Research Article

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# The Relationship Between The Polymorphism of Beta A (Ba) Sheet of Inhibin Gene and Semen Characteristics in Holstein Bulls

**Aboud, Qusay Mohammed, Younis Laith***

Department of Obstetrics, Faculty of Veterinary Science, Al Fallujah University, Al Fallujah, Iraq.

*Corresponding Author: laythsufyan@uofallujah.edu.iq.

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**Abstract**

This study aimed to test a linkage between Inhibin beta A (INHβA) polymorphism and semen quality in Holstein bulls in Iraqi Artificial Insemination Center. The experiment trial was carried out on (12 n) pure Holstein breed bulls Holstein breed bulls. The study period started from January 2022 until February 2022. The Ejaculates were collected and its characteristics were evaluated immediately, additionally, 10 ml of blood were collected from each bull (12 samples) for genomic DNA extraction to determine the single nucleotide polymorphisms (SNPs) and compared with semen quality. The findings showed presence four variants at Exon 2; C (903) T, C (916)Y, G (917)K, and G (966) R in Exon 2 when compared with Gene sequence ID: XM_024990466.1. Two out of four Exon 2 SNPs were non-sense mutations(C (903) T and G (966) R), while the remains were missense which made the change in Arginine > phenylalanine at position (306). Regarding the volume of ejaculates, the results recorded that non-significant difference was showed in whole alleles of the loci, except in one locus (G (966) R), the bulls that had a wild allele (A) recorded maximum collected volume ($p < 0.001$). In addition, the bulls that represented wild genotypes showed a significant increase in sperm concentration and both mass and individual motility than mutant genotypes in all loci. Morevere, The results of the dead sperm percentage showed a non-significant correlation between the genotypes of all loci except in C (903) T, which demonstrated the superiority of the wild allele over the mutant. Lastly, the results prevailed that the mutant genotype related to the bad semen quality for the bulls, while, the wild genotypes showed good semen parameters, this results may be due to the effect of SNPs on the expression of INHA or the increase its activity.

**Keywards:** Inhibin A, Polymorphism, Ba Sheet, Fertility, Bulls.

**العلاقة بين التغاير للصفيحة (βA) لجين Inhibin وخصائص السائل المنوي لثيران الهولشتاين**

الخلاصة

هدفت هذه الدراسة إلى اختبار العلاقة التغايرفي صفيحة beta A (INHβA) وجودة السائل المنوي في ثيران الهولشتاين في مركز التلقيح الاصطناعي العراقي. أجريت التجربة على (عدد 12) ثورمن سلالة الهولشتاين النقية. بدأت فترة الدراسة من يناير 2022 حتى فبراير 2022. تم جمع السائل المنوي الطازج وتقييم خصائصه آنيا, اضافة الى ذلك, تم جمع 10 مل من الدم من كل ثور (12 عينة) لاستخراج الحمض النووي الجيني لتحديد التغايرات (SNPs) ومقارنتها بجودة السائل المنوي. أظهرت النتائج وجود أربعة متغيرات في Exon 2 ؛ T (903) C و Y (916) C و G (917) K و R (966) G في Exon 2 بالمقارنة مع معرف التسلسل الجيني: XM_024990466.1. كان اثنان من أصل أربعة من SNPs Exon 2 طفرات غير محسوسة T (903) C و R (966) G) ، في حين كانت البقية محسوسة حيث احدثت تغيير في Arginine> phenylalanine في الموقع (306). فيما يتعلق بحجم السائل المنوي ، أظهرت النتائج فرقًا غير معنوي في كل الأليلات للمواقع، باستثناء موضع واحد R (966) G) ، حيث سجلت الثيران التي لديها أليل غير طافر (A) أقصى حجم للسائل المنوي تم تجميعه ($P<0.001$). فضلاً عن ذلك, أظهرت الثيران التي مثلت الأنماط الجينية البرية زيادة معنوية في تركيز الحيوانات المنوية وحركتها الجماعية والفردية مقارنة بالأنماط الجينية الطافرة في جميع المواقع. علاوة على ذلك, أظهرت النتائج بأن النسبة المئوية للنطف الميتة علاقة غير معنوية بين الطرز الوراثية لجميع المواقع باستثناء T (903) C ، مما يدل على تفوق الأليل الغير الطافر على الطافر. علاوة على ذلك ، تم تسجيل نسبة عالية من التشوهات في كل من الأنماط الجينية الطافرة غير المتجانسة و المتجانسة ، على التوالي ، ولكن تم تسجيل ارتباط غير معنوي بين الأنماط الجينية G(917)K. بالنهاية، كشفت النتائج أن التركيب الوراثي الطافر مرتبط بالجودة السلبية للسائل المنوي للثيران، بينما أظهرت الطرز الوراثية الغير الطافرة معايير جيدة للسائل المنوي، وقد تكون هذه النتائج بسبب تأثير SNPs على التعبير عن INHA أو زيادة نشاطها.

## Introduction

Artificial insemination (AI) is an assisted reproductive technology that has significantly improved the genetic efficiency of breeding herds by allowing the successful use of selected breeding males, the cryopreserved sperm is the most widely used procedure for cattle reproduction worldwide. (1-3).

However, various factors are influencing the performance of AI programs and conception rate, including managemental; feeding system, latitude, housing system, and the time of artificial insemination (4), semen processing and addatives (5,6). Several studies pointed out that semen quality is affected significantly by genetic factors (7,8).

The Inhibins consider a main beta-transforming growth factor, a gonad particular glycoprotein hormones, size 30-kilo dalton (9). Inhibins are considered heterodimeric or dimeric and consist of α and either βA or βB subunits which form inhibin (A) or inhibin (B) (9,10).

Several studies indicated the presence of mRNA and protein of βA inhibin/activin subunit in the seminiferous tubules and Sertoli cells of adult ram testes, it suggested can act synergistically to maintain FSH plasma concentrations (11). The testicular inhibin A may act as a spermatogenesis regulator (12). Even though α, βA, and βB subunits are expressed in ram testes (in the same types of cell), they only seem to produce inhibin A, not inhibin B, and then export into the circulation (11).

Moreover, the testicular cell proliferation (Sertoli and germ cells) under the control of inhibin, regulates this process at different levels (13). In the adult testis, inhibin is synthesized in Leydig cells but in small amounts, while, the Sertoli cells are mainly responsible for producing the major amount in the adult testis (14,15).

The Inhibin A gene consists of α and β subunits, the location of the β subunit in the fourth chromosome (16), a total size of about 13.8 KB (consisting of two exons and a single intron) (17,18).

Inhibin A considers a marker of fertility because it reflects the efficiency of the testes, in bulls, the abnormal sperm quality (low motility and sperm morphological abnormalities) is related significantly ($p < 0.05$) with the lower inhibin A levels in prepuberal and puberal bulls (19).

Several studies candidate the encoding of INHA, and INHBA genes as a fertility-related marker by influencing the expression of INHBA, the transcription factors, and binding sites (20-22).

Because the studies in this aspect are scanty, and because the Iraqi AI center aimed to improve semen quality by selecting potent progeny bulls and excluding the weak bulls, the present study aimed to determine a relation between βA sheet polymorphism and semen quality in Holstein bulls.

## Material and methods

### Experimental animals

This study was conducted on (n 12) bulls born in Iraq at the AI center in Abou-Ghareeb, west of Baghdad. All bulls were maintained in the same management, feeding, and watering conditions throughout the study which started from January 2022 until February 2022.

Ejaculates were collected weekly by using the artificial vagina method. A total of 48 ejaculates from (12) bulls were studied during the period of this experiment. After semen collection, the samples were straightway brought to the AI center laboratory, samples placed in a (37-38˚C) water bath for macroscopic evaluation (Volume and color) and microscopic estimation (Sperm

concentration, Mass activity, Individual motility, Dead, Abnormality).

**Evaluation of semen characteristics.**

**Macroscopical evaluation**

Directly after collection, the volume of semen was accurately measured from the graduated semen collecting tube. The semen color was classified as Watery/opalescent, milky white, yellowish, and creamy were used to describe the color of the semen according to Sarder (23).

**Microscopical evaluation**

Firstly, the sperm concentration was calculated directly after semen collection by analyzing a drop of fresh semen using an account cell bovine photometer with a diluter and printer. Additionally, the Mass activity percentage was calculated by taking a drop of fresh semen on a warmed slide at 10X magnification under a microscope with an attached stage warmer.

Moreover, Individual motility was Assessed by mixing 1:2 drops of semen with sodium citrate solution (2.9%) on a warm slide (37˚C), and the score of motility was done according to Baril et al. (24).

Furthermore, the dead sperms percent was measured using Eosin (1.67 gm) –Nigrosin (10 gm) stain that included (Eosin (0.167 gm) – Nigrosin (1 gm) stain and 0.29 gm Sodium citrate in 10 ml double distilled water) and examined under a light microscope (40X).

Finally, the sperm abnormalities were calculated on the same slide used for counting dead sperm percent but under (100X) magnification with oil emersion use. The types of abnormalities in the head and tail and any defect in the normal shape of sperm that can see under a light microscope were included (25).

*PCR and genotyping*

*Genomic DNA extraction*

Ten ml f blood samples were collected from 12 bulls via syringe through a vena puncture of the jugular vein and evacuated into vitamin K-contained collection tubes, then transported to the laboratory (Biotechnology Center Company / AL- Harthiya, Baghdad). The samples were stored in a refrigerator at 5 °C until DNA extraction. Genomic DNA was isolated from blood samples by DNA extraction kit (Promega, USA) according to the instructions of the kit.

**Primer design and PCR condition**

Pair of primers were designed manually to amplify the particular DNA region in exon 2 of the INHBA gene online via the Integral DNA technology program for primer design of the gen bank ID: ENSBTAG00000048508 (Table 1).

**Table 1:** Primer sequences, melting temperature, product size, and length (bp).

| gene | Primer | Sequence | Tm (°C) | GC (%) | Product size | length |
|---|---|---|---|---|---|---|
| | Forward | 5'-GAGCCTGGTTAGAGATGATTTG - 3' | 61 | 45. 5 | | 22bp |
| | Reverse | 5'-AGTGAAAGGAGAGGGATGAG - 3' | 61 | 50 | | 20bp |

The PCR tube containing the final reaction volume of 25 µl; 5µl of PCR Master Mix (INtRON/ Korea), 1.5µl DNA, and 2µl pair primer (10 pmol/µl) dissolved in 16.5 µl ddH2O. The exon 2 cycles for the fragment was 34, the PCR optimization program was; 94°C for 3 min, 94°C for 30 sec, 61 °C for 30 sec, 72°C for 40 sec, and 72°C for 10 min for Initial denaturation (one cycle), Denaturation, Annealing, Extension and final extension (one cycle), respectively.

The ethidium bromide-stained 0.02 g was added to agarose gel during the electrophoresis of the target PCR product, then transport to trans-illuminar (miniPCR Bio/ USA) to monitor the results.

### Sequencing and genotyping

The amplicon of each sample (PCR product) was transmission to Korean Macrogen Corporation for made Sanger DNA sequencing by using the sequencer, the sequence of each sample was augmented online by the BLAST tool, which is available on National Center Biotechnology Information website.

### Statistical Analysis

Duncan's Multiple Range test (ANOVA) and T-test were used to compare between means in this study. The Chi-square test was used to compare percentages (a significant level was set at 0.05 probability) in this study.

## Results and Discussion

### Gene Amplification of β subunit of Inhibin A

A part of exon II was amplified by using two primers, the amplification target was the β subunit of the INHA gene for the pure Holstein breed. The fragment size of the maximized Bos taurus INHBA gene was (835 bp).

### Determined the genetic varying of Inhibin βA

Four variants (SNPs) were identified at bovine INHBA-Exon 2; C (903) T, C (916)Y, G (917)K and G (966) R After DNA alignment with the NCBI gene sequence ID: XM_024990466.1 (Figure 1).

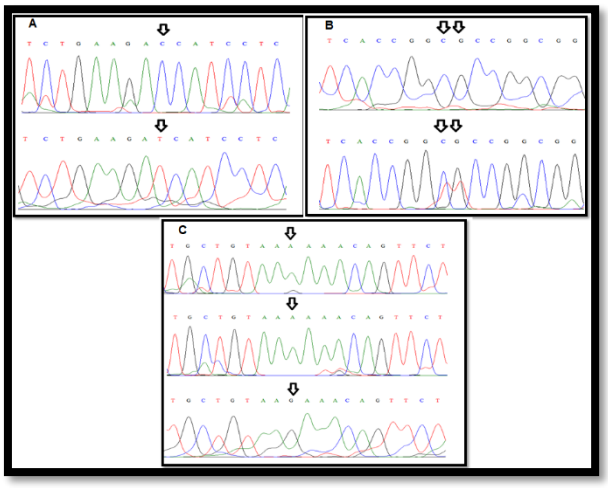


**Figure 1: The INHBA sequencing SNP between the samples. A,B and C showed: C (903) T, C (916)Y, G (917)K and G (966) R respectively.**

Two out of four Exon 2 SNPs were non sense mutations, while the remains were missense, that changed Arginine > phenylalanine at position (306) (Table 2).

**Table 2:** Type of substitution in part of exon II of *Bos taurus* or bovine INHBA gene in Holstein bulls.

| | locus | Code change | Amino Acid change | Predicted effect | Type of mutation |
|---|---|---|---|---|---|
| 1. | C (903) T rs797512549 | GAC > GAT | (Aspartic acid > Aspartic acid (301) | Non sense | Transition |
| 2. | C (916)Y G (917)K | CGC > CGC and TTC | Arginine > phenylalanine (306) | missense | Transversion |
| 3. | G (966) R | AAG > AAG and AAA | Lysine > Lysine (322) | Non Sense | Transition |

Various alleles and genotypes were noticed for all loci of the bovine INHBA gene after making a comparison between the sequences of each sample. According to table 4, highly significant variations were recorded between the genotypic frequencies of C (916)Y, G (917)K, and G (966) R while non-significant relation was monitored for the first locus C (903) T in analyzed population.

**Table 3:** Genotype distribution and allele frequency of mutation

| | Locus | Genotype | Number | Percentage (%) | Allele frequency |
|---|---|---|---|---|---|
| INHBA | C (903) T | CC | 7 | 58.3 | C: 0.79 T: 0.21 |
| | | TT | 5 | 41.7 | |
| | | Chi-Square ($\chi 2$) | 12 | 2.56 NS | |
| | C (916)Y | CC | 8 | 66.7 | C: 0.83 T: 0.17 |
| | | CT | 4 | 33.3 | |
| | | Chi-Square ($\chi 2$) | 12 | 11.56** | |
| | G (917)K | GG | 8 | 66.7 | G: 0.83 T: 0.17 |
| | | GT | 4 | 33.3 | |
| | | Chi-Square ($\chi 2$) | 12 | 11.56** | |
| | G (966) R | GG | 3 | 25 | G: 0.62 A: 0.37 |
| | | AA | 6 | 50 | |
| | | GA | 3 | 25 | |
| | | Chi-Square ($\chi 2$) | 12 | 12.5** | |

** (P≤0.01), NS: Non-Significant.

**Effects of INHBA gene variants on semen quality in Holstein bulls**

The semen quality in bulls was significantly influenced by the INHBA gene SNPs. The interconnection between the INHBA variants and semen quality in Holstein bulls was mentioned in Tables (4,5,6 and 7). Regarding the volume of ejaculates, a non-significant correlation was demonstrated between the genotypes of all loci, except one locus (G (966) R), Whether the finding appeared that bulls with wild genotype (homozygote allele) GG was higher significantly increment compared with the mutant homozygote and heterozygote alleles.

These outcomes agreed with Nikitkina et al. (19) results, who mentioned that the differences in ejaculate volume were no-significantly between the wild and mutant alleles of INHA gene SNP. Additionally, bulls with the wild allele of INHBA had the highest ejaculation volume than the other mutant allele ($p < 0.05$), while Chandra et al. (26) outcomes find a significant relationship between the genotypes.

The bulls that represented wild genotypes showed a significant rise to mutant genotypes in sperm concentration and both mass and individual motility (P≤0.05) in all loci. These findings agreed with Sang et al. (27) findings, who mentioned that polymorphism in the INHBA gene significantly correlated with sperm motility and concentration. Also, it came constant with Nikitkina et al. (22) and Chandra et al. (26), who reported that bulls that possess the wild homozygote genotype of INHA showed a higher spermatozoal concentration than bulls that had the mutant hetero and homozygote respectively, while non-significant difference recorded in motility between the same genotypes.

The results of the dead percentage showed a non-significant correlation between the genotypes of all loci. A high percentage of abnormalities was recorded in both hetero and

homozygote mutant genotypes respectively, but a non-significant correlation was recorded between the G (917)K genotypes, however, Sang et al. (27) reported a non-significant correlation.

**Table 4 :** Effects of Single Nucleotide Polymorphisms (SNP) of C (903) T in INHBA gene on fresh semen quality (Mean ± SE)

| No. | Fresh Semen characteristic | Genotypes (Mean ± SE) | | |
|---|---|---|---|---|
| | | CC N= 7 | TT N= 5 | Sig. |
| 1. | Volume (ml) | 4.00±0.30 a | 3.80±0.15 a | 0.07 NS |
| 2. | Concentration (x10$^6$) | 1824.6±85.3 a | 425.5±32.2 b | 0.00012 * |
| 3. | Mass motility % | 40.0±1.10 a | 10.6±2.20 b | 0.0001 * |
| 4. | Individual motility % | 57.5±0.90 a | 18.1±2.60 b | 0.00015 * |
| 5. | Dead % | 21.8±5.30 a | 22.6±3.90 a | 0.912 NS |
| 6. | Abnormality % | 4.60±0.80 b | 16.1±3.20 a | 0.004 * |

*** (P≤0.05), NS: Non-Significant.**

**Table (5):** Effects of Single Nucleotide Polymorphisms (SNP) of C (916)Y in INHBA gene on fresh semen quality (Mean ± SE)

| No. | Fresh Semen characteristic | Genotypes (Mean ± SE) | | |
|---|---|---|---|---|
| | | CC N= 8 | CT N= 4 | Sig. |
| 1. | Volume (ml) | 5.30±0.52 a | 4.40±0.20 a | 0.130NS |
| 2. | Concentration (x10$^6$) | 1589±162 a | 1291±182.0 a | 0.912 * |
| 3. | Mass motility % | 45.0±1.80 a | 22.5±1.60 b | 0.0003 * |
| 4. | Individual motility % | 58.1±0.90 a | 33.7±1.80 b | 0.0003 * |
| 5. | Dead % | 24.7±4.70 a | 23.3±3.20 a | 0.56 NS |
| 6. | Abnormality % | 4.70±1.20 b | 15.0±3.20 a | 0.015 * |

*** (P≤0.05), NS: Non-Significant.**

**Table 6 :** Effects of Single Nucleotide Polymorphisms (SNP) of G (917)K in INHBA gene on fresh semen quality (Mean ± SE)

| No. | Fresh Semen characteristic | Genotypes (Mean ± SE) | | |
|---|---|---|---|---|
| | | GG N= 8 | GT N= 4 | Sig. |
| 1. | Volume (ml) | 5.70±0.20 a | 5.50±0.50 a | 0.75NS |
| 2. | Concentration (x$10^6$) | 1382±32.0 a | 1129±83.0 b | 0.014* |
| 3. | Mass motility % | 31.3±3.50 a | 19.4±4.00 b | 0.044* |
| 4. | Individual motility % | 44.4±4.80 a | 29.4±4.80 b | 0.047* |
| 5. | Dead % | 24.9±4.50 a | 23.0±3.00 a | 0.38 NS |
| 6. | Abnormality % | 8.10±2.00 a | 6.10±1.50 a | 0.49NS |
| * (P≤0.05), NS: Non-Significant. | | | | |

The SNPs in INHBA seem to be effect negative on semen quality. Because of the inhibitory role of INHA hormone on FSH. The mutations lead to an increase in the activity of INHA by altering the transcription and/or expression of the INHBA gene, therefore, this activity exerts negative feedback on FSH that reflects the worsening of semen quality. This opinion agreed with many previous speculations; Barakat et al. (28) revealed that the possible explanation for the effect of INHBA protein on testes, may be to regulate the gonocytes action specially spermatogonia and round spermatids for immature and adults testis, additionally, it can effect on the Sertoli cell population. In constant, INHBA polymorphism effect postively on fertility in cow (29).

Moreover, the INHA represents a marker of the testicular function, and in particularly Sertoli cell health status and function. During puberty, the INHA hormone was lower for the bulls that had poor semen assessment (low motility rate and high abnormalities) in comparison to bulls with good semen quality (12).

The INHA governed the spermatogonia development, Leydig cells production of testosterone, and modulates the pituitary FSH, therefore, it candidate the encoding INHA, and INHBA genes as a fertility-related marker (21). Giesecke et al (22) mentioned that an INHBA gene SNP may affect the expression of INHBA by altering the transcription factors and binding sites, it firstly candidate the INHBA gene as a marker for stallion fertility (23). The INHA mutation in the Bulls is related to a decline in cell concentration, motility, and ejaculate volume. the bulls with the wild allele had greater motility and collected volume than the bulls that genetically carried the mutant allele, which had the poorest semen quality (24).

**Table 7 :** Effects of Single Nucleotide Polymorphisms (SNP) of INHBA gene G (966) R on fresh semen quality (Mean ± SE)

| No. | Fresh Semen characteristic | Genotypes (Mean ± SE) | | | |
|---|---|---|---|---|---|
| | | GG N= 3 | AA N= 6 | GA N= 3 | Sig. |
| 1. | Volume (ml) | 8.40±0.30 a | 3.40±0.10 b | 3.40±0.40 b | 0.0001 * |
| 2. | Concentration (x10$^6$) | 1780±175.0 a | 1276±69.0 b | 1479.6±76.0 ab | 0.020 * |
| 3. | Mass motility % | 45.0±1.80 a | 12.5±1.60 c | 23.7±7.00 b | 0.0002 * |
| 4. | Individual motility % | 62.5±1.60 a | 22.5±1.60 c | 32.5±2.90 b | 0.0004 * |
| 5. | Dead % | 25.8±2.30 a | 26.3±8.50 a | 28.0±4.70 a | 0.350 NS |
| 6. | Abnormality % | 4.00±0.80 b | 18.3±2.30 a | 8.40±1.20 b | 0.0003 * |

*** (P≤0.05), NS: Non-Significant.**

## Conclusions

The mutant genotype recorded low semen quality in bull, while, the wild genotypes showed superior semen parameters than the wild. It can use the mutation in the mutation in C (903) T, C (916)Y, G (917)K, and G (966) R loci as a genetic marker to improve fertility by excluding the bulls that have these SNPs from the breeding programs.

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## Conflict of interest

There are no conflicts of interest to be declared.

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