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Original Article

Effect of Point Mutation in the Growth Differentiation Factor 9 Gene of Oocytes on the Sterility and Fertility of Awassi Sheep

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ABSTRACT

Growth differentiation factor 9 (GDF9) plays a critical role in ovarian follicular development and ovulation rate. The present study aimed to investigate the correlation between the single-nucleotide polymorphism (SNP) of the *GDF9* gene and reproductive performance variables, such as fertility and sterility in Awassi sheep. Forty pairs of ovaries from a total of 40 slaughtered Iraqi Awassi ewes were used in this study. Twenty of the ovaries were collected from sterile ewes and the other 20 ovaries were taken from fertile ewes for genomic DNA extraction, polymerase chain reaction, and sequencing to detect *GDF9* gene polymorphism. Follicles and oocytes of all the 40 ovaries were evaluated and compared with the results of genotyping. Furthermore, histopathological and microscopic evaluations were performed for 40 ovarian tissues of the two groups. The sequence analysis revealed that exon I had three SNPs, including T(114)C, G(129)R, and G(199)A. The first two SNPs were silent mutations and the last mutation was missense responsible for the substitution of glutamic acid with lysine at position 67. The current study showed a significant increase ($P \leq 0.01$) in GG, AA, CC, GA, and GG genotypes at G(129)R, G(199)A, T(114)C, G(129)R, and G(199)A loci, respectively. Moreover, the TT genotype in locus T(114)C was recorded to significantly augment ($P \leq 0.05$) in the fertile ewes. Mutant GA genotype of the G(129)R locus led to a significant ($P \leq 0.05$) increase in the percentage of follicles (4-8 mm) and oocytes number, compared to wild GG. On the other hand, a significant decrease was recorded in the mutant AA genotype in G(199)A, compared to wild GG. Differences between CC and TT genotypes at T(114)C locus were not significant. Histopathological examination revealed hypoplasia in the ovarian tissue of sterile ewes accompanied by fibrous connective tissue invasion and follicles degeneration. However, in the fertile ewes, the ovarian tissues were normal with the presence of numerous corpus albicans and degenerative corpus luteum. According to the findings of this study, the homozygote mutation in fertile ewes minimized the number of follicles and oocytes leading to sterility, while the heterozygote mutation was reported in the fertile Awassi ewes.

Keywords: Fertility, Heterozygote, Homozygote, Infertility

Effet de la Mutation Ponctuelle dans le Gène du Facteur de Différenciation de Croissance 9 des Ovocytes sur la Stérilité et la Fertilité des Moutons Awassi

Résumé: Le facteur de différenciation de croissance 9 (GDF9) joue un rôle essentiel dans le développement folliculaire ovarien et le taux d'ovulation. Cette étude visait à étudier la corrélation entre le polymorphisme mononucléotidique (SNP) du gène *GDF9* et les variables de performance de reproduction, telles que la fertilité et la stérilité chez les moutons Awassi. A cet effet, 40 paires d'ovaires ont été prélevées à partir de 40 brebis Awassi irakiennes abattues. Au total, 20 ovaires ont été prélevés sur des brebis stériles et les 20 autres sur des brebis fertiles. Les échantillons prélevés ont été ensuite soumis à une extraction d'ADN génomique suivie d'une réaction en chaîne par polymérase et d'un séquençage pour détecter le polymorphisme du gène *GDF9*.

Les follicules et les ovocytes des 40 ovaires ont été ensuite évalués et comparés aux résultats du génotypage. De plus, des évaluations histopathologiques et microscopiques ont été réalisées pour les 40 tissus ovariens des deux groupes. L'analyse des séquences a révélé que l'exon I avait trois SNPs, en l'occurence T (114) C, G (129) R et G (199) A. Les deux premiers SNP étaient des mutations silencieuses et la dernière était une mutation faux-sens, responsable de la substitution de l'acide glutamique par la lysine en position 67. L'étude actuelle a montré une augmentation significative (P≤0.01) des génotypes GG, AA, CC, GA et GG à G (129) R, G (199) A, T (114) C, G (129) R, et G (199) A loci, respectivement. De plus, le génotype TT dans le locus T (114) C, était significativement augmenté (P≤0.05) chez les brebis fertiles. Le génotype mutant GA du locus G (129) R a conduit à une augmentation significative (P≤0.05) du pourcentage de follicules (4-8 mm) et du nombre d'ovocytes, par rapport au GG sauvage. En revanche, une diminution significative a été enregistrée dans le génotype AA mutant dans G (199) A, comparé au GG sauvage. Il n'y avait aucune différence significative entre les génotypes CC et TT au locus T (114) C. L'examen histopathologique a révélé une hypoplasie dans le tissu ovarien des brebis stériles accompagnée d'une invasion du tissu conjonctif fibreux et d'une dégénérescence des follicules. Cependant, les tissus ovariens étaient normaux avec la présence de nombreux corps albicans et de corps jaune dégénératif chez les brebis fertile. Selon les résultats de cette étude, la mutation homozygote chez les brebis fertiles a diminué le nombre de follicules et d'ovocytes qui ont conduit à la stérilité, tandis que la mutation hétérozygote a été rapportée chez les brebis fertiles Awassi.

Mots-clés: Fertilité, Hétérozygote, Homozygote, Infertilité

INTRODUCTION

Growth differentiation factor-9 (GDF9) is a transforming growth factor from β superfamily (Elvin et al., 2000). The GDF9 is secreted particularly from oocytes during folliculogenesis and works synergistically with bone morphogenetic protein 15 (BMP15) to enhance the proliferation of granulosa cells, increase inhibin production, and suppress progesterone secretion (McNatty et al., 2005). The GDF9 maintains normal fertility in mammalian females (de Castro et al., 2016). In addition, it stimulates ovine follicular growth and granulosa cells luteinization (Juengel et al., 2002). Oocyte GDF9 acts as paracrine to control several enzymes of granulosa cells (Vitt et al., 2000), which are crucial for ovulation, fertilization, and successful reproduction. Block follicular progression and complete infertility occur following the *GDF9* gene deletion. It is due to the occupation of ovaries by abnormal follicles with a single layer of granulosa cells arranged asymmetrically and the theca cell layer prominently missing in the primary one-layer follicle (Dong et al., 1996). The ovine *GDF9* gene is located in chromosome 5 (Sadighi et al., 2002). This gene spans about 2.5 kb, contains two exons and one 1126-bp intron intermediating between the two exons. Exon I spans 397 bp and encodes 1-134 amino acids, while exon II spans 968 bp and encodes 135-456 amino acids (Bodensteiner et al., 1999). Sheep litter size (ovulation rate) is an important fertility variable with economic value (Notter, 2008). Awassi sheep is representing more than half of Iraqi sheep (Al-Barzinji and Othman, 2013) and about 9% of Iraqi Awassi ewes lamb twice per year (Lafi et al., 2009). Distinguishing by DNA markers can prompt the hereditary enhancement and promotes selection for reproduction hastening (Asadpour and Joozani, 2012). The *GDF9* gene has a critical role in ovulation rate enhancement and improves the litter size of female sheep (Souza et al., 2014). Diverse mutations were recognized in the *GDF9* gene of various sheep breeds, which are significantly associated with infertility or ovulation rate improvement (Hanrahan et al., 2004). As a result, the enhancement of sheep fertility traits through selection is among the major goals of researchers (Kumm, 2009). The available information regarding this aspect in Iraq is insufficient. With this background in mind, the current study aimed to evaluate the relationship

between single-nucleotide polymorphisms (SNPs) in the *GDF9* gene, reproductive sterility, and fertility in Awassi breed.

MATERIAL AND METHODS

Animals. Forty slaughtered Awassi ewes were used to recover ovaries from Al-Shoala abattoir, Baghdad. Forty pairs of ovaries were collected and transferred to the laboratory of Biotechnology Research Center, Al-Nahrain University, Baghdad within 2-3 h. An equal number of samples were collected from the two groups of fertile and sterile ewes. Fertility condition was determined based on the history of normal or abnormal reproductive efficiency, gross evaluation of the ovaries, and the genitalia condition after slaughtering. The ewes with a history of poor post-partum follicles were considered as sterile ewes. On the other hand, the ewes were regarded as fertile in the case of normal cycles characterized by the presence of follicles, corpus albicans, or corpus luteum.

Follicles and Oocytes Evaluations. A total of 70 follicles with the diameters of 4-8 mm were measured using a micrometer (Mitutoyo, Japan) immediately after transferring the ovaries to a petri dish containing normal saline. Cumulus oocyte complexes (COCs) (n=88) were collected by aspiration from follicles (4–8 mm) and slicing the ovaries. Next, they were examined under a microscope to assess the quality and number of recovered oocytes.

DNA Extraction and Gene Amplification. Genomic DNA was isolated from the ovarian tissue of fertile and sterile groups applying the G-spin Kit (iNtRON, Korea) according to the protocols of the manufacturer. Two primers utilized to amplify a part of Exon I of ovine GDF9 included the forward primer of 5-GAAGACTGGTATGGGGAAATG-3 and the reverse primer 5-CCAATCTGCTCCTAC ACACCT-3. The reaction program consisted of 35 cycles as initial denaturation at 94°C for 5 min, denaturation at 94°C for 45 s, annealing at 58°C for 40 s, and extension at 72°C for 1 min, followed by final extension at 72°C for 10 min (Kasiriyan et al., 2011).

Sequencing and Genotyping. The polymerase chain reaction (PCR) products (Amplicon) were sequenced successfully by Macrogen Corporation, Korea through the Sanger sequencing method. A homology search was conducted by the BLAST option, which is available online on NCBI. Moreover, the SNPs were determined using the BioEdit program and NCBI.

Histopathological Evaluation. Twenty ovaries from each group of fertile and sterile ewes were selected. Each ovarian sample was stabilized in neutral buffered formalin (10%) (BDH, England) after being dissected. Next, the samples were transmitted into ethanol 70% (BDH, England) followed by dehydration and embedding in paraffin wax. Afterwards, the samples were dissected into several sections of 5 mm and the sections were floated into slides and left several hours to dry at 50°C. The sections were dewaxed and rehydrated in a graded series of ethanol solutions. Finally, the morphological inspection was performed after hematoxylin and eosin staining (vector, USA) of the sections as described by Elvin et al. (1999) and Nicol et al. (2009).

Statistical Analysis. The SAS software (2012) was applied to analyze the effect of factors on the parameters of the present study. The Chi-square test and t-test were utilized to compare the percentages and means, respectively (Ray, 2012).

RESULTS

PCR Amplification and Sequencing. The PCR amplification of the *GDF9* gene (exon I) in the samples collected from Awassi ewes demonstrated uniform fragments with a size of 462 bp when electrophoresed in 1% agarose gel (Figure 1). Sequencing revealed three SNPs in the exon I of the *GDF9* gene, including T(114)C, G(129)R, and G(199)A, compared to the *GDF9* gene of Norwegian white sheep breed with the Sequence ID of HE866499.1.

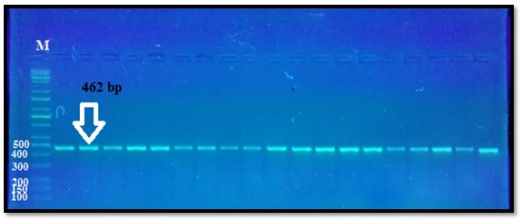

Figure 1. PCR product of exon I *GDF9* gene; M: DNA ladder 100–10000 bp; Lane 1–19: s PCR samples.

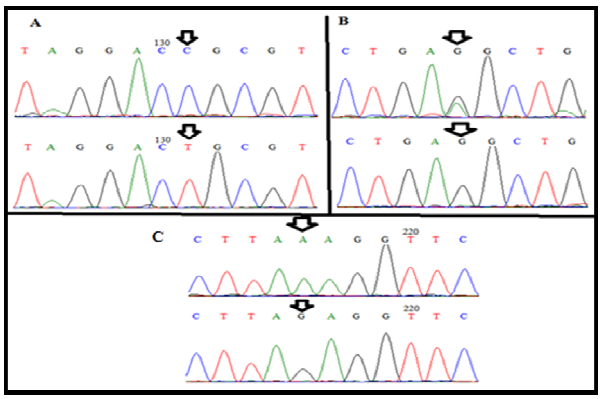

Figure 2. Wild-type and new variants of the exon I of *GDF9* gene; (A): T(114)C, (B): G(129)R, and (C): G(199)A.

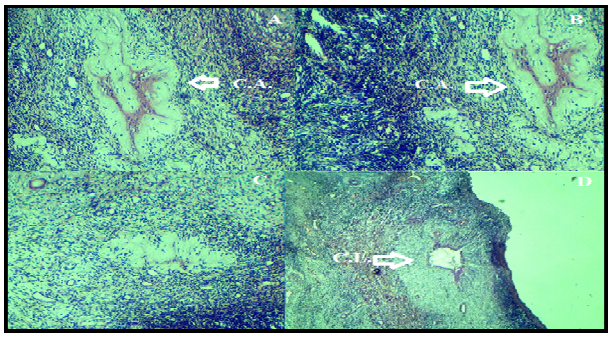

Figure 3. Ovarian histological sections of sterile ewes; (A) hypoplatic ovarian tissue; (B) follicular cells degeneration (H&E ×40).

The first two SNPs were silent mutations and the third one was a missense mutation (Figure 2) (Table 1).Genotypic frequencies, allele distribution, and sequence polymorphisms of the exon I of *GDF9* gene in Awassi ewes is shown in Table 2.

Relationship between Genotypes and Reproduction (Sterility and Fertility). According to Table 3, the homozygote G and mutant A alleles (GG and AA genotypes) were significantly($P \leq 0.01$) higher than the heterozygote G allele (GA genotype) in G(129)R locus and homozygote G allele (GG genotype) in G(199)A locus, respectively. In addition, the C allele was observed significantly($P \leq 0.05$) more than the T allele in T(114)C locus. The results of fertile ewes indicated that the mutant heterozygote G allele (GA genotype) in G(129)R locus and homozygote G allele (GG genotypes) in G(199)A locus were significantly ($P \leq 0.01$) more frequent than homozygote G and A alleles in G(199)A, respectively. However, the C allele was significantly ($P \leq 0.05$) less than the T allele in locus T(114)C.

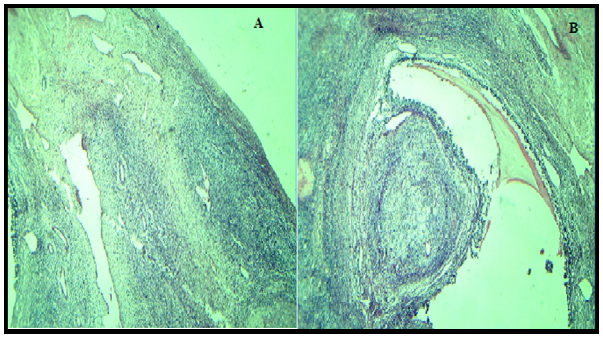

Figure 4. Histological analysis of the ovaries of normal fertile ewes; (A) normal follicular development with the presence of many corpus albicans; (B) degenerative corpus luteum.

Relationship between Genotypes, Follicles, and Oocytes. The percentage of GA and GG genotypes in follicles obtained from ovarian samples was significantly ($P \leq 0.05$) higher than the wild GG and mutant AA genotypes in G(129)R and G(199)A loci, respectively. On the other hand, the differences found between TT and CC genotypes of T(114)C locus were not significant (Table 4). Significant differences ($P \leq 0.05$) were reported between GG and GA genotypes, as well as between GG and AA genotypes in the oocytes completely and partially enclosed with cumulus cells (CCs) in G(129)R and G(199)A loci, respectively. However, the differences between TT and CC genotypes in T(114)C locus were not significant (Table 4).

Table 1. Type and position of the substitution of the exon I of *GDF9* gene in Awassi ewes

	Location of SNP (exon I)	Nucleotide change	Amino acid change and number	Predicted effect	Type of mutation
1	T(114)C	ACT>ACC	threonine>threonine (38)	Transition	Silent Mutation
2	G(129)R	GAG>GAGand GAA	glutamic acid>glutamic acid (43)	Transition	Silent Mutation
3	G(199)A	GAG>AAG	glutamic acid>lysine (67)	Transition	Missense

Table 2. Frequency of genotypes and alleles structure of exon I in the *GDF9* gene of Awassi ewes

	Locus	Genotype	Observed genotypes	Genotypic frequency %	Allele frequency		Chi-square
1	T(114)C	TT	18	45	T Allele	0.45	4.392 *
	Homozygote	CC	22	55	C Allele	0.55	
2	G(129)R	GG	13	32.5	A Allele	0.33	10.053 **
	Heterozygote	GA	27	67.5	G Allele	0.66	
3	G(199)A	GG	16	40	G Allele	0.4	7.25 **
	Homozygote	AA	24	60	A Allele	0.6	

Table 3. Correlation of genotypes with sterility (n=20) and fertility in ewes (n=20)

	Parameters	T(114)C		G(129)R		G(199)A	
1	**Sterile**	**TT**	**CC**	**GG**	**GA**	**GG**	**AA**
		9 (55%)	11 (45%)	13 (65%)	7 (35%)	8 (40%)	12 (60%)
	Chi-square	4.392 *		9.783 **		7.25 **	
2	**Fertile**	11 (55%)	9 (45%)	8 (40%)	12 (60%)	14 (70%)	6 (30%)
	Chi-square	4.392 *		7.25 **		11.25 **	

Table 4. Comparison of the number of follicles and oocytes surrounded by CCs with GDF9 genotypes

	Parameters	T(114)C		G(129)R		G(199)A	
		TT	**CC**	**GG**	**GA**	**GG**	**AA**
1	**Number of follicles With the size of 4-8 mm**	31 (44.28%)	39 (55.71%)	32 (45.71%)	38 (54.28%)	37 (52.85%)	33 (47.14%)
	Chi square	1.644 NS		4.327 *		4.398*	
2	**Oocytes surrounded completely and partially by CCs**	41 (46.59%)	47 (53.4%)	40 (45.45%)	48 (54.54%)	49 (55.68%)	39 (44.31%)
	Chi-square	3.381 NS		4.278 *		4.564 *	

Relationship between Genotypes and Ovarian Histopathology. Ovarian tissues obtained from the sterile ewes that possessed mutant A allele in G(199)A locus showed severe hypoplastic changes. Hypoplasia was characterized by diminished developmental follicles (the different phases of follicles are absent) mostly replaced with stromal connective tissue (Figure 3A). Furthermore, follicular remnants undergo severe degeneration followed by the proliferation of fibrous connective tissue, which contains eosinophilic material (Figure 3B). On the other hand, the histopathology of ovaries taken from fertile ewes with a mutant heterozygote G allele in G(145)R demonstrated normal follicular development along with numerous corpus albicans in all samples and degenerative corpus luteum (Figure 4).

DISCUSSION

In the current study, the polymorphic variations of *GDF9* gene coding were analyzed. This investigation was essential in terms of folliculogenesis and female Awassi breed fertility. Three new variant SNPs were observed by sequencing and the analysis of the exon I of *GDF9* gene, including T(114)C, G(129)R, and G(199)A. Two of the aforementioned polymorphisms, namely threonine (38) for T(114)C and glutamic acid (43) for G(129)R were nucleotide changes that do not result in alterations in amino acids. The remaining nucleotide G(199)A gave rise to amino acid variation (conservative changes) of negatively-charged polar glutamic acid to positively-charged polar lysine (67). The latter SNPs have not been described before.

Comparing between genotypes and the phenotypic traits of this study revealed that the effect of genotype on fertility and infertility was noticeable. These results are consistent with the findings of Bahrami et al. (2014), who reported polymorphisms in the exon I region of the *GDF9* gene in Hisari sheep using restriction enzyme HhaI. Moreover, the present results are in line with the investigation performed by Hanrahan et al. (2004). These authors reported a missense SNP of 260G>A in the exon I of the *GDF9* gene in Belclare and Cambridge Sheep breeds (rs410123449). This SNP substitutes one basic-charged polar group Arginine with another polar charge (Histidine) at amino acid residue (87).

Relationship between Genotypes, Fertility, and Sterility. Findings of the present study (Table 3) showed C mutant allele for T(114)C locus in sterile ewes and wild T allele in the fertile cases. Furthermore, the A mutant allele in G(199)A locus was demonstrated in sterile samples, while the wild G allele was detected in fertile ewes. These SNPs were not described before and such findings are consistent with the study completed by Kasiriyan et al. (2011). The latter authors mentioned the C to T transitional mutation in the exon I of Sangsari sheep, which is related to reproduction, fertility, and sterility. Regarding G(129)R, the sterile ewes had wild homozygote G allele, while fertile ewes possessed mutant heterozygote G allele. The present study is in agreement with other studies carried out by Galloway et al. (2000), Davis et al. (2006), Kasiriyan et al. (2011). They reported that homozygous genotypes led to sterility or reduced fertility rate. In addition, the current investigation is in line with the results of Barzegari et al. (2010). The mentioned authors indicated that heterozygous GDF9 (G>A) genotypes result in higher ovulation rates in Ghezel and Moghani Sheep. Nonetheless, they did not find a significant relationship between mutation and sterility. Moreover, the present study agrees with Hanrahan et al. (2004), who concluded that *GDF9* SNPs in exons I and II were associated with elevated ovulation rate. Subsequently, these SNPs might account for the enhancement of litter size in heterozygous alleles and sterility in homozygotes in Belclare and Cambridge breeds. These events happened due to failure in the normal development of ovarian follicles. The three SNPs found in exon I reflect a stronger effect on GDF9 function influencing folliculogenesis. These facts are in congruence with the findings of Khodabakhshzadeh et al. (2016). They concluded that the high grade of genetic diversity observed in the ovine *GDF9* gene coding region may affect folliculogenesis and the fertility of ewes.

Relationship between Genotypes, Follicles, and Oocytes. Our findings showed a direct relationship between the SNPs of GDF9 and the number of normal follicles and oocytes retrieved from ovaries. A number of follicles recorded in the mutant GA genotype (Table 4) were significantly higher than GG in G(129)R. These results seem to be consistent with the findings of Mullen and Hanrahan (2014). The latter researchers suggested that the missense mutation in GDF9 leads to the diminished biologic impact of GDF9 mature protein. Moreover, causes a reduction in the capacity of acting as a feedback inhibitor for FSH leading to enhanced ovulation rate in Finn sheep breed. In addition, Vitt et al. (2000) noted that GDF9 inhibits FSH-induced cAMP synthesis in rats. As a result, the SNP in *GDF9* of sheep may cause an increment in the sensitivity of ovarian follicles to FSH exerting a positive effect on the number of developing follicles and CCs expansibility. Furthermore, GDF9 reduces the synthesis of the mRNA of LH receptors, imposes a particular influence on CCs through inducing the expansion of CCs, and improves the expression of hyaluronan synthase-2 and cyclooxygenase-2. These events lead to the augmented ovulation rate in G(129)R SNP. Moreover, we observed that the mutant AA genotype of G(199)A locus recorded a marked diminish in the average number of follicles, as well as in the number of oocytes enclosed by CCs. These results are in agreement with the study performed by Nicol et al. (2009), which indicated that homozygote

mutation in the oocyte *GDF9* gene had a negative effect on the fertility of Thoka sheep breed.

Histopathological Differences between the Ovarian Tissues of Sterile and Fertile Ewes. Histopathological examination showed clear histopathological differences between the two groups. As explained before, follicular degeneration and hypoplasia were prevalent among sterile ewes. In addition, different phases of follicles are absent in the case of mutant homozygote A alleles in G(199)A locus.

These results were confirmed by Nicol et al. (2009), who suggested that ovaries from homozygous ewes are characterized by minimal follicle numbers beyond the primordial stage and most of the follicles passing primary stage were abnormal and underwent degeneration. Ovarian tissues of fertile ewes with heterozygote G allele of G(129)R had normal follicles, numerous normal corpus albicans, and degenerative corpus luteum proving that the ewes were cyclic and fertile.

According to the results of the present study, the three SNPs in Awassi ewes have different effects on follicles and oocyte number and subsequently on fertility and sterility. The homozygote mutation of G(199)R is associated with decreased follicles and oocyte numbers, which is accompanied by sterility. On the other hand, the heterozygote mutation of G(129)R could be related to normal fertility in Awassi ewes.

Ethics

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

Conflict of Interest

The authors declare that they have no conflict of interest.

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