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Sperm Properties Associated with Polymorphism of the FSH Beta Subunit Gene in Different Ages of Local Iraqi Rams

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Abstract | The ram is a crucial member of the flock, yet it is often the most neglected. Ram contributes half of the genetics to the flock, and its success as a breeder will go a long way towards ensuring a profitable lamb crop. This study aimed to evaluate the relationship between polymorphism of the FSH beta-subunit gene (FSH β) and sperm properties in different age groups of local Iraqi rams, to improve the breed through genetic improvement of the herd. Testicular samples (n=50 per age group) were collected from the Al-Shoa'alla slaughterhouse in western Baghdad. Methods: A total of 50 testicular samples were collected for each age group and distributed across genotypes as follows: (AA n=16, AG n=17, GG n=17) in 1-year-old, >1-year-old, and 2-year-old rams, respectively and Age was estimated based on dental eruption patterns and information provided by the owners. Each caudal of epididymis was injected with 20 mL of the diluent Botu-Turbo™ warmed at 37°C. The spermatozoa were collected by the method of slicing using an automatic syringe and put in graduated tubes, then in a water bath at 37°C to evaluate the physical properties of epididymal fluid, including individual motility %, dead sperms %, and abnormal sperms %. Blood samples were collected prior to slaughter for genetic analysis; this included DNA extraction, PCR using specific primers, and genotyping via Sequencing or PCR-RFLP. The results of association analysis between the FSH β subunit gene in rams and sperm properties traits showed that the heterozygote with the AG genotype had a significantly higher sperm individual motility (P<0.05) than the GG and AA genotypes in 2-year-old rams as compared with other age groups. Conclusion: It is concluded from this study that the genotype AG of the FSH β subunit gene in local rams is associated with improved sperm motility, making it a useful genetic marker for selective breeding programs for herd improvement.

Keywords | Abnormal sperms, Dead sperms, Individual motility, Polymorphism of FSH beta-subunit, Sperm properties

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INTRODUCTION

Climatic conditions can significantly affect semen quality. Indeed, sexual behavior, semen quality, and

quantity are key factors limiting male reproductive efficiency throughout the year. These factors could vary according to different environmental and physiological factors such as climate (Hu *et al.*, 2020); latitude, and breeding season of

the year (Saleh and Abdul-Ameer, 2019). Semen quality can be influenced by factors such as breed, age, nutritional status, and environmental conditions, including ambient temperature, humidity, and daylight hours (Saeed and Zaid, 2019; Goshme *et al.*, 2020). Selection of young rams for fertility can be accomplished through selecting some important reproductive traits such as age at puberty, body condition score, body growth rate, scrotal circumference, scrotal growth rate, and semen quality (Zaher *et al.*, 2020; Maquivar *et al.*, 2021). In general, the sexual development of rams appears to be more closely related to body growth than to their age (Andreeva *et al.*, 2020).

The age of onset of puberty in ram lambs varies across breeds and climatic conditions (Maquivar *et al.*, 2021). However, it is distinguished by a sequence of endocrine processes which activate the reproductive axis, making the Sertoli and Leydig cells susceptible to the effects of gonadotropins (FSH and LH), thereby initiating spermatogenesis. The photoperiod influences the timing of puberty in ram lambs, with longer daylight periods generally promoting earlier sexual maturation. Typically, puberty occurs between 20 and 28 weeks of age, when the animal has attained approximately 65% of its mature body weight (Li *et al.*, 2024).

The anterior pituitary gland produces the glycoprotein hormones follicle-stimulating hormone (FSH) and luteinizing hormone (LH). In males, FSH primarily targets Sertoli cells in the seminiferous tubules to support spermatogenesis, while LH stimulates testosterone production in the Leydig (interstitial) cells (Banihani 2020; Duan *et al.*, 2021). The FSH hormone consists of α and β -subunits; β -subunits play a role in determining the specificity of bonds with receptors (FSHR) (Ulloa-Aguirre *et al.*, 2018; Obaid *et al.*, 2022). Spermatogenesis is a highly regulated and continuous process that takes place in the seminiferous tubules by which normal diploid cells transform to sperm (haploid). Spermatogenesis depends on stem cells, spermatogonia (Madeja *et al.*, 2021).

In males, FSH promotes spermatogenesis, the process of sperm development, in the seminiferous tubules primarily via actions on Sertoli cells (Rougier *et al.*, 2019; Barquero *et al.*, 2021). For this reason, polymorphisms within the FSH β gene and its promoter are of particular interest to male fertility. FSH activates the proliferation of Sertoli cells during fetal and neonatal development as well as during the pubertal phase. Since each Sertoli cell can only support a limited number of developing male germ cells at a time, it is important that we understand how polymorphisms of the FSH β subunit gene can affect male fertility (Recchia *et al.*, 2021).

Sperm motility is the most widely tested semen quality trait, which requires the use of an intact plasma membrane and a certain chemical gradient of ions and other soluble components to operate correctly (Jabulani *et al.*, 2020). An unstained semen sample may be subjected to a manual microscopic examination to determine the percentage of total and/or increasingly motile sperm (Touré *et al.*, 2021; Johannisson *et al.*, 2024). It is a simple and fast approach that does not require costly equipment. Photographic analysis or computer assisted semen analysis (CASA) can be used to analyze sperm concentration, motility, motion, and to some degree sperm head morphology as an alternative to subjective visual motility evaluation (Tanga *et al.*, 2021).

Sperm morphology is usually determined by spermatogenesis (Johannisson *et al.*, 2024) or events that occur following spermiation, and improper handling techniques or problems during cooling and freezing, which can often harm the acrosome or cause sperm tail reflection (Ngcobo *et al.*, 2020; Savvulidi *et al.*, 2021). The objectives of this study are to provide a basis for identifying and selecting rams with superior reproductive performance and desirable genetic traits, and to evaluate the genetic potential of local Iraqi rams by examining their semen characteristics. Selection of breeds based on genetic traits is not currently practiced in Iraq, which supports future breeding programs.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

Samples were collected from Al-Shoáalla slaughterhouse positioned in western Baghdad as follows: A total of 50 testicular samples were collected for each age group and distributed across genotypes as follows: (AA n=16, AG n=17, GG n=17) in 1-year-old, >1-year-old, and 2-year-old rams, respectively; age is determined as possible according to the dental figures (Miller and Robertson, 1975) and information from the owners. The samples belong to local rams aged nearly (12-24) months as determined by dental formula (Albrkaty, 2024). Blood samples were collected from the jugular vein before slaughtering the animals for estimation molecular study for molecular analysis of the FSH-beta subunit gene. The epididymis was washed in tap water to remove blood and any other debris. Each caudal of epididymis was injected with 2mL of the diluent Botu-TurboTM (semen extender) warmed at 37°C, the spermatozoa were collected by the method of slicing: the visceral layer of the tunica vaginalis covering the cauda epididymis (CE) was carefully removed to prevent any blood contamination. The exposed CE was then rinsed with phosphate-buffered saline (PBS), excised, and finely sliced using a sterile scalpel in a Petri dish containing 3 mL of semen extender. The sliced tissue was left immersed in the extender in water bath at 37-38 °C to facilitate

spermatozoa release. Subsequently, the suspension was gently rinsed with an additional 2 mL of semen extender and filtered through sterile gauze sheets to obtain a clear sperm sample and evaluate the physical properties of epididymal fluid, including individual motility %, dead sperms %, and abnormal sperms %.

Collection of blood before the slaughter for evaluation of a genetic study; DNA extraction of blood, PCR by using primers (Table 1), and Sequencing or Alleles by PCR-RFLP. Collection of blood samples: Investigation of the FSH-beta subunit gene. Blood Genomic DNA was extracted from the collected blood samples using the Favor Prep Blood genomic DNA extraction Mini kit.

Table 1: Primers applied for analysis of FSH β -gene.

Gene	Sequence 5' - 3'	Amplicon size
FSH β	F CTT CCA GAC TAC TGT AAC TCA TC R GTA GGC AGT CAAA GC ATC CG	313bp

DNA EXTRACTION FROM BLOOD

According to the manufacturer's instructions, the protocol

designated for extraction of genomic DNA from whole blood, serum, and plasma samples was applied to extract of DNA from the frozen EDTA-blood samples of the study rams.

PCR AMPLIFICATION

The amplified fragments represented the desired product of FSHB-subunit gene with a molecular weight of 313 bp (Figures 1 and 2).

PRIMER PREPARATION

To prepare the working primer solution from stock primer, a total of 250 μ l nuclease-free water was added to each tube of primers (forward and reverse) (Table 1), mixed well by vortex, and saved deep frozen.

MASTERMIX PREPARATION

PCR is a technique that selectively amplifies many copies of a particular segment of DNA using a specific enzyme, polymerase, and a specific primer (s) (Joshi *et al.*, 2010). According to manufacturer instructions, AccuPower PCR-PreMix kit (Biotechnology, Korea) was employed in the present study to prepare of MasterMix. The tubes of PreMix have a ready-to-use PCR reagent that is optimized

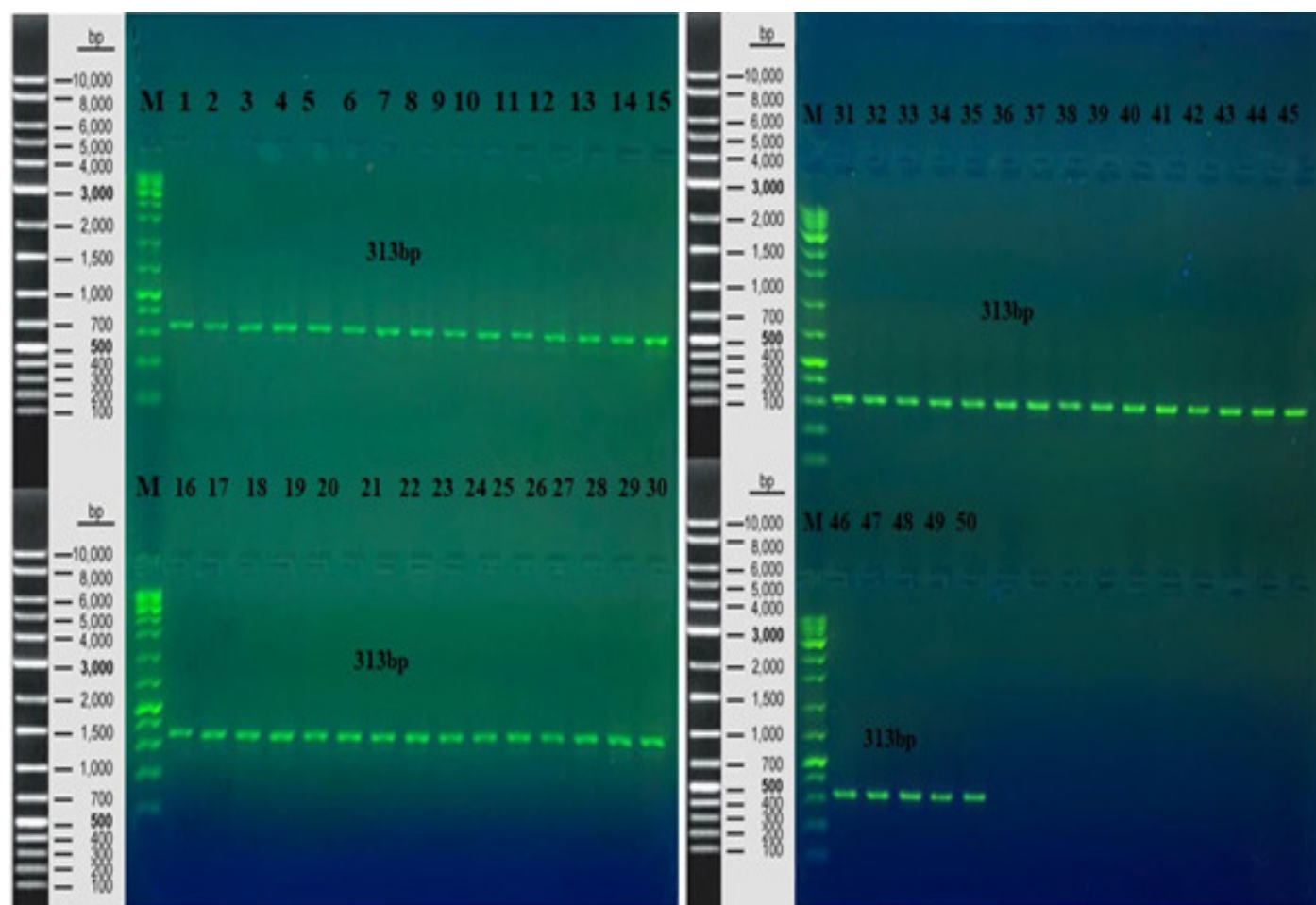


Figure 1: PCR result for the FSH gene's 313 base pair band. The electrophoresis product was run for 90 minutes on Agarose gel (2.0%) electrophoresis at 5 volts per centimeter and 1 $\times$ TBE buffer.

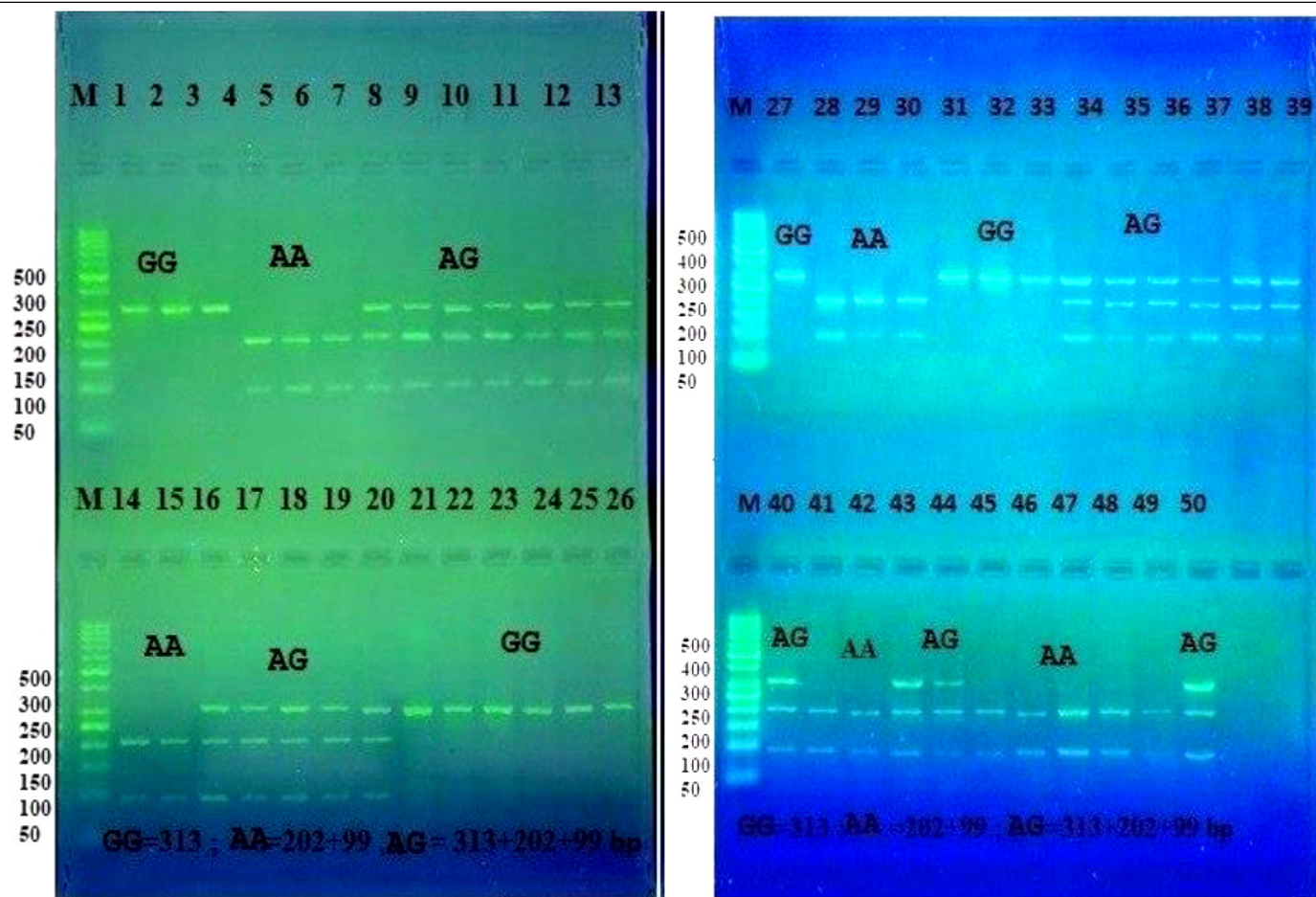


Figure 2: Agarose gel electrophoresis showing PCR-RFLP patterns of the studied gene in rats. Lane M is the 100 bp DNA marker. Lanes 1–4 and 21–26 represent the GG genotype (single band at 313 bp). Lanes 5–10 and 14–15 show the AA genotype (two bands at 202 bp and 99 bp). Lanes 11–13 and 16–20 show the AG genotype (three bands at 313 bp, 202 bp, and 99 bp).

for more accurate amplification, as they are characterized by speed, stability, reproducibility, and simplicity. According to manufacturer instructions, the MasterMix of each sample was performed at a final volume of 25 μ l. Then, the tubes of MasterMix were mixed by Exispin vortex (Digisystem, Germany) at 3000 rpm for 3 minutes and transferred to the thermal Cycler. PCR primers were designed based on a previously performed reference (Yang *et al.*, 2011) and provided by Macrogen Company, Korea. Primers were applied for analysis of the FSHB-Subunit gene as demonstrated in Table 1.

STATISTICAL ANALYSIS

Data were analyzed using one-way analysis of variance (ANOVA) to evaluate statistically significant differences among the experimental groups. Data were organized and tabulated using Microsoft Excel, and statistical analyses were performed using IBM SPSS Statistics for Windows (version 26.0; IBM Corp., Armonk, NY, USA) following the procedures described by George and Mallery (2019). Differences among means were considered statistically significant at $P < 0.05$.

RESULTS

SPERM PROPERTIES AND GENOTYPES OF FSH β SUBUNITS IN RAM OF 1-YEAR AGE

According to the study's age groups, the findings, as revealed in Table 2, showed a significant association ($P < 0.05$) between sperm properties and the studied genotypes of FSH β subunits. For the 1-year age group, significant increases in values (mean $\pm$ SE) of individual motility were detected in the AG genotype (70.5 ± 3.73) compared to values of AA (62.5 ± 4.82) and GG (60.19 ± 4.5) genotypes, which showed insignificant differences ($P > 0.05$) in their values. The percentage of dead (17.19 ± 1.08) and abnormal sperms (6.72 ± 0.73) was lowest significantly ($P < 0.05$) in the AG genotype in comparison with the values of AA (28.46 ± 1.5 and 14.39 ± 2.06 , respectively) and GG genotypes (25.81 ± 2.03 and 13.55 ± 1.75 , respectively) of FSH β subunits respectively. However, the results of AA and GG genotypes showed no significant differences ($P < 0.05$) in values of dead and abnormal sperms.

Table 2: Association of sperm properties and genotypes of FSH β subunits in ram of 1-year age group rams.

Geno- type	Sperm properties (%)		
	Individual motility	Dead sperms	Abnormal sperms
AA	62.5 $\pm$ 4.82 B	28.46 $\pm$ 1.5A	14.39 $\pm$ 2.06 A
GG	60.19 $\pm$ 4.5 B	25.81 $\pm$ 2.03A	13.55 $\pm$ 1.75 A
AG	70.5 $\pm$ 3.73 A	17.19 $\pm$ 1.08B	6.72 $\pm$ 0.73 B

Variation in large vertical letters refers to significant differences (P<0.05). AA= Adenine Adenine, GG= Guanine Guanine, AG= Adenine Guanine.

SPERM PROPERTIES (%) AND GENOTYPES OF FSH β SUBUNITS IN RAMS OF MORE THAN ONE-YEAR AGE GROUP

For more than one year (>1year) age group, Table 3 showed a significant increases (P<0.05) in values (mean $\pm$ SE) of individual motility were reported in AG (79.08 $\pm$ 3.52) genotype of FSH β subunits in comparison with values of AA (67.31 $\pm$ 4.18) and GG (65.22 $\pm$ 3.94) genotypes both were showed no significant differences (P>0.05) in their values. Regarding the percentage of dead and abnormal sperms, respectively, values were a significantly decline in AG genotype (12.4 $\pm$ 1.26 and 5.15 $\pm$ 1.01) and increased significantly (P<0.05) in the AA (21.73 $\pm$ 2.03 and 12.98 $\pm$ 1.64, respectively) and GG (20.15 $\pm$ 1.51 and 12.45 $\pm$ 1.82, respectively) genotypes, which both showed insignificant variation (P>0.05) between their values.

Table 3: Association of sperm properties (%) and genotypes of FSH β subunits in rams of more than one year (>1 year) age group.

Gen- otype	Sperm properties (%)		
	Individual motility	Dead sperms	Abnormal sperms
AA	67.31 $\pm$ 4.18 B	21.73 $\pm$ 2.03A	12.98 $\pm$ 1.64 A
GG	65.22 $\pm$ 3.94 B	20.15 $\pm$ 1.51A	12.45 $\pm$ 1.82 A
AG	79.08 $\pm$ 3.52 A	12.4 $\pm$ 1.26B	5.15 $\pm$ 1.01 B

Variation in large vertical letters refers to significant differences (P<0.05). AA= Adenine Adenine, GG= Guanine Guanine, AG= Adenine Guanine.

SEMINAL CHARACTERISTICS AND GENOTYPES OF FSH β SUBUNITS IN RAMS OF 2-YEARS AGE GROUP RAMS

The results in Table 4 revealed that the values of individual motility showed a significant elevation in the AG genotype group (87.29 $\pm$ 3.36) and a decrease in AA (77.45 $\pm$ 4.23) and GG (76.82 $\pm$ 4.19) genotypes. Concerning the percentage of dead and abnormal sperms, respectively, though the values of both AA and GG were recorded no significant differences between their values; the AG genotype group (6.98 $\pm$ 0.89 and 3.22 $\pm$ 0.54, respectively) showed a significant reduction in its values as compared with other groups.

Table 4: Association of seminal characteristics and genotypes of FSH β subunits in rams of 2-years age group rams.

Gen- otype	Sperm properties (%)		
	Individual motility	Dead sperms	Abnormal sperms
AA	77.45 $\pm$ 4.23 B	15.12 $\pm$ 1.64 A	8.99 $\pm$ 1.16 A
GG	76.82 $\pm$ 4.19 B	12.71 $\pm$ 1.33 A	9.34 $\pm$ 1.25 A
AG	87.29 $\pm$ 3.36 A	6.98 $\pm$ 0.89 B	3.22 $\pm$ 0.54 B

Variation in large vertical letters refer to significant differences (P<0.05). AA= Adenine Adenine, GG= Guanine Guanine, AG= Adenine Guanine.

DISCUSSION

The association analysis revealed that rams with the heterozygous AG genotype had significantly higher sperm individual motility (P<0.05) than GG and AA genotypes in ram of 2-years age-old (Table 4) as compared with other age groups (Tables 2 and 3), these result was in agreement with Yang *et al.* (2011), Iyer and Rajkumar (2019) and Pascal *et al.* (2023), consistent with previous reports that polymorphisms of the FSH β subunit gene were significantly associated with individual motility. Ram fertility is significantly impacted by polymorphisms in genes linked to semen quality. These genes also influence important traits such as sperm count, motility, and morphology in other species (Abril-Parreño *et al.*, 2023). The Follicle-Stimulating Hormone Beta Subunit (FSH β) confers the distinct biological action of FSH and its specific interaction with the FSH receptor. In the testes' seminiferous tubules, it promotes spermatogenesis, or the generation of sperm. It acts predominantly on Sertoli cells, which in turn support the meiosis of spermatocytes. Understanding male fertility requires an understanding of polymorphisms (variations) in the FSH β gene, which can impact the hormone's activity (Dalvi *et al.*, 2018; Wu *et al.*, 2023). Consistent with findings of Primasari *et al.* (2021) in bulls, where low genetic diversity in the FSH β gene was associated with poor semen quality and fertility, our results suggest that polymorphisms in this gene may play a crucial role in regulating testicular function and sperm motility with different ages (1-year and >1-year old). The observed differences among genotypes in our study could reflect varying levels of FSH β gene expression or receptor interaction efficiency. Therefore, maintaining adequate genetic variability at this locus may be essential for sustaining optimal reproductive performance in rams (Kim *et al.*, 1988).

Numerous studies have shown that genetic factors influence reproduction and semen quality traits in bulls (Fonseca *et al.*, 2020), goats (Alberto *et al.*, 2018), and boars (Krupa *et al.*, 2023). Ram sperm properties are a quantitative trait controlled by multiple genes. The identification of these

genes using molecular techniques will ultimately lead to the development of novel tools for analyzing complex genetic traits, thereby improving the selection efficiency of rams (Salleh *et al.*, 2018; Iyer and Rajkumar, 2019). There were many reports regarding the use of the candidate gene as a marker for semen quality and fertility in bulls (Ghorbani *et al.*, 2017; Özbek *et al.*, 2021). Dai *et al.* (2009) reported that FSH- β gene polymorphisms also affect semen quality and fertility. Our results indicate that by 2 years of age, rams undergo a cascade of morphological, physiological, and behavioral events leading to increased gonadal activity, so the result in this study showed AG genotype had a significantly higher than GG and AA genotype in sperm individual motility, and decreased in dead and abnormality ($P < 0.05$) of ram 2-years age-old (Taylor *et al.*, 2018; Camara *et al.*, 2020). The β -subunit determines the biological specificity of gonadotropins, and the transcriptional differences of genes encoding the β -subunit directly affect the synthesis and secretion of hormones (Simoni *et al.*, 2020; Wang *et al.*, 2021).

The current results corroborate earlier studies that shown the crucial function of the FSH β gene in controlling the fertility and quality of semen in all mammals. According to Kim *et al.* (2011), POBA cattle with limited genetic diversity of the FSH β gene had lower fertility and semen quality, indicating that this locus is being influenced by selection pressures that affect reproductive performance. Also, according to research by Lamminen *et al.* (2005) and Grigorova *et al.* (2010), allele A of the FSH β gene is associated with improved sperm motility and viability in both fresh and post-thawed semen. One possible explanation for this positive impact is that the encoded protein undergoes changes to its biological activity and hormonal function as a result of non-synonymous single nucleotide polymorphisms (SNPs). Consistent with these results, the present investigation showed that compared to rams with the GG and AA genotypes, AG genotypes had substantially smaller percentages of dead and defective spermatozoa and considerably better sperm individual motility. This indicates that variations in the FSH β gene's genetic code can influence spermatogenesis and testicular function by altering the interactions between FSH and receptors as well as the amounts of gene expression. Thus, it may be crucial to optimize reproductive efficiency and direct selection programs for better breeding rams by maintaining sufficient genetic variety at this locus.

Selection of breeding rams would be the prerequisite for getting better fertility, which can be accomplished through selecting some important reproductive traits (Li *et al.*, 2023; Sa *et al.*, 2024). There is a range of moderate to high heritability in sperm motility (Omolaoye *et al.*, 2022). Accordingly, it is anticipated that enhanced fertility

through assisted selective breeding would result from a deeper comprehension of the genomic architecture of complex semen-related variables. To improve breeds, it's critical to find polymorphisms that can act as genetic markers for enhanced reproductive performance (Ramirez-Diaz *et al.*, 2023).

Specifically, at 2 years of age, rams with the AG genotype of the FSH β gene showed far better sperm motility and lower percentages of dead and deformed spermatozoa compared to rams with the GG and AA genotypes. Alterations to the Hypothalamic-Pituitary-Gonadal (HPG) Axis with Age: The secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) increases in sexually mature rams at 2 years of age, which improves spermatogenesis and amplifies the phenotypic manifestation of advantageous genotypes. Sperm motility and morphology are impacted by aging-related changes in gene expression that pertain to mitochondrial function, oxidative stress, lipid metabolism, and membrane dynamics. The quality of the semen may be impacted by these alterations' interactions with the FSH β genotype. Semen characteristics including motility and viability can be affected by changes in protein function caused by non-synonymous mutations in the FSH β gene. Under the hormonal milieu that exists at 2 years of age, the AG genotype may give a functional benefit. This research confirms what previous research has shown, which is that sheep and other mammals exhibit semen quality attributes linked to genetic variants in the FSH β gene. One example is the work of Liang *et al.* (2024), who demonstrated how gene polymorphisms affect mammalian fertility and sperm quality. The intricate genetic structure of male fertility was further demonstrated by the findings of Khan *et al.* (2024), who found strong associations between semen quality features and polymorphisms in genes such as FSHR and INHA. The frequency of the pattern is largely unknown, but Sustainable breeding efforts, especially in indigenous ram populations, require genetic variety maintenance, even though the AG genotype is a potential target for enhancing fertility.

CONCLUSION AND RECOMMENDATIONS

In conclusion, the AG genotype of the FSH β gene is associated with superior semen quality in 2-year-old rams. This is likely due to an interaction between genetic factors and age-related physiological changes. These findings can guide efforts to improve sheep reproductive efficiency through selective breeding. Although the AG genotype represents a promising target for improving fertility, maintaining genetic diversity is essential for sustainable breeding programs, particularly in indigenous ram populations.

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NOVELTY STATEMENT

The true novelty of this research lies not only in the “discovery” of a new gene but also in its practical application to improve a local breed by linking genetic variation to an important production trait, taking into account the age factor, thus providing an effective and accurate tool for breeding programs.

AUTHOR'S CONTRIBUTION

The first author designed the study, supervised the experimental work, and contributed to data interpretation, Second author performed the laboratory analyses, collected samples, and carried out the molecular and statistical procedures, third author assisted in data analysis, prepared the figures and tables, and contributed to writing the initial draft of the manuscript and the Forth author as corresponding and contributed to data interpretation, critical revision of the manuscript, and improvement of the final scientific content. All authors read and approved the final version of the manuscript.

ETHICAL APPROVAL

This study was conducted in the Animal House of the College of Medicine, University of Fallujah, and was approved by the Ethical Committee (ethical approval number: 8) before commencing the research work. All information is anonymized and used for research only.

GENERATIVE AI AND AI-ASSISTED TECHNOLOGY STATEMENT

Generative AI tools (such as ChatGPT) were used only to assist in minor language editing and improving the clarity of a few non-scientific sections of the manuscript (e.g., novelty statement). No AI tools were used to generate, analyze, or interpret scientific data, or to write the Introduction, Methods, Results, or Discussion. All content has been thoroughly reviewed and approved by the authors, who take full responsibility for the accuracy and integrity of the final manuscript.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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