Research Article

Association of *FST* Gene Polymorphism and Litter Size with Reproductive Hormones in Iraqi Awassi Ewes During Pregnancy and Postpartum

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Abstract | Many factors affect reproductive traits, including ovulation rate, hormones, and genetics. Genetics and endocrine signal transpositions between the pituitary and the ovary play a crucial role in the reproductive traits of sheep. Genetic polymorphism of candidate genes is associated with economic traits. Follistatin (*FST*) gene is one of the most notable potential candidate genes linked to economic traits. This study aimed to assess the genetic variation of the *FST* gene and reproductive hormones in Awassi sheep. Genomic DNA was isolated from 232 sheep (123 with a single pregnancy and 109 with twins); genotyping and sequencing were used to confirm the variants in the amplified fragment of the *FST* gene (exon 4). Results of the genotyping technique identified three genotypes: CC, CG, and GG. Sequencing analysis revealed a novel mutation c. 25760691 C>G in the CG genotype. Association analysis revealed significant differences (P ≤ 0.05) between CC and other genotypes in reproductive hormone levels. Estrogen and progesterone were significantly higher (P≤0.05) and follicle-stimulating hormone (FSH) levels were lower in the CC genotype (85.10 ± 3.11) (pg/ml), (8.82 ± 0.52) (ng/ml), and (13.95 ± 2.71) (ng/ml) respectively compared to a CG and GG genotypes in twins versus singleton gestations. In conclusion, *FST* gene polymorphism significantly influences reproductive hormone levels in Awassi ewes. Excluding sheep that carry the GG genotypes of *FST* gene polymorphism suggests being a future study, as this gene could be associated with high reproductive success.

Keywords | *FST* gene polymorphism, Iraqi Awassi sheep, Pregnancy, Reproductive hormones

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INTRODUCTION

Among all livestock species, sheep (*Ovis aries*) are of significant importance in the global economy due to their high productivity characteristics (Ma et al., 2017; Al-Thuwaini, 2021a). The litter size is considered a valuable characteristic in all sheep production systems (Yavarifard et al., 2015; Abd-Allah et al., 2019). In addition, reproductive performance is a critical determinant of flock efficiency (Mazinani and Rude, 2020). Aktaş et al. (2015) attribute the increased production efficiency of sheep flocks to their better reproductive performance. Genetic polymorphisms of candidate genes influence these economic traits (Ajafar et al., 2022a, b). The follistatin (*FST*) gene is an incredibly significant candidate gene for economic traits; reside on chromosome 5 of humans, chromosome 13 of mice, chromosome 20 of cows, and chromosome 16 of sheep (Ma et al., 2017; Zhu et al., 2022). Follistatin is

a glycoprotein encoded by this gene. It is expressed in various tissues, including the pituitary, adrenal cortex, ovary, and the granulosa cells of the developing ovary (Xia et al., 2010; Lee et al., 2015; Ma et al., 2017). This protein binds and neutralizes proteins in the transforming growth factor-beta (TGF-β) superfamily, especially activins and myostatin, growth differentiation factor 8 (GDF8), GDF9. It also binds to and neutralizes bone morphogenetic proteins (BMP) 2, 5, 7, and 8 (Zheng et al., 2017). Activin stimulates the pituitary to produce FSH and enhances the action of FSH on granulosa cells (Jones et al., 2007). Activin is neutralized by follistatin by binding with high affinity (Xia et al., 2010). FST has also been shown to neutralize BMP systems, which regulate folliculogenesis and ovulation within the ovary (Zheng et al., 2017). According to Kashimada et al. (2011), FST blocks BMP2 signaling in developing follicles.

The Awassi breed is well adapted to harsh conditions and provides high meat, milk, and carpet wool production (Ajafar et al., 2022a). However, it has a lower ovulation rate and unbalanced productivity compared to the other surrounding breeds of sheep in the Middle East (Üstüner and Oğan, 2013). Reproductive traits are responsible for flock productivity (Ibarra et al., 2000), and are regulated by several candidate genes, including the *GREM1* gene in Awassi sheep (Imran et al., 2020), the prolactin gene (Al-Thuwaini, 2021b), the *OLR1* gene in Awassi sheep (Mohammed et al., 2022), and numerous new candidates (Zlobin et al., 2019), one among them being *FST* gene. *FST* gene polymorphisms have been linked to a variety of economic traits in livestock that include wool quality traits in Chinese Merino sheep (Ma et al., 2017), chicken growth traits (Dushyanth et al., 2020), and the litter size of Dazu black goats (Zhu et al., 2022). The relationship between *FST* variants and reproductive hormones in livestock has been investigated a little bit. A limited amount of information is available on *FST* gene polymorphism and how they relate to sheep reproductive traits. This study investigated the genetic polymorphisms of the *FST* gene in Awassi sheep and their association with reproductive hormone levels. It is the first study in Iraq to report that the *FST* gene variant influences reproductive hormones in a worthwhile sheep population during pregnancy and postpartum.

MATERIALS AND METHODS

Animals, blood sampling, and hormonal assay

The study was conducted at Al-Qasim Green University from July 2021 to April 2022 and followed international guidelines for animal care and use (Agri, No. 015,7,20). There were 232 healthy, sexually mature ewes of three to four years old in the study. Randomly selected ewes were taken from two sheep stations – Babylon and Karbala. Of these 123 ewes had single pregnancies and 109 had twins that were classified according to litter size after parturition and their weight ranged between 40-60 kg. Four, five-month pregnant ewes, and the month following delivery participated in the study. Animals were fed concentrates in proportion to 2.5% of their weight, including 59% barley and 40% bran, and 1% salt. Each animal received three kg of green alfalfa and one kg of straw. Drinking water was accessible at all times. The sheep's blood (5 ml) was collected by puncturing the external jugular vein with a disposable 18-gauge sterile needle, the serum was separated from the blood by centrifugation at 2,000 xg for 15 minutes and frozen at -20°C until the hormone levels were determined. The Enzyme-linked Immune-sorbent Assay (ELISA) kit E0047Sh, E0015Sh, E0105Sh, and E0106Sh from Bioassay Technology Laboratory (Shanghai, China) was used to measure reproductive hormones (estrogen, progesterone, FSH, and LH hormones).

DNA extraction, genotyping, and sequencing reaction

The rapid salting-out method was applied for extracting genomic DNA from whole blood (Al-Shuhaib, 2017). DNA samples were tested with the NanodropμLITE spectrophotometer (Biodrop, UK) before conducting a polymerase chain reaction (PCR). Ten pmol of each primer, 50 ng of genomic DNA, 50 dNTPs, 10 mM Tris-HCl (pH 9.0), 30 mM KCl, 1.5 mM $MgCl_2$, and one unit of Top DNA polymerase were used in PCR reaction (Al-Thuwaini, 2021b). GenBank ovine sequence (NC_019458.2) was used to design primers for the *FST* gene (exon 4). The primer sequence of the *FST* gene was F-5′-TCCTTCCTCAATCCAGAATACCT-3′ and R-5′ TCAGAGACCTGTCGGGATG T-3′. PCR PreMix from Bioneer (South Korea) was used in this experiment. Denaturation was carried out at 94°C for 4 minutes, annealing at 59.8°C for 45 seconds, elongation at 72°C for 45 seconds, and a final extension at 72°C for 5 minutes in the PCR amplification. The PCR products were visualized on a 2 % agarose gel (Al-Thuwaini, 2020).

After this, polymerase chain reaction-SSCP (PCR-SSCP) was performed according to Imran et al. (2020). Each amplicon was loaded with equal amounts of denaturing-loading buffer SSCP. The samples were denatured for 7 minutes and cooled for 10 minutes on wet ice before loading onto 8% polyacrylamide gels in 0.5X TBE buffer. During electrophoresis, constant current and voltage of 200 mA and 100 V were used for 4 hours, and gel staining according to Byun et al. (2009). Sequencing and editing of the detected nucleic acid polymorphisms were performed at Macrogen Geumchen, followed by viewing in Bioedit (version 7.1) (DNASTAR, Madison) and SnapGene Viewer ver. 4.0.4 (www.snapgene.com). In addition, ensemble genome browser 96 (https://asia.ensembl.org/ index.html) was used to confirm the novelty of *FST*

variants observed.

Statistical analysis

The data were analyzed using SPSS, version 23.0, SPSS Inc., Chicago, IL, with repeated measures ANOVA. The following model was used; $Y_{ijk} = \mu + G_i + L_j + P_k + (GLP)_{ijk} + pk(_j) + e_{ijk}$, where μ is the overall mean, G_i is the main effect of genotype (CC, CG, GG) (fixed w/$\sum$i G_i=0), L_j is the main effect of progeny type (single, twin) (fixed w/$\sum$j L_j=0), P_k is the main effect of the physiological stage (gestation and postpartum) (fixed w/$\sum$k P_k =0), $pk(_j)$ is the main effect of subjects $N(0, \sigma 2\, p)$, and e_{ijk} is random error assumed e_{ijk} ~ N (0; σ2). A Tukey-Kramer test was used for comparing the main factors' means. A 0.05 P-value was used to measure significance. In a preliminary statistical analysis two factors interactions, season and station had no significant effect on phenotypic traits, resulting in a no match in the general linear model. Popgen32 software, version 1.31, was used to measure genetic data, allele frequencies, and Hardy-Weinberg disequilibrium (Yeh et al., 1999).

RESULTS AND DISCUSSION

PCR amplification was performed on all 232 samples (Figure 1). The PCR-SSCP patterns for exon 4 were investigated, and three distinct PCR-SSCP patterns were found. The sequencing reactions revealed that the c. 25760691 C>G SNP occurred only in one of the SSCP variants, indicating the presence of heterogeneity in exon 4. Based on the c. 25760691 C>G substitution, the identified variants were assigned CC, CG, and GG in homozygous C/C and G/G and the heterozygous C/G patterns of the 254th position PCR amplicons (Figure 1). Based on polymorphism information content results (low polymorphism for PIC values less than 0.25, median polymorphism for PIC values between 0.25 and 0.5, and high polymorphism for PIC values >0.5), this study showed moderate polymorphism levels at the c. 25760691 C>G locus. When comparing polymorphisms of the *FST* gene to the HWE, the Chi-square test indicated a significant difference ($P \leq 0.05$).

Genetic diversity analysis and genotyping analysis indicated the polymorphism in exon 4 of the *FST* gene in Awassi sheep. Several studies in livestock have examined *FST* gene variations. Ma et al. (2017) detected seven SNPs in the *FST* gene in Chinese Merino sheep (Junken Type) using a sequencing analysis. Dushyanth et al. (2020) found polymorphisms in the coding regions (exons 2 and 5) of the *FST* gene in white leghorn chickens (PD-1) and Native American (Aseel) chickens. By using SNaPshot, Zhu et al. (2022) detected twenty-six single nucleotide polymorphisms (SNPs) and one deletion in UTRs of 186 Dazu black goats. Unfortunately, there is a lack of literature on *FST* polymorphism in Awassi sheep.

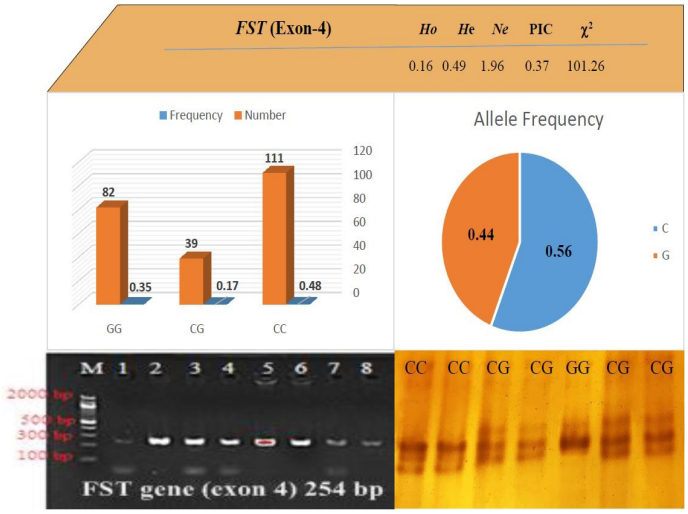

Figure 1: Genotyping and genetic diversity of the *FST* gene in Awassi ewes. *Ho*: observed heterozygosity; *He*: expected heterozygosity; *Ne*: effective allele frequency; PIC: polymorphism information content; χ^2: Chi-square. All Chi-square tests have two degree of freedom and are within the significance level ($P \leq 0.05$).

In terms of the effects of *FST* genotypes on reproductive hormone levels, the three genotypes had significant differences ($P \leq 0.05$) in reproductive hormone levels (Table 1). Comparing twin-pregnant ewes to single-pregnant ewes, CC genotypes had higher estrogen, progesterone, and lower FSH levels (85.10 ± 3.11) (pg/ml), (8.82 ± 0.52) (ng/ml), and (13.95 ± 2.71) (ng/ml) as compared to CG and GG genotypes, respectively.

The present study demonstrated that CC genotype individuals in twin pregnancy had higher estrogen, progesterone, and lower FSH levels than CG and GG genotypes when compared with single-pregnant ewes. Therefore, the c. 25760691 C>G mutation negatively affected the reproductive hormones of Awassi ewes. Hormones have been implicated as regulators of multiple reproductive processes in mammals, including sheep. Hypothalamic-pituitary-gonadal (HPG) factors contribute to most of these reproductive processes (Juengel et al., 2018; Al-Thuwaini, 2022). At the anterior pituitary level, activin, follistatin, and inhibin factors are known to play a role in autocrine/paracrine regulation of anterior pituitary FSH secretion (Padmanabhan and Cardoso, 2020). In mammals, follistatin regulates oocyte maturation, follicular cell proliferation, differentiation, steroidogenesis, and corpus luteum function (Stangaferro et al., 2014). Activin-follistatin affects the production of gonadotropin receptors and steroids, which control antral follicle growth and differentiation (Tian et al., 2022). The amount of activin produced by a follicle decreases when it becomes a dominant follicle, while inhibin and

Table 1: The association between *FST* genetic polymorphism and litter size with reproductive hormones levels during pregnancy and postpartum in Awassi ewes.

Hormones	Genotypes	Progeny type (LSM ± SE)						P value
		Single (123)			Twin (109)			
		4th Month	5th Month	Post parturition	4th Month	5th Month	Post-parturition	
Estrogen (pg/ml)	CC	61.34 ± 3.16^{Ba}	34.60 ± 2.04^{Aa}	41.98 ± 2.42^{Aa}	85.10 ± 3.11^{Aa}	34.06 ± 3.02^{Aa}	43.71 ± 2.12^{Aa}	0.04
	CG	48.32 ± 3.69^{Bb}	38.33 ± 2.45^{Aa}	42.21 ± 2.21^{Aa}	67.34 ± 1.65^{Ab}	37.61 ± 2.45^{Aa}	43.87 ± 2.06^{Aa}	0.03
	GG	29.74 ± 1.69^{Bc}	32.99 ± 2.33^{Aa}	45.41 ± 2.42^{Aa}	42.47 ± 1.42^{Ac}	35.36 ± 2.31^{Aa}	40.36 ± 4.20^{Aa}	0.01
Progesterone (ng/ml)	CC	6.15 ± 0.80^{Ba}	3.03 ± 0.43^{Aa}	2.69 ± 0.34^{Aa}	8.82 ± 0.52^{Aa}	3.01 ± 0.33^{Aa}	2.66 ± 0.34^{Aa}	0.02
	CG	3.29 ± 0.55^{Bb}	2.94 ± 0.33^{Aa}	2.61 ± 0.28^{Aa}	4.50 ± 0.35^{Ab}	2.97 ± 0.16^{Aa}	2.70 ± 0.14^{Aa}	0.03
	GG	2.72 ± 0.64^{Bc}	2.70 ± 0.19^{Aa}	2.68 ± 0.32^{Aa}	4.12 ± 0.42^{Ac}	2.78 ± 0.42^{Aa}	2.65 ± 0.27^{Aa}	0.02
FSH (ng/ml)	CC	11.57 ± 2.97^{Bc}	17.21 ± 2.31^{Aa}	7.89 ± 1.28^{Aa}	13.95 ± 2.71^{Ac}	18.35 ± 2.60^{Aa}	10.09 ± 2.07^{Aa}	0.04
	CG	16.85 ± 1.78^{Bb}	19.08 ± 2.14^{Aa}	8.77 ± 1.43^{Aa}	17.56 ± 2.37^{Ab}	18.23 ± 2.34^{Aa}	9.13 ± 1.96^{Aa}	0.02
	GG	19.25 ± 2.52^{Ba}	19.36 ± 1.78^{Aa}	9.10 ± 1.53^{Aa}	21.92 ± 1.89^{Aa}	17.08 ± 2.22^{Aa}	9.79 ± 2.11^{Aa}	0.02
LH(ng/ml)	CC	11.37 ± 0.32^{Aa}	10.49 ± 0.28^{Aa}	8.54 ± 0.14^{Aa}	11.84 ± 0.24^{Aa}	10.41 ± 1.11^{Aa}	8.46 ± 0.07^{Aa}	0.13
	CG	10.72 ± 0.22^{Aa}	10.97 ± 0.13^{Aa}	8.62 ± 0.17^{Aa}	10.87 ± 0.16^{Aa}	10.89 ± 0.13^{Aa}	8.57 ± 0.05^{Aa}	0.24
	GG	10.46 ± 0.25^{Aa}	10.12 ± 0.16^{Aa}	8.41 ± 0.20^{Aa}	10.32 ± 0.21^{Aa}	10.74 ± 0.09^{Aa}	8.34 ± 0.01^{Aa}	0.35

LSM ± SE, Least square means ± Standard error. ^{A,B} different capital letters indicate a significant difference in the raw within each classification (P ≤0.05). ^{a,b} different lowercase letters indicate a significant difference in columns within each classification (P ≤0.05).

follistatin are produced (Muttukrishna et al., 2004; Tian et al., 2022). Additionally, follistatin inhibits FSH secretion by inhibiting activins' internalization and degradation, which reduces their bioavailability (Cash et al., 2009). Follistatin is recognized first as an inhibitor of the FSH (Zheng et al., 2017). The administration of recombinant human follistatin to sheep has been shown to suppress FSH concentrations but not luteinizing hormone (LH), according to Padmanabhan and Sharma (2001).

Based on the results of the *FST* gene association study, twin-pregnant ewes with a CC genotype showed higher estradiol, progesterone, and lower FSH levels in comparison to single-pregnant ewes. The reason behind this is that follistatin plays a crucial role during development and early neonatal life and during pregnancy in sheep (McFarlane et al., 2002). According to the study by Lee et al. (2009), follistatin treatment accelerates development to the blastocyst stage in early embryos, suggesting that follistatin derived from the oocyte is crucial for determining the competence of oocytes in cattle. O'Connell et al. (2016) reported that activin and follistatin work on numerous reproductive processes, including luteinization to facilitate luteinization, and implantation of the conceptus. In mammals, the deletion of the follistatin gene causes various fertility defects that include reduced litter size, impaired fertilization and ovulation, and elevated levels of FSH and LH (Jorgez et al., 2004). As a result, a lower frequency of c. 25760691 C>G is associated with better reproductive traits in Awassi ewes. Therefore, the current study in the Awassi breed explains the superiority of CC genotype in twin-pregnant ewes compared to single-pregnant ewes.

CONCLUSIONS AND RECOMMENDATIONS

Genetic variations in the *FST* gene were linked to reproductive hormone levels in Iraqi Awassi sheep. These results suggest that *FST* gene polymorphism contributes to sheep reproductive traits. It is crucial to select sheep with CC genotypes and conduct future studies to determine whether this gene is associated with high prolificacy.

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NOVELTY STATEMENT

This study investigated a genetic variation within the *FST* gene in Awassi sheep for the first time. Researchers found that the *FST* gene can be selected to improve Awassi sheep's reproductive traits, especially litter size, through assisted selection.

AUTHOR'S CONTRIBUTION

All authors contributed equally.

Conflict of interest

The authors have declared no conflict of interest.

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