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Identification the Effect of Inhibin βA/Activin A Genes Polymorphism on Superovulation (Calving Rate) in Holstein Friesian Cows

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ABSTRACT
This study performed to test the influence of β subunit polymorphisms for inhibin A (αβ) and Activin A (ββ) on the superovulation characteristic. Also to investigate the effect of β subunit polymorphisms on inhibin and FSH concentration during the estrus phase for Singleton birth (SB) and Dizygotic twin birth (DZTB) cows.
Thirty-six mature pregnant Iraqi Holstein Friesians cows were utilized, in the confined period (between July 2018-June 2019), these cows were equally disconnected into two groups according to a type of birth; SB cows and DZTB cows. The DZTB trait indicates that superovulation has occurred. Five pairs primers were utilized to magnificate the whole exon 2 of the β sheet of inhibin A and Activin A by polymerase chain reaction (PCR) after extracting the DNA from blood specimens. The single nucleotide polymorphism (SNPs) were detected through sequencing by matching with the Bos taurus inhibin subunit beta A, NCBI Gene ID: 281867 and Ensembl gene browser ENSBTAT00000066382.1.
The data analysis revealed presence of four missense SNPs in exon 2; G(554)S, G(866)R, C(883)S, A(877)R, and 3 prime UTR variant C(1865)Y. The first four SNPs caused amino acid changes, while the fifth wasn't encode amino acid. Two genotypes were detected for each SNP; (GG and GC), (GG and GA), (CC and CG), (AA and AG), (CC and CT) for G(554)S, G(866)R, C(883)S, A(877)R, and C(1865)Y loci respectively.
Higher significant increase (P<0.01) were showed in mutant genotypic frequencies as a compared with the wild in A(877)R and C(1865)Y loci, a significant variance (P<0.05) were registered between the genotypic frequencies of A(877)R locus, while non-significant variation were noticed between the G(554)S and G(866)R genotypes.
The distribution of genotypic frequencies according to type of birth; the genotypic frequencies for mutant genotypes of recorded a higher significant increment (P<0.01) in dizygotic twin birth cows when compared with the wild genotypes of the G(554)S, G(866)R and A(877)R loci, a significant rise (P<0.05) were viewed in mutant genotypes of the C(883)S C(1865)Y loci. While, higher significant rise (P<0.01) were detected in wild when compared with mutant genotypes in C(883)S, A(877)R, C(1865)Y, and a considerable enhance (P<0.05) in wild against the mutant genotypes in G(554)S and G(866)R for SB cows groups.
In addition, a significant rise (P<0.05) in ovulation rate (calving rate) were noticed in heterozygote mutant genotypes of C(883)S and A(877)R loci.
The hormonal evaluation revealed that measured inhibin A was lower in the DZTB group than SB cows groups at the estrus phase. While the finding showed that mean FSH concentration were higher in the DZTB than SB cows groups.
The distribution of hormonal concentration according to genotypes revealed that the inhibin A concentration was lower (P<0.05) in the mutant heterozygote genotype against wild homozygote genotype in A(877)R locus, otherwise, the FSH level recorded a significant rise (P<0.05) in the mutant heterozygote genotypes when compared with wild homozygote genotypes in C(883)S and A(877)R loci.
In conclusion, the exon II SNPs affect positively on superovulation, besides, C(883)S and A(877)R SNPs affect the calving rate. Furthermore, the finding reported that inhibin A was lesser in DZTB cows than in the SB cows, while, FSH was higher in DZTB cows when compared with SB cows. Moreover, A(877)R SNP contributes to inhibin A concentration (mutant genotypes decrease inhibin A level), C(883)S and A(877)R SNPs participate in the increase of FSH level. The bovine superovulation trait can be enhanced via genetic selection for specific genetic markers. The detected Inhibin βA/Activin A SNPs especially C(883)S and A(877)R can be considered as genetic markers to improve the superovulation in cows for embryo transfer and long term genetic selection.
The focus of the two thieves was lower in the first group than in the second

Keywords: Superovulation, Inhibin A, FSH, DZTB cows, SB cows, β sheet polymorphism.

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INTRODUCTION
Nowadays, more than 500 000 of the cattle embryos are generated per year from superovulated cattle, therefore, the superovulation is a most important step for accomplishing bovine embryo transfer technique (utilized in combination with embryo transfer) to create a good and viable embryo adopted in cattle breeding programs (Mapletoft and Hasler, 2005). A noteworthy, all the techniques used to induce superovulation in cattle are dependent on prompting FSH to maximize the number of ovarian growing follicles (superovulation response) (Kanitz et al., 2002; Baruselli et al., 2016).
Inhibins are gonadal specific hormones, its a glycoprotein in nature (belonging to β-transforming growth factor) with average size 30000 dalton (Vale et al., 1990). Inhibins are considered as heterodimeric or dimeric consist of α and either βA or βB subunit connected by two disulfide binds to create inhibin (A) or inhibin (B), while activins are formed from binding two β chains (βA & βB) to consist either activin A or B (Vale et al., 1990; Mason et al., 1996; Hayes et al., 1998).
There are two active inhibins forms in circulation; The inhibin A is detected in the follicular fluid of cow by ELISA technique (Beg et al., 2016). Although the inhibin B levels aren't noticeable in bovine follicular fluid or serum (Beg et al., 2016; Groome et al., 2001), the βB mRNA is expressed in bovine follicles (small antral) and manufactured a noticeable level of inhibin B.
Inhibins are exclusively expressed in ovine follicular cells (granulosa and theca cells) under the FSH effect (Campbell and Baird, 2001). In the pig, both (α and βA) mRNA are elevated in antral follicles that tend to ovulation, while the reduction happens after ovulation and luteal tissue formation (Guthrie et al., 1992). On the other hand, the small antral follicles are express the mRNA of the βB subunit (Schwall et al., 1990; Fraser et al.,

1993), but the inhibin type B isn't detected in cow plasma or serum (Ireland and Ireland, 1994).

In the cow, the inhibin type A pattern excess gradually with follicular progress, then its decrease to low quantity as soon as after ovulation and luteinization (Hillard et al., 1995).

Inhibins are more ability than activin to bind with inhibin and activin type II receptor because it possess inhibin co-receptor or binding molecules (Lebrun and Vale, 1997) that promote inhibin to bind with activin receptor via its β subunit and block activin binding (Lewis et al., 2000).

Inhibin A involve in folliculogenesis and maturation of oocytes via controlling granulosa cell development (Sirotkin, 2011), but the main function of inhibin A is a dose- based suppress FSH function through diminishing the mRNA of FSH receptor in granulosa cells (autocrine negative regulator of FSH receptors and short feedback loop at gonad level) and inhibin A block estradiol and progesterone-induced by FSH in culture (Lu et al., 2009). Additionally, inhibin A decline the FSH secretion by alter the FSH amplitude and pulse frequency without disrupting the LH level (negative-feedback control), also decrease the pituitary sensitivity to GnRH (Rivier et al., 1991b).

According to Woad et al. (2009), a mutation in has been recorded in inhibin A gene which led to a decrease within the level of inhibin A, this decline led to rising in the level of FSH, which increase the chance of premature ovarian insufficiency or failure (POF) occurrence as a result of premature exhaustion of the follicle pool. Therefore, the main function of inhibin A to prevent POF by negative FSH control (Shelling et al., 2000).

Inhibin A function has been tested via immunization in different animal models; inhibin antiserum treatment in sheep can clearly enhance the ovulation rate and induce multiple ovulations in inhibin immunized ewes (Naqvi et al. 2009). In goats, active and passive inhibin antisera caused a rise in the plasma FSH level, follicles number and the ovulation rate in comparison with control, therefore, it can be utilized to improving superovulation in goats (Sasaki et al., 2006). In addition, the mRNA level of FSH was increased in the pituitary gland of inhibin-immunized rats (Attardi et al., 1992). Moreover, the inhibin-neutralization led to increasing the follicles growth and number of corpora lutea in response to the superovulation program in cattle, this in turn, to enhance the quantity of the transferable embryos in cows or heifers (Takedomi et al., 2005).

The Inhibin A gene consist from α and β subunits, the α subunit gene is located in chromosome 2 (NCBI Reference Sequence: NM_174094.4 and NC_037329.1), it has two exons intermediated by single intron, all of which are presented inside a 3067 bp genomic region (gene size), the exon I size is 322 bp, exon II 860 bp and intron I 1,836 bp (Scarlet et al., 2017; Stangaferro et al., 2014). Whereas the location of the β subunit in the fourth chromosome with Gene ID: 281867 and NCBI Reference Sequence: NM_174363.2 (Zimin et al., 2009), its span 13813 bp (total size) and its consist from two exons and single intron; the exon I, exon II and intron I size are 1388 bp, 3434 bp and 9852 bp respectively (Glister et al., 2010; Stangaferro et al., 2014).

The Inhibin A gene polymorphism has a strong relationship with enhancement the heterozygous twinning rate by effect on FSH level, this feature make it a potent candidate gene to improve breeding in domestic animal; a single nucleotide polymorphisms (SNPs) were related with increase the super ovulatory response in Holstein cows that treated with gonadotropin to induce the ovulation (Tang et al., 2011). Moreover, in Holstein bull, the SNP in βA subunit of IHA gene related with enhancement the sperm standards such as sperm concentration, total volum, sperm motility and acrosome integrity rate, so it is suggested to used for improving of sperm quality by increase the biological efficiency on FSH in testis (Sang et al., 2011).

Several genes influence litter size (ovulation rate) by direct and indirect influence the FSH; several reports pointed out that the Growth differentiation factor 9 (GDF9) gene mutations responsible for increasing the litter by effect increasing the follicles and oocytes number that surrounded by cummulus cells (Hanrahan *et al*., 2004; Al-Mutar et al., 2018). While Leptin gene polymorphism enhance the litter size by effect on LH (Younis et al., 2018).

This study designed to investigate the possible role of β subunit polymorphisms that encode the inhibin A (αβ) and Activin A (ββ) in the superovulation trait of the Holstein Friesians breed, also the relation between the β subunit polymorphisms and inhibin, FSH concentrations.

MATERIALS AND METHODS

Animal management and experimental design (Determined of Traits)

Thirty-six mature multiparous Iraqi Holstein Friesians cows were utilized in this study with an average age of 3-4 years. The cows were reared in several cows stations in Baghdad province at latitude 33 and longitude 44. The experiment was extended from July 2018 to June 2019.

The animals were split into two groups; first group selected according to the case history of Dizygotic twin birthing trait (DZTB) (n =18), while the second group represented by the cows that delivered single birth/per birth, that referred as Singleton birth (SB) cows (n =18).

The blood samples were collected from each cow of the two groups for genotyping by sequencing technique. Another blood collection was at the time of the estrus phase as soon as the end of puerperium for Inhibin and FSH hormones analysis.

The signs of estrus were observed by owners and co-workers. When the mother of DZTB became pregnant, the rectal examination was performed to detect the twin's (uterine horns were symmetrically swollen, corpora lutea were present in each ovary, and four cases, both corpora lutea lie on the same ovary) (only the confirmed twins pregnant cows were selected in the second group).

DNA Isolation and βA subunit for *Bos taurus* Inhibin A/ Activin A (Exon 2) Amplification

The Genomic DNA was firstly purified according to standard procedures using proteinase K and RNase A, thats available with G-spin-DNA extraction kit (Promega/ USA) followed by DNA binding, washing and elution according to Kit manual. Many primers (5n pairs) were experimentally designed and utilized to amplified the

limited region between the exon 2 and 3'-UTR of Inhibin βA/Activin A. The primers were designed manually by Integrated DNA Technologies (IDT) program- Primer design, depend on ensemble gene bank: ENSBTAG00000048508 of Inhibin subunit beta A of cow (Table 1).

Table 1: The primers of Inhibin A/ Activin A-β subunit gene amplication

	Primer	Length	Amplicon Length	Start-stop	Tm	GC%
1	F:GAGCCTGGTTAGAGATGATTTG R:AGTGAAAGGAGAGGGATGAG	F:22 R:20	835 bp	482 to 1317	61°C	F:45.5% R:50%
3	F:AAGGTCAACATCTGCTGTAAG R:GTGTGTGCTGGGTATGTATG	F:21 R:20	880 bp	1157 to 2037	60°C	F:42.9% R:50%
2	F:TGACCCTCTCTCTGTGTATATC R:TCTCAAGCCTGAACACAATC	F:22 R:20	910 bp	1952 to 2862	60°C	F:45.5% R:45%
4	F:GTCTGTCTGTGGTTGGTATC R:CTCATTCCTGTTTCCCATTCT	F:21 R:20	872 bp	2774 to 3646	60°C	F:50% R:42.9%
5	F:CGCAGCTGGACTCAATAAT R:GGCTTCAGTAGATGGCATAC	F:19 R:20	964 bp	3593 to 4503	60°C	F:47.4% R:50%

The optimization for the anealing was match the Tm of each primers. The total volume of the reaction was 25 in eppendorf tube; 16.5 µl ddH2O, 1.5µl DNA, 5µl of PCR Master Mix (INtRON/ Korea) and 2µl pair primer (10 pmol/µl). The exon 2 Inhibin/ Activin βA subunit gene expansion cycles for the five fragments were 34; A. Initial denaturation at 94°C for 3 min, B. Denaturation at 94°C for 30 sec, C. Anealing was 61 °C for 30 (fragment 1), while the rest fragments was 60 °C for 30, D. Extension at 72°C for 40 sec, E. The final extension was 72°C for 10 min. The amplified products were effectively electrophoresed after loaded on the ethidium bromide-stained 0.02 g agarose gel and monitored on the tran illuminar apparatus (CBS Scientific/ USA).

Genotyping **of βA subunit for Bos ta**urus Inhibin /Activin (Exon 2)

By using Sanger sequencing method, the PCR amplicones were successfully processed (Macrogen company/ Korea). The genotyping was determined by utilized Bio Edit program and used "BLAST" option which was obtainable at NCBI.

Plasma Inhibin and FSH Assay

The peripheral Inhibin and Activin hormones for the both cow's samples groups were analyzed by used Bovine Inhibin A and FSH ELISA kits (Mybiosource / USA) according to kits manuals. The sensitivity range for Inhibin A and FSH ELISA Kits were 62.5-2000 pg/ml and 0.625 -20 ng/ml respectively. According to kits company commitment; there were no significant cross reactivity or interference between the sub units.

Statistical analysis

The significant compare between the genotypes of the Bos taurus Inhibin A/Activin A (βA-subunit) were done by used chi-square test on the Statistical Analysis System SAS (2012), while T test was applied to compare between calving rate and hormonal levels.

RESULTS

β subunit of Inhibin A/Activin A Gene Amplication

Five pairs of primers that replicate the particular regions (whole exon II and 3 UTR) of the Holstein Friesians breed Inhibin gene (β subunit). The magnified *Bos taurus* Inhibin βA/Activin A gene for Holstein Friesians breed showed as five different size fragments (835 bp, 880 bp, 910 bp, 872 bp, and 964 bp) after electrophoresed in 0.02 agarose gel (Figure 1), whereas the target fragments which came consistent with the predicted sizes.

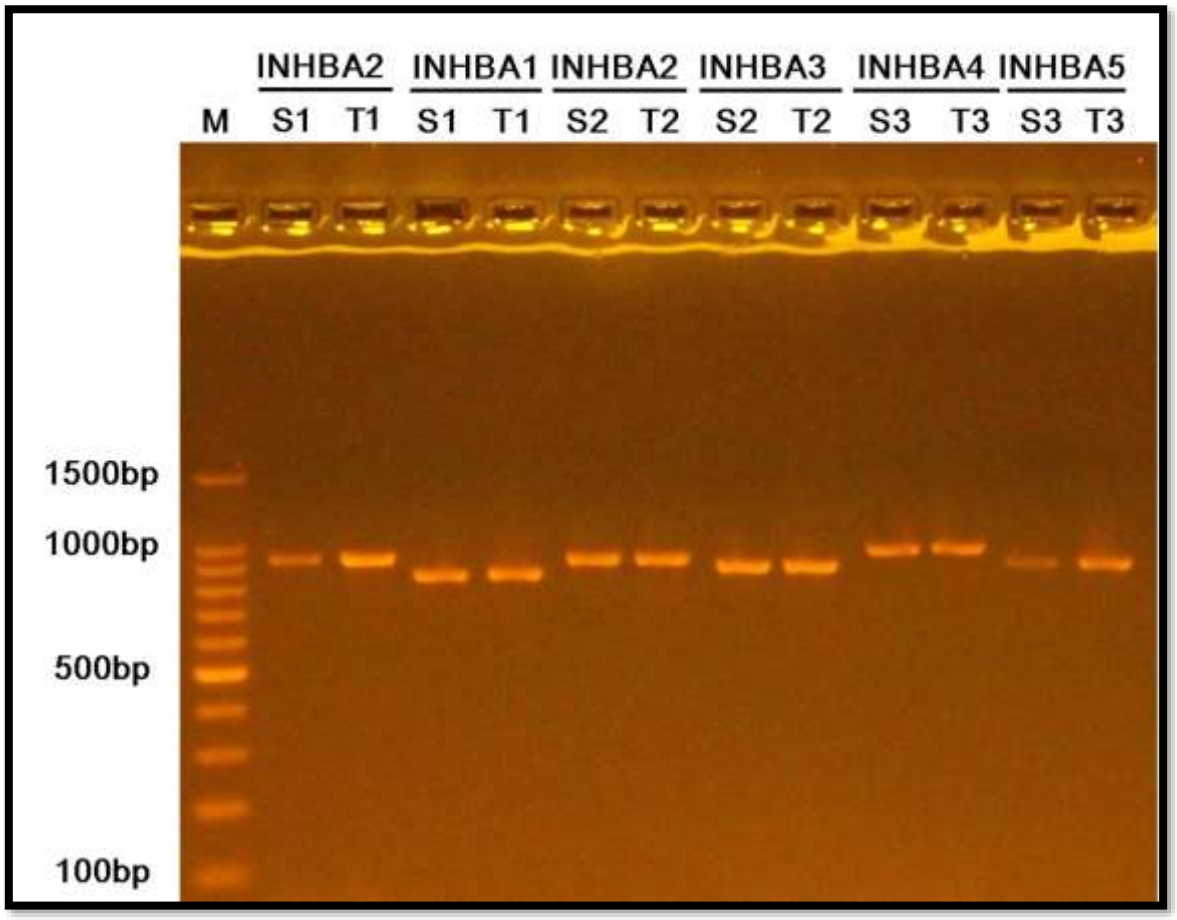

Figure 1: The amplicon fragments of Inhibin βA/Activin A gene, S: Singleton birth. T: Dizygotic twin birth cows. M: DNA ladder.

Genetic variability determine of **Inhibin βA**/Activin A (**β subunit**)

The polymorphisms of bovine Inhibin βA/Activin A were detected by PCR- Sequencing. After DNA alignment with the NCBI sequences, four variants were identified at Exon 2 and one SNPs in 3 UTR of Inhibin βA/Activin A gene for Holstein Friesians breed; G(554)S, G(866)R, C(883)S, A(877)R in Exon 2 and C(1865)Y in noncoding 3 UTR when compared with Gene ID: 281867 and ENSBTAT00000066382.1 (Figure 2). The Exon 2 SNPs were missense mutations (not detected before), and that chinged Serine > Threonine, Arginine > Lysine, Glutamine > Glutamic acid and Methionine > Valine respectively, while the last C(1865)Y was recorded before; rs448995057 (Table 2).

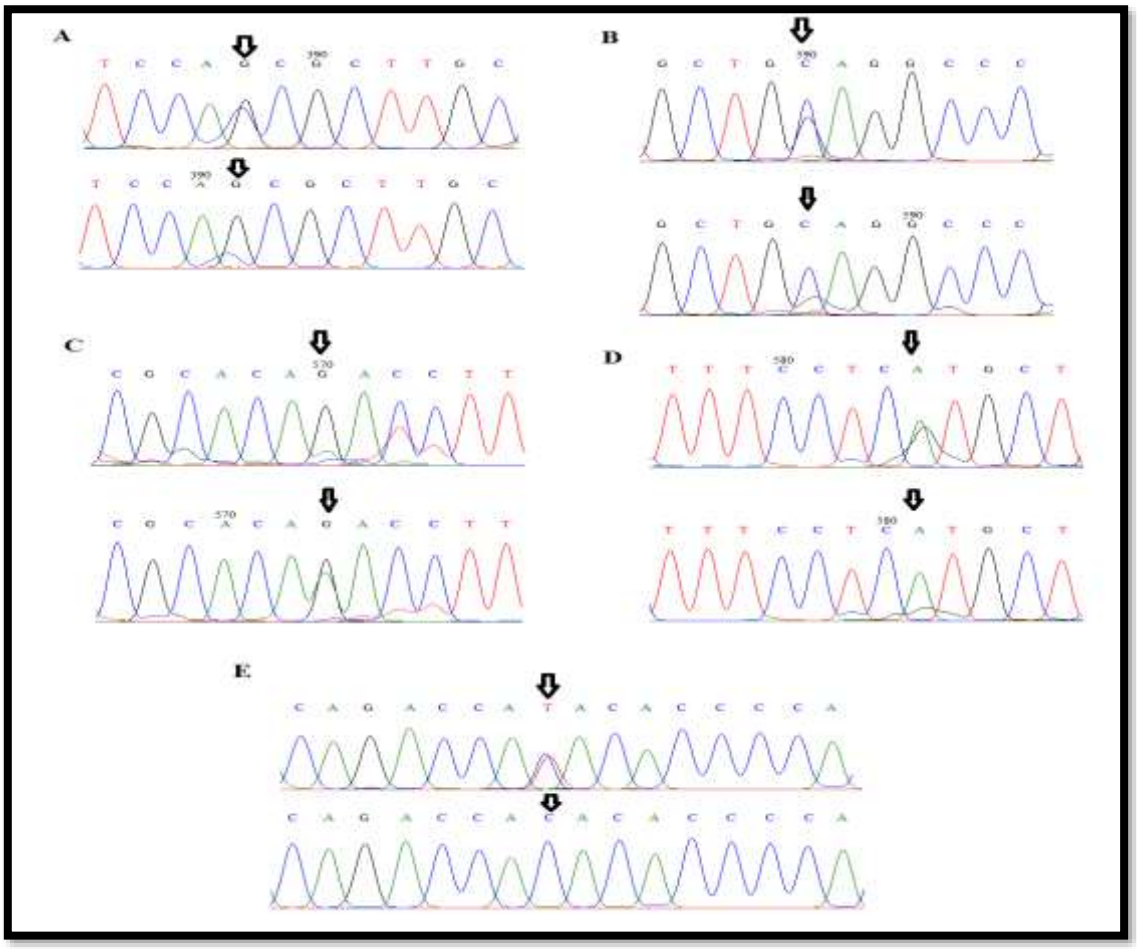

Figure 2: The Inhibin βA/Activin A sequencing variats between the Holstein Friesians cow samples. A,B,C and D: represent the Exon 2 SNPs, E: 3 UTR variant.

Table 2: The Exon 2 and 3 UTR variants of Holstein Friesians cows Inhibin βA/Activin A gene

	SNP Location	Code change	Amino Acid change	Predicted effect	Type of mutation
1	G/S 554	AGC> ASC	185 Serine > Threonine	Transversion	Missense
2	G/R 866	AGA>ARA	289 Arginine > Lysine	Transtion	Missense
3	C/S 883	CAG>SAG	295 Glutamine > Glutamic acid	Transversion	Missense
4	A/R 877	ATG> RTG	293 Methionine > Valine	Transtion	Missense
5	C/Y 1865	3 prime UTR variant			

According to these variants; two different genotypes and alleles were observed for each locus of bovine Inhibin βA/Activin A gene when compared between the sample sequences. The Inhibin βA/Activin A gene frequencies of genotypes and alleles of in analyzed population showed in (Table 3).

Table 3: The Alleles and Genotypes of Inhibin βA/Activin A gene loci (Exon 2 and 3UTR)

	Locus	Genotypes	Observed Genotypes	Genotypes Frequency	Allele Frequency		Chi Square
1	G(554)S	GG	19	52.80	G	0.76	1.274 NS
		GC	17	47.20	C	0.24	
2	G(866)R	GG	18	50.00	G	0.75	0.00 NS
		GA	18	50.00	A	0.25	
3	C(883)S	CC	13	36.00	C	0.68	8.931 **
		CG	23	64.00	G	0.32	
4	A(877)R	AA	16	44.40	A	0.72	4.389 *
		AG	20	55.60	G	0.28	
5	C(1865)Y	CC	15	41.70	C	0.71	6.025 **
		CT	21	58.30	T	0.29	

*(P<0.05), ** (P<0.01).

According to table 3, non-significant relationship was noticed between genotypes and alleles frequencies of G(554)S and G(866)R loci in exon 2, otherwise, a higher significant differences (P<0.01) were identified between the alleles and genotypes of C(883)S locus in exon 2 and C(1865)Y locus in 3UTR, additionally, the genotypes and alleles of A(877)R locus showed significant variations (P<0.05).

The distribution of **Inhibin βA**/Activin A genotypes according to the type of birth and calving rate

The finding of correlation between the Inhibin βA/Activin A mutations and calving rate (superovulation) phenomenon in Iraqi Holstein Friesians cows were mentioned in (Table 4); A higher significantly increase (P<0.01) were detected in mutant genotypes (heterozygote alleles) as compared to wild genotypes (homozygote alleles) at C(883)S, A(877)R and C(1865)Y loci in the cows that had twin birth (Dizygotic). Furthermore, a significant rise (P<0.05) was showed in mutant genotypes for G(554)S and G(554)S when compared with wild genotypes at the same cow's group.

On the other hand, in cows that had Singleton birth; the wild genotypes and alleles were higher significantly excessed (P<0.01) as compared with mutant genotypes in G(554)S, G(866)R and A(877)R loci, Conversely, the wild genotypes demonstrated a significantly increment (P<0.05) in comparison with the rest genotypes in C(883)S and C(1865)Y loci.

The reproductive performance (calving rate) for Iraqi Holstein Friesians cows was calculated according to genotypes of loci, a significant increased (P<0.05) in calving rate were monitored in the mutant CG and AG genotypes when compared with wild CC and AA at C(883)S and A(877)R loci respectively. While, non-significant variations in calving rate were recorded among the genotypes of the G(554)S, G(866)R and C(1865)Y loci.

Table 4: Comparative between the Inhibin βA/Activin A genotypic distribution in DZTB and SB cows groups with the calving rate

Locus	Genotype	Genotypic Frequency for DZTB cows	Chi Square	Genotypic Frequency for SB cows	Chi Square	Calving rate	T- Test
G(554)S	GG	8 (44.4%)	4.735 *	11 (61%)	7.502 **	1.42	0.249 NS
	GC	10 (56.6%)		7 (39%)		1.58	
G(866)R	GG	7 (39%)	7.502 *	11 (61%)	7.502 **	1.38	0.255 NS
	GA	11 (61%)		7 (39%)		1.61	
C(883)S	CC	3 (17%)	13.647 **	10 (56%)	4.735 *	1.23	0.271 *
	CG	15 (83%)		8 (44%)		1.65	
A(877)R	AA	4 (28%)	12.461 **	12 (67%)	9.233 **	1.25	0.266 *
	AG	14 (72%)		6 (33%)		1.65	
C(1865)Y	CC	6 (33%)	9.233 **	9 (56%)	4.735 *	1.4	0.256 NS
	CT	12 (67%)		9 (44%)		1.65	

* (P<0.05), ** (P<0.01).

The comparative between plasma Inhibin A level in singleton and multiple birth Holstein Friesians cows at estrus

The outcomes of the present experiment showed that the mean plasma Inhibin A concentration in mothers of DZT was significantly lower (P<0.05) than SB cows during the estrus phase. In addition, The DZTB cows group recorded a significantly raised in mean plasma FSH concentration in comparison to SB cows group at same period (Table 5).

Table 5: Inhibin A and FSH levels in DZTB and SB cows during estrus phase

Hormones level		DZTB cows (Group 1)		SB cows (Group 2)		P-value
Inhibin A pg/ ml	Estrus (0 h)	155.2	165.8	180.6	192.8	18.304 *
	Estrus (24 h)	176.4		205		
FSH ng/ml	Estrus (0 h)	13.2	8.15	11.1	6.25	1.085 *
	Estrus (24 h)	3.1		1.4		

* (P<0.05).

Correlation between **Inhibin βA/Activin A** gene polymorphism and mean Inhibin A and FSH levels

However, the outcomes displayed that a significant increased (P<0.05) in Inhibin A concentration for AG genotype as a compared with AA for A(877)R locus at estrus, the other loci recorded non-significant differences in Inhibin A concentration between their genotypes, which mean that's SNPs had not effect on Inhibin A concentration in the same period. Moreover, there were a significant increased (P<0.05) in FSH level for CG and AG genotypes when compared with CC and AA for C(883)S and A(877)R respectively at estrus period, otherwise, non-significant differences were recorded in FSH level between the genotypes of G(554)S, G(866)R and C(1865)Y loci at estrus phase.

Table 6: The Mean Inhibin A and FSH levels according to Inhibin βA/Activin A genotypes

Locus	Genotypes	Mean Inhibin A at estrus (pg/ml)	T- Test	Mean FSH at estrus (ng/ml)	T- Test
G(554)S	GG	184	0.197 NS	6.95	0.662 NS
	GC	173		7.25	
G(866)R	GG	178	0.205 NS	6.8	0.428 NS
	GA	174		7.11	
C(883)S	CC	186	0.273 NS	6.61	0.594 *
	CG	159		7.38	
A(877)R	AA	191	16.338 *	6.55	0.602 *
	AG	157		7.46	
C(1865)Y	CC	170	11.08 NS	6.92	0.533 NS
	CT	162		7.44	

* (P<0.05).

DISCUSSION

The present study clarified that the 5 detected variants in Inhibin βA/Activin A gene represented the genetic variety, the first four variants were missense; G(554)S chinged polar, non-charged Serine to polar, non-charged Threonine, G(866)R converted positively charged, polar and hydrophilic Arginine to Lysine (same chemical properties), C(883)S chinged polar, non-charged Glutamine to negatively charged, polar; hydrophilic Glutamic acid, A(877)R altered polar, non-charged Methionine to non-polar, aliphatic residues Valine. The fifth variant does not code amino acid.

Two different genotypes were appeared in each SNP locus of Holstein Friesians cow Inhibin βA/Activin A gene with significant differences between the genotypes of the last three loci (Table 3), while non-significant differences were exhibited between the genotypes of the first two loci.

Regardless the missense mutation was happened in G(554)S and G(866)R loci, but the chemical properties of the resulting amino acids does not differ from the previous, which may interpret the non-significance relationship between the genotypic frequencies of the G(866)R and G(554)S loci. On the other hand, the genotypic frequencies of the C(883)S and A(877)R loci noticed significant differences may because of the massive divergence between the chemical characteristics of the original and altered amino acids.

The exon 2 mutations not previously determined, this finding comes constant with Yang et al (2014), which identified one SNP in the intron region of Inhibin βA/Activin A gene of Chinese Holstein cows.

In addition, several nucleotide mutations of exon 1 and 2 of the Inhibin βA/Activin A gene were identified in different sheep breeds (Chu et al., 2007).

The correlation between **Inhibin βA/**Activin A genotypes and type of birth in Holstein Friesians cows

According to table 4, in DZTB cows group; the major variations (P<0.01) between the genotypes were noticed in C(883)S, A(877)R and C(1865)Y loci (heterozygote mutant genotypes exhibited higher significantly increment than homozygote wild genotypes). While in the SB group; the wild homozygote genotypes showed higher significant raise (P<0.01) in G(554)S, G(866)R and A(877)R loci.

These findings lead to the belief that the mentioned mutations have a relative effect on increasing the proportion of twin births, in a more accurate sense, an increase in the ovulation rate in Holstein Friesians breed.

A few researchers have highlighted the effect of Inhibin βA/Activin A mutations on reproductive performance in cows and bulls, this study agreed with Sang et al (2011), they demonstrated that Intron 1 SNP had an effect on enhancing sperm motility in Chinese-Holstein bulls.

Yang et al (2014) results did not exhibit a correlation between the polymorphism in the Inhibin βA/Activin A gene and the superovulation in Holstein breed, this may be due to the fact that the variant occurs in the intron, an area that does not encode amino acid, whereas, in the present study, four SNPs detected in exon 2, and that the resulting amino acids differ in their chemical properties from original amino acids of the wild codon.

Furthermore, 3' untranslated region SNP of Inhibin B was detected in Hu sheep breed by Chu et al (2011) and claimed that effect on litter size (ovulation rate). Moreover, Chu et al (2012) referred that the exon 2 polymorphism of the goats Inhibin B gene was related to litter size.

The calving rate reflects the ovulation rate in the cow. In this study, a significant increase (P<0.05) was noticed in mutant heterozygote genotypes of C(883)S and A(877)R, and non-significant relationship was recorded between the genotypes of the rest loci.

The genetic factor contributes mainly in determining the ovulation rate in cattle. Because of the inhibin A paly main role in control FSH and follicular growth, therefore, the Inhibin A mutation effect on ovulation rate and subsequently on calving rate.

These outcomes came consonant with several previous reports which confirmed the role of genetic impact on the ovulation rate and calving rate (twinning rate); Echternkamp et al (1990) informed that heritability has a role in increasing the cattle twinning rate. Van Vleck et al (1991) that the twinning rate trait inherited by selection to first-generation heifers.

Comparative between plasma Inhibin A level in singleton and multiple birth Holstein Friesians cows at estrus

As expected, Table 5 showed the mean inhibin A level in the DZTB group was lesser significantly (P<0.05) than SB group and the FSH level in DZTB cows was significantly superior (P<0.05) than SB cows.

This finding can be explained by the fact of the superovulation resulting from dropping in inhibin A level in DZTB cows in comparison with SB cows which a rising of FSH level in DZTB cows. The FSH is very important for the growth and maintenance of the primary follicles also protects the growing follicles from atresia. Therefore, cows require more FSH for eliciting and select more than one antral follicle (superovulation).

This hypothesis agreed with Lopez et al (2005), who showed that inhibin level was lower in heifers that emerged double predominant follicles than had single predominant follicle emergence. The lower level of inhibin led to a rising FSH level; the peripheral FSH concentration was higher in cows group that develop double dominant follicles compared with mono dominant follicle.

In addition, this observation agrees with Baliarti's (2013) study, which demonstrated that the FSH level in DZTB cows which had double dominant follicles was much higher than single emergence follicles in SB cows.

Furthermore, Lambalk et al (1998) results demonstrated that DZT produces because endogenous FSH hyper stimulation to ovary through pituitary or hypothalamic mechanisms in women, but didn't record a significant fluctuations in inhibin A and B levels between the control and DZT.

Correlation between **Inhibin βA**/Activin A gene polymorphism and mean Inhibin A and FSH levels

The inhibin A levels during the estrus phase record a significant variance (P<0.05) between the genotypes of A(877)R locus, Whilst non-significant variations were recorded in the rest loci. Furthermore, the FSH level recorded a significant variation (P<0.05) between the genotypes of C(883)S and A(877)R loci, whilst, no significant differences monitored in the remaining loci.

The heterozygote alleles A and G effect negatively on inhibin A levels at the estrus phase. It may be because the changes in amino acid (Methionine to Valine) made Structural changes in mature protein shape and in chemical characteristic especially in polarity because the valine is a polar amino acid, this reflects the inhibin A action or decrease expression of this hormone, the decline in inhibin A level reflect positively on FSH level (Inverse relationship), whereas the FSH recorded a significant increase in the same mutant genotype.

Otherwise, Activin A consists of two βA sheet, so the βA sheet encoded A(877)R mutation may affect Activin quality or overexpression than normal and that may be led to extra stimulation of FSH in DZTB cow.

The FSH level excesses led to super stimulation of follicular growth and led to induced superovulation as happens in the case of DZTB cow.

This hypothesis agreed with Tang et al (2011), who supposed that an Inhibin α sheet gene mutation caused a decline in inhibin level by decrease inhibin gene expression), and that caused rise FSH. even though Tang's studied mutation was nonsense, they assumed that affects inhibin function through effect on protein folding.

The findings of present study corresponded with Sang et al (2011), it is proposed that C(7639)T SNPs (rs43408735) of the Inhibin A gene related with enhancement the sperm parameters in bull such as; sperm volume, motility and sperm concentration although even though the SNP locate in intron 1(non-coding region), as a compared to our results, four SNPs were detected in coding region that made changes in amino acids.

The decrease in the inhibin A concentration or level due to the mutation in the inhibin gene encoding, or due to a lack of secretion as a result of other factors, as in the case of immunization, lead to a prompt FSH. Likewise, any increase in the activin A secretion leads to an increase in the FSH secretion. These assumptions were agreed with Bleach et al (2001), whereas the results of their research results confirmed that the producing inhibin A by dominant follicle had a potential role as a negative feedback regulator in termination of the FSH surge in the cow. In addition, the Sasaki et al (2006) results showed that immunization of inhibin A led to increment FSH plasma level and subsequently the follicular growth and ovulation rate in doe.

Furthermore, activin A, as we mentioned above, stimulate FSH secretion, Bernard and Tran (2013) showed that activin A induces pituitary FSH secretion in gilts by regulated the FSH beta subunit transcription.

CONCLUSION

The bovine superovulation trait can be enhanced via genetic selection for specific genetic markers. It should be emphasized that β sheet compose inhibin A (αβ) and activin A (ββ) (that has been studied in this research), for this reason, the polymorphism may decline in inhibin A and or improve activin activity, that led to overstimulation to FSH. The detected Inhibin βA/Activin A SNPs especially C(883)S and A(877)R can be considered as genetic markers to improve the superovulation in cows for embryo transfer and long term genetic selection.

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