Investigation the polymorphism of gonadotropin releasing hormone receptor gene in Iraq sheep

Hayder Abdul-Kareem Hasan AL-Mutar
Department of Surgery and Obstetrics, College of Veterinary Medicine, Baghdad University, Iraq.
E-mail: almutar.haydar@gmail.com

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Summary

In the present study, the investigation includes the estrus activity after synchronization of estrus by using 20 mg impregnated sponges with Medroxy Progesterone Acetate for 12 days with 400 IU I/M injection of pregnant mare serum gonadotropin 24 hrs. before sponges withdrawal. All 30 Iraqi sheep's showed (100%) estrus after sponge withdrawal. Twin percentage was 40% while single percentage was 60%. The polymorphisms of gonadotropin-releasing hormone receptor gene were analyzed as a genetic marker in 30 Iraqi sheep. There were two noticed genotypes (GA and GT), and single nucleotide polymorphisms in the exon2 region of gonadotropin-releasing hormone receptor gene as genetic marker and correlated with high litter size after hormonal super ovulation response in sheep. The frequencies of alleles G,A and G,T in Iraqi ovine breeds were 0.50 and 0.50, respectively. The result revealed that GA, GT genotype was related with better litter-size in Iraqi ovine breeds. Therefore, these outcomes recommend that gonadotropin-releasing hormone receptor gene be a powerful candidate gene that impacts litter-size in sheep.

Keywords: Estrus synchronization, Gonadotropin releasing hormone, Polymorphisms, Sheep.

Introduction

Estrus synchronizations are a key factor of all techniques and have a major influence to increase the overall efficiency of these programs (1 and 2). The technique most widely used to control production during the reproduction season is the progestogen impregnated intravaginal sponge, kept in situ for 10 to 14 days (3). A single administration of pregnant mare serum gonadotropin (PMSG) hormone after progesterone treatment increased the ovarian response, fertilization rates and increased the amount of several births (4). The hypothalamic gonadotropin-releasing hormone (GnRHR) is a key regulator of the reproduction, leading to the synthesis and release of LH and FSH in the pituitary. Control of GnRH receptors is essential because the quality of LH released in reaction to a physical task with GnRH is reliant on the concentricity of GnRH receptors on the plasma membrane of gonadotropes (5 and 6). In the sheep, GnRH is released in pulsatile style from the hypothalamus gland (7) and is a regulator of homologous of its own receptor. Decrease in the number of GnRH receptors by roughly 50%. However, it is not known whether the decrease in number of GnRH receptors is caused by receptor internalization, mediated at the degree of appearance of the GnRH receptor gene, or both. In the sheep, estrogen stimulates appearance of the GnRH receptor gene and number of GnRH receptors in the lack of GnRH (8). The connections of GnRH and its receptor is an important occasion in the hormonal control of breed (9). Due to the main part of GnRHR in controlling gonadotropin synthesis and release. Therefore the aim of study is to search for single nucleotide polymorphisms (SNPs) of GnRHR gene and associations between GnRHR polymorphisms and single, twinning parturition of the Iraq sheep.

Materials and Methods

The present study was conducted on sheep breeds. A total of 30 Iraqi sheep with history of single and twin births from farms. Healthy females sheep in ages from (2.5-4) years old and fertile ram about 2.5-3 years. Estrous synchronization was done by a sponge saturated with 20 mg Medroxy Progesterone Acetate (MAP) (Intervet -Dutch). And 400 IU PMSG (Folligon/Intervet-Dutch).Each treated sheep had received intravaginal sponge, containing 20 mg of MAP and coated with an antiseptic cream. Although, sponge was left for 12 days and injected I/M 400 IU PMSG 24 hrs. before sponges withdrawal. Estrous was detected and observed after progestin

treatment and Insemination of all sheep by natural mating. Five ml blood was collected aseptically from jugular vein of sheep into tubes containing anticoagulants (0.5 M EDTA). DNA was produced by using the standard protocol by intron kit (Korea) procedure. Two conserved primers (forward) 5-CCT ACA GTT ATA CAT CTT TGG GA-3 and (reverse): 5-GAG AAA TAC ATA CTG TGG GGA T-3. The thermal cycling conditions were done as follows: Denaturation at 95 °C for 3 min., followed by 35 cycles of 94 °C for 30s, 55 °C for 30s and 72 °C for 35s with final incubation at 72 °C for 7 min. using a thermal Cycler (Gene Amp, PCR system 9700; Applied Biosystem). The Polymerase chain reaction (PCR) products were extracted by 2% agarose gel electrophoresis and visualized by contact with ultra violet light (302 nm). Sequence of nucleotides of exon 2 of GnRHR gene by using BioEdit program, which was performed by National Instrumentation Center for Environmental Management (NICEM) online at (http://nicem.Snu .ac. kr/main/ ?en skin =index.html), biotechnology lab, machine is DNA sequencer 3730XL, Applied Biosystem), Homology look for was conducted using Basic Local Alignment Search Device(BLAST) program which was available at the National Center Biotechnology Information (NCBI) online at (http://www.ncbi.nlm.nih.gov). Data were submitted to statistical analysis using chi-Square test, spss program (10).

Results and Discussion

During progestin treatment no sign of estrous was observed. In Post-treatment all (100%) experimental animals showed estrous signs within 24-60 hrs**.** The present results agreed with other study shows that the expression of estrous behavior was correlated with chronologically preovulatory secretion of luteinizing hormone and ovulation (11 and 12). As well as the manifestation of estrous behavior might vary either the duration or intensity, consisting on the breed, age, health status, season, and the presence of males**.** The 20 mg of MAP which used in this research was enough to suppress the production of gonadotropin out of breading season (12). While removal the blockage of progesterone lead to release of gonadotropin and sequent estrous and ovulation in female treated with progesterone. Also estrous response to the application of intravaginal sponge depends on breed, co-treatment system, management and mating (11). Smith, (13) described that the ovulation in the female caprine usually happen 30-36 hrs. after beginning of estrous. Also, the same outcomes was described (14 and 15) when the PMSG hormone given 24 hrs. before the removal of sponge, the does come in heat during the 12-36 hrs. by removing the sponge**.** This finding agree with suggested that doses of commercial formulation (MAP 20 mg) followed by (400 IU) PMSG injected at day before sponge withdrawal was sufficient to induce and synchronize estrus suggesting (16). Fertility result in this study was evaluated by kidding rate which proved that 25 sheep out of 30 (75 %) gave birth. However, the percentage of does returning to estrus was 5 (25%) (Table, 1) which might be attributed to fertilization failure or early embryonic mortality. The 400 I.U PMSG hormone injection and method of mating used could be increased the percentage of twining (Table, 2). Similar observation was recorded by (17). The present protocol was recommended outside of breeding response by (18) to improve ovulation rate claimed that PMSG has long half-life about 4-6 days in turn, the long action of biological activity causing to continually release of a few number of un ovulated follicles (19 and 20). The distribution of parturition in pregnant animals according to type of birth showed 15 out of 25 (60%) pregnant ewe had a twin birth and 10 out of 25 (40%) had single birth (Table, 2).

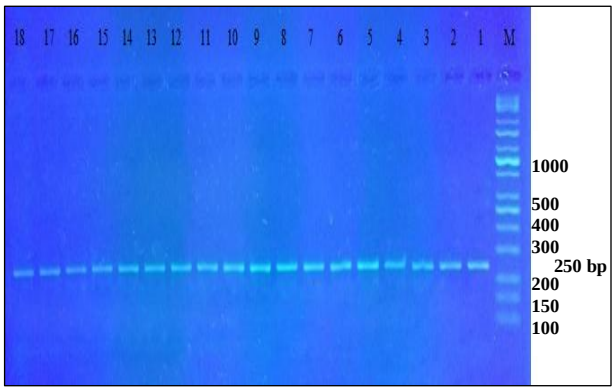
Table, 1: Distribution of study according to pregnant and non-pregnant in ewes.

Animal	Number	Percentage (%)
Pregnant	25	75%
Non-pregnant	5	25%
Total	30	100 %

Table, 2: Distribution of parturition of pregnant animal according to offspring in ewes.

offspring	Number	Percentage (%)
Twin	15	60%
Single	10	40%
Total	25	100 %

DNA was extracted from the blood samples of all animals (30 ewes) by AccuPrep® kit/ intron the procedure was very efficient and showed sharp band by gel electrophoresis (Fig. 1). Genomic DNA of white blood cells was also used for amplification of GnRHR gene using PCR specific primers for exon 2. The amplified fragment which is yielded of single band of the desired product with a molecular weight of 240 base pair appeared sharp in agarose gel through Gel electrophoreses technique and loaded with (100-1000bp) DNA ladder (Fig. 1).



Figure, 1: The product was electrophoresis on 2% agarose gel at 5 volt/cm^2, 1x TBE buffer for 2 hours. M: DNA ladder (100-10000bp), lane (1-18) PCR product of band size 240bp. visualized under U.V light after staining with red stain safe.

The sequencing of amplified product of exon 2 GnRHR gene from 30 sheep (only kidding animals), 25 out of them appeared 100 % compatibility with standard Ovis aries breed gonadotropin-releasing hormone receptor (GnRHR) gene, exon 2 from 571 to 747 number of nucleotide from gene of gene Bank results as shown in (Fig. 2), Sequence ID: ref|NM_001009397.1||, and have number score (327) bits whereas the single cases and 10 out of them appeared 95% compatibility with standard Ovis aries breed gonadotropin-releasing hormone receptor (GnRHR) gene, from 576 to 764 number of nucleotide from gene of gene Bank results as shown in (Fig. 3), Sequence ID: ref|NM_001009397.1||, and have number score (292) bits whereas the towing, Ovis aries gonadotropin releasing hormone receptor (GNRHR), mRNA Sequence ID: ref|NM_001009397.1|

In total, twin cases of does had six type of transversion substitution in location 581A>T, 590A>C, 606 A>T, 621 C>G, 655 T>A and 755 A>T and three types of transition substitution in 586 T>G, 600 T>C and 756 T>C, in exon 2 of GnRHR gene (Table, 3). (21) Reported that exon 2 of GnRHR gene in Small Tail Han Sheep (Chinese) have two substitution (transversion A>C and transition A>G), many studies referred to polymorphic fragment amplified of GnRHR gene and there was one substitution (G→C) at cDNA 198 of GnRHR gene and the mutation resulted in amino acid change (Gln→Arg and Gly→Arg) in Gansu Meat ovine New Breed Population of Chinese (22). (23) Showed that is three mutations at location (552T→G, 653C→G, 654G→A) and change of amino acids (Arg→Ser) in Hu sheep. (24) Referred to polymorphism that changes amino acid lysine to glutamic acid in Goat of GnRHR gene.

Score	Expect	Identities	Gaps	Strand
327 bits	3e-86	177/177(100%)	0/177(0%)	Plus/Plus

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Query  7    ACAGTTATACATCTTTGGGATGATCCATTTAGCAGATGACTCTGGACAGACTGAAGGTTT  66
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  571  ACAGTTATACATCTTTGGGATGATCCATTTAGCAGATGACTCTGGACAGACTGAAGGTTT  630

Query  67   CTCTCAATGTGTAACACACTGCAGTTTTCCACAGTGGTGGCATCAAGCCTTTTATAACTT  126
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  631  CTCTCAATGTGTAACACACTGCAGTTTTCCACAGTGGTGGCATCAAGCCTTTTATAACTT  690

Query  127  TTTCACCTTCAGCTGCCTCTTCATCATCCCTCTTCTCATCATGCTGATCTGCAATGC  183
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  691  TTTCACCTTCAGCTGCCTCTTCATCATCCCTCTTCTCATCATGCTGATCTGCAATGC  747
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Figure, 2: Sequencing of sense flanking the exon 2 of GnRHR gene, for cases of ewes, obtained from Gene Bank. Query represents of sample; Sbject represent of database of National Center Biotechnology Information (NCBI).
Ovisaries gonadotropin releasing hormone receptor (GNRHR), mRNA
Sequence ID: ref|NM_001009397.1|

Score	Expect	Identities	Gaps	Strand
292 bits(158)	1e-75	179/189(95%)	1/189(0%)	Plus/Plus

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Query  2    TATACTTCTTGGGGCTGATCCATTCAGCAGTTGACTCTGGACAGAGTGAAGGTTTCTCTC  61
            |||||  ||||  ||||  ||||||||  |||||  ||||||||||||||  ||||||||||||||
Sbjct  576  TATACATCTTTGGGATGATCCATTTAGCAGATGACTCTGGACAGACTGAAGGTTTCTCTC  635

Query  62   AATGTGTAACACACTGCAGATTTCCACAGTGGTGGCATCAAGCCTTTTATAACTTTTTCA  121
            ||||||||||||||||||  ||||||||||||||||||||||||||||||||||||||||
Sbjct  636  AATGTGTAACACACTGCAGTTTTCCACAGTGGTGGCATCAAGCCTTTTATAACTTTTTCA  695

Query  122  CCTTCAGCTGCCTCTTCATCATCCCTCTTCTCATCATGCTGATCTGCAATGCAAAAATCT  181
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  696  CCTTCAGCTGCCTCTTCATCATCCCTCTTCTCATCATGCTGATCTGCAATGCAAAAATCA  755

Query  182  CCTTC-CCC  189
              ||||  |||
Sbjct  756  TCTTCACCC  764
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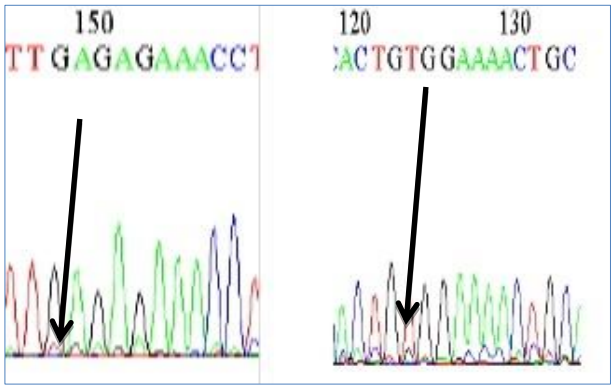
Figure, 3: Sequencing of sense flanking the exon 2 of GnRHR gene, for cases of ewes , obtained from Gene Bank.

Table, 3: Type of polymorphism and amino acid change in sense of GnRHR gene in sheep.

location of gene bank	Nucleotide change	Amino acid change	Predicted effect	Type of mutation
A581T	ACA>ACT	Threonine> Threonine	Silent	Transversion
T586G	TTG>TGG	Leucine>tryptophan	Missense	Transition
A590C	GGA>GGC	Glycine>glycine	Silent	Transversion
T600C	TTA>TCA	Leucine>serine	Missense	Transition
A606T	GAT>GTT	Aspartic acid>valine	Missense	Transversion
C621G	ACT>AGT	Threonine>serine	Missense	Transversion
T655A	AGT>AGA	Serine>arginine	Missense	Transversion
A755T	ATC>TCC	Isoleucine>serine	Missense	Transversion
T756C	ATC>TCC	Isoleucine>serine	Missense	Transition

Genotype distribution and allele frequency of GnRHR gene in Iraqi sheep by the use of sequence of nucleotides of exon 2 of GnRHR gene which have been utilized to determine the existence or absence of substitution. Initial genotyping data using the curve method revealed 25 (100%) clear G/A homozygotes, 10 (40%) and 15 (60%) out of them single and twin birth and 25 (100%) clear G/T heterozygotes but only one from them had single and twin birth, as showed in (Table, 4 and 5), (Fig. 4) shows sample curves that represent each of the one genotypes (G/A).



Figure, 4: Nucleotide sequence of genotype of exon2 GnRHR gene. The right arrow is a G/A heterozygote, and the left arrow is a G/T heterozygote.

In the present study, the homozygous A/A and G/G genotypes were not detected in the GnRHRgene. The present observation was agreed with a previous finding in another breed of goats which observed two types of genotype (AA and AC), (25) and (GG and AG) or (GG and GT), (26) and agreed with (27). In conclusion, this genotypes (A/A) may have a negative effect on reproduction, so that sheep with this genotype might have been eliminated through the process of artificial selection and breeding program or it is also possible that A/A genotype was not found in the study population due to its low allele frequency that results from low populations. It is certain that the percentage distribution of genetic structures varies, when compared between different strains and this follows in most cases, to prefer the environment for the installation of the latest (suitability to the environment) or as a result of the preference breeders certain amount of the appearance of such level and then reflected on the distribution of genotypes rates. Amounts of genotype and allele frequency of G, A and G, T that returned to exone 2 GnRHR gene in the present study on Iraqi sheep were 0.50 and 0.50, respectively (Table, 6).

Table, 4: Distribution of genotype G757A according of type of parturition (No. and %) in sheep.

Genotype G757A	No. of animal		Percentage (%)	
	Single	Twin		
GA	25		100%	
	10	15	40%	60%
AA	0		0.00	
GG	0		0.00	
Total	25		100 %	

Table, 5: Distribution of genotype G891T according of type of offspring (No. and %) in sheep.

Genotype G891T	No. of animal		Percentage (%)	
	Single	Twin		
GT	25		100%	
	10	15	40%	60%
GG	0		0.00	
TT	0		0.00	
Total	25		100 %	

Table, 6: The allelic and genotypic frequencies for sequence polymorphisms in the *GnRHR* gene in sheep.

Locus	Genotype	Allele frequency
G757A	GA (25)	G= 0.5
G757A		A=0.5
G891T	GT (25)	G=0.5
G891T		T=0.5

The researchers (28) found two different breed of goat that G and T allele frequencies were (0.90 and 0.10), respectively. While (29) referred to allele frequency of G and A in the *GnRHR* gene in Boer goats 0.925 and 0.075 respectively at 757 location of nucleotide of gene. However allele frequency of G and T were 0.951 and 0.049 respectively at 891 location of the *GnRHR* gene in Boer goats. (30) Referred to two allele frequencies C and A in Iraqi goat breeds which were 0.85 and 0.15. In conclusion, the present study identified two new polymorphisms (G891T and G757A) in the sheep *GnRHR* gene of Iraqi sheep.

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التحري عن التغايرات الوراثية لمستقبلات جين هرمون المحفز غدد القند للأغنام العراقية

حيدر عبد الكريم حسن المطر

فرع الجراحة والتوليد، كلية الطب البيطري، جامعة بغداد. العراق.

E-mail: almutar.haydar@gmail.com

الخلاصة

تضمنت الدراسة إستحداث الشبق باستعمال الاسفنجات المشبعة بـ 20 ملغم من (ميدروكسي بروجسترون اسيتيت) لمدة 11 يوماً مع حقن (500 وحدة دولية) في العضل من هرمون المشيمة للفرس الحامل. أظهرت جميع الاغنام (30) الشبق (100٪) بعد سحب الاسفنجات. وكانت نسبة ولادة التوائم 40% والولادة المفردة 60%. وقد حُللت الأشكال من مستقبلات هرمون المحفز لغدد القند كعلامة وراثية في 30 من الأغنام العراقية ولوحظ اثنين من التراكيب الوراثية (GA و GT)، القواعد النيتروجينية المنفردة في المنطقة المعبرة الثانية من جين مستقبل هرمون المحفزللغدد التناسلية واستعمال هذا الجين كعلامة وراثية وربطه بعدد المواليد مقارنة مع مستوى الاستجابة لفرط الاباضة باستعمال العلاجات الهرمونية في الأغنام العراقية. كانت تغايرات الأليلات G,A و G,T في سلالات الأغنام العراقية 0.50 و 0.50 على التوالي. وبينت النتائج الوراثية أن GA وGT لهما علاقة مع نسبة الولادات في سلالات الأغنام العراقية. ومن ثَمَ، فان هذه النتائج بينت إن جينات مستقبل هرمون المحفز للغدد التناسلية هي مرشحة في نسبة ولادات الأغنام.

الكلمات المفتاحية: توحيد الشبق، مستقبلات هرمون المحفز لغدد القند، تغايرات وراثية، الاغنام.