Inspection the Linkage between Bovine GDF9 SNPs and Calving Rate (Superovulation) in Holstein Friesians Cows

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Abstract

This study prepared to explore the relation between Growth Differentiation Factor 9 (GDF9) genotypes and calving rate, Follicle-stimulating hormone (FSH) and Estradiol (E2) in Iraqi Holstein Friesians breed. Fifteen blood samples collected from a mother of DZTB (with high calving rate records), another blood sample collected from 15 SB cows, the DNA extracted and six primers designed for PCR and sequencing analysis. The FSH and E2 level tested through estrus phase for the two groups (10 n for each group). The sequence evaluation revealed that presence 2 single nucleotide polymorphisms (SNPs) in exon II; A (1109) T and G (1133) A. The genotypic frequency for mutant genotypes was higher significantly (P<0.01) in DZTB cows (with calving rate) than wild at the same loci. On the other hand, the wild genotypes recorded higher significant increment (P<0.01) for SB cows when compared with mutant genotypes in the same loci. Moreover, a significant rise (P<0.05) was reported in E2 and FSH levels for DZTB cows and mutant genotypes (P<0.01) against SB cows and wild genotypes respectively in 0 and 24 h of estrus phase. Non-significant differences recorded in E2 concentration between the same genotypes at same period.

In conclusion, the GDF9 exon II SNPs increase the calving rate in Holstein Friesians cows. The blood FSH and E2 concentrations are higher in the mother of DZTB may control the superovulation. Finally, these SNPs can be considered as markers to accelerate the breeding programs and used in embryo transfer and in vitro embryo production for Iraqi Holstein Friesians cows breed.

Keywords: Calving rate, Dizygotic twinning, GDF9 polymorphism, FSH, Estradiol.

Introduction

Iraqi Holstein Friesians cows breed consider a multi-purposes livestock animals. Because of the high reproductive performance of this breed as compared to other breeds, they had been used for an economic point in Iraq (1). Therefore, it's necessary to upgrade the information about their physiology of reproduction especially the folliculogenesis to improve the reproductive in these animals.

There are many genes contributed directly to folliculogenesis and ovulation such as GDF 9, bone morphogenetic protein 15 (BMP15) (2), these genes involved with fertility and fecundity.
Oocytes specific GDF9 or FecG is a part of the transforming growth factors that belong to β superfamily that is expressed particularly during folliculogenesis, it's a vital factor for female fertility and folliculogenesi (3).

In cattle, both GDF9 and BMP15 act together to control the development of ovarian follicles, and the immunization (neutralization) of GDF9 alone or with BMP15 resulted in suppressed in cow folliculogenesis (3).

The mRNA of GDF9 is detected (expressed) particularly in the bovine ovarian oocytes, granulosa cells, cumulus cells in primary and antral follicles (4). The expression and continued during fertilization and cleavage until 8-cell stage, as well as, GDF9 is expressed in the bovine pituitary tissues (5). This factor contributed in female fertility through regulation of the folliculogenesis and control the ovulation rate by several mechanisms after binding to own receptor (TGFβ-R1) (6). Therefore, this gene has been vastly investigated and was candidate as a marker to improve the reproductive efficiency in cattle and other domestic animals.

The role of GDF9 in folliculogenesis and ovulation well observed, and without GDF9, the folliculogenesis and subsequently fertility diminished. A block in folliculogenesis and infertility noticed when GDF9 gene knock-down at the primary stage in mice (7). Spicer et al. (2008) (8) study demonstrated that GDF9 treatment caused decline both androstenedione and progesterone production in bovine thecal cells that induced by luteinizing hormone (LH). Additionally, GDF9 administration stimulates proliferation of theca cell and prevent early follicular development by prohibiting theca interna premature differentiation.

Moreover, Choi et al. (2014) (9) mentioned that paracrine secretion of pituitary GDF9 mediates mRNA expression of FSH by in gonadotrope cells under effect of Gonadotropin releasing hormone (GnRH), additionally, the exogenous GDF9 administration prompt FSH secretion in the same cells. Whilst, antibodies neutralization of GDF9 was minimized the FSH mRNA expression.

BOS Taurus GDF9 gene located in fifth bovine autosomal chromosome. The length about 2754 base pairs (BP), it contains two different size exons discrete by a single intron (1126) BP. I size is 397 BP, while exon II spans 1000 BP. The exon I encode 134 amino acids, and exon II encodes 456 amino acids (10).

Because of the site of GDF9 mRNA expression (in pituitary and ovarian cells), and because the vital role of GDF9 in folliculogenesis, numerous studies have referenced to the role of GDF9 gene in ovulation rate and twinning. In sheep, Hanrahan et al. (2004) (2) find eight mutations in GDF9 codon, and these nucleotide substitutions enhancing the litter size and another concerned with sterility. Besides, Santos-Biase et al. (2012) (11) results showed a correlation between GDF9 SNP with the number of collected oocytes from the cow follicles.
Non-identical or Dizygotic twin originate from two different spermatozoa that successfully fertilize two entirely different oocytes as a result of superovulation, a dizygotic twinning in cattle uncommonly occurred, it's inherited phenomenon (12). The genetic selection program for twining trait achieved through selection in from 0.6 to 4% in the sixth parity Norwegian cow breeds (13). Even though twinning in cattle associated with complications like shorter gestation length, reduced calf weight, dystocia, increase risk of retained of placenta and lowering the fertility (14). But the superovulation an important to bovine embryo transfer and cattle breeding programs (15).

As mention above, the twining in cattle is under genetic control. Therefore, this study aimed to inspect the polymorphism in bovine GDF9 gene and to found a possible relationship with twining rate or calving rate through an effect on FSH level and folliculogenesis.

**Material and methods**

**Animal management and data collection**

A total of 30 n normal multiparous Holstein Friesians cows utilized in this experiment with an average age 4.5 years.

For sequencing and genotypic analysis; Group 1 (15 n) were chosen from double births cows (non-identical phenotypically twin births with two separated placenta) that calved recently with case history DZTB every year with high calving rate records. In opposite, 15 n SB cows chosen as a control (Group 2). For hormonal Assay, the samples taken from 10 cows of the group 1 and from 10 cows of the group 2 every 24 hours at the beginning of estrus phase of the first estrus after puerperium.

The estrus detected by observed the cows for signs of estrus with hormonal detection of the progesterone (began to increase more than basal concentration then rise up to 1 ng/ml) and E2 levels. When the cows of the 1st group became pregnant, the rectal examination used to detect the twin (two corpora lutea and symmetrically swollen of the uterine horns). Also, the pregnancy and twin pregnancy confirmed by ultrasound (Bondway BW570V/ China). All cows that’s not give twin birth were excluded from the experiment. The samples taken from several cattle stations in Salah Aldin province, and the experimental interval extends from February/ 2018 to August/ 2019.

The blood samples (5 ml per cow) were aseptically aspirated by veterinarians from jugular venous puncture into the heparin-collection tube and gel tube (APTACA/Italy) for genotyping and hormonal assay respectively, then the plasma and serum preserved at -20 °C.

**DNA extraction and BOS Taurus GDF9 gene amplification**
The DNA of blood samples (single and multiple birth animals groups) was extracted by using DNA isolation G-spin Kit (Promega/ USA) as stated by manufacturer’s kit protocol. The DNA concentration tested by Nanodrop (LG/ Korea) on wavelength 260/280 nm.

Bovine GDF9 gene consists of two exons, according to Hanrahan et al (2004) (2), Exon 1 codons do not involve in mature protein because the cleavage occurs in the mature peptide that exon I encoded it. Therefore, exon I was excluded from this study. Exon 2 amplified by designed six specific primers. Two primers designed manually in this study (fragment 3), and another four designed as stated by Tang et al (2013) (16), based on Gene Bank ID: 282574, Reference Sequence: NC_037334.1 and ENSBTAT00000012476.2 as follows (Table 1).

**Table 1: The primer's information that used for amplification and sequencing of bovine GDF9**

<table>
<thead>
<tr>
<th>Fragments</th>
<th>Primer</th>
<th>Primers sequence</th>
<th>Tm (°C)</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragment 1</td>
<td>F1:</td>
<td>TGG CAT TCC CTC CAC CCT AA</td>
<td>58°C</td>
<td>Part 1 of exon II</td>
</tr>
<tr>
<td></td>
<td>R1:</td>
<td>TCC AGT TGT CCC ACT TCA GTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fragment 2</td>
<td>F2:</td>
<td>CTC CTC AGT GCC AAG ACC AT</td>
<td>61°C</td>
<td>Part 2 of exon II</td>
</tr>
<tr>
<td></td>
<td>R2:</td>
<td>GAT AGA TGC CAC AGA ATA CGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fragment 3</td>
<td>F3:</td>
<td>GAG CCT GGT TAG AGA TGA TTT G</td>
<td>61°C</td>
<td>Most of exon II</td>
</tr>
<tr>
<td></td>
<td>R3:</td>
<td>AGT GAA AGG AGA GGG ATG AG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The PCR reaction with total volume consists of DNA 1.5μl, PCR Master Mix Kit (INtRON/ Korea) 5μl, two primer 2μl (10 pmol/μl), ddH2O 16.5 μl. The amplification cycles for the three fragments were 34. The initial denaturation at 94 ⁰C for 3 min, denaturation at 94 ⁰C for 30 sec, annealing at 58°C for 30 sec. (fragment 1) and 61°C for 30 sec (fragment 2 and 3). The extension at 72 ⁰C for a min, then followed by final expansion at 72 ⁰C for 10 min. After GDF9 fragments amplification, the Amplicons successfully detected after electrophoresis (CBS Scientific/ USA) on 0.02 Agarose gel stained by Ethidium bromide.

**BOS Taurus GDF9 sequencing and genotyping**

The Amplicons (PCR products) were automatically processed by Macrogen company/ Korea according to the Sanger sequencing method, and aligned with the reference-
The genome sequence of BOS Taurus GDF9 by using online BLAST option in NCBI. The SNPs determined by visual inspection of samples on Bio Edit program and NCBI.

**Hormonal Assay**

The serum concentrations of FSH and Estradiol β17 was assayed via utilizing a Bovine FSH and Estradiol β17 ELISA Kits (Sun Long Biotech\ China), according to the kit manual procedure. Progesterone level at estrus detected by IMMULITE 2000 XPI (Siemens/ Germany).

**Statistical analysis**

The significant compare between genotyping frequencies analyzed through direct using the Statistical Analysis System- SAS program with the chi-square test. The significant compare between litter size of animals determined by utilized T test.

**Results**

**PCR amplification**

Six primers that replicated the whole exon II of Holstein Friesians cows GDF9 gene. The amplified BOS Taurus GDF9 gene (exon II) for Holstein Friesians cows appeared different size fragments (940 BP, 255 BP and 283 BP) when electrophoresis in 1.5% Agarose gel (Fig. 1). The target products (replicated fragments) were proportionate with the predicted size and NCBI BOS Taurus GDF9 gene.

**Fig. 1:** The PCR fragments of Holstein Friesians cows GDF9 gene-exon II. M = DNA ladder, 100–1500 BP. T: Twin birth cow samples, S: Single birth cow samples.
Sequencing, genotyping and allele frequencies (Genetic variability)

Depending on the sequence analysis, two SNPs revealed in exon II of Holstein Friesians cows GDF9 gene (Fig. 2). These SNPs; A(1109)T and G(1133)A when compared with Sequence ID: XM_027546514.1 of BOS Taurus growth differentiation factor 9 (GDF9) and ensemble ENSBTAT00000012476.2.

The detected SNPs were missense mutation that substitute amino acids; Isoleucine > Asparagine and Aspartic acid > Asparagin at position 370 and 376 respectively (Table 1).

**Fig. 2:** The sequence match of the altered nucleotide bases of GDF9 in Holstein Friesians cows samples. A: samples of single birth cows gene, B: samples of twin birth cows gene

<table>
<thead>
<tr>
<th>SNP Location</th>
<th>Code change</th>
<th>Amino acid change</th>
<th>Type of mutation</th>
<th>Predicted effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A/T 1109</td>
<td>ATT &gt; AAT</td>
<td>370 Isoleucine &gt; Asparagine</td>
<td>Missense</td>
</tr>
<tr>
<td>2</td>
<td>G/A 1133</td>
<td>GAC &gt; AAC</td>
<td>376 Aspartic acid &gt; Asparagine</td>
<td>Missense</td>
</tr>
</tbody>
</table>
Two different genotypes find in each locus; AA and TT for A (1109) T locus, while GG and AA for G (1133) A locus. The genotypic and allele frequencies of exon II polymorphisms in Holstein Friesians cows GDF9 gene showed in Table 2.

Table 3: The genotypic and allele structure frequencies of the Holstein Friesians cows GDF9 gene

<table>
<thead>
<tr>
<th>Locus</th>
<th>Genotypes</th>
<th>The observed number of genotypes</th>
<th>Genotypic Frequencies</th>
<th>Allelic Frequencies</th>
<th>Chi-Square</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (1109) T</td>
<td>AA</td>
<td>18</td>
<td>60.00</td>
<td>0.6</td>
<td>7.250 **</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>12</td>
<td>40.00</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>G (1133) A</td>
<td>GG</td>
<td>19</td>
<td>63.33</td>
<td>0.63</td>
<td>9.147 **</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>11</td>
<td>36.66</td>
<td>0.36</td>
<td></td>
</tr>
</tbody>
</table>

** (P<0.01).

The genotypic distribution of GDF9 gene polymorphism in the single and twin birth Holstein Friesians cows

According to table 3; a higher significantly increase (P<0.01) observed in genotypic frequencies of TT and AA (mutant alleles) for the twin birth cows as a compared with wild genotypes and alleles in the detected loci respectively. Otherwise, the AA and GG genotypes (wild alleles) showed a higher significant increment (P<0.01) in single-born cows compared with mutant genotypes and alleles in same loci. Additionally, the mutant genotypes recorded a significant rise (P<0.05) in calving rate in comparison with wild genotypes (Table 4).
**Table 4:** A comparison between the genotypic frequency of GDF9 in twin and single birth Holstein Friesians cows

<table>
<thead>
<tr>
<th>Locus</th>
<th>Genotypes</th>
<th>Genotypic Frequency for Twin birth cows</th>
<th>Chi-Square</th>
<th>Genotypic Frequency for Single birth cows</th>
<th>Chi-Square</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (1109) T</td>
<td>AA</td>
<td>5 (33.3%)</td>
<td>9.362 **</td>
<td>13 (86.6 %)</td>
<td>13.521 **</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>10 (66.6%)</td>
<td></td>
<td>2 (13.3 %)</td>
<td></td>
</tr>
<tr>
<td>G (1133) A</td>
<td>GG</td>
<td>6 (40%)</td>
<td>7.250 **</td>
<td>13 (86.6 %)</td>
<td>13.521 **</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>9 (60%)</td>
<td></td>
<td>2 (13.3 %)</td>
<td></td>
</tr>
</tbody>
</table>

**(P<0.01).**

**Table 5:** The calving rate for the Holstein Friesians cows bovine GDF9 genotypes

<table>
<thead>
<tr>
<th>Locus</th>
<th>Genotypes</th>
<th>Cows number</th>
<th>Calves number</th>
<th>Calving rate</th>
<th>T- Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (1109) T</td>
<td>AA</td>
<td>18</td>
<td>23</td>
<td>1.27</td>
<td>0.336 *</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>12</td>
<td>22</td>
<td>1.83</td>
<td></td>
</tr>
<tr>
<td>G (1133) A</td>
<td>GG</td>
<td>19</td>
<td>25</td>
<td>1.31</td>
<td>0.309 *</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>11</td>
<td>20</td>
<td>1.81</td>
<td></td>
</tr>
</tbody>
</table>

**(P<0.05).**

The comparative between mean hormonal concentrations (FSH and estradiol) during estrus in twin and single birth Holstein Friesians

The twin birth cows group exhibited a significant rise (P<0.05) in the peripheral concentration of FSH and E2 as compared with single birth cows group throughout estrus phase in 0 and 24 h (Table 5) (Fig 2).
Table 6: The hormones level in twin and single birth cows during estrus
* (P<0.05)    ** (P<0.01).

<table>
<thead>
<tr>
<th>Hormones level</th>
<th>Twin birth cows (Group 1)</th>
<th>Single birth cows (Group 2)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH ng/ml</td>
<td>1st Day of estrus (0 h)</td>
<td>13.3 ± 0.893 *</td>
<td>8.25 ± 0.57</td>
</tr>
<tr>
<td></td>
<td>After 24 h</td>
<td>3.2 ± 0.42</td>
<td>1.3</td>
</tr>
<tr>
<td>Estradiol pg/ml</td>
<td>Day of estrus (0 h)</td>
<td>65 ± 56 **</td>
<td>74 ± 0.74</td>
</tr>
<tr>
<td></td>
<td>After 24 h</td>
<td>83 ± 68</td>
<td>68</td>
</tr>
</tbody>
</table>

Fig. 3: Peripheral plasma FSH and E2 concentration at estrus in Holstein Friesians cows

The linkage between GDF9 genotypes and hormonal concentration (FSH and Estradiol) at estrus phase in Holstein Friesians cows

The table 6 showed a higher significant increment (P<0.01) in the FSH concentration for mutant genotypes as a compared with wild during heat period, whereas, non-significant differences recorded in E2 concentration between the genotypes at same period.
Table 7: Comparison between the different genotypes of Holstein Friesians cows GDF9 in FSH and E2 concentration (Mean ± SE)

<table>
<thead>
<tr>
<th>Locus</th>
<th>Genotypes</th>
<th>Mean Plasma FSH level during estrus ng/ml</th>
<th>T-Test</th>
<th>Mean Plasma estradiol level during estrus pg/ml</th>
<th>T-Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (1109) T</td>
<td>AA</td>
<td>6.99 ± 0.68</td>
<td>1.063 **</td>
<td>56.90± 5.47</td>
<td>0.0944 NS</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>8.11 ± 0.54</td>
<td></td>
<td>66.80 ± 4.98</td>
<td></td>
</tr>
<tr>
<td>G (1133) A</td>
<td>GG</td>
<td>6.68 ± 0.61</td>
<td>0.884 **</td>
<td>59.00 ± 6.06</td>
<td>0.218 NS</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>7.94 ± 0.52</td>
<td></td>
<td>71.00 ± 10.08</td>
<td></td>
</tr>
</tbody>
</table>

** (P<0.01).

Discussion

In the present study, the genetic variability represented by detected of two SNPs not been described before in codon (exon II) of GDF 9 gene. The first new variant A(1109)T and was missense mutation that altered a non-polar amino acid residue Isoleucine into polar Asparagine at position 370. The second SNP was G(1133)A missense that switched negatively charged Aspartate into polar Asparagine in position 376. By comparing the genotypes of Holstein Friesians cows GDF9 with reproductive parameter and hormonal levels, the GDF9 effect noticed. These facts agree with Hanrahan et al (2004) (2) finding, which detected seven SNPs in exon II of Ovine GDF9 as well as one in exon I, they find a linkage between exon II SNPs with increasing ovulation rate and sterility. While, they proposed that amino acids that are encoded from exon I don't involve in mature protein, so it excluded the exon I from this study.

Association between GDF9 gene polymorphism and calving rate in Holstein Friesians cows

The results of the table 3 showed that mutant homozygote alleles noticed majorly in twin birth cows, whilst wild homozygote A and G alleles showed more frequently in single birth cows for the detected loci. In addition, the calving rate or ovulation rate increased in mutant alleles for the same loci (Table 4), these findings lead to speculation that the exon II polymorphism in bovine GDF9 gene effect positively on calving rate in Holstein Friesians cows.
At first, this finding comes constant with Karlsen et al (2000) (13) study, which revealed that the bovine twinning rate is heritable, and the selection for 18 years in the Norwegian cattle breeds caused an increase in calving rate.

The GDF9 called fecundity gene because it's have a dynamic effect on folliculogenesis alone or a combination with BMP15, so its play a fundamental role in superovulation. The current outcomes agreed with previous reports that described an effect of mutation in GDF9 gene on increasing ovulation rate in ruminant. Tang et al (2013) (16) find two SNPs in bovine GDF9 gene (A485T and A625T) and assume that responsible for superovulation in Chinese Holstein breed, but didn't find SNP in exon II. Furthermore, the current study agrees with Santos-Biase et al (2012) (11), which demonstrated that the genetic variability effect on the number of growing follicles and collected ova.

In any case, the studies of bovine GDF9 variants have been generally uncommon. In small ruminant (sheep and goat), the GDF9 mutations remarkably correlated with ovulation rate increment. The exon II SNPs of GDF9 gene are related with multiple births in Île-de-France sheep breed (17).

The GDF9 SNPs not considered as a genetic marker in cows only but also in the improvement of bull reproductive performance. According to Tang et al (2013) (16), the GDF9 variants also influence the sperm concentration and Acrosome integrity in Holstein bull, this report reflects the positive function of GDF9 polymorphism on reproduction in the bull.

The importance of GDF9 as a fecundity gene it came from the set of expression and its function. According to Reineri et al (2018) (18), GDF9 is expressed in oocyte, cumulus, granulosa and follicular cells of cows ovary to act in a local pattern (autocrine and paracrine) to coordinate the folliculogenesis. By binding to own receptors in granulosa cells, the GDF9 mediate GnRH and LH stimulation the ovulation in cows (4).

**The comparative between hormonal concentrations (FSH and estradiol) during estrus in twin and single birth Holstein Friesians**

According to table 5, the FSH level recorded a significant in mothers of twins when compared with singleton cows at estrus phase, additionally, E2 recorded the same results. These results lead to speculate that FSH and E2 levels in mothers of a dizygotic twin were higher than the control (mothers of a singleton).

This hypothesis agrees with earlier findings by Lopez et al (2005) (19) about the of FSH and E2 levels. They argue that peripheral blood concentration of these hormones are greater in heifers that had double dominant follicles as a compared with heifers that had a single dominant during two days before and two days after estrus. Besides, the study agrees with Lambalk et al (1998) (20) finding, which revealed that the mean FSH levels in mothers of twins were more than control, but no elevation recorded in E2 for twins.
mothers groups. This elevation came from the rise of endogenous FSH pulse frequency and led to hyper-stimulation of follicles growth in dizygotic twin mothers.

Moreover, the results are also identical with Martin et al (1984) (21), who displayed that FSH level was significantly higher in DZ twins women than women with no DZ twins. The elevation of FSH level may be responsible for increasing the ovulation rate, this mostly occur because the fecundity gene (GDF9) mutation. According to Hafez and Hafez (2013) (22), who mentioned that twining rate per lambing was high in some sheep breeds like Finn sheep and Booroola Merino, this was linked by effect of fecundity genes on FSH level and led to superovulation.

The FSH is essential in maintaining and develops the follicles and also used in superovulation programs because induces and maximize follicular growth. Mapletonot and Bo (2014) mentioned that exogenous FSH administration led to super stimulation of follicular growth in cows. In addition, Hesser at al (2011) (23) demonstrated that exogenous FSH is necessary to elicit and encourage the development of numerous follicles to become dominance and subsequently ovulation in cows.

The linkage between GDF9 genotypes and hormonal concentration (FSH and Estradiol) at estrus phase in Holstein Friesians cows

The hormonal level distribution according to GDF9 genotypes showed a strong relationship between the GDF9 genotypes and FSH concentration at the peak of follicular phase. The FSH level in cows that possess TT and AA genotypes recorded a higher significant increase than cows that have AA and GG genotypes according to A (1109) T and G (1133) A loci respectively. While, non-significant variation noticed between the E2 level and the same genotypes. As mentioned in table 2; the genotypic frequency of TT and AA were higher in twin birth cows, while the genotypic frequency of AA and GG were higher in non-twin birth cows group at the same loci.

The intra ovarian and intra-pituitary roles of GDF9 protein on FSH and steroids specially E2. Therefore, many investigators explore the GDF9 polymorphism effect on reproductive performance in domestic animals.

According to observations, this study suggests that missense mutation causes changes in two amino acids, that effect on the final shape and lead to enhance GDF9 function. Because the GDF9 role in regulating FSH level, the study supposes that polymorphism in GDF9 has a significant effect on rising FSH level without an effect on E2 level. This argues is agreed with Hafez and Hafez (2013) (22), who mentioned that the mechanism of fecundity genes to elicit superovulation done through increasing FSH level. Hosoe et al (2011) (5), illustrate that mRNA of GDF9 were detected in pituitary tissue as well as ovarian tissues (oocyte and follicular tissues). Furthermore, the exogenous GDF9 increased FSH β expression without effect on LH β in rat gonadotrope cells of pituitary tissue culture, that stimulate GnRH- prompt FSH expression by a synergistic
relationship between GnRH and GDF9 (9). Same report noticed that GDF9 resemble Activin by effect on FSH protein expression in pituitary cells.

The intra ovarian action of GDF9 represent by stimulating theca cells proliferation in small and large follicles, in addition, GDF9 suppress androstenedione and progesterone production under the effect of Insulin-like growth factor 1 (IGF-1) and LH (8). These events may strengthen the FSH because these steroids exert negative feedback loop on FSH.

Depending on table 6, there was no significant relationship between the GDF9 polymorphism and E2 level. A little evidence mentioned the synergetic mechanism between E2 and GDF9, however, Sugiura et al (2010) (24) clarified that GDF9 and E2 act together to regulate the cumulus cells expansion.

**Conclusion**

The current study infers that; 1) The exon II polymorphisms in GDF9 increase the calving rate (ovulation rate) in Holstein Friesians cows. 2) The peripheral blood FSH and E2 concentration are higher in mother of dizygotic twin than mother of non-dizygotic twin. 3) The FSH concentration is higher in mutant GDF9 genotypes. 4) lastly, these SNPs can be considered as molecular DNA markers for superovulation to improve and accelerate the breeding programs and assisted reproductive techniques such as embryo transfer and in vitro embryo production in Iraqi Holstein Friesians cows breed. Researchers recommend the use of primers of the fragment 3 exclusively because it covers most of the exon II.

**Authors' contribution**

L. S. Y. design the experiment, made the ultrasound, sequencing, writing and hormonal evaluations. Q. M. A. prepared the animals, collect the data and made the rectal and ultrasound examination along with L. S. Y., also contributed in writing. S. T. R. contributed to collected data, statistical analysis, writing and management.

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**Conflict of interest**

There is no conflict of interest.

**Ethics**

I hereby declare all ethical standards have been respected in preparation of the submitted article.
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