Archives of Razi Institute, Vol. 76, No. 4 (2021) 1035-1045

DOI: 10.22092/ari.2021.354310.1632

Original Article

Association between Bovine GDF9 SNPs and Calving Rate (Superovulation) in Holstein-Friesian Cows

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Received 7 May 2021; Accepted 29 May 2021
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Abstract

The present study aimed to assess the relationship of Growth Differentiation Factor 9 (GDF9) genotypes with calving rate, Follicle-stimulating hormone (FSH), and Estradiol (E2) in the Iraqi Holstein-Friesian breed. A number of 15 blood samples were collected from a mother of dizygotic twin birth (DZTB) (with high calving rate records), and another blood sample was collected from 15 single birth (SB) cows. The DNA was extracted and six primers were designed for PCR and sequencing analysis. The FSH and E2 levels were tested through the estrus phase for the two groups (n=10 in each group). The sequence evaluation revealed the presence of two 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), as compared to wild genotypes at the same loci. On the other hand, the wild genotypes recorded a significant increment (P<0.01) for SB cows, when compared to 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 in 0 and 24 h of estrus phase, respectively. Furthermore, non-significant differences were recorded in E2 concentration among the same genotypes at the same period. In conclusion, the GDF9 exon II SNPs increased the calving rate in Holstein-Friesian cows. The blood FSH and E2 concentrations were higher in the DZTB cows and control the superovulation. Finally, these SNPs can be regarded as markers to accelerate the breeding programs and used in embryo transfer and in vitro embryo production for Iraqi Holstein-Friesian cow breed.

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

1. Introduction

Iraqi Holstein-Friesian is a multi-purpose cow breed widely used owing to its high reproductive performance, as compared to other breeds (1). Therefore, it is necessary to upgrade the information about their physiology of reproduction, especially the folliculogenesis, to improve reproductive in these animals. Many genes, such as growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) (2), which contributed directly to folliculogenesis and ovulation are involved in fertility and fecundity.

Oocyte-specific GDF9 or FecG is a part of the transforming growth factor which belongs to the β superfamily and is expressed particularly during folliculogenesis; moreover, it is a vital factor for female fertility and folliculogenesis (3). In cattle, both GDF9 and BMP15 act together to control the development of ovarian follicles, and the immunization (neutralization) of GDF9 alone or in combination with BMP15 results

in the suppression of cow folliculogenesis (3).

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

The role of GDF9 in folliculogenesis and ovulation has been well observed, and without GDF9, the folliculogenesis, and subsequently, fertility diminished. A block in folliculogenesis and infertility was observed when the GDF9 gene was knock-down at the primary stage in mice (7). In their study, Spicer, Aad (8) demonstrated that GDF9 treatment declined the production of both androstenedione and progesterone in bovine thecal cells that are induced by luteinizing hormone (LH). In addition, GDF9 administration stimulates the proliferation of theca cells and prevents early follicular development by prohibiting premature differentiation of theca interna cells.

In the same context, Choi, Wang (9) indicated that paracrine secretion of pituitary GDF9 mediates mRNA expression of follicle-stimulating hormone (FSH) in gonadotrope cells under the effect of Gonadotropin-releasing hormone (GnRH); moreover, the exogenous GDF9 administration prompt FSH secretion in the same cells. On the other hand, the GDF9 neutralizing antibody minimized the FSH mRNA expression.

BOS Taurus GDF9 gene is located in the fifth bovine autosomal chromosome. The length is about 2754 base pairs (BP), and it contains two different-sized exons discrete by a single intron (1126) BP. The exon I sizes 397 BP, while exon II spans 1000 BP. Exon I encode 134 amino acids, and exon II encodes 456 amino acids (10). Due to the site of GDF9 mRNA expression (in pituitary and ovarian cells) and the vital role of GDF9 in folliculogenesis, numerous studies have assessed the role of the GDF9 gene in ovulation rate and twinning. Hanrahan, Gregan (2), found eight mutations in the GDF9 codon in sheep, and these nucleotide substitutions enhanced the litter size and another was concerned with sterility. Along the same lines, the results of a study by Santos-Biase, Biase (11) demonstrated a correlation between GDF9 single nucleotide polymorphisms (SNP) and the number of collected oocytes from the cow follicles.

Non-identical or dizygotic twins originate from two different spermatozoa that successfully fertilize two entirely different oocytes as a result of superovulation (12). The genomic selection program for the twining trait is achieved through selection from 0.6%-4% in Norwegian cow breeds in the sixth parity (13). Twinning in cattle is associated with some complications, such as shorter gestation length, reduced calf weight, dystocia, increased risk of retained placenta, and lowered fertility (14). Nonetheless, superovulation is important to bovine embryo transfer and cattle breeding programs (15).

As mentioned above, the twining in cattle is under genetic control. In light of the aforementioned issues, the present study aimed to assess polymorphism in the bovine GDF9 gene and identify its possible relationship with twining rate or calving rate through an effect on FSH level and folliculogenesis.

2. Material and Methods

2.1. Animal Management and Data Collection

A total of 30 normal multiparous Holstein-Friesian cows with an average age of 4.5 years were used in this experiment. For sequencing and genotypic analysis, Group 1 (n=15) was selected from twin birth cows (non-identical phenotypical twin births with two separated placenta) that calved recently with a case history of Dizygotic twin birth (DZTB) every year with high calving rate records. On the other hand, 15 single-birth cows were chosen as the control (Group 2). For hormonal

assay, the samples were taken from 10 cows of group 1 and 10 cows of group 2 every 24 h at the beginning of the estrus phase of the first estrus after puerperium.

The estrus was detected by observing progesterone changes (began to increase more than basal concentration then rise to 1 ng/ml) and Estradiol (E2) levels. When the cows of the first group became pregnant, the rectal examination was used to detect the twin (two corpora lutea and symmetrically swollen of the uterine horns). Moreover, the pregnancy and twin pregnancy were confirmed by ultrasound (Bondway BW570V/ China). All the cows that did not give twin births were excluded from the experiment. The samples were taken from several cattle stations in Salah Aldin province, and the experimental interval extends from February 2018-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; thereafter, the plasma and serum were preserved at -20°C.

2.2. DNA Extraction and BOS Taurus GDF9 Gene Amplification

The DNA of blood samples (single and multiple birth animals groups) was extracted using DNA isolation G-spin Kit (Promega/ USA) as stated by the manufacturer. The DNA concentration was tested by Nanodrop (LG/ Korea) on wavelength 260/280 nm. The bovine GDF9 gene consists of two exons, according to Hanrahan, Gregan (2), Exon 1 codons are not involved in mature protein since the cleavage occurs in the mature peptide encoded by exon I. Therefore, exon I was excluded from this study. Exon 2 is amplified by six specific primers. Two primers were designed manually in this study (fragment 3), and the other four were designed as stated by Tang, Yang (16) based on Gene Bank ID: 282574, Reference Sequence: NC_037334.1, and ENSBTAT00000012476.2 as follows (Table 1):

Table 1. Information on primers used for amplification and sequencing of bovine GDF9

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

2.3. Polymerase Chain Reaction

(PCR) 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°C for 3 min, denaturation at 94°C for 30 sec, annealing at 58ºC for 30 sec (fragment 1), and 61ºC for 30 sec (fragment 2 and 3). The extension was at 72°C for a min and followed by a final expansion at 72°C for 10 min. After GDF9 fragment amplification, the Amplicons were successfully detected after electrophoresis (CBS Scientific/ USA) on 0.02 Agarose gel stained by Ethidium bromide.

2.4. 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 genome sequence of BOS Taurus GDF9

using the online BLAST option in NCBI. The SNPs were determined by visual inspection of samples on the Bio Edit program and NCBI.

2.5. Hormonal Assay

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

2.6. Statistical Analysis

The agreement of genotyping frequencies was analyzed using the Statistical Analysis System (SAS) program and the chi-square test. The litter size of animals was compared by the t-test.

3. Results

3.1. Polymerase Chain Reaction Amplification

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

3.2. Sequencing, Genotyping, and Allele Frequencies (Genetic Variability)

Depending on the sequence analysis, two SNPs were revealed in exon II of the Holstein-Friesian cow GDF9 gene (Figure 2). These SNPs included: A(1109)T and G(1133)A, as compared to Sequence ID: XM_027546514.1 of BOS Taurus (GDF9 and ensemble ENSBTAT00000012476.2. The detected SNPs were missense mutations that substitute amino acids; Isoleucine > Asparagine and Aspartic acid > Asparagine at the positions of 370 and 376, respectively (Table 1).

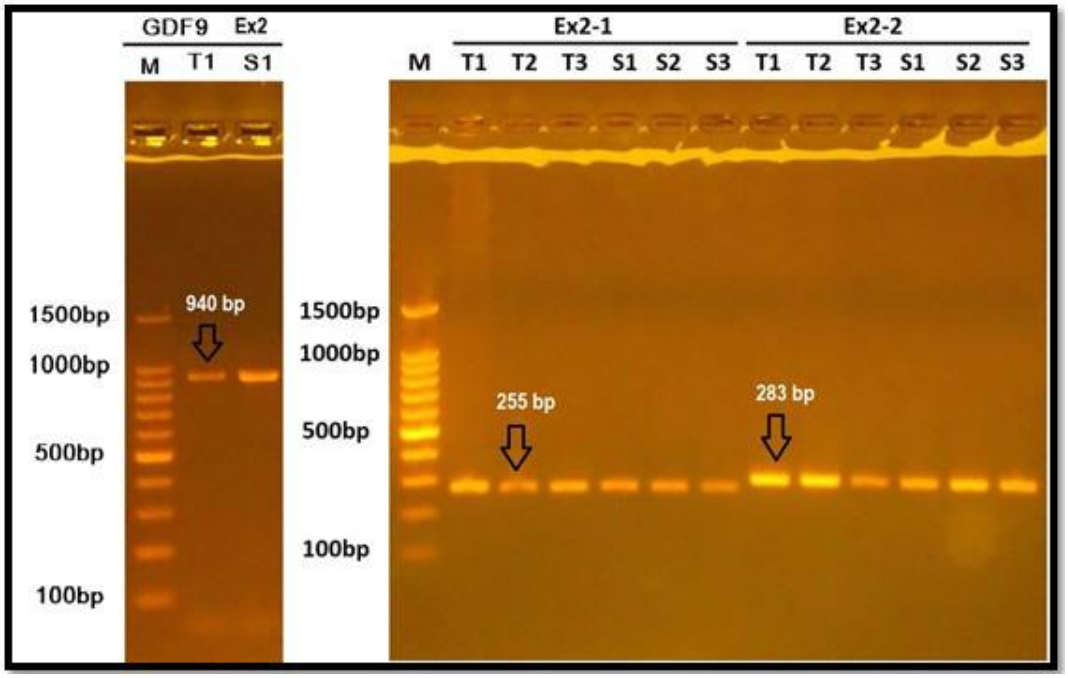

Figure 1. Polymerase chain reaction fragments of Holstein-Friesian cow GDF9 gene-exon II
M=DNA ladder 100-1500 bp, T: Twin birth cow samples, S: Single birth cow samples

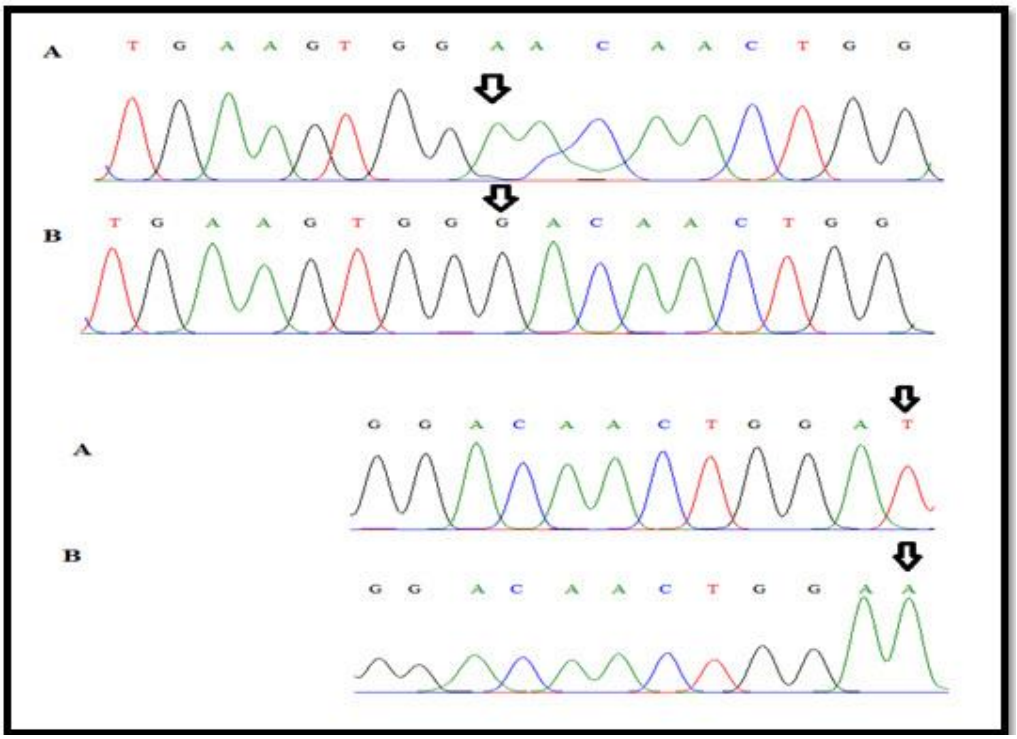

Figure 2. Sequence match of the altered nucleotide bases of GDF9 in Holstein-Friesian cow samples**, A:** samples of single birth cows gene, **B:** samples of twin birth cows gene

Two different genotypes were found 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 the Holstein-Friesian cow GDF9 gene are displayed in table 2.

3.3. Genotypic Distribution of GDF9 Gene Polymorphism in the Single and Twin Birth Holstein-Friesian Cows

According to table 3, a significant increase (P<0.01) was observed in genotypic frequencies of TT and AA (mutant alleles) for twin birth cows, as compared to wild genotypes and alleles in the detected loci, respectively. On the contrary, the AA and GG genotypes (wild alleles) showed a significant increment (P<0.01) in single-born cows, in comparison with mutant genotypes and alleles in the same loci. In addition, the mutant genotypes recorded a significant rise (P<0.05) in calving rate, as compared to wild genotypes (Table 4).

Table 2. Detected SNPs, amino acid changes, and mutation type in the GDF9 gene in the Holstein-Friesian cow population

	SNP Location	Code change	Amino Acid change	Type of mutation	Predicted effect
1	A/T 1109	ATT > AAT	370 Isoleucine > Asparagine	Missense	Transversion
2	G/A 1133	GAC > AAC	376 Aspartic acid > Asparagine	Missense	Transition

Table 3. Genotypic and allele structure frequencies of the Holstein-Friesian cow GDF9 gene

Locus	Genotypes	Observed number of genotypes	Genotypic Frequencies	Allelic Frequencies	Chi- Square
A (1109) T	AA	18	60.00	0.6	7.250 **
	TT	12	40.00	0.4	
G (1133) A	GG	19	63.33	0.63	9.147 **
	AA	11	36.66	0.36	

** (P<0.01).

Table 4. A comparison between the genotypic frequency of GDF9 in twin and single birth Holstein-Friesian cows

Locus	Genotypes	Genotypic Frequency For Twin birth cows	Chi-Square	Genotypic Frequency for Single birth cows	Chi-Square
A (1109) T	AA	5 (33.3%)	9.362 **	13 (86.6 %)	13.521 **
	TT	10 (66.6%)		2 (13.3 %)	
G (1133) A	GG	6 (40%)	7.250 **	13 (86.6 %)	13.521 **
	AA	9 (60%)		2 (13.3 %)	

** (P<0.01).

3.4. Comparison of Mean Hormonal Concentrations (FSH and Estradiol) During Estrus in Twin and Single Birth Holstein-Friesians

The group of twin birth cows exhibited a significant rise (P<0.05) in the peripheral concentration of FSH and E2, as compared to single birth cows throughout the estrus phase in 0 and 24 h (Table 5; Figure 2).

3.5. Linkage between GDF9 Genotypes and Hormonal Concentration (FSH and Estradiol) at Estrus Phase in Holstein Friesians Cows

Table 6 pointed to a higher significant increment (P<0.01) in the FSH concentration for mutant genotypes, as compared to wild genotypes during the heat period, whereas non-significant differences were recorded in E2 concentration between the genotypes at the same period (Figure 3) (Table 7).

Table 5. Calving rate for the Holstein-Friesian cow bovine GDF9 genotypes

Locus	Genotypes	Cows number	Calves number	Calving rate	T- Test
A (1109) T	AA	18	23	1.27	0.336 *
	TT	12	22	1.83	
G (1133) A	GG	19	25	1.31	0.309 *
	AA	11	20	1.81	

* (P<0.05).

Table 6. Hormones level in twin and single birth cows during estrus

Hormones level		Twin birth cows (Group 1)		Single birth cows (Group 2)		P-value
FSH ng/ml	**1st Day of estrus (0 h)**	13.3	8.25 ± 0.57	11.4	6.35 ± 0.42	0.893 *
	After 24 h	3.2		1.3		
Estradiol pg/ml	**Day of estrus (0 h)**	65	74	44	56	0.0081 **
	After 24 h	83		68		

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

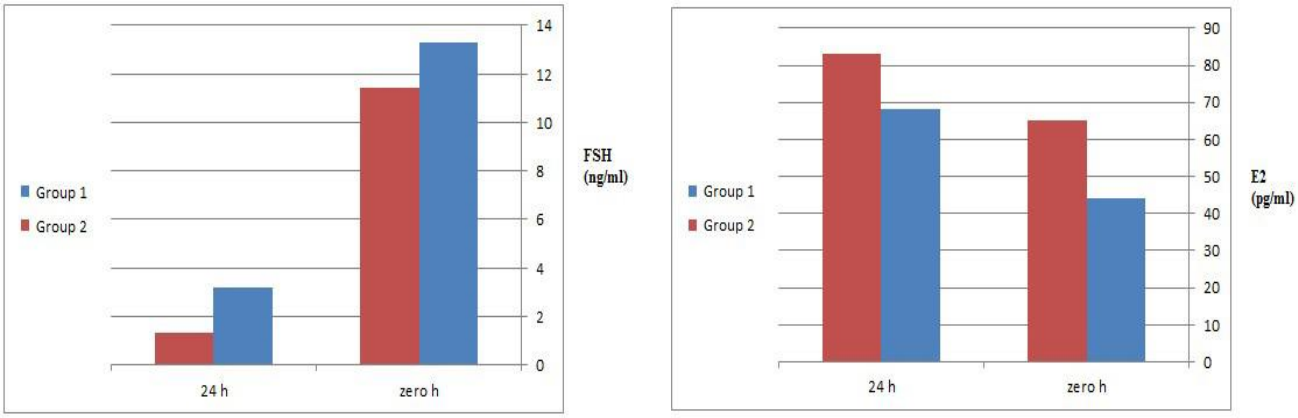

Figure 3. Peripheral plasma FSH and E2 concentration at estrus in Holstein-Friesian cows

Table 7. Comparison between the different GDF9 genotypes of Holstein-Friesian cow in case of FSH and E2 concentration (Mean±SE)

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

** (P<0.01).

4. Discussion

In the present study, the genetic variability was represented by the detection of two SNPs which have not been described before in codon (exon II) of the GDF 9 gene. The first new variant A(1109)T was a missense mutation that altered a non-polar amino acid residue Isoleucine into polar Asparagine at position 370. The second SNP was G (1133) A which is a missense that switched negatively charged Aspartate into polar Asparagine in position 376. The GDF9 effect was noticed by detecting the association of the genotypes of Holstein-Friesian cow GDF9 with reproductive parameters and hormonal levels. These facts agree with the findings obtained by Hanrahan, Gregan (2) who detected seven SNPs in exon II of Ovine GDF9, as well as one in exon I; moreover, they pointed to the association of exon II SNPs with increasing ovulation rate and sterility. Nonetheless, they proposed that the amino acids that are encoded from exon I are not involved in mature protein; therefore, exon I was excluded from this study.

4.1. Association between GDF9 Gene Polymorphism and Calving Rate in Holstein Friesians Cows

As demonstrated in table 3, mutant homozygote alleles were mostly noticed in twin birth cows, while 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 led to the speculation that exon II polymorphism in the bovine GDF9 gene exerted a positive effect on the calving rate in Holstein-Friesian cows. This finding is in line with those obtained by Karlsen, Ruane (13) who revealed that the bovine twinning rate is heritable, and an 18-year selection of Norwegian cattle breeds caused an increase in calving rate.

The GDF9 is called fecundity gene since it has a dynamic effect on folliculogenesis alone or in combination with BMP15; therefore, it has a critical role to play in superovulation. The current outcomes were in accordance with previous reports that pointed to the effect of a mutation in the GDF9 gene on the enhancement of ovulation rate in ruminants. Tang, Yang (16) found two SNPs in the bovine GDF9 gene (A485T and A625T) and assumed that it is responsible for superovulation in the Chinese Holstein breed; nonetheless, they found no SNP in exon II. Furthermore, the results of the current study were in agreement with those indicated by Santos-Biase, Biase (11) who demonstrated 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 ruminants (sheep and goats), the GDF9 mutations were remarkably correlated with ovulation rate increment. The exon II SNPs of the GDF9 gene are related to multiple births in the Île-de-France sheep breed (17). The GDF9 SNPs are not only considered a genetic marker in cows but also in the improvement of bull reproductive performance. According to Tang, Yang (16), the GDF9 variants also influence the sperm concentration and Acrosome integrity in Holstein bull. This report reflects the positive effect of GDF9 polymorphism on reproduction in the bull.

The importance of GDF9 as a fecundity gene came from the set of expression and its function. According to Reineri, Coria (18), GDF9 is expressed in the oocyte, cumulus, granulosa, and follicular cells of cow ovary to act in a local pattern (autocrine and paracrine) to coordinate the folliculogenesis. By binding to own receptors in granulosa cells, the GDF9 mediates Gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) ovulation stimulation in cows (4).

4.2. 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 increase in mothers of twins, as compared to singleton cows at the estrus phase; in addition, E2 recorded the same results. These results lead to

speculate that FSH and E2 levels in mothers of a dizygotic twin were higher, in comparison with those in the controls (singleton mothers).

This hypothesis agrees with earlier findings suggested by Lopez, Sartori (19) about the increase of FSH and E2 levels. They argued that peripheral blood concentration of these hormones is greater in heifers that had double dominant follicles, as compared to heifers that had a single dominant during two days before and two days after estrus. Moreover, the results of this study are in line with those suggested by Lambalk, Boomsma (20) who revealed that the mean FSH levels in mothers of twins were more than controls; nonetheless, no elevation was recorded in E2 for twin birth mothers. This elevation came from the rise of endogenous FSH pulse frequency and led to hyper-stimulation of follicles growth in dizygotic twin mothers.

Furthermore, the results are also in accordance with those obtained by Martin, El Beaini (21) who displayed that FSH level was significantly higher in DZ twin women, as compared to that in women with no DZ twins. The elevation of FSH level may be responsible for the enhancement of the ovulation rate, and this mostly occurs as a result of fecundity gene (GDF9) mutation. According to Hafez and Hafez (22), who mentioned that twining rate per lambing was high in some sheep breeds, such as Finn sheep and Booroola Merino, this was linked by the effect of fecundity genes on FSH level and led to superovulation.

The FSH is essential in the maintenance and development of follicles; moreover, it is used in superovulation programs since it induces and maximizes follicular growth. Hopper (15) pointed out that exogenous FSH administration led to super stimulation of follicular growth in cows. In addition, Hesser, Morris (23) demonstrated that exogenous FSH is necessary to elicit and encourage the development of numerous follicles to become dominant, which in turn, leads to ovulation in cows.

4.3. The Linkage between GDF9 Genotypes and Hormonal Concentration (FSH and Estradiol) at Estrus Phase in Holstein-Friesian Cows

The hormonal level distribution according to GDF9 genotypes displayed a strong relationship between the GDF9 genotypes and FSH concentration at the peak of the follicular phase. The FSH level in cows that possess TT and AA genotypes recorded a significant increase, as compared to that in the cows that have AA and GG genotypes according to A (1109) T and G (1133) A loci, respectively. However, a non-significant variation was noticed between the E2 level and the same genotypes. As displayed 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 at the same loci. Therefore, many investigators explore the GDF9 polymorphism effect on reproductive performance in domestic animals.

According to observations, the present study suggested that missense mutation causes changes in two amino acids, affecting the final shape and enhancing GDF9 function. Due to the role of GDF9 in the regulation of FSH level, the current study supposed that polymorphism in GDF9 has a significant effect on the elevation of FSH level without exerting any effect on the E2 level. This finding is in line with those indicated by Hafez and Hafez (22) who reported that the mechanism of fecundity genes to elicit superovulation is exerted by the elevation of FSH level. Hosoe, Kaneyama (5) illustrated that mRNA of GDF9 were detected in pituitary tissue and ovarian tissues (oocyte and follicular tissues). Furthermore, the exogenous GDF9 increased FSH β expression without exerting any 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). The same report noticed that GDF9 resembles Activin by exerting an effect on FSH protein expression in pituitary cells.

The intra-ovarian action of GDF9 is represented by the stimulation of theca cell proliferation in small and large follicles. In addition, GDF9 suppresses androstenedione and progesterone production under the effect of Insulin-like growth factor 1 (IGF-1) and LH (8). These events may strengthen the FSH since these steroids exert a negative feedback loop on FSH. As depicted in Table 6, there was no significant relationship between the GDF9 polymorphism and E2 level. The evidence pointed to the synergetic mechanism between E2 and GDF9; however, Sugiura, Su (24) clarified that GDF9 and E2 act together to regulate the cumulus cell expansion.

5. Conclusion

As demonstrated by the results of the current study, it can be concluded that: 1) The exon II polymorphisms in GDF9 increase the calving rate (ovulation rate) in Holstein-Friesian 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, and 4) these SNPs can be considered 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-Friesian cow breed. Researchers recommend the exclusive use of primers of fragment 3 since it covers most of exon II.

Authors' Contribution

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

Ethics

All the procedures were approved by the Ethics Committee at the Fallujah University, Baghdad, Iraq

Conflict of Interest

The authors declare that they have no conflict of interest.

Grant Support

There is no financial support

Acknowledgment

Our deepest appreciation goes to the ASCO Center in Baghdad / Iraq for supporting us with the necessary equipment. Moreover, we thank Vet. College- Fallujah University.

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