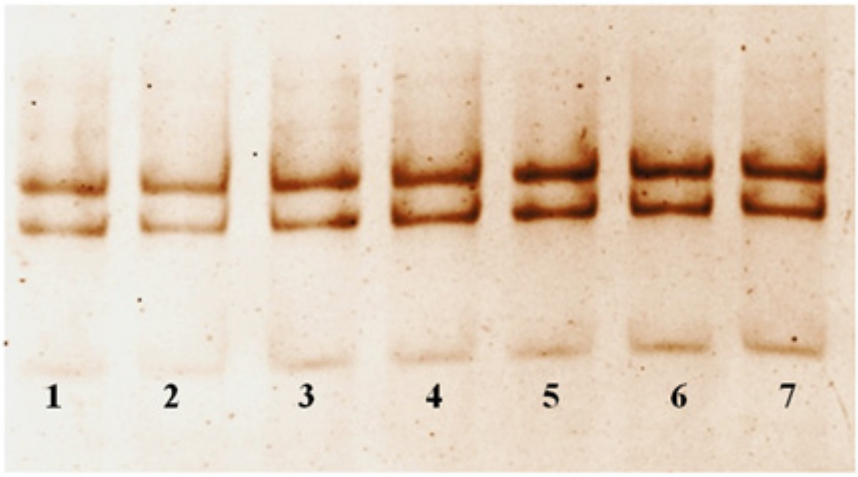

Fig.3. PCR-SSCP patterns of OPN gene in buffalo bulls.

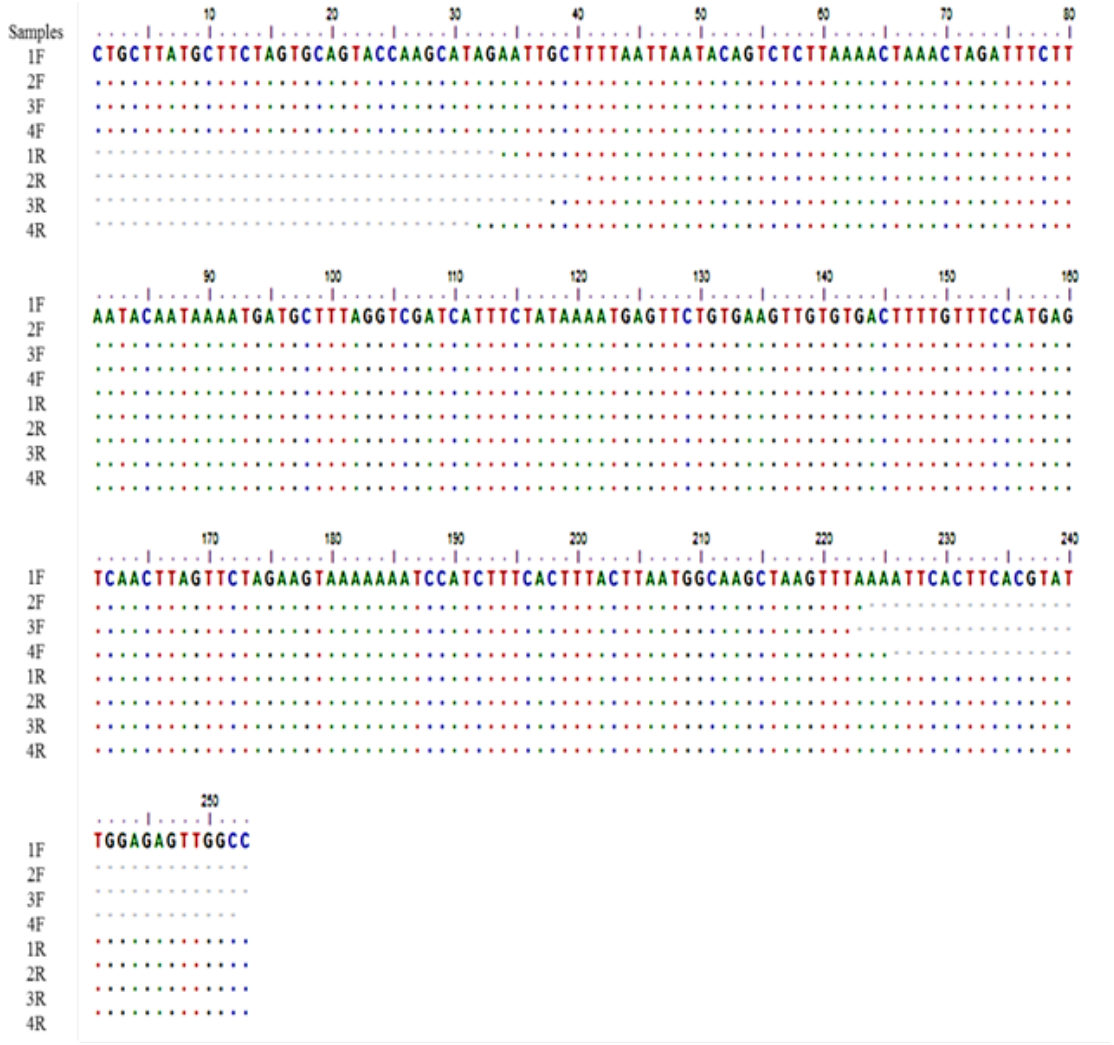

Fig. 4. Osteopontin gene multiple sequence alignment in Egyptian buffalo bulls in 8 samples using BioEdit software showing 100% similarity among bulls

Score	Expect	Identities	Gaps	Strand
436 bits(236)	3e-118	248/253(98%)	3/253(1%)	Plus/Plus

```
Query  1     GCCTGCTATGCTTCTAGTGCAGTACCAAGCATAGAATTGCTTTTAATTAATACAGTCTCT  60
Sbjct  3570  .........G.................................-....-..........  3627

Query  61    TAAAACTAAACTAGATTTCTTAATACAATAAAATGATGCTTTAGGTCGATCATTTCTATA  120
Sbjct  3628  .-.............................................A............  3686

Query  121   AAATGAGTTCTGTGAAGTTGTGTGACTTTTGTTTCCATGAGTCAACTTAGTTCTAGAAGT  180
Sbjct  3687  ............................................................  3746

Query  181   aaaaaaaTCCATCTTTCACTTTACTTAATGGCAAGCTAAGTTTAAAATTCACTTCACGTA  240
Sbjct  3747  ............................................................  3806

Query  241   TTGGAGAGTTGGC  253
Sbjct  3807  .............  3819
```

Fig. 5. The sequence alignment of the amplified fragment of OPN gene in Egyptian buffalo bulls, by NCBI/ BLAST/blastn suite, with the sequence ID: DQ899755.1 for buffalo OPN gene in the GenBank.

aligned with the reference sequence of buffalo with ID: DQ899755.1 including three nucleotide insertions (T 44, A49 and A62) and two nucleotide substitutions (G11>C and A108>G) as shown in Fig.5. The sequence data was then submitted for the GenBank and provided accession No. (MZ359640). The sequencing results confirmed the monomorphic pattern of that fragment of OPN gene with no genetic variation in the studied population.

Discussion

The current study aimed to investigate the genetic polymorphisms of OPN gene in Egyptian buffalo bulls with high and low quality semen. The quality of semen can be used as a physiological indicator for bull fertility [4]. The percentage of living spermatozoa determines the ejaculate quality and there is a positive correlation between the conception rate and the viability of sperm [36]. The rate of semen dilution at semen processing centers is also determined by progressive forward motility and sperm concentration [37].

In view of the semen examination as a bull fertility parameter in the current study, the percentage of individual motility, live spermatozoa and sperm concentration were significantly increased in the high quality than low quality semen group. However, the ejaculate volume, the percentage of live spermatozoa, the sperm concentration and the percentage of sperm abnormalities lie within the reference range values for buffaloes in both the low and high quality semen as previously discussed [3, 38]. The progressive forward motility reflects the physiological status of bull spermatozoa after semen collection or cryopreservation and is commonly used as an indicator of sperm viability and its ability to pass the barrier in the female reproductive tract to reach the oocyte [39]. Also, the higher percentage of progressive forward motility in fertile than sub-fertile bulls suggested that motility can be considered as an important attribute to classify fertile and infertile animals [40]. The progressive forward motility reported using CASA system is correlated to field fertility in bovine (29).

In the current study, the PCR-RFLP technique revealed no genetic polymorphism in the targeted fragment (250 bp) of OPN gene in both groups of Egyptian buffalo bulls. This could be attributed to the fact that RFLP examines the nucleotide sequence within the restriction frame of the enzyme used [34]. SSCP, on the other hand, considers the whole nucleotide sequence of the amplified segment, regardless of restriction frame [35]. However, no genetic variation was recorded and all the samples had the same pattern. We confirmed our results by applying nucleotide sequencing for that region of OPN gene. Our findings assumed, this may be a highly conserved sequence in the intron 3 region of osteopontin gene in Egyptian buffalo bulls. The sequence in the present work (ID: MZ359640) varied from the reference sequence of the buffalo OPN gene in the GenBank (ID: DQ899755.1). Our investigation discovered five mutations including three nucleotide insertions (T 44, A49 and A62) and two

nucleotide substitutions (G11>C and A108>G) along the 250 bp amplicon. In this respect, there are no data in the literature for comparison of our findings for osteopontin gene polymorphism in Egyptian buffalo bulls. More research with a greater number of populations and additional regions of the osteopontin gene are recommended for better understanding its influence on semen quality and fertility traits of the buffalo bulls.

Few reports in other countries are available till now, whether the genetic variation of osteopontin gene affects semen quality of buffalo and cattle. In this context, Rolim Filho et al [28] identified seven SNPs in OPN gene in water Buffaloes in the Brazilian Amazon. The SNP at bp 6690 in the intron 5 region was significant for scrotal circumference, sperm concentration, motility and pathology. Meanwhile, the SNP at bp 6737 in the intron 5 also was significant for scrotal volume. Moreover, Rorie et al. [30] identified SNP at bp 3379 within the promoter region of OPN gene that was associated with increased sperm motility in Angus and Angus x Brahman bulls. In addition, Hernawati et al. [29] determined the polymorphism in the promoter region of OPN gene in dairy bull Peranakan Holstein Friesian. They observed deletion in 10098 bp and a transition (T-C) in 10054 bp was associated with low quality frozen semen.

Conclusion

This is the first study in Egyptian buffalo bulls that reported the monomorphic pattern of OPN gene. This may be a highly conserved sequence in the studied population. Further studies on different regions of OPN gene with greater number of populations are recommended for more identification of OPN gene polymorphisms and understanding their influences on the semen quality of the Egyptian buffalo bulls.

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Conflicts of interest

None

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التعرف على التباين الوراثي (SNPs) في intron 3 لجين الاستيوبنتين (Osteopontin) في طلائق الجاموس المصرية ذات الجودة العالية والمنخفضة من السائل المنوي

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الاستيوبنتين هو بروتين سكري موجود بشكل كبير في بلازما السائل المنوى للطلائق عالية الخصوبة. هدفت هذه الدراسة إلى التحقق من تعدد الأشكال الوراثية لجين الاستيوبنتين في طلائق الجاموس المصري في محاولة لإيجاد ارتباطه بجودة السائل المنوي .تم تجميع ٢٢٨ قذفة منويه لعدد ٥٧ حيوان و تم فحصهم مباشرة لكل من حجم القذفة و نسبة الحركة الفردية النشطة ونسبة الحيوانات المنوية الحية و نسبة الحيوانات المنوية المشوهة و أيضا تركيز السائل المنوي. تم تصنيف السائل المنوي للطلائق وفقًا للحركة الفردية إلى جودة عالية ومنخفضة . تم استخلاص الحمض النووى DNA من عينات السائل المنوى و عمل تفاعل البلمرة المتسلسل لقطعة طولها 250 نيوكليتيدة لجين الاستيوبنتين واستخدام إنزيم القطع BsgI لتقنية RFLP ثم استخدمت تقنية SSCP لتعدد الأشكال وعمل تحليل للتتابع النيوكلتيدى.

أثبتت النتائج زيادة معنويا في الحركية الفردية ، الحيوانات المنوية الحية وتركيز الحيوانات المنوية للسائل المنوي عالي الجودة عن السائل المنوي منخفض الجودة. كما لوحظ سيادة نمط جينى واحد لجين الاستيوبنتين فى طلائق الجاموس المصرى. ومع ذلك ، تم اكتشاف خمس طفرات منها إضافة لثلاث نيوكليوتيدات (T 44 و A49 و A62) واستبدال لعدد اثنين نيوكليوتيدات (C >11G و G >108A) عند مقارنة التتابع النيوكلتيدى مع المرجع الجينى الجاموس في بنك الجينات.

و الخلاصة أن جين الاستيوبنتين هو تسلسل محفوظ و ثابت في طلائق الجاموس المصري. و يوصى بدراسات مستقبلية على مناطق أخرى من جين الاستيوبنتين مع عدد أكبر من الحيوانات لتقييم تعدد الأشكال الجينية للجين و مدى ارتباطه بجودة السائل المنوي لطلائق الجاموس.

الكلمات الدالة: طلائق الجاموس - تقييم السائل المنوي - جين الاستيوبنتين - تعدد الأشكال.